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Patterns of Cell Division, DNA Base Compositions, and Fine Structures of Some Radiation-Resistant Vegetative Bacteria Found in Food†

S. W. SANDERS AND R. B. MAXCY*
Department of Food Science and Technology, University of Nebraska, Lincoln, Nebraska 68583

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Representative highly radiation-resistant *Moraxella-Acinetobacter* (M-A), *Pseudomonas radiora, Micrococcus radiodurans,* and *Micrococcus radiophilus* exhibited a wide variety of division systems and cell wall characteristics. However, the most resistant M-A possessed unusually thick cell walls, indicating a possible role of the cell wall in radiation resistance in the M-A. Thick septation was present in most of the bacteria studied, but was absent in *P. radiora,* thus excluding this as a necessity for high resistance. Reliable determination of the number of division planes of the M-A for use as a taxonomic criterion was achieved by the direct observation of dividing cells. The highly resistant M-A were found to divide in multiple planes and had base compositions of 54.0 to 57.5%, unlike typical *Moraxella* and/or *Acinetobacter* species. The taxonomic position of most highly resistant bacteria remains unclear.

Vegetative bacteria show a wide range of resistance to ionizing radiation (22, 29). This apparently continuous range of resistance may indicate the participation of many factors in the resistance phenomenon.

Numerous mechanisms have been proposed to help explain high radiation resistance. Efficient recombination repair (24-26) and excision repair (4, 17) were found in *Micrococcus radiodurans.* The physiological nature of these very efficient systems, however, is not understood. Thornley and Glauert (30) suggested that resistance to permeability changes associated with radiation damage may affect the ability of an organism to repair damaged DNA. Such permeability changes have been shown to occur with irradiated cultures of *M. radiodurans* (23). Thornley and Glauert (30) reported that the radiation resistance of some moderately resistant *Acinetobacter* strains correlated with the presence of a layer of wrinkled material observed interior to the outer membrane. Their most resistant isolates divided by septation, whereas the more sensitive types dividef by constriction. In a possibly related phenomenon, Adler and Hardigree (1) determined that a gene locus that influenced sensitivity to UV irradiation also influenced the ability of a bacterium to form septa after irradiation.

In addition to cell envelope-related aspects, the presence of intracellular or extracellular protective substances may also influence the radiation resistance of bacteria (9, 10, 16). Also, Kaplan and Zavarine (12) found that among relatively radiation-sensitive bacteria, resistance to X rays correlated inversely with guanine-plus-cytosine (G+C) contents. This relationship did not hold, however, when the more highly resistant bacteria such as *M. radiodurans* were considered.

The vegetative bacteria most resistant to ionizing radiation include *M. radiodurans* (2), *Micrococcus radiophilus* (18), certain members of a group characterized as *Moraxella-Acinetobacter* (M-A) (32, 35), a gram-positive rod isolated from the feces of a giant panda (14), *Arthrobacter radiotolerans* (38), *Pseudomonas radiora* (11), and *Micrococcus radioproteolyticus* (15). Although these organisms are believed to be relatively inactive and unimportant in current food systems from a public health or spoilage standpoint, the possible development of a process of radiation pasteurization or sterilization may increase their significance (22, 31, 35).

The present work, carried out on a number of these vegetative bacteria, is a comparative study emphasizing patterns and mode of cell division (i.e., planes of division, septum formation), DNA base composition, and cell wall fine structure. Patterns of cell division, such as number of division planes, was of particular interest in the genera *Moraxella* and *Acinetobacter.* They have been characterized as dividing in only one

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plane, whereas *Neisseria* spp. are generally characterized as dividing in two planes (5). DNA base composition was determined to understand better the possible taxonomic relationships between these radiation-resistant bacteria and other bacterial groups. Electron microscopy of ultrathin sections was performed to help determine whether a particular cell wall morphology may be conducive to radiation resistance. In addition, cell wall structure can be a useful aid in the classification of gram-variable bacteria such as some of the M-A group (7).

MATERIALS AND METHODS

Bacteria. The following organisms were used in the electron microscopy and DNA base composition studies: M-A isolates 4, 5, 7, and 13 (35); *M. radiophilus* (18); *P. radiora* no.0-1 (11); *Moraxella osloensis* ATCC 19963; and *Moraxella nonliquefaciens* ATCC 17953. In addition to the above, other M-A types representative of the various subgroups classified by Welch and Maxcy (35) were observed by phase-contrast microscopy. Cultures were grown to maximum log phase for the electron microscope and base composition studies and to either the mid-log or stationary phase for the division observations in m-plate count broth (Difco Laboratories, Detroit, Mich.) or tryptic soy broth (Difco).

Patterns of cell division. A thin layer of molten plate count agar (Difco) was applied to a clean microscope slide with a Pasteur pipette, using aseptic techniques. After solidification, a loopful of broth culture diluted with phosphate buffer (34) was spread on the agar and allowed to absorb. A square cover slip was then placed on top, the protruding agar was cut away with a razor blade, and the edges were sealed with petroleum jelly.

The confined cells were limited to two-dimensional growth (33); therefore, single versus multiple plane division could be easily differentiated. Active growth was apparent, as judged by a generation time of less than 2 h at 25°C. Growth of a single cell or a pair of cells was observed with a Leitz microscope with phase-contrast optics and recorded on 35-mm Kodak high-contrast copy film with a Leitz automatic camera.

DNA base composition. DNA was extracted and purified by methods derived from various literature sources and personal communication with T. L. Thompson, Department of Life Sciences, University of Nebraska. Approximately 1 g of wet-packed cells was suspended in 10 ml of 0.01 M sodium diphosphate plus 0.002 M ethylenediaminetetraacetate, pH 7. The cells were lysed by treatment with 100 μg of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml at 37°C for 30 min with occasional gentle shaking, followed by treatment with 100 μg of *Streptomyces griseus* protease (Sigma) per ml for 3 to 4 h at 37°C. Those cultures showing lysozyme resistance were subjected to 1 ml of 25% sodium lauryl sulfate until adequate viscosity was obtained. The lysate was cleared by centrifugation. Deproteinization was accomplished by gentle shaking with an equal volume of 0.1 M phosphate buffer-saturated phenol (pH 7.5) for 15 min. After separation into two phases by centrifugation at 1,800 × *g* for 10 min, the aqueous phase was carefully removed and further deproteinized by two repetitions of the same process. The nucleic acids were precipitated with 2 volumes of cold 95% ethanol, collected on a glass rod, and dissolved in 0.015 M NaCl plus 0.0015 M trisodium citrate (saline-citrate). Ribonuclease (Sigma) was then added to a final concentration of 50 μg/ml, and the solution was incubated at 37°C for 30 min. The solution was deproteinized once more, precipitated as before, redissolved in about 4 ml of saline-citrate, and dialyzed against 1 liter of saline-citrate for 48 h with one change of saline-citrate after 24 h. The resulting DNA solution was centrifuged at 1,800 × *g* for 10 min to obtain a clear supernatant and stored at 5°C.

The mean percent G+C content was determined by estimation of the midpoint (T_m) of the optical melting curve at 260 nm (21). The following reference formula of Mandel et al. (20) was used to calculate mean percent G+C contents from the T_m values: $GC_x = GC_{std} + 0.0199(T_{mx} - T_{std})$. The standard was based on *Escherichia coli* B having a G+C content of 0.51.

Thin-section electron microscopy. Bacteria were fixed by the methods of Kellenberger and Ryter (13) and embedded in Araldite (Ladd Research Industries, Burlington, Vt.). An LKB or Sorvall ultramicrotome was used to obtain sections showing silver-gray interference colors. Some sections were further stained with lead citrate; however, this did not result in a noticeable change in the appearance of the cell walls. A Phillips electron microscope was operated at 60 kV to obtain electron micrographs.

RESULTS

Patterns of cell division. Phase-contrast microscopy showed that the most highly resistant M-A isolates 4, 7, and 13, as well as less resistant M-A isolates 1, H, J, and *M. radiophilus*, divide regularly in at least two planes, thus indicating a common characteristic with *Neisseria*. The remaining bacteria studied were observed to divide in only one plane, likening them to *Moraxella* and *Acinetobacter* (Table 1). A typical single-plane division pattern of a coccobacillus is shown in Fig. 1. The sliding motion during growth often led to the formation of tetrads (Fig. 1h through l). When comparable tetrads were observed in a Gram stain, they appeared to have grown by multiplane division. Multiplane division of M-A isolate 4 is shown in Fig. 2. These would be expected to appear as tetrads in a Gram stain. Thus, the sliding motion during growth can lead to confusion in differentiation between single and multiplane division as determined by Gram stains.

DNA base composition. The most resistant M-A isolates 4, 7, and 13 (35) had percent G+C contents ranging from 54.0 to 57.5 (Table 1), which were higher than the 40 to 47% range for *Moraxella* spp. and the 39 to 47% range for

Acinetobacter spp. as reported in the literature (5). The percent G+C of *M. radiophilus* was 56.1, which was lower than the value (82.88) reported by Lewis et al. (19). In light of this incongruity, additional controls consisting of *M. osloensis* ATCC 19963, *M. nonliquefaciens* ATCC 17953, and *M. radiodurans* ATCC 13939 were run. The data in Table 1 indicate that our results were essentially the same as those published by De Ley (6).

Thin-section electron microscopy. A variety of cell wall types were present in these highly radiation-resistant bacteria, indicating that no unique cell wall structure is common to all highly resistant bacteria. The simplest structure observed was that of M-A isolate 5 (Fig. 3). It possessed a thin outer membrane and plasma membrane separated by a lighter-staining thick area. A dense intermediate layer, attributed to mucopeptide substances (7), was not apparent. The cell wall structures of the most resistant M-A isolates 4, 7, and 13 were nearly identical to one another. All three possessed an outer membrane, an electron-dense intermediate layer, and a plasma membrane, as illustrated in Fig. 4. The dense intermediate layer of M-A isolates 4, 7, and 13 was apparent as a trilaminar structure with two dark lines intermittently separated by a light area (Fig. 4). In addition, a very narrow dense layer could be resolved in micrographs of M-A isolate 4 between the outer membrane and the dense intermediate layer (Fig. 4). The cell walls of *P. radiora* and *M. osloensis* differed from M-A isolates 4, 7, and 13 in that the dense layer did not appear trilaminar and was positioned very close to the outer membrane (Fig. 5). M-A isolates 4, 5, and 7 commonly possessed unidentified dark-staining cytoplasmic inclusions of various sizes (see Fig. 6 and 8). These usually appeared to be adjacent to or within the light-staining nuclear regions.

Thick septa were observed in all of the bacteria except M-A isolate 5, *P. radiora*, and *M. osloensis* (Fig. 6). Division of *P. radiora*, M-A isolate 5, and *M. osloensis* under these culture and fixation conditions was primarily by constriction, followed by ingrowth of all cell wall layers (Fig. 7 through 9). A few M-A 5 isolates showed some septum formation; however, this was never apparent until the cells were greatly constricted (Fig. 10).

The mature cell wall thickness was estimated by measuring from the inner surface of the dense intermediate layer to the outermost surface. The large values in Table 2 were attributed to a thick layer outside the dense intermediate layer. A thin overall cell wall in M-A isolate 5 was measured, as no dense layer could be detected and the measurements reflected only the thickness of the outer membrane. M-A isolate 5, however, possessed a large light-staining area between the outer membrane and the plasma membrane.

TABLE 1. *G+C content and division planes of strains observed*

Organism	Division (no. of planes)	mol% G+C
4	≥2	57.5
7	≥2	57.5
13	≥2	54.0
5	1	
1	≥2	
K	1	
17	1	
0	1	
3	1	
A	1	
D	1	
2	1	
H	≥2	
N	1	
E	1	
J	≥2	
M. osloensis 19963	1	44.3 (45.9)[a]
M. nonliquefaciens 17953	1	44.6 (46.0)
M. radiophilus	≥2	56.1
M. radiodurans	≥2	66.3 (66.4)

[a] Numbers within parentheses are from De Ley (6).

DISCUSSION

Most research reports dealing with mechanisms of bacterial radiation resistance have dealt with a single species. Their conclusions have offered explanations for high radiation resistance but without proof of general applicability among different bacterial species. For example, Glauert and Thornley (8) associated the resistance of *Acinetobacter* spp. with a wrinkled cell wall positioned between the outer membrane and a dense intermediate layer, as well as division by thick septa. Similarly, cells of highly radiation resistant M-A (35) divided by septation and had unusually thick cell walls. This thickness was attributed to a cell wall layer corresponding in position to the wrinkled layer in the most resistant *Acinetobacter* strains observed by Glauert and Thornley (8). Conversely, *P. radiora*, a highly radiation-resistant pseudomonad (11), had an apparently typical gram-negative cell wall morphology and did not form visible septa. Thus, cell wall structure would not appear to provide a full explanation for radiation resistance.

The high G+C content and multiple-plane division of the radiation-resistant M-A raise some doubt as to the logic of their being placed in the genus *Moraxella*. Confusion also exists

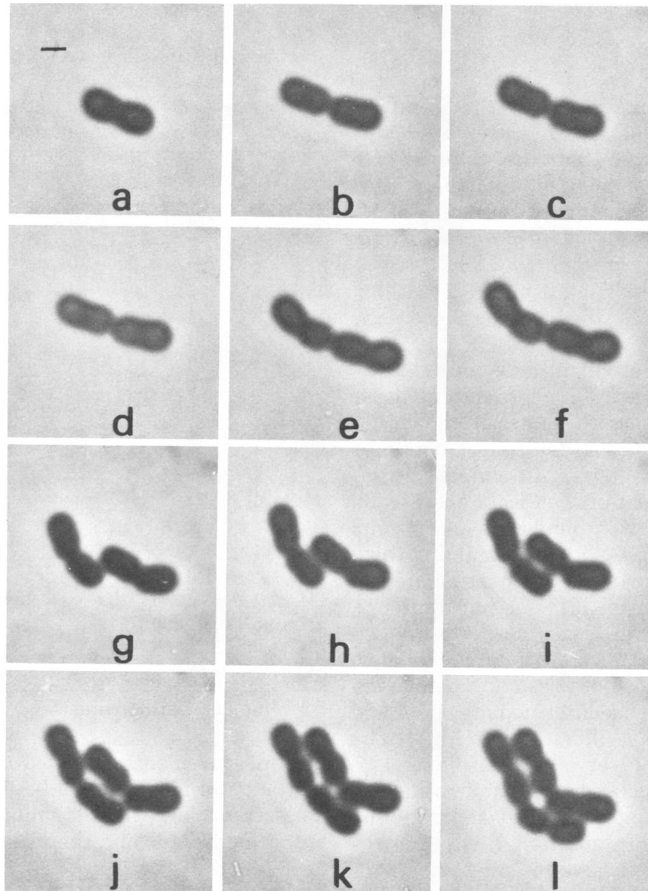

FIG. 1. *(a through l) Phase-contrast photomicrograph series of M-A isolate 5 showing one-plane division. Bar = 1 μm.*

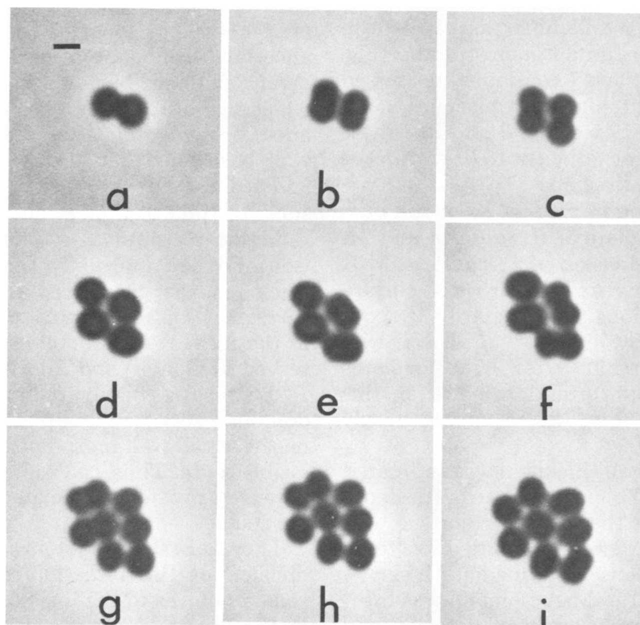

FIG. 2. *(a through i) Phase-contrast photomicrograph series of M-A isolate 4 showing two-plane cell division. Bar = 1 μm.*

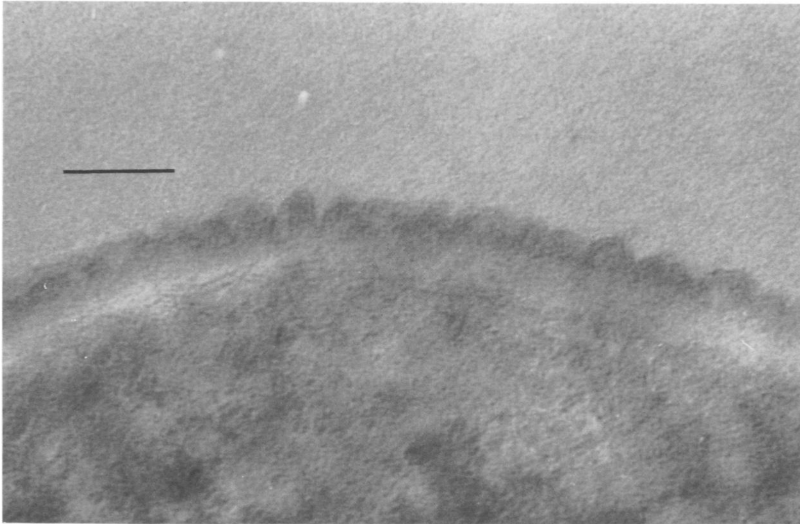

FIG. 3. *Electron micrograph of M-A isolate 5 showing thin outer membrane and plasma membrane. Bar = 57.6 nm.*

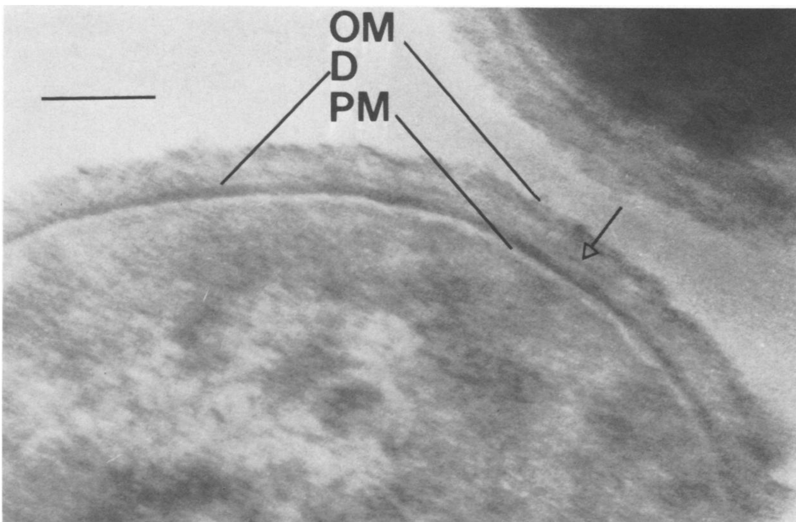

FIG. 4. *Electron micrograph of M-A isolate 4. Note the thin dense layer (arrow) outside the thick dense intermediate layer. OM, Outer membrane; D, dense intermediate layer; PM, plasma membrane. Bar = 32.9 nm.*

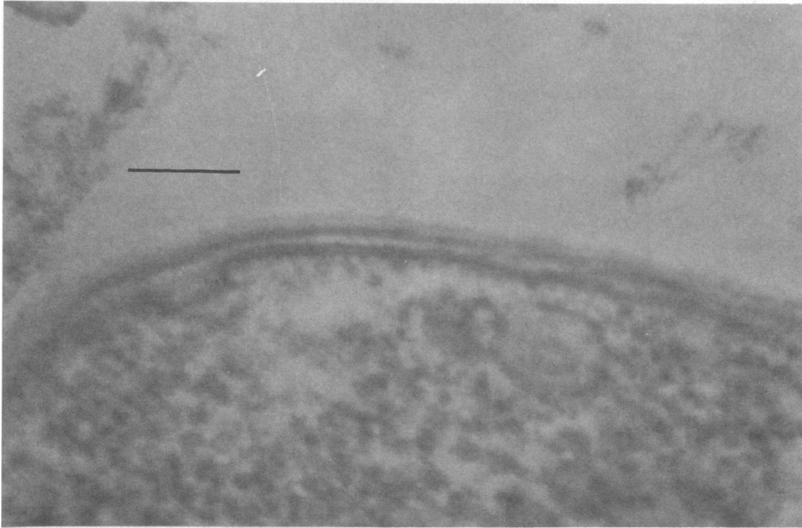

FIG. 5. *Electron micrograph of P. radiora. Note the heavily stained plasma membrane. Bar = 32.9 nm.*

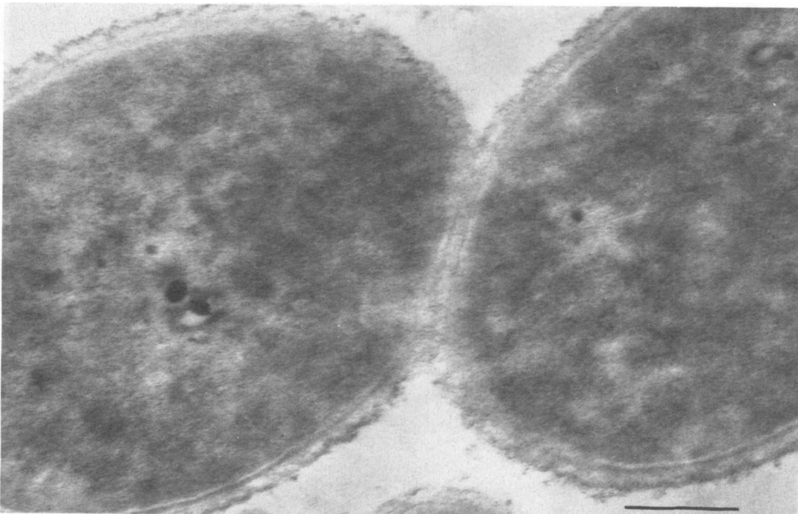

FIG. 6. *Electron micrograph of M-A isolate 4 showing septum. Note small black inclusions. Bar = 210 nm.*

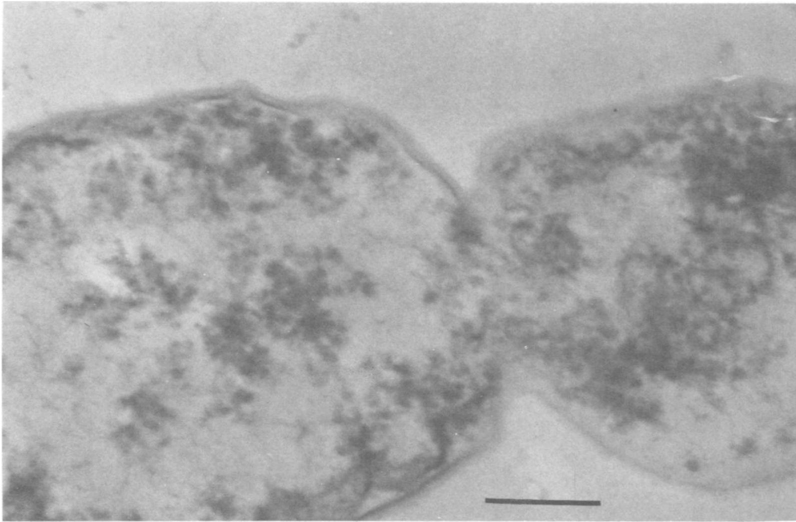

FIG. 7. *Electron micrograph of P. radiora showing a pinching-type division with no apparent septum. Bar = 210 nm.*

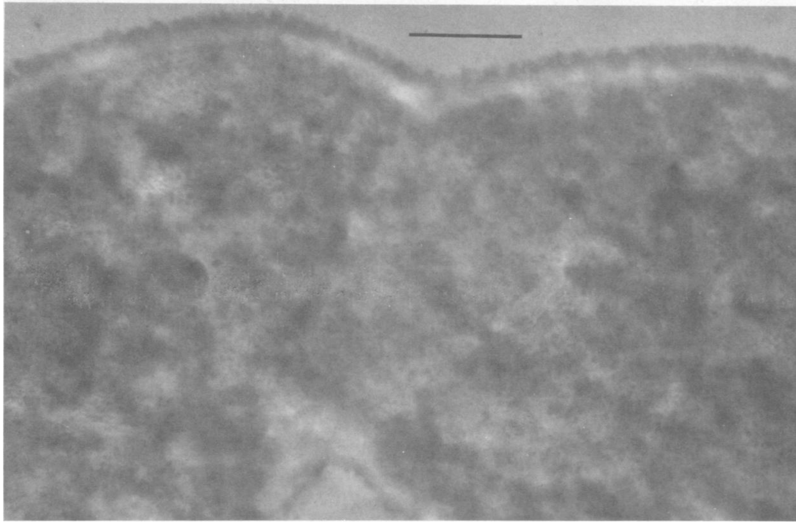

FIG. 8. *Electron micrograph of M-A isolate 5 showing division similar to P. radiora. Note dark-staining inclusions. Bar = 210 nm.*

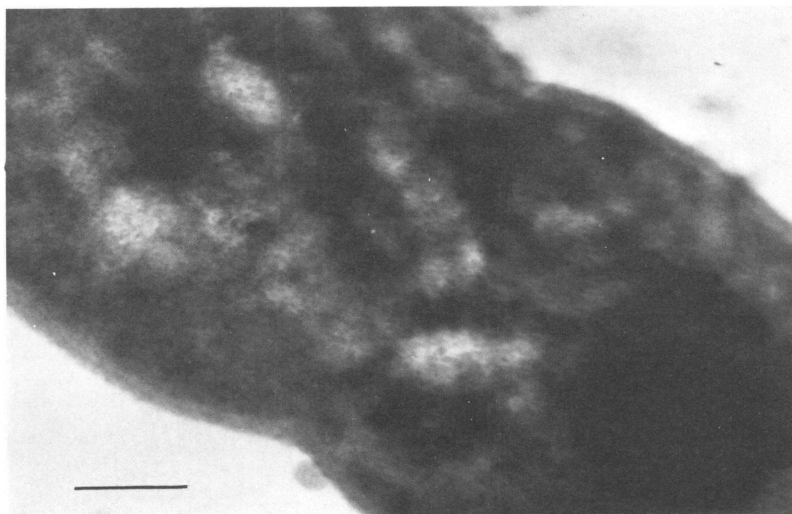

FIG. 9. *Electron micrograph of M. osloensis ATCC 19963 showing pinching-type division. Bar = 210 nm.*

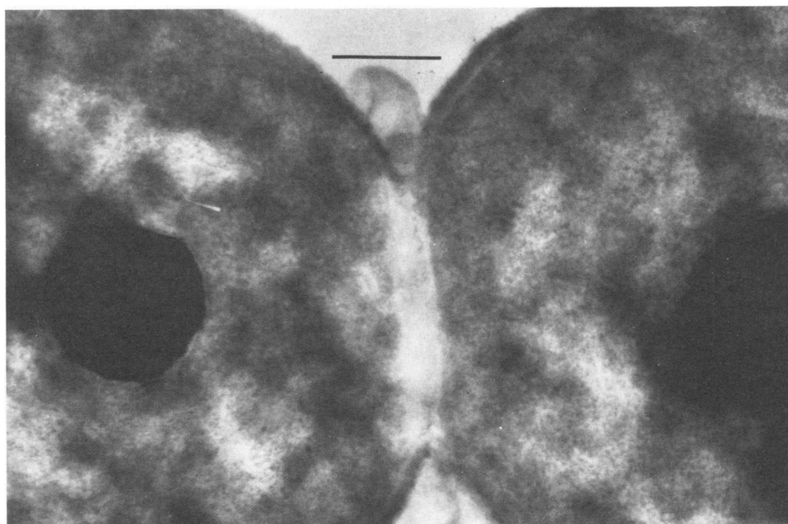

FIG. 10. *Electron micrograph of M. osloensis ATCC 19963 showing septum formation after extensive pinching. Bar = 210 nm.*

TABLE 2. *Total average cell wall thickness of some highly resistant vegetative bacteria and M. osloensis ATCC 19963*

Organism	Cell wall thickness (nm)
5	5.2
4	52.0
7	43.9
13	48.2
M. radiophilus	43.9
P. radiora	26.3
M. osloensis	21.9

concerning the taxonomic position of *M. radiodurans* and other highly resistant pigmented micrococci (5). Baird-Parker (3) suggested placing *M. radiodurans* into an unnamed gram-negative genus based on the presence of lipoprotein and a wide range of amino acids in its cell wall, as well as G+C content somewhat low for *Micrococcus*. There are some striking similarities in characteristics between the M-A and pigmented micrococci. All are relatively salt sensitive (2, 35) and were found to divide in multiple planes and to have similar G+C values. Perhaps the resistant M-A could be included in such a genus.

Confusion regarding the nature of these unusual highly radiation-resistant bacteria has contributed to the reluctance toward approval of radiation processing of foods. Study of their taxonomic relationships and significance is necessary before widespread adoption of commercial food irradiation.

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