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Characterization of Radiation-Resistant Vegetative Bacteria in Beef[1]

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Ground beef contains numerous microorganisms of various types. The commonly recognized bacteria are associated with current problems of spoilage. Irradiation, however, contributes a new factor through selective destruction of the microflora. The residual microorganisms surviving a nonsterilizing dose are predominantly gram-negative coccobacilli. Various classifications have been given, e.g., *Moraxella*, *Acinetobacter*, *Achromobacter*, etc. For a more detailed study of these radiation-resistant bacteria occurring in ground beef, an enrichment procedure was used for isolation. By means of morphological and biochemical tests, most of the isolates were found to be *Moraxella*, based on current classifications. The range of growth temperatures was from 2 to 50 C. These bacteria were relatively heat sensitive, e.g., D_{10} of 5.4 min at 70 C or less. The radiation resistance ranged from D_{10} values of 273 to 2,039 krad. Thus, some were more resistant than any presently recognized spores. A reference culture of *Moraxella osloensis* was irradiated under conditions comparable to the enrichment procedure used with the ground beef. The only apparent changes were in morphology and penicillin sensitivity. However, after a few subcultures these bacteria reverted to the characteristics of the parent strain. Thus, it is apparent that these isolates are a part of the normal flora of ground beef and not aberrant forms arising from the irradiation procedure. The significance, if any, of these bacteria is not presently recognized.

Meats contain a broad spectrum of microorganisms arising from the carcass and the processing operations involving equipment and human contact. Fresh meat provides a favorable environment for microorganisms which constitute public health and organoleptic spoilage problems. To alleviate these problems, various processing techniques have been investigated. Gamma irradiation is an example of a potential commercial process to extend shelf life of meat (15) and aid in public health protection (8).

Some members of the microflora of commercially processed red meat are sensitive to gamma radiation whereas others are quite resistant to comparable doses (13). The vegetative cells of bacteria most resistant to gamma radiation do not appear to be a part of the commonly recognized spoilage flora (14). Tiwari and Maxcy (14), working with radiation-resistant organisms from meat, made a general classification with minimal characterization of these bacteria as *Moraxella-Acinetobacter* (M-A). These, or very similar bacteria, have been shown to be widespread, occurring in various types of unprocessed foods, e.g., dairy products (6), fish (9), and vegetables (10).

Microorganisms of the M-A type may have gone unnoticed by food microbiologists, since these bacteria have not been associated with problems and are present in relatively small numbers (13). However, irradiation processing with nonsterilizing, low-dose gamma radiation to reduce total numbers of organisms brings the M-A into prominence. Many members of the M-A group have an inherent resistance to gamma radiation at least twice that of most other vegetative bacteria. The mechanism of this resistance is unknown.

The purpose of this investigation was to isolate the most radiation-resistant vegetative cells from meat and to study them in detail with the hope of obtaining a better understanding of their significance.

MATERIALS AND METHODS

Isolation procedures. Samples of ground beef were obtained from various sources. These involved animals from numerous feed lots and carcasses from various abattoirs and processing operations. These

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samples involved a wide geographical area, including imported beef and seasonal variations of summer and winter.

Ground beef was irradiated in vacuum packages and in atmospheric packages at approximately −30 C at various dose levels so that a 1:10 dilution yielded approximately 10 colonies of bacteria when cultured on plate count agar. Incubation was carried out at 32 C under aerobic and anaerobic conditions. Individual colonies were subcultured, observed for morphology and colony characteristics, and then tested for oxidase, catalase, reaction in litmus milk, and proteolysis on skim milk agar. Approximately 60 isolates that appeared to be different on the basis of any one of these reactions were used for further study.

To select the most radiation-resistant bacteria, isolates were grown in pure culture, then combined, and added to ground beef. The inoculated product was then irradiated as described above to obtain a second enrichment. Nineteen different organisms surviving the second enrichment process were maintained in pure cultures for detailed study. For comparative purposes, 10 isolates from a previous study (14) obtained from a single enrichment were included in this study and denoted by the prefix M-A.

Characterization of isolates. Gram strains were prepared from cultures on plate count agar and plate count broth previously incubated for 16 to 24 h at 32 and 37 C and examined for microscopy morphology. Isolated colonies on plate count agar, Trypticase soy agar (TSA), TSA containing 5% defibrinated sheep blood, TSA with 5% bovine serum, chocolate blood agar with 5% sheep blood, and brain heart infusion agar containing 5% defibrinated rabbit blood were observed periodically from 16 h to 7 days.

Tests and media used to group and identify the isolates were: oxidase reaction; catalase production; motility (microscopy examination of a hanging drop preparation, darkfield, motility test agar, and SIM agar); reactions on triple sugar iron agar; oxidation-fermentation of glucose (Hugh and Leifson [4] O-F basal medium containing 1% glucose, cystine trypticase agar with 1% glucose and phenol red agar base with 1% glucose); nitrate reduction (nitrate broth); gelatin hydrolysis (nutrient gelatin); indole production (1% tryptone broth and SIM agar); litmus milk; citrate utilization (Koser citrate); urease activity (urea broth); and phenylalanine deaminase activity.

Presence or absence of growth on basal mineral medium, O-F without carbohydrate, MacConkey agar, eosin methylene blue agar, and *Shigella-Salmonella* agar and growth on TSA with 2.5 and 6.5% NaCl were observed. Penicillin susceptibility was tested by the Bauer-Kirby technique (1), using 10-U disks. Thermal limits of growth were determined by culturing bacteria on TSA and incubating for 24 to 48 h at temperatures ranging from 0 to 50 C. An anionic surfactant, Ultrawet (Atlantic Refining Co., Chicago, Illinois), was incorporated into TSA and Trypticase soy broth (TSB) at concentrations of 0.01, 0.1, and 1.0% to test susceptibility to surfactant.

To determine radiation resistance, pure cultures were grown in plate count broth or TSB, quick-frozen in a dry ice-alcohol bath, and treated with various doses of gamma radiation to give a 7- to 8-log cycle reduction in population. To determine radiation resistance in meat, ground beef was irradiated with 200 krad, after which enough cells of the cultures to be studied were added to provide approximately 10^6 cells per g of meat. A cobalt-60 source provided approximately 12 krad of gamma radiation per min. Operation of the equipment has been previously described (13).

Heat resistance of pure cultures in broth was determined by heating cultures in the plate count broth or TSB in which they were grown for various temperature-time combinations. To determine heat resistance in meat, ground beef was irradiated with an essentially sterilizing dose (2 Mrad) of gamma radiation. Then approximately 10^6 cells per g were ground with the meat and formed into patties 3-mm thick in polyethylene bags, after which the patties were immersed in a water bath for various temperature-time combinations. A separate patty was used for each time interval.

Comparative alteration of Moraxella osloensis by irradiation. The reference culture of *M. osloensis*, which had not been previously exposed to radiation, was grown in TSB overnight on a rotary shaker at 37 C. Cultures were quick-frozen in a dry ice-alcohol bath and treated with various doses of gamma radiation to provide a 7- to 8-log cycle reduction in population. A colony surviving the highest dose was grown in broth and subjected to the same irradiation process. Colony selection and irradiation were repeated for the third time. Survivors of the repeated irradiations were grown in pure culture and observed as described below.

RESULTS

Nature of isolates. Isolates of bacteria obtained from aerobic plating of beef irradiated either in vacuum packages or under atmospheric conditions appeared to be the same. When irradiated beef was cultured anaerobically, total counts obtained were less than 1% of those obtained by aerobic plating. None of the isolates obtained by anaerobic incubation was an obligate anaerobe.

Colony morphology was similar on various types of media. Colonies were mainly circular, convex, entire, smooth, opaque, dull or glistening, and butyrous. After 18 to 24 h of growth at 32 to 37 C, colonies were pinpoint and white. After 1 or 2 additional days of incubation, they were 1 to 3 mm in diameter and white, cream colored, light buff, or pale yellow. Four of the isolates produced a bright orange to red pigment and were considered to be *Brevibacterium*. The majority of the isolates had no effect on blood agar medium or produced only indeterminate reactions. One isolate, MA-27, produced a lavender-green discoloration of the erythrocytes in blood agar medium. No beta-like hemolysis was detected.

Microscopy examination of Gram stains showed short, plump, gram-negative rods usually in pairs or short chains. A few isolates showed some resistance to decolorization. Those considered to be *Brevibacterium* were gram positive. All the isolates appeared to be coccobacilli but frequently appeared as diplococci suggestive of *Neisseria*. Some isolates had a marked tendency toward pleomorphism (Fig. 1).

Glucose utilization was tested on several media, each of which has been used by various workers. Some isolates oxidized or fermented glucose in certain media, but not in others. In addition, some reactions were weak or delayed (Table 1). None of the isolates produced H_2S, indole, urease, or phenylalanine deaminase. Isolates were grouped on the basis of morphology, cultural characteristics, biochemical reactions (Table 2), and growth on selective media (Table 3).

All of the isolates grew at temperatures between 20 and 37 C and most had a much broader range of growth temperatures (Table 4).

Radiation and heat resistance. Although all the isolates were relatively radiation resistant, the range was from D_{10} of 273 to 2,039 krad (Table 5). The D_{10} values were calculated by the method of least squares. None of the isolates was particularly heat resistant (Table 5).

Irradiation of reference M. osloensis. To determine if our isolates had acquired radiation resistance by exposure during the enrichment procedure, a reference culture of *M. osloensis* was irradiated using conditions similar to those used for irradiation of meat. The D_{10} value of the reference culture was determined to be 137 krad (lines located by the method least squares). A pure culture from a colony that had survived 1,000 krad had a D_{10} of 145. When a pure culture from a colony surviving the second irradiation treatment of 1,000 krad was observed, the D_{10} value was 146. When slopes of lines were determined by the method of least squares, it was apparent they were approximately the same (Fig. 2).

Prior to irradiation the *M. osloensis* used as the reference culture were predominantly coccoid, 1 μm in diameter, or diplobacilli, 0.8 by 2 μm. Some larger diplococci, 2 by 3 μm, or even bacillary forms, 0.5 by 6 μm, were seen. Occasionally a long bacillus, up to 10 μm in length, was noted (Fig. 3). When one colony surviving a 1,000-krad dose was Gram stained, long cells were predominant (Fig. 4). Many cells were 15 to 20 μm long and it was not unusual to find a single cell 26 to 30 μm. Often cells appeared to be two bacteria lying end to end and these forms measured up to 55 μm. After the irradiated *M. osloensis* had been subcultured a few times on TSA the morphology appeared to be similar to the parent reference culture.

When penicillin sensitivity of the reference *M. osloensis* was tested by the standard Bauer-Kirby technique, there was a 32-mm zone of inhibition around a 10-U disk and a 13-mm zone of inhibition around a 2-U disk. A pure culture from a colony surviving the second irradiation treatment of 1,000 krad showed a 20-mm zone around the 10-U disk and no zone of inhibition

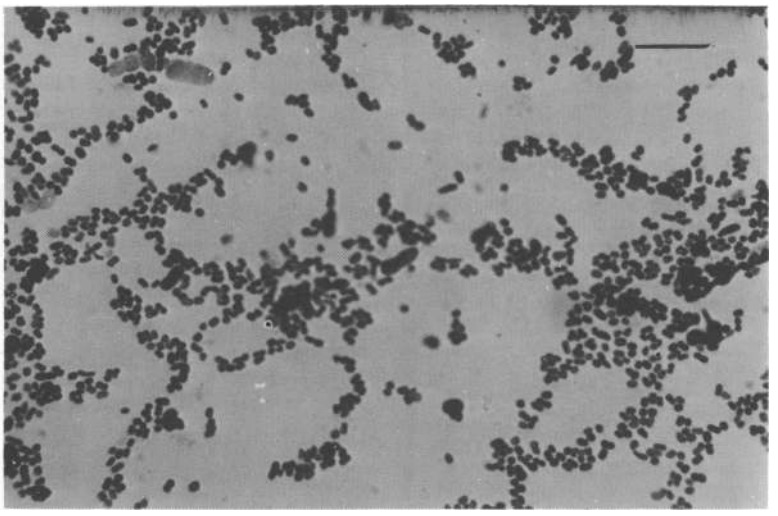

FIG. 1. *Gram-negative bacteria isolated from irradiated ground beef. Coccobacillary forms were predominant, but some pleomorphic forms were present. Marker indicates 10 μm.*

TABLE 1. *Glucose utilization*[a]

Classification	Isolate no.	TSI	O-F + 1% glucose		CTA + 1% glucose		PRA + 1% glucose
			Aerobic	Anaerobic	Aerobic	Anaerobic	
Moraxella nonliquefaciens	3	K/N	N	N	K	N	K/N
	13	N/N	N	N	K	N	K/N
	D	K/N	N	N	K	N	K/N
	MA-5	K/N	N	N	N	N	N/N
	MA-16-5	K/N	N	N	N	N	K/N
	MA-16-7	N/N	N	N	N	N	K/N
	MA-16-8	N/N	N	N	N	N	(A/A) 3
	MA-16-17	N/N	N	N	N	N	N/N
Moraxella osloensis	5	K/N	N	N	K	N	K/N
	17	K/N	N	N	(A) 3	(A) 3	A/N
	A	K/N	N	N	K	N	K/N
	K	K/N	N	N	K	N	K/N
	N	K/N	N	N	N	N	K/N
	MA-16-2	K/N	N	N	N	N	N/N
M-5	4	K/N	N	N	N	N	(A/A) 5
	7	K/N	N	N	N	N	(A/A) 5
M-4f	MA-27	K/N	N	N	N	N	N/N
Acinetobacter calcoaceticus	1	NG					(A/A) 5
	E	A/AA	A	A	A	A	A/A
	I	A/A	N	N	A	A	A/A
	MA-3	K/N	N	N	K	N	N/N
Intermediates[b]	H	A/A	N	N	(A) 3	(A) 3	A/A
	J	A/A	N	N	(A) 3	(A) 3	A/A
	MA-2	K/N	(A)4	(A)4	(A)4	N	A/A
	MA-4	K/N	(A)4	(A)4	(A)6	N	A/A
Brevibacterium	2	(A/A) 5	N	N	N	N	(K/A) 7
	6	(A/A) 5	N	N	N	(A) 9	(A/A) 3
	9	(A/A) 5	N	N	N	N	A/N
	0	(A/A) 5	(A) 14	(A) 14	(A) 14	(A) 21	(K/A) 3

[a] TSI, Triple sugar iron agar; CTA, cystine Trypticase agar; PRA, phenol red agar; K, alkaline; N, neutral; A, acid; NG, no growth. Reactions denoted as occurring in 1 or 2 days. Many neutral reactions later became alkaline. Parenthesis indicates delayed reaction with number showing the day positive.

[b] Characteristics of these organisms did not fit with recognized groups.

around a 2-U disk. After a few subcultures on TSA, penicillin sensitivity was essentially the same as that of the parent culture.

DISCUSSION

Gram-negative, aerobic, nonsporeforming coccobacilli are widely distributed in nature and have been implicated as being important in irradiated foods (5, 7, 14). Thornley (12), working with organisms surviving the irradiation of poultry, found them to have characteristics similar to the M-A group and classified them as *Alcaligenes-Achromobacter*. These organisms were described by Thornley as being "gram-negative or gram-variable, nonmotile coccobacilli which have a doubtful taxonomic position." It is quite possible that the author was working with the M-A group of microorganisms, since members of the *Alcaligenes-Achromobacter* group are now considered to be gram-negative, motile, nonfermentative bacteria. Tiwari and Maxcy (14) recognized that the M-A group of microorganisms was present in beef and was found in a greater proportion in irradiated beef. These examples serve to illustrate the lack of availability of an adequate, universally accepted classification.

To our knowledge this is the first investiga-

TABLE 2. *Biochemical reactions*[a]

Classification	Isolate	Oxidase	Catalase	Gelatin hydrolysis	Nitrate reduction	Litmus milk	Koser citrate
Moraxella nonliquefaciens	3	+	+	I	+	I	I
	13	+	+	I	+	I	I
	D	+	+	I	+	I	I
	MA-5	+	+	I	+	I	NG
	MA-16-5	+	+	I	+	I	I
	MA-16-7	+	+	I	+	I	I
	MA-16-8	+	+	I	+	I	I
	MA-16-17	+	+	I	+	I	I
Moraxella osloensis	5	+	+	I	+	I	I
	17	+	+	I	+	I	I
	A	+	+	I	+	I	NG
	K	+	+	I	+	I	I
	N	+	+	I	+	I	I
	MA-16-2	+	+	I	+	I	I
M-5	4	+	+	I	−	I	NG
	7	+	+	I	−	I	I
M-4f	MA-27	+	+	+	−	A	K
Acinetobacter calcoaceticus	1	−	+	I	+	I	I
	E	−	−	I	−	I	I
	I	−	+	I	+	AC	NG
	MA-3	−	+	+	+	I	I
Intermediates[b]	H	+	+	+	+	P	NG
	J	+	+	+	+	P	NG
	MA-2	−	+	I	−	A	K
	MA-4	+	+	I	−	A	K
Brevibacterium	2	+	+	+	−	I	I
	6	+	+	+	−	I	I
	9	+	+	+	−	I	I
	0	+	+	−	−	I	I

[a] None of the isolates produced H_2S, indole, urease, or phenylalanine deaminase. +, Reaction positive; −, reaction negative; I, Inactive (growth but no change); NG, no growth; K, alkaline; A, acid; C, curd; P, peptonization.

[b] Characteristics of these organisms did not fit with recognized groups.

tion specifically designed to obtain radiation-resistant bacteria from beef. Various enrichment techniques were used. Irradiation was carried out under atmospheric and vacuum-packed conditions. In addition, aerobic and anaerobic incubation was used for isolation. Detailed characterization was sought for a more adequate classification based on recent literature (2, 11).

Various media have been used to study carbohydrate utilization. The results obtained are not always identical, due to the different chemical composition of the media, e.g.. amount of nitrogen and presence of more than one carbohydrate. Some investigators (11) suggest using triple sugar iron medium to obtain preliminary information and then differentiating nonfermentative and fermentative bacteria by use of open and sealed tubes of O-F medium containing 1% glucose.

Even though the isolates could be placed in groups based on commonly used morphological and physiological characteristics, it can be noted from Tables 1, 2, and 3 that they were a heterogeneous group. It is recognized that bacteria belonging to this group have not been extensively studied and, to date, many are still unnamed. More attention has been given to organisms isolated from clinical specimens. The *Manual of Clinical Microbiology* (11) lists and characterizes a number of unnamed bacteria not recognized by *Bergey's Manual of Determinative Bacteriology* (2). However, *Bergey's Manual* (2) mentions "oxidase positive sac-

charolytic strains showing *Moraxella* morphology," but *Moraxella* spp. are described as not utilizing carbohydrates. These strains, which conform to the generic descriptions of both *Moraxella* and *Acinetobacter*, have been found in cold-stored poultry and fish, as well as from human pathological specimens (2).

Some of the isolates investigated in this study had a range of growth temperatures from 2 to 50 C. It is apparent that these organisms could grow in foods stored at refrigeration temperatures or ambient temperatures and represent a potential spoilage problem. Tiwari and Maxcy (14) reported that the M-A group decreased in importance during storage of ground beef at 5 and 25 C because of overgrowth by less radiation-resistant organisms. None was particularly resistant to heat in comparison to spores. Few would survive pasteurization at 63 C for 30 min.

Although the method of isolation dictated that all isolates be radiation resistant, it is apparent from the data in Table 5 that there was considerable variation in D_{10} values. Certain isolates, however, were as much as four to five times more radiation resistant than *Micrococcus radiodurans* or any endospore (12).

To determine if our isolates had acquired radiation resistance by exposure during the

TABLE 3. *Growth on selective media*[a]

Classification	Isolate	BMM	O-F without glucose	Mac-Conkey	SS	EMB	TSA plus:		Penicillin sensitivity[b]
							2.5% NaCl	6.5% NaCl	
Moraxella nonliquefaciens	3	–	+	+	–	+	+	+	33
	13	–	–	–	–	–	+	+	40
	D	–	+	+	–	–	+	+	25
	MA-5	–	+	–	–	–	+	+	18
	MA-16-5	–	+	–	–	–	+	–	38
	MA-16-7	–	+	–	–	–	+	–	29
	MA-16-8	–	+	–	–	–	+	–	25
	MA-16-17	–	+	–	–	–	+	–	28
Moraxella osloensis	5	+	+	+	–	–	+	+	22
	17	+	+	–	–	+	+	+	25
	A	K	+	–	–	–	–	–	40
	K	K	+	–	–	–	+	–	24
	N	K	+	–	–	–	–	–	22
	MA-16-2	+	+	–	–	–	+	+	20
M-5	4	–	+	–	–	–	–	–	55
	7	–	+	–	–	–	–	–	40
M-4f	MA-27	K	+	+	+	+	+	–	10
Acinetobacter calcoaceticus	1	–	+	–	–	–	+	+	60
	E	+	K	–	–	+	+	+	0
	I	–	K	–	–	+	+	+	25
	MA-3	–	K	+	–	–	+	–	0
Intermediates[c]	H	–	+	–	–	+	+	+	14
	J	–	K	–	–	+	+	+	12
	MA-2	+	K	+	–	+	+	–	0
	MA-4	K	K	+	–	+	+	–	0
Brevibacterium	2	–	–	–	–	–	–	–	33
	6	–	–	–	–	–	+	–	32
	9	–	–	–	–	–	+	–	33
	0	–	+	–	–	–	–	–	22

[a] BMM, Basal mineral medium; SS, *Shigella-Salmonella* agar; EMB, eosin methylene blue agar; +, growth; –, no growth; K, alkaline reaction; A, acid; N. neutral; I, inactive.

[b] Diameter of zone of inhibition of growth surrounding a 10-U disk of penicillin. Zone > 22 mm = sensitive; 11- to 21-mm = intermediate; <11 = resistant.

[c] Characteristics of these organisms did not fit with recognized groups.

TABLE 4. *Range of growth temperatures*

Classification	Isolate	Temp in degrees centigrade
		0 2 5 10 20 32 37 42 45 50
Moraxella nonliquefaciens	3	
	13	
	D	
	MA-5	
	MA-16-5	
	MA-16-7	
	MA-16-8	
	MA-16-17	
Moraxella osloensis	5	
	17	
	A	
	K	
	N	
	MA-16-2	
M-5	4	
	7	
M-4f	MA-27	
Acinetobacter calcoaceticus	1	
	E	
	I	
	MA-3	
Intermediates[a]	H	
	J	
	MA-2	
	MA-4	
Brevibacterium	2	
	6	
	9	
	0	

[a] Characteristics of these organisms did not fit with recognized groups.

TABLE 5. *Radiation and heat resistance*[a]

Classification	Isolate	Radiation D_{10} in krad	Heat resistance	
			D_{10} (min)	Temp of heating (C)
Moraxella nonliquefaciens	3	583	5.2	55
	D	539	2.9	60
Moraxella osloensis	5	1,000	1.5	60
	17	671		
	A	582	5.5	60
	K	764	2.5	63
	N	477	3.0	60
M-5	4	1,672	5.4	70
	7		7.8	65
Acinetobacter calcoaceticus	1	814	6.7	65
	E	405	6.3	65
	I		4.5	65
Intermediates[a]	H	480	5.8	60
	J	273	5.6	60
Brevibacterium	2	485	2.5	55
	0	642	2.8	55

[a] Characteristics of these organisms did not fit with recognized groups.

enrichment procedure, a reference culture of *M. osloensis* was irradiated using conditions similar to those used for irradiation of meat. When the reference culture of *M. osloensis* was subjected to radiation comparable to that used in enrichment and isolation, there was no appreciable change in D_{10}. Therefore, it is unlikely that resistance to irradiation had been induced during isolation procedures. The only differences noted between the parent reference culture of *M. osloensis* and the culture subjected to irradi-

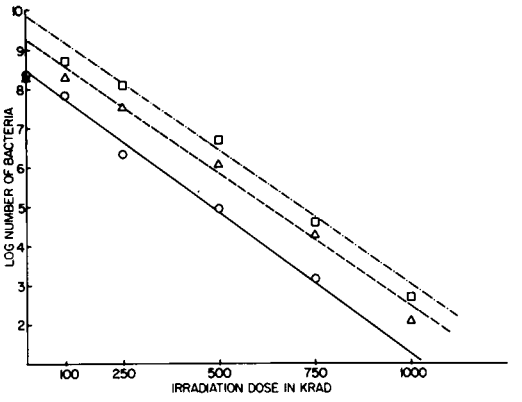

FIG. 2. *Irradiation of reference M. osloensis. Comparative radiation resistance of reference M. osloensis and subcultures surviving irradiation treatments.* ○, *Reference culture;* □, *subculture of colony surviving first irradiation treatment;* △, *subculture of colony surviving second irradiation treatment.*

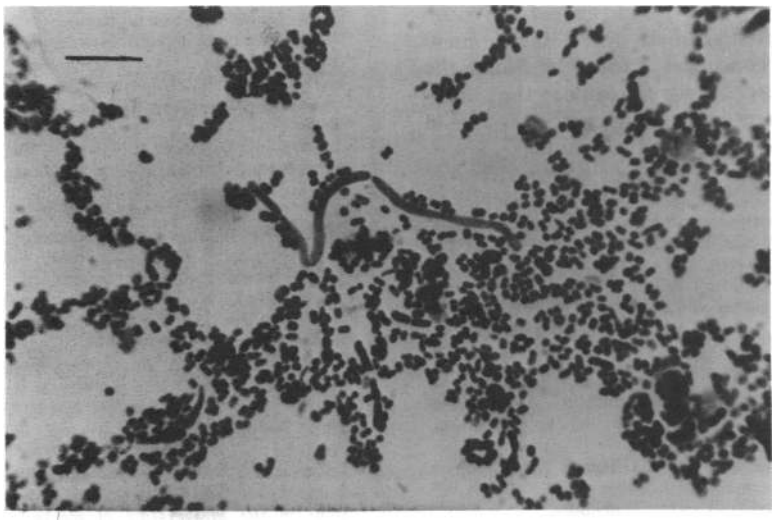

FIG. 3. *Reference culture of M. osloensis before irradiation. Very few long bacilli (10 μm in length) were seen. Marker indicates 10 μm.*

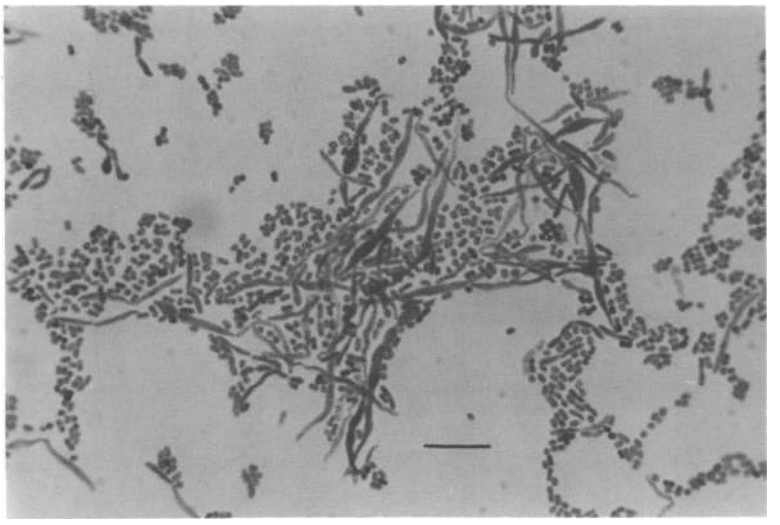

FIG. 4. *Gram stain of colony of M. osloensis surviving 1,000 krad. Long cells were predominant. Marker indicates 10 μm.*

ation were the great increase in numbers of very elongated forms and some loss of sensitivity to penicillin. The elongated forms apparently resulted from a failure of the bacteria to divide, which correlated with reduction of sensitivity to penicillin. Reversion to the same morphological form as the parent culture and sensitivity to penicillin occurred after a few subcultures.

Although these bacteria are prevalent in nature and are found in various food products, they have not been recognized as being significant in organoleptic spoilage or in food-borne diseases. On the other hand, an extensive review by Henriksen (3) lists over 150 references to pathological conditions caused by *Moraxella* and *Acinetobacter*. It would appear that additional work is necessary to delineate the role of these organisms in our environment.

ACKNOWLEDGMENTS

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