Observations by Electron Microscopy on Pig Muscle Inoculated and Incubated with *Pseudomonas fragi*

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Myofibrils from pig muscle inoculated and incubated with *Pseudomonas fragi* showed an extremely disrupted appearance as compared to uninoculated controls. There was an almost complete absence of material in the H zone, marked disruption of the A band (probably myosin), and some loss of dense material from the Z line. These changes indicated that marked proteolysis had occurred. Bacteria observed in spoiled muscle tissue exhibited protrusions or blebs on the outer surface of the cell walls. The blebs appeared to form detached globules that migrated into the muscle mass. Bacteria grown in non-muscle-containing media did not produce blebs, which indicates the blebs were induced by growth on muscle tissue. The possibility that the blebs and globules may contain a proteolytic enzyme responsible for myofibrillar disruption is discussed.

Jay and Kontow (7, 8) concluded that primary muscle proteins cannot be utilized for growth by meat spoilage organisms. However, others (2–5) have indicated that some protein breakdown occurs during microbial spoilage of meat. More recently, Tarrant et al. (13) demonstrated the loss of many salt-soluble protein bands on disc-gel electrophoresis of meat inoculated with *Pseudomonas fragi* and stored at 10 C for 20 days. They also found a change in the electrophoretic protein-banding pattern 8 days after inoculation with *P. fragi*. However, the effects of spoilage organisms at the ultrastructural level have not been elucidated.

The present study was undertaken to determine whether ultrastructural changes occur in the muscle proteins during bacterial spoilage, and, if so, to determine the nature of such changes.

**MATERIALS AND METHODS**

**Sampling procedures.** A market weight (ca. 100 kg live) pig obtained from the Michigan State University Swine Farm was slaughtered in a conventional manner, except that special techniques were utilized to obtain essentially aseptic muscle samples (4, 5).

**Inoculation.** A culture of *P. fragi* ATCC 4973 was grown in all-purpose plus Tween (APT) broth for 48 hr at 25 C and diluted 100-fold in buffered dilution blanks just before addition to the meat. The diluted cultures were added to the meat during grinding (0.01 ml per g of meat). The excised muscle samples were ground through a sterile, prechilled grinder, placed in presterilized sample jars, and covered loosely with sterilized lids. The operator wore sterilized, disposable plastic gloves at all times during the grinding and inoculation procedure. Control samples were ground in the same manner.

Bacteria were grown on media containing no intact muscle tissue (APT) and media containing no protein material (nonprotein media) to ascertain whether alterations in bacterial ultrastructure are attributable to growth on muscle tissue. Two loops of an undiluted, 48-hr culture of *P. fragi* were transferred to 15 ml of APT broth and 15 ml of nonprotein medium. The nonprotein medium contained 1% NH₄Cl, 0.5% glucose, 0.2% Na₂HPO₄, 0.08% MgSO₄·7H₂O, 0.004% NaCl, 0.004% Fe SO₄·7H₂O, and 0.014% Mn Cl₂·4H₂O (pH of 7.4). Two transfers were made in both APT broth and nonprotein medium after 48 hr at 25 C before inoculation at 10 C.

**Control samples.** Control samples of muscle were treated identically to the inoculated samples, except they were not inoculated. Thus, by observing differences between control and inoculated samples at the end of each incubation period, it was possible to determine the changes resulting from bacterial action as compared to autolytic changes caused by incubation.

**Incubation.** Control and inoculated muscle samples were incubated at 10 C for 0, 8, and 20 days. Bacteria
grown in APT were incubated for 6 days at 10°C; bacteria grown in nonprotein medium were incubated at 10°C for 12 days.

**Bacterial counts.** Standard plate counts were performed for control and inoculated muscle samples after 0, 8, and 20 days of incubation. Numbers were obtained by using the methods described by the American Public Health Association (1). APT agar was used as the plating medium, and all plates were incubated at 25°C for 48 hr.

Bacterial counts were not taken for the APT and nonprotein media. However, large numbers of bacteria were observed to be present in both media upon examination under a phase-contrast microscope.

**Electron microscopy.** Samples of spoiled muscle tissue were taken from the surface of the ground muscle mass and trimmed to a size no larger than 3 mm³ for fixing. Bacteria from the nonmuscle media were sampled by pipetting 4 ml of the bacteria-containing media into 10 ml of fixative. All samples were fixed for 2 hr in 1.25% glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.; biological grade 99%) solution buffered with 0.007 M NaH₂PO₄ and 0.041 M Na₂HPO₄. The fixing solution had a pH of 7.4 and contained 0.043 M NaCl. Samples were then washed with two changes of a pH 7.4 solution containing 0.013 M NaH₂PO₄, 0.081 M Na₂HPO₄, and 0.086 M NaCl. Subsequent to washing, samples were postfixed in 1% osmium tetroxide for 1 hr, as described by Sjöstrand (12), and dehydrated in ethanol. Fixation and dehydration were accomplished at room temperature. Dehydrated samples were washed in two changes of propylene oxide, embedded in Epon 812 (10) and sectioned on an LKB 4801A ultramicrotome. Sections were mounted on Formvar-coated copper grids and stained in uranyl acetate (12) and lead citrate (11). Electron micrographs were obtained with a Philips EM 300 transmission electron microscope operated at an accelerating voltage of 80 kV and recorded on Kodak 70-mm, fine-grain positive film.

**RESULTS AND DISCUSSION**

**Bacterial growth and meat spoilage.** The log bacterial numbers per gram of muscle were 5.05, 9.48, and 10.34 for the inoculated muscle samples after incubation for 0, 8, and 20 days, respectively. Bacterial growth was not detected (<10 organisms per gram) in control samples incubated for 0, 8, and 20 days. Inoculated samples were judged to be spoiled after incubation for 8 days by the presence of off-odors and the appearance of slime on the meat surface. However, a greater amount of spoilage (off-odors and slime) was evident after 20 days of incubation. The major portion of bacterial growth appeared to be on the surface of the ground meat, which is not surprising, as *P. fragi* is an aerobe.
Changes in muscle ultrastructure attributable to spoilage. The ultrastructure of control and inoculated samples at 0 incubation were similar and essentially the same as for normal pig muscle at 24 hr postmortem (T.R. Dutson, unpublished data).

Although more bacterial growth and spoilage were evident in inoculated samples incubated for 20 days, the ultrastructure did not appear to be any different than that in inoculated samples after incubation for 8 days. This probably reflects the fact that most spoilage occurred on the outer surface of the muscle mass. Thus, it appears that maximum tissue disruption occurs on the surface of the sample by the end of 8 days of incubation.

There was marked alteration of the myofibrils in muscle inoculated and incubated with P. fragi as compared to uninoculated controls, as can be seen by comparing Fig. 1 (uninoculated control) and Fig. 2 (inoculated and incubated sample). The myofibrils from muscle inoculated and incubated with P. fragi (Fig. 2) showed an extremely disrupted appearance in the A band region, with the H zone being almost devoid of material. Few if any thick (myosin) filaments are still evident and most of the dense material from the Z line has been lost. The actin filaments of the I band are fairly distinct, however, and appear to extend into the A band, where they become less apparent and show a more granular appearance.

The present study shows that breakdown of myofibrils occurs as a result of spoilage by P. fragi and supports the results of other investigators (2, 3, 13), who have shown that proteolysis of myofibrillar proteins occurs in muscle inoculated with this bacterium. In addition, results of the present study show specific disruption of components in the A band region, probably myosin, and removal of material from the Z line as a consequence of bacterial growth.

Tarrant et al. (13) found some change in the electrophoretic banding pattern of myofibrillar proteins after 8 days of incubation with P. fragi, but complete breakdown of the myofibrillar fraction was not evident until 20 days. These investigators indicated that, because of the growth of P. fragi on the surface of the meat, proteolysis might have occurred at an early stage, but was not detected until spoilage had proceeded further and affected the entire mass of meat. Results of the present study show equal disruption of the myo-
Fibrils at both 8 and 20 days of incubation and suggest that proteolysis commences at an early stage.

Myofibrillar disruption was probably detected early because of the fact that the samples were taken at the surface of the muscle mass where bacterial growth was greatest. Tarrant et al. (13) recently demonstrated proteolytic activity in muscle inoculated and incubated with P. fragi. The enzyme(s) contributing to the proteolytic activity in spoiled muscle is probably responsible for the marked myofibrillar disruption observed in the present study.

Bacterial ultrastructure. Bleblike evaginations or protrusions were observed on the surface of P. fragi organisms growing on spoiled muscle. The blebs, shown in Fig. 3, contain dense granular material, which appears to be present on the entire surface of the bacteria. Globules containing dense granular material similar to that in the blebs can be seen in close proximity to the bacterial cell. This suggests that these globules may be formed from the surface blebs. A high magnification of a surface bleb is presented in Fig. 4. The granular material in the blebs is surrounded by a membrane, which appears to be continuous with the outer surface of the bacterial cell wall.

Knox et al. (9) have shown that a lipopolysaccharide is secreted in globules formed from blebs on the surface of Escherichia coli organisms. Hitchins and Sadoff (6) have also shown that material involved in the formation of a cyst capsule is released by Azotobacter vinelandii in the form of protrusions or blebs that later separate from the cell to form globules. The blebs observed in the present study have the same appearance as those described by other authors (6, 9), which indicates that they may contain a specific substance (possibly a proteolytic enzyme).

Certain nutritional and physiological conditions have been shown to induce the formation of blebs on the cell wall of some pseudomonads (14, 15). Thus, it is possible that the formation of blebs in the present study may be the result of growing the bacteria in muscle tissue.

To determine whether bleb formation was caused by growing the bacteria on muscle tissue,
FIG. 4. A very high magnification of a bleblike evagination from the surface of a Pseudomonas fragi organism in muscle tissue inoculated and incubated at 10 C for 8 days. B = bacterial cell, g = dense granular material, and arrows point to the apparent continuation of the bleb membrane with the outer surface of the cell wall. X 918,000.

FIG. 5. Pseudomonas fragi organisms that were grown in APT media at 10 C for 6 days. B = bacterial cell. X 46,000.
bacteria were grown in APT and nonprotein media. The bacteria grown in these media were prepared for examination by electron microscopy in an identical manner to those grown in muscle tissue. Electron micrographs of bacteria grown in APT and nonprotein media are presented in Fig. 5 and 6, respectively. The P. fragi organisms grown on nonmuscle media did not contain blebs or surface evaginations like those observed on organisms grown in muscle tissue. Thus, results of the present study suggest that the bleblike evaginations and globules are formed at the surface of the organisms as a result of their being grown on muscle tissue.

The present study showed that marked alteration in the myofibrillar ultrastructure occurred as a result of the growth of P. fragi on muscle tissue. It is postulated that proteolytic activity is responsible for myofibrillar disruption. The enzyme(s) may be secreted into the blebs on the bacterial surface and later form globules. The globules then probably release their contents into the muscle tissue surrounding the bacteria.

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