APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1982, p. 905-915
0099-2240/82/040905-11$02.00/0
Vol. 43, No. 4

# Scanning Electron Microscope Study of *Pseudomonas fragi* on Intact and Sarcoplasm-Depleted Bovine Longissimus Dorsi Muscle

R. Y. YADA AND B. J. SKURA*

*Department of Food Science, University of British Columbia, Vancouver, British Columbia V6T 2A2 Canada*

Received 31 August 1981/Accepted 16 December 1981

Intact bovine longissimus dorsi muscle strips used 24 h postmortem were washed to remove sarcoplasmic fluid or left intact and were either left uninoculated or inoculated with *Pseudomonas fragi* ATCC 4973. The effects of decreased sarcoplasm concentration on growth of *P. fragi* and consequent microstructural changes of beef muscle during aerobic storage at 4°C for 12 days were evaluated. *P. fragi* grew slower on washed muscle than on intact muscle. Scanning electron micrographs revealed surface degradation of both intact inoculated and washed inoculated muscle only in areas of localized colonization. Extracellular fibrils appeared to mediate adhesion of *P. fragi* to the muscle surface as well as cell-to-cell attachment within microcolonies. *P. fragi* was also observed growing between muscle fibers.

In nature, adhesion of bacteria to surfaces is a widespread phenomenon. Information concerning the mechanisms of bacterial attachment and adhesion, particularly to food myosystems, is limited. Recent investigations (8, 17, 20, 21) into the attachment of bacteria to myosystems have shown that the presence of an extracellular polysaccharide, the glycocalyx, appears to mediate attachment. In contrast, Thomas and McMeekin (29) have suggested that bacteria on chicken skin are not attached by extracellular bridging substances.

Various factors have been thought to influence the attachment process. Flagellated bacteria attach more readily to chicken skin (20), pork skin, and lamb and beef muscle (4) than do nonflagellated bacteria. Temperature and pH were important factors in one study of chicken skin (20), whereas immersion time, pH, and temperature of the attachment medium were not significant factors in a study of pork skin and lamb and beef muscle (4). Notermans and Kampelmacher (21) reported that a proportion of the bacterial flora associated with chicken skin is actually present in a water film surrounding the skin and can be easily removed by adequate rinsing. The bacterial flora in the water film may be of great importance, since they appear to play a key role in the attachment of bacteria to chicken skin (18, 21). The attachment of bacteria to myosystems is proportional to the number of bacteria present (4, 21).

Marshall et al. (15), studying the sorption of marine bacteria to inert surfaces, stated that attachment entails an instantaneous reversible phase and a time-dependent irreversible phase. The reversible phase involves a weak attachment of bacteria to a surface through London-van der Waals attractive forces (15). In this state, bacteria exhibit Brownian motion and can be removed from the surface by being washed off with 2.5% NaCl (15). During the time-dependent irreversible phase, which involves the formation of polymers between the bacteria and the surface as a result of bacterial metabolism, the bacteria are firmly attached to the surface, exhibit no Brownian motion, and are not removable with 2.5% NaCl (15). Working with membrane filters (Millipore Corp., Bedford, Mass.) as an attachment surface, Fletcher and Floodgate (10) found that the initial stage of irreversible adhesion involves contact between the surface and the primary polysaccharide surrounding the bacterium before the formation of secondary fibrous acidic polysaccharide.

Studies (16) of the adhesion of bacteria to epithelial cell surfaces within the reticula of cattle have revealed intermittent colonization of the surface of epithelial cells. Ruthenium red staining has shown the presence of carbohydrate material (glycocalyx) on both the epithelial cells and the adhering bacteria. The glycocalyx of the bacteria appears to mediate attachment of the bacteria to the epithelium, food particles, and one another (16). After bacteria initially attach to cow teats, the strength of attachment increases, the rate being faster at higher storage temperatures (19). Polymers in the form of thin

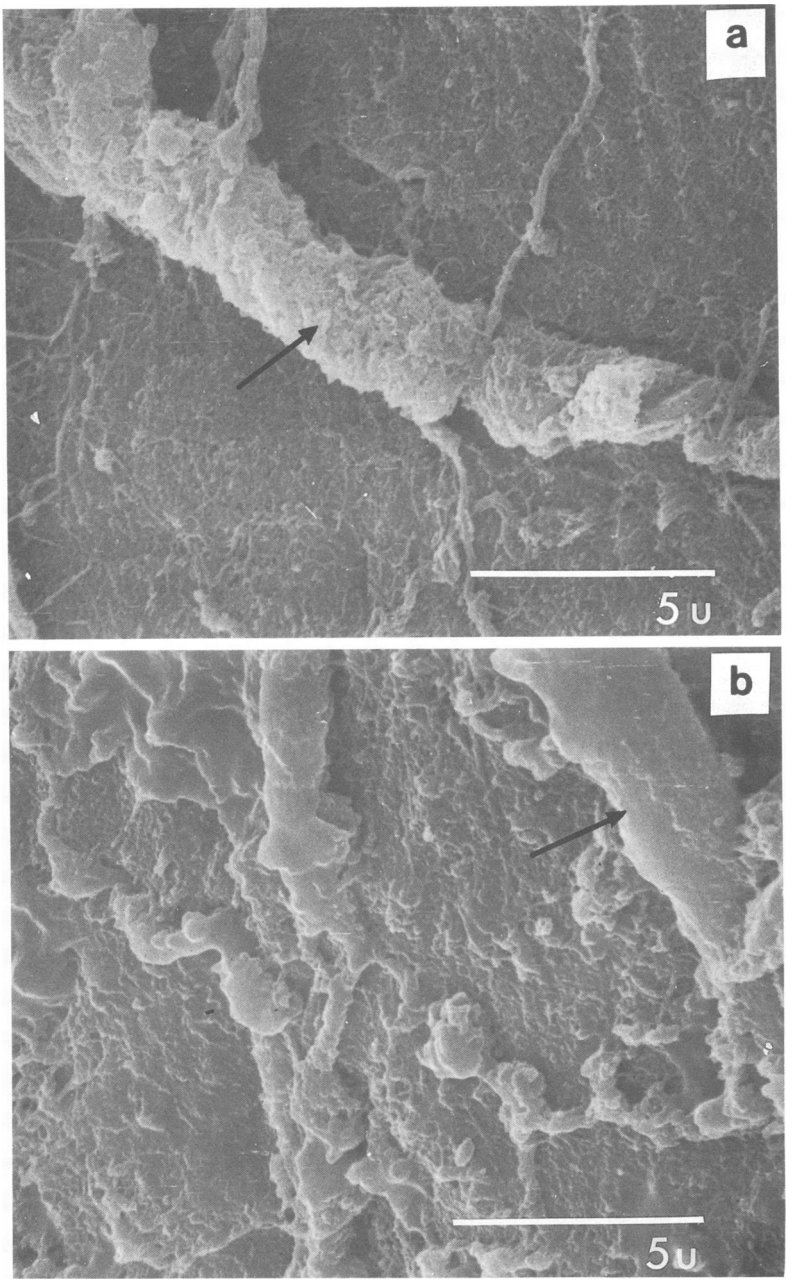


FIG. 1. Micrographs (day 0) of uninoculated intact (a) and uninoculated washed (b) muscle. Collagen and reticular fibrils (arrows) cover the muscle surface.

fibers, observed by scanning electron microscopy (SEM), are produced during the storage of inoculated teats (8). These fibers eventually thicken and form slime. Acidic polysaccharides (pure polysaccharides, glycoproteins, and other mixed polymers) are thought to be involved in the adhesion process (6).

A better understanding of the mechanisms of attachment and adhesion of bacteria to myosystems may aid in the selection or development of procedures for reliably estimating numbers and types of bacteria on muscle surfaces as well as the development of techniques for decreasing bacterial numbers on muscle and thereby increasing shelf life. In this study, SEM was used to determine microstructural changes occurring

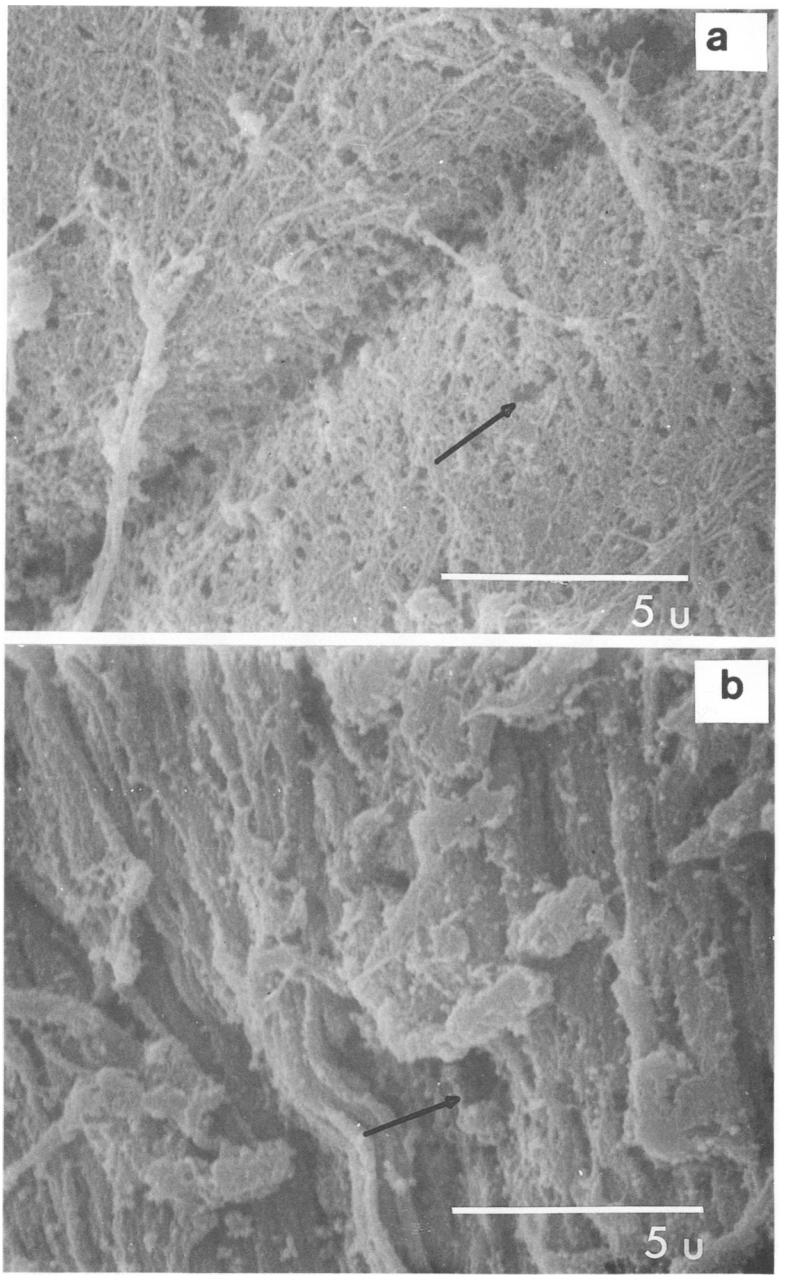


FIG. 2. Micrographs (day 12) of uninoculated intact (a) and uninoculated washed (b) muscle. Arrows show areas of autolytic deterioration of tissue.

on the surface of intact and sarcoplasm-depleted bovine longissimus dorsi muscle during growth of *Pseudomonas fragi* at 4°C.

(This paper was presented at the 23rd annual Canadian Institute of Food Science Technology Conference, Toronto, Ontario, Canada, 1 June 1980 to 4 June 1980.)

## MATERIALS AND METHODS

Bovine longissimus dorsi muscle, used 24 h postmortem, was obtained from a local abattoir and transported on ice to the laboratory. Before being sectioned, the muscle was sprayed with a 70% ethanol solution to reduce surface bacterial contamination (13). Thin slices (3 mm) were removed with a Hobart

delicatessen slicer (Don Mills, Ontario, Canada) which had been previously sprayed with 70% ethanol. Upon completion of every fifth slice, the slicer was sprayed with 70% ethanol.

The slices were randomly organized into two groups. One group did not receive further treatment (intact muscle), and the other group was subjected to a washing procedure (30) to extract water-soluble components (washed muscle). Intact and washed muscle slices were sterilized with 10 kGy of gamma-radiation, inoculated with *P. fragi*, and incubated for up to 12 days at 4°C (30). Sterile controls of intact and washed muscle were also prepared.

**SEM.** Muscle tissue was cut into pieces (ca. 2 by 2 cm) and fixed in 2.5% (vol/vol) electron microscopic-grade glutaraldehyde (CAN-EM Chemicals, Guelph, Ontario, Canada)–0.05 M phosphate buffer (pH 7.0; 61 ml of 0.05 M $Na_2HPO_4$ + 39 ml of 0.05 M $KH_2PO_4$) overnight. The tissue was then washed twice in 0.05 M phosphate buffer (pH 7.0) before being postfixed in 1% (wt/vol) osmium tetroxide (CAN-EM Chemicals)–0.05 M phosphate buffer (pH 7.0) for 1 h (26). Samples were subsequently washed three times with 0.05 M phosphate buffer (pH 7.0). This step was followed by dehydration through an ascending series of ethanol: 50, 70, and 80% (vol/vol) ethanol for 5 min each; two changes with 90% ethanol for 10 min each; and three changes with 100% ethanol for 20 min each. All ethanol dilutions were made with distilled, deionized water. Samples were then subjected to infiltration by a graded series of amyl acetate (Fisher Scientific Co., Fairlawn, N.J.): one change each with 25, 50, and 75% (vol/vol) amyl acetate diluted with absolute ethanol (10-min duration each) and one change with 100% amyl acetate (1-h duration). Samples were critical point dried in a Parr-bomb (Parr Instrument Co., Moline, Ill.) and mounted on aluminum stubs with silver paste (Structure Probe Inc., West Chester, Penn.) Samples were then coated with gold-palladium in a sputter coater (Technics Inc., Alexandria, Va.) and observed with a Hitachi S-500 scanning electron microscope at an accelerating voltage of 20 kV. Images were recorded on Ilford Pan F 135 fine-grain black-and-white film (Ilford Ltd., Essex, England). Micrographs of the uninoculated controls (intact and washed) were taken at the same times as those at which micrographs of the inoculated muscle were taken.

## RESULTS AND DISCUSSION

Washing the sarcoplasm from the beef muscle did not produce any noticeable changes of the surface microstructure (Fig. 1). Since there were no extensive changes in the surface structure of the controls during the 12 days of incubation, only micrographs of controls at days 0 (Fig. 1) and 12 (Fig. 2) are shown. Perforations, indicative of autolytic tissue degradation, appeared on the surface of both intact and washed controls after 12 days of storage at 4°C (Fig. 2). Such indications of tissue deterioration were extremely variable from site to site on the same sample. Schaller and Powrie (25), in a SEM study of skeletal muscle from various sources, found some surface degradation on bovine longissimus dorsi muscle during the commercial aging of beef.

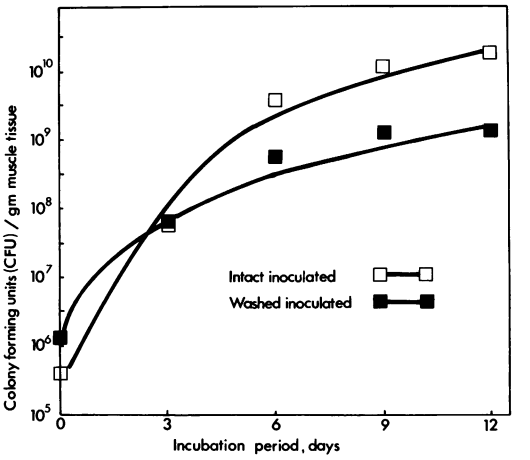


FIG. 3. Bacterial population of intact and washed muscle samples that were inoculated with *P. fragi* and stored at 4°C. Each point represents the mean of quadruplicate determinations.

Figure 3 shows the growth profiles of *P. fragi* on intact and washed muscle. *P. fragi* grew significantly slower on washed muscle than on intact muscle (30). Although $4.2 \times 10^5$ and $1.3 \times 10^6$ *P. fragi* cells per $cm^2$ were found on intact and washed muscles, respectively, no bacteria could be observed on any of the intact or washed samples at day 0. The lack of detectable bacteria may have been due to the removal of *P. fragi* from the muscle during fixation for SEM. McMeekin et al. (18) noted that in their experiment, some bacteria had been probably washed off the chicken skin during preparation for SEM. During the primary stages of attachment, bacteria can be easily removed from surfaces (15).

Limited numbers of bacteria were seen on the surfaces of inoculated intact and inoculated washed muscle by day 3. The presence of observable bacteria at day 3 (Fig. 4) was probably due to the population of *P. fragi* increasing (Fig. 3) and, consequently, the probability of detecting bacteria increasing. Although there is no evidence in the micrographs, it is possible that some extracellular fibers produced by *P. fragi* may have mediated the adhesion of the bacterial cells to the muscle surface. McCowan et al. (16) stated that in their experiments, the "fine slime fibers" which mediate bacterial adhesion were below the limit of resolution of the scanning electron microscope and could not be seen except in aggregated form.

After 6 days at 4°C, the presence of what appeared to be extracellular material was observed on the surface of *P. fragi* on both inocu-

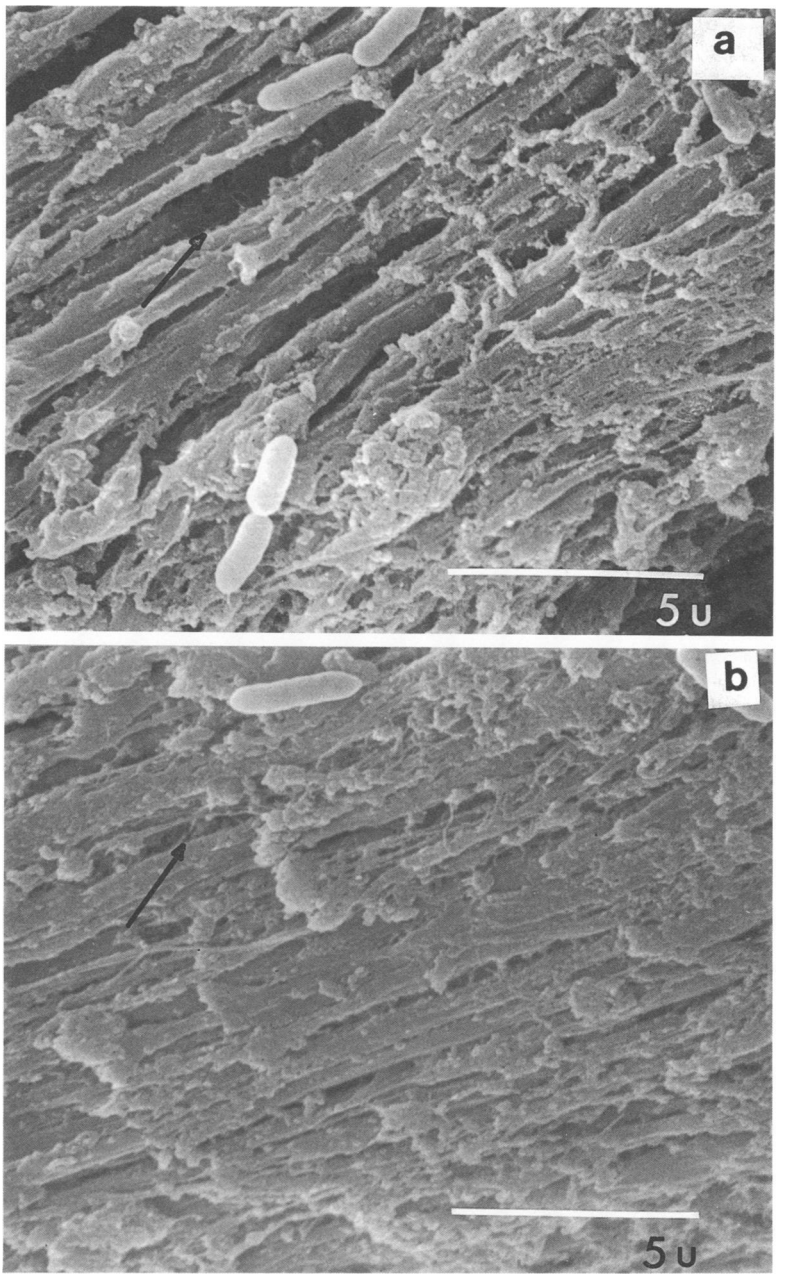


FIG. 4. Micrographs (day 3) of inoculated intact (a) and inoculated washed (b) muscle showing crevices (arrows) which may trap bacteria.

lated intact and inoculated washed muscle (Fig. 5). Extracellular fibers seen by SEM appeared to be severely condensed, radically dehydrated remnants of the extracellular polysaccharide. These fibers, therefore, may represent areas where large masses of glycocalyx were located. Marshall et al. (15) stated that time-independent primary attachment is followed by a time-dependent secondary attachment during which the strength of attachment increases owing to the production of extracellular acidic polysaccharides by the primary attached bacteria (10, 15). Extracellular cellulose fibrils have been found on many gram-negative bacteria (7). McCowan et al. (16) observed carbohydrate coats that surrounded adherent bacteria and appeared to

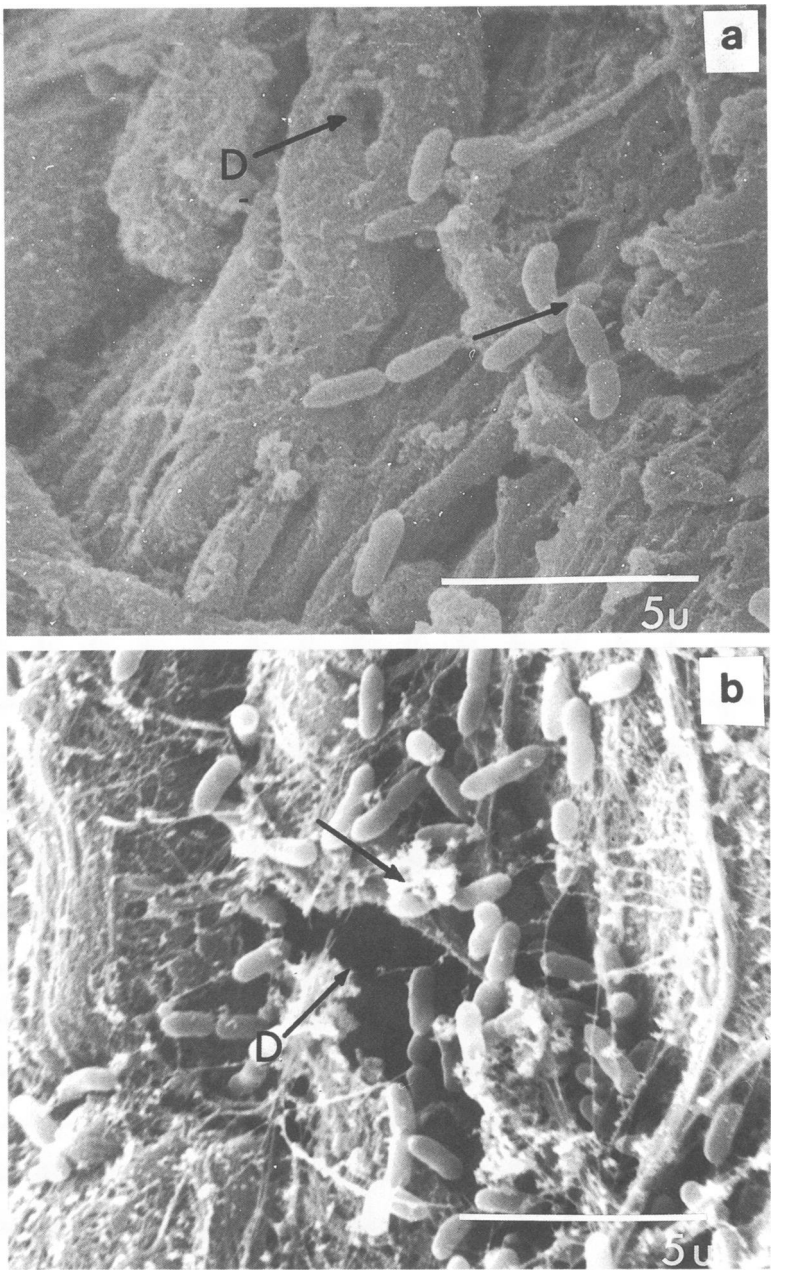


FIG. 5. Micrographs (day 6) of inoculated intact (a) and inoculated washed (b) muscle. Extracellular fibrils (arrows) can be seen on the surface of *P. fragi*. Areas of degradation (D) are evident on the muscle surface.

mediate the attachment of bacteria to the epithelium of the reticula of cattle, to food particles, and to one another.

Degradation of intact inoculated and washed inoculated muscle tissue in the areas of bacterial growth was apparent by day 6 (Fig. 5). These changes in muscle microstructure were, however, not detectable in protein extractability profiles or electrophoretic patterns of various muscle protein fractions (30).

The amount of intact inoculated and washed inoculated muscle surface degradation increased during storage beyond day 6. By day 9, more bacteria were attached to one another and were only indirectly attached to the muscle surface (Fig. 6). The adherent bacterial population was a

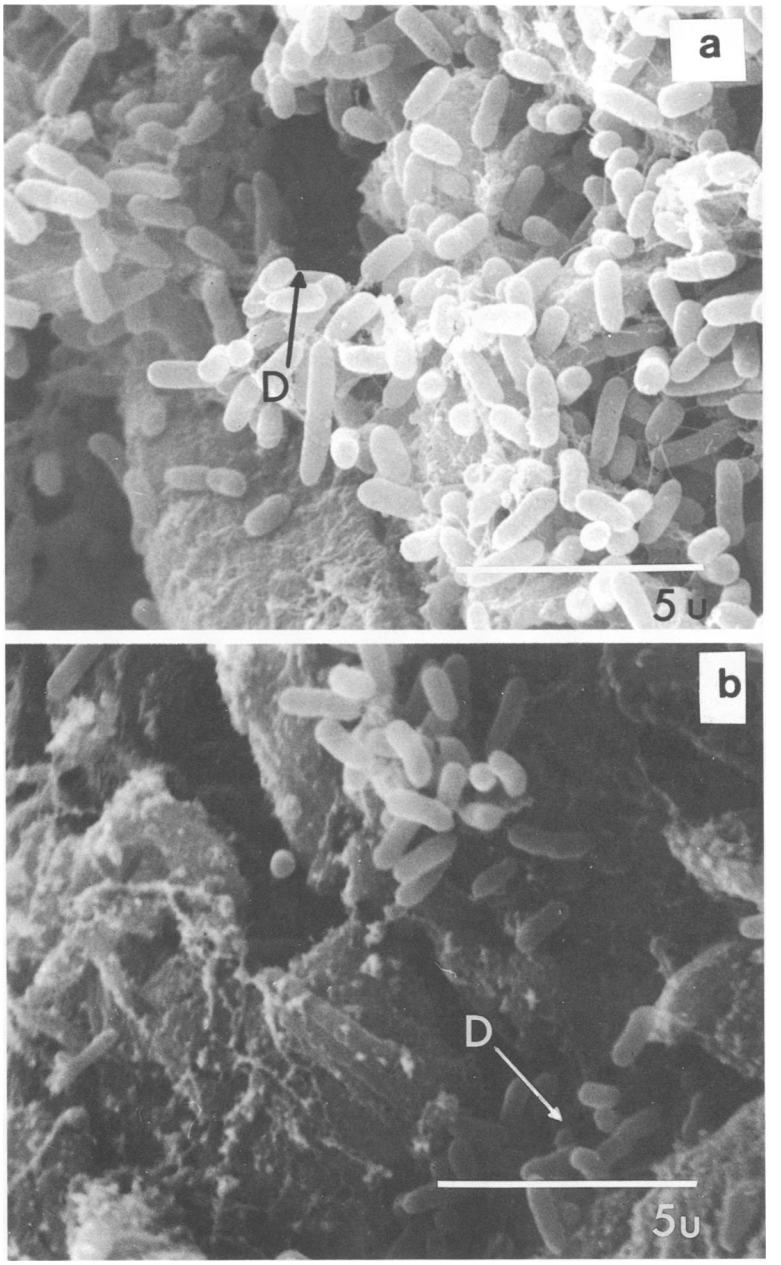


FIG. 6. Micrographs (day 9) of inoculated intact (a) and inoculated washed (b) muscle. Possible areas of surface degradation (D) are indicated.

complex mass of cells. Areas of surface degradation were evident in both intact inoculated and washed inoculated muscle.

The distribution of bacteria on the muscle surface after day 12 of incubation was nonuniform. A similar colonization pattern was reported in a study of the colonization of bovine tongue (17). A higher population of cells in a given microenvironment could suggest that physical and chemical conditions of the locale are more favorable for the active uptake of nutrients (9, 27, 31). Although possibly an artifact of the sample preparation, a well-defined extracellular fiber network appeared to hold the microcolonies on the washed inoculated muscle together, whereas the extracellular fibers of the

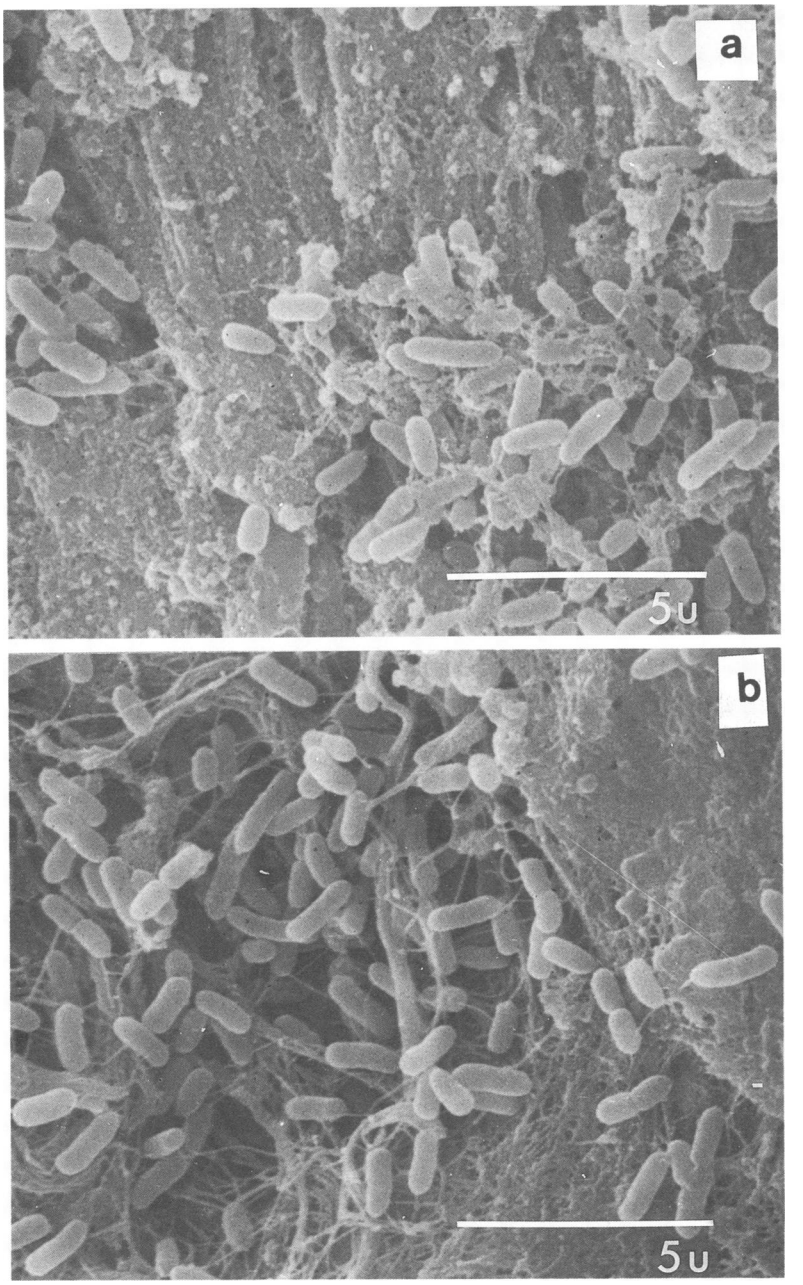


FIG. 7. Micrographs (day 12) of inoculated intact (a) and inoculated washed (b) muscle.

intact inoculated muscle were amorphous in nature (Fig. 7). If not an artifact, the fiber network may have resulted from differences in available nutrients (8). Although the washed muscle was an adequate growth medium (as indicated by the *P. fragi* growth curve shown in Fig. 3), removal of the majority of water-soluble components (30) probably made the washed muscle an ecosystem which supported the growth of *P. fragi* with more difficulty than intact muscle. Bacteria, when provided with complex and simple nutrient sources, will invariably utilize the simpler nutrients (amino acids, nucleotides, or carbohydrates) instead of the more complex nutrients such as proteins (11, 12, 14). Costerton et al. (5) stated that polymers produced by bacteria may not only position the bacteria but may also conserve and concentrate

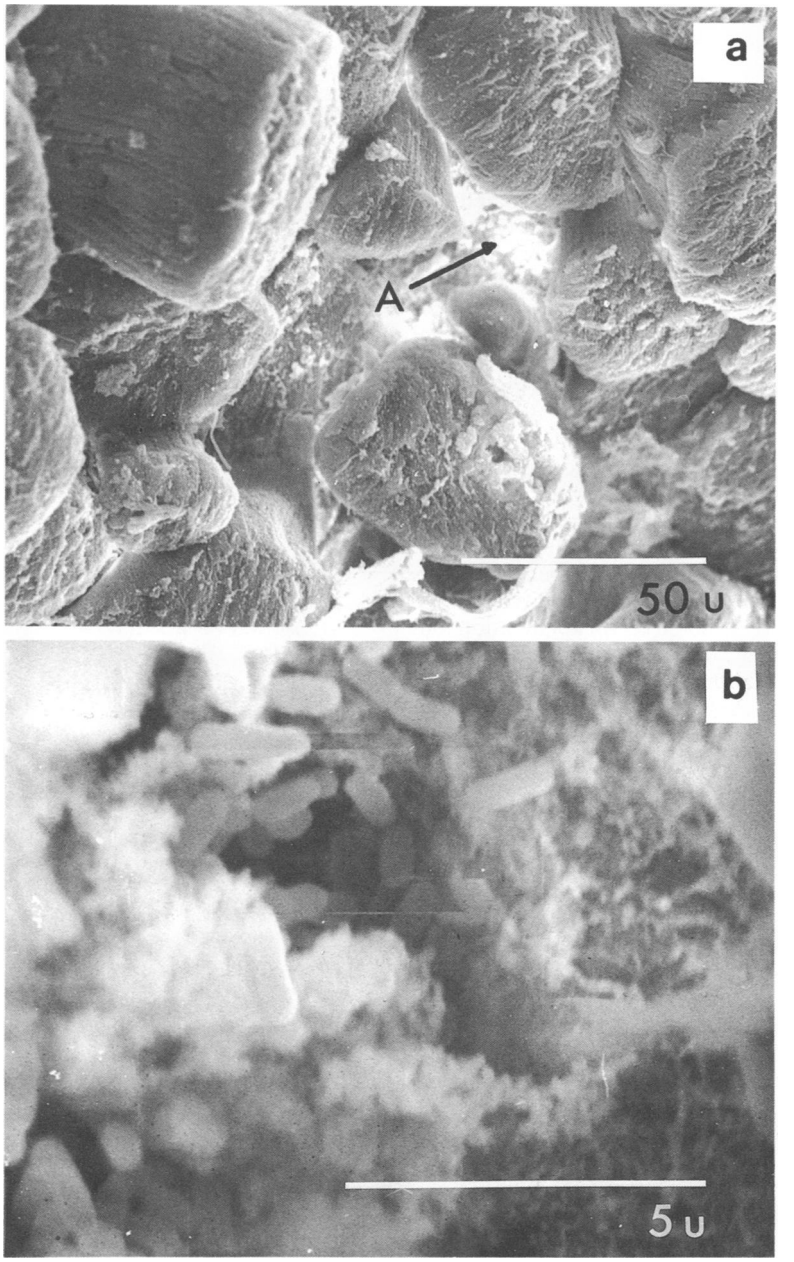


FIG. 8. Micrographs showing the presence of *P. fragi* (b) between muscle fibrils (a). ([b] is a higher magnification of the area indicated by A in [a].)

digestive enzymes and serve as a food reservoir. ZoBell (31) found slime production by attached bacteria to be influenced primarily by the microecosystem, more slime being produced in ecosystems in which it is difficult for bacteria to survive.

In addition to attractive forces, the microtopography of the muscle surface may play an important role in attachment. Figure 8a demonstrates the irregularity of the muscle surface. A closer examination of the area between the muscle fibrils (Fig. 8a, arrow) revealed a microcolony of *P. fragi* (Fig. 8b). Firstenberg-Eden et al. (8), studying the attachment of bacteria to cow teats, reported that bacterial adherence may result from bacteria becoming locked in

small holes or in skin tissue. By this process, bacteria may encapsulate themselves in surface irregularities, thereby making detachment difficult. Bacterial adherence may explain the practical difficulty in obtaining accurate estimates of bacterial numbers by nondestructive methods such as swabbing and rinsing (1, 21, 22, 24).

Physical degradation of intact inoculated and washed inoculated muscle tissue was extensive in colonized areas (Fig. 6 and 7). Degradation of proteins, presumably the stromal and myofibrillar fractions, would explain the changes in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoretic patterns of the urea-insoluble, urea-soluble, and salt-soluble proteins of the intact inoculated muscle after 12 days at 4°C (30). Other studies have reported the proteolysis of myofibrillar proteins of bovine muscle by *P. fragi* (2, 3, 28), and the degradation of stromal proteins of bovine muscle by a *Pseudomonas* species was observed in another study (23).

Thomas and McMeekin (29) stated that areas of depression around bacteria on skin and muscle surfaces were not due to degradation but rather to shrinkage of the water film between the bacterial cells and the surface. In light of the fact that extensive degradation of proteins was demonstrated in a previous report (30) by electrophoresis and protein extractability studies, it is apparent that the depressions surrounding colonies of bacteria were due to degradation of the muscle surface by *P. fragi* (Fig. 6 and 7). This conclusion is supported by the observation that such depressions (i.e., depressions due to shrinkage) were not observed on intact inoculated muscle after 3 days at 4°C (Fig. 4), although *P. fragi* cells were observed on the muscle surface. After day 6 of incubation, signs of protein degradation were revealed by SDS-polyacrylamide gel electrophoresis and protein extractability studies (30).

Localized degradation of muscle tissue was also observed on the washed inoculated muscle (Fig. 6 and 7), but only minimal evidence of proteolysis was detected by SDS-polyacrylamide gel electrophoresis (30). It is possible that because proteolysis was restricted to localized areas and because the numbers of *P. fragi* cells were insufficient to affect the entire muscle mass, proteolysis was not readily detected by SDS-polyacrylamide gel electrophoresis (30). Thus, although degradation was apparent by SEM, the degradative changes were not always detectable by SDS-polyacrylamide electrophoresis or protein extractability data, as is indicated by a previous report (30).

In summary, we have shown that sarcoplasm-depleted, intact bovine longissimus dorsi muscle was not as good a substrate for growth of *P. fragi*. Extracellular fibrils, however, were still produced by *P. fragi* on the depleted muscle, and signs of surface degradation around colonized areas were revealed by SEM. The use of depletion-repletion studies combined with SEM and other analytical methods may provide an insight into the mechanisms of growth and the attachment of bacteria to food myosystems.

## ACKNOWLEDGMENTS

The support of the Natural Sciences and Engineering Research Council of Canada through postgraduate scholarships to R.Y.Y. is acknowledged. This work was supported by a Natural Sciences and Engineering Research Council of Canada operating grant.

## LITERATURE CITED

1. **Avens, J. S., and B. F. Miller.** 1970. Quantifying bacteria on poultry carcass skin. Poult. Sci. **49**:1309–1315.
2. **Borton, R. J., L. J. Bratzler, and J. F. Price.** 1970. Effects of four species of bacteria on porcine muscle. 1. Protein solubility and emulsifying capacity. J. Food Sci. **35**:779–782.
3. **Borton, R. J., L. J. Bratzler, and J. F. Price.** 1970. Effects of four species of bacteria on porcine muscle. 2. Electrophoretic patterns of extracts of salt soluble protein. J. Food Sci. **35**:783–786.
4. **Butler, J. L., J. C. Stewart, C. Vanderzant, Z. L. Carpenter, and G. C. Smith.** 1979. Attachment of microorganisms to pork skin and surfaces of beef and lamb carcasses. J. Food Prot. **42**:401–406.
5. **Costerton, J. W., G. G. Geesey, and K. J. Cheng.** 1978. How bacteria stick. Sci. Am. **238**:86–95.
6. **Costerton, J. W. J. M. Ingram, and K. J. Cheng.** 1974. Structure and function of the cell envelope of gram-negative bacteria. Bacteriol. Rev. **38**:87–110.
7. **Deinema, M. H., and L. P. T. M. Zevenhuizen.** 1972. Formation of cellulose fibrils by Gram-negative bacteria and their role in bacterial floculation. Arch. Mikrobiol. **78**:42–57.
8. **Firstenberg-Eden, R., S. Notermans, F. Theil, S. Henstra, and E. H. Kampelmacher.** 1979. Scanning electron microscopic investigations into attachment of bacteria to teats of cows. J. Food Prot. **42**:305–309.
9. **Fletcher, M.** 1979. A microautoradiographic study of the activity of attached and free-living bacteria. Arch. Mikrobiol. **122**:271–274.
10. **Fletcher, M., and G. D. Floodgate.** 1973. An electron-microscopic demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. J. Gen. Microbiol. **74**:325–334.
11. **Gill, C. O.** 1976. Substrate limitation of bacterial growth at meat surfaces. J. Appl. Bacteriol. **41**:401–410.
12. **Gill, C. O., and K. G. Newton.** 1977. The development of aerobic spoilage flora on meat stored at chill temperatures. J. Appl. Bacteriol. **43**:189–195.
13. **Hasegawa, T., A. M. Pearson, J. F. Price, J. H. Rampton, and R. V. Lechowich.** 1970. Effect of microbial growth upon sarcoplasmic and urea-soluble proteins from muscle. J. Food Sci. **35**:720–724.
14. **Jay, J. M., and L. A. Shelef.** 1976. Effect of microorganisms on meat proteins at low temperatures. J. Agric. Food Chem. **24**:1113–1116.
15. **Marshall, K. C., R. Stout, and R. Mitchell.** 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. J. Gen. Microbiol. **68**:337–348.
16. **McCowan, R. P., K. J. Cheng, C. B. M. Bailey, and J. W. Consterton.** 1978. Adhesion of bacteria to epithelial cell surfaces within the reticulo-rumen of cattle. Appl. Environ. Microbiol. **35**:149–155.
17. **McCowan, R. P., K. J. Cheng, and J. W. Costerton.** 1979. Colonization of a portion of the bovine tongue by unusual filamentous bacteria. Appl. Environ. Microbiol. **37**:1224–1229.

18. **McMeekin, T. A., C. J. Thomas, and D. McCall.** 1979. Scanning electron microscopy of microorganisms on chicken skin. J. Appl. Bacteriol. **46**:195–200.
19. **Notermans, S., R. Firstenberg-Eden, and M. van Schothorst.** 1979. Attachment of bacteria to teats of cows. J. Food Prot. **42**:228–232.
20. **Notermans, S., and E. H. Kampelmacher.** 1974. Attachment of some bacterial strains to the skin of broiler chickens. Br. Poult. Sci. **15**:573–585.
21. **Notermans, S., and E. H. Kampelmacher.** 1975. Further studies on the attachment of bacteria to skin. Br. Poult. Sci. **16**:487–496.
22. **Notermans, S., E. H. Kampelmacher, and M. van Schothorst.** 1975. Studies on sampling methods used in control of hygiene in poultry processing. J. Appl. Bacteriol. **39**:55–61.
23. **Ockerman, H. W., V. R. Cahill, H. H. Weiser, C. E. Davis, and J. R. Siefker.** 1969. Comparison of sterile and inoculated beef tissue. J. Food Sci. **34**:93–99.
24. **Patterson, J. T.** 1972. Microbiological sampling of poultry carcasses. J. Appl. Bacteriol. **35**:569–575.
25. **Schaller, D. R., and W. D. Powrie.** 1971. Scanning electron microscopy of skeletal muscle from rainbow trout, turkey and beef. J. Food Sci. **36**:552–559.
26. **Sjostrand, F. S.** 1967. Electron microscopy of cells and tissue. Academic Press, Inc., New York.
27. **Stotzky, G., and L. T. Rem.** 1966. Influence of clay minerals on microorganisms. 1. Montmorillonite and kaolinite on bacteria. Can. J. Microbiol. **12**:547–563.
28. **Tarrant, P. J. V., A. M. Pearson, J. F. Price, and R. V. Lechowich.** 1971. Action of *Pseudomonas fragi* on the proteins of pig muscle. Appl. Microbiol. **22**:224–228.
29. **Thomas, C. J., and T. A. McMeekin.** 1981. Spoilage of chicken skin at 2°C: electron microscopic study. Appl. Environ. Microbiol. **41**:492–503.
30. **Yada, R. Y, and B. J. Skura.** 1981. Some biochemical changes in sarcoplasmic depleted, intact beef muscle inoculated with *Pseudomonas fragi*. J. Food Sci. **46**:1766–1773 and 1776.
31. **ZoBell, C. E.** 1943. The effect of solid surfaces upon bacterial activity. J. Bacteriol. **46**:39–56.