ISOLATION OF A *CLOSTRIDIUM* SPP. FROM SPOILED VACUUM-PACKAGED REFRIGERATED BEEF AND ITS SUSCEPTIBILITY TO BACTERIOCIN FROM *PEDIOCOCCUS ACIDILACTICI*

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INTRODUCTION

Spoilage of vacuum-packaged refrigerated fresh beef results from growth of psychrotrophic facultative anaerobes, predominantly lactobacilli, *Leuconostoc* spp. and *Brocothrix thermosphacta*. The spoiled beef has a sour and cheesy odor (Gill, 1986). *Alteromonas putrefaciens* and one *Lactobacillus* strain produced H_2S and caused greening of meat (Shay and Egan, 1981). We reported recently about a large scale spoilage of vacuum-packaged fresh beef at 2C by a *Clostridium* spp. (Kalchayanand et al., 1989). We report here isolation, characterization and growth inhibition by pediocins (bacteriocins of *Pediococcus acidilactici* strains) of this *Clostridium* spp.

MATERIALS AND METHODS

Characteristics of spoiled beef.
Spoiled vacuum-packaged fresh beef samples were received from the commercial producers. The samples were examined for the volume and odor of gas and purge, color, odor, and texture of meat, pH of the purge, and presence of H_2S in gas with a lead acetate paper. The purges were also examined by phase contrast microscopy for tissue fragments and microbiological evaluation. In addition, the purges were examined by pour plating for aerobic plate counts (35C, 2d), psychrotrophic counts (10C, 7d) and psychrotrophic lactic acid bacteria (pH 5, 10C, 7d). Materials were also stained by specific methods to determine gram-characteristics, spores and flagella of the bacterial cells (Kalchayanand et al., 1989).

Isolation of the *Clostridium* spp.
Two methods were used. Initially the purge from spoiled beef containing the characteristic bacterial cells was serially diluted and aliquots from several dilutions were pour plated in agar media and inoculated in broth media. Some of the media were: brain heart infusion agar and broth with 0.1% Na-thyoglycollate and/or 0.05% cysteine, anaerobic agar and broth, cooked meat agar and broth and trypicase peptone glucose yeast extract agar and broth. The plates and broth were incubated anaerobically at 2C up to 4wk and cells from colonies or broth suspensions were examined under a phase contrast microscope. In the second method, purge containing spores was either treated with ethanol for 45 min or heated at 85C for 10 min and transferred in thioglycollate broth, incubated anaerobically at 2C up to 4wk and the materials from broth showing growth were examined with a phase contrast microscope.

Characteristics of the Clostridial isolate.
The pure culture obtained from the spores was examined for growth in several broth and agar media. Also influences of temperatures on growth, viability loss, sporulation and germination, biochemical characteristics and carbohydrate fermentation patterns were studied.

Characteristics of pediocins from *P. acidilactici* strains.
P. acidilactici strains H, E, F, M, from our culture collection, were known to produce bacteriocins (pediocins). The strains were grown in trypicase-glucose-yeast extract (TGE) broth at 37C for 24h, the cells were removed and the supernatant fluids (20 l) were examined for a clear zone of activity by disc assay against a lawn of *Lactobacillus plantarum* NCDO 955. The effects of pH, heat, drying, catalase, proteolytic

enzymes and ethanol on the activity of pediocins were also determined by disc assay using L. plantarum as an indicator. Molecular weights of the partially purified pediocins with $(NH_4)_2SO_4$ precipitates were determined by 10-20% gradient SDS-PAGE (Bhunia et al., 1987 and 1988 and Ray et al., 1989). Finally, growth inhibition of several food-spoilage and pathogenic bacteria by the pediocins was determined by disc assay on lawns prepared with the test strains.

Susceptibility of Clostridial isolate to pediocins.

Several studies were conducted to determine susceptibility of this isolate from spoiled beef to pediocins. Because of the poor relationship between cell viability and colony forming ability in agar media, cell viability was studied differently by first exposing a certain number of cells to pediocin and then separating the cells and incubating in a suitable broth for growth. The cells were also incubated in a broth containing pediocins. Germination and outgrowth of spores were examined either by exposing spores first to pediocin or by incubating spores in broth containing pediocin. To determine influence of pediocin to inhibit spoilage, fresh beef steaks were vacuum-packaged with Clostridium cells along with or without pediocin, stored at 2C up to 8wk and examined for characteristic spoilage.

RESULTS

Characteristics of spoiled beef.

The characteristics of the spoiled samples are listed in Table 1. The spoilage was associated with accumulation of large quantities of foul smelling gas including H_2S, produced from the microbial metabolism of the sulfur-containing amino acids. Initial bright red color of the meat that changed to reddish-green in about 6 to 8wk were most likely associated with H_2S production and its reaction with metmyoglobin. The microorganisms responsible for the spoilage also produced extracellular proteolytic enzymes and proteolysis of the beef. The soft texture of the beef, large accumulation of purge and presence of muscle myofibrils in the purge were associated with the proteolytic action. The pH of the purge ranged between 5 to 6. Also psychrotrophic lactic acid bacteria counts were high; further examination revealed them mostly Leuconostoc spp. Although the spoilage generally occurred in 4 to 6wk, in some cases spoilage was detected within 1wk under commercial operation.

Phage contrast microscopy of the purge revealed the predominant bacterial cells were large, thick rods with straight and tumbling motion. Some cells, especially from samples 6 to 8wk old, had spores. The spores were large, oval, and terminal giving the cells a drumstick appearance. The cells were gram-positive and had large numbers of flagella in peritrichous arrangements. In addition, microscopic examination revealed the presence of some bacterial cells different from the predominant type, and later identified as Leuconostoc spp.

Isolation of Clostridium spp. and its characteristics.

The above observations suggested that the predominant type could be a Clostridium spp. Pour plating and subculturing of the purge in several agar and broth media followed by anaerobic incubation at 2C up to 4wk produced colonies in agar and turbidity in broth media. Microscopic examination of the cells from large numbers of colonies and broth followed by biochemical testing, indicated these to be Leuconostoc spp. However, cells from thioglycollate broth that was subcultured with either heat treated or alcohol treated purge containing spores, revealed the presence of one type of cells that were motile, gram-positive rods, with peritrichous flagella and some with oval terminal spores (data not

presented).

The characteristics of this isolate revealed the pure culture grew well in many broth and agar media, especially under anaerobic conditions (Table 2). However, pour plating of a cell suspension in many different media allowed about 5% of the cells to form colonies even under good anaerobic incubation. The cells grew as low as -3C, grew rapidly between 10 to 20C (in 2d) but did not grow at or above 25C. Exposure of 10^7 cells in a broth at 50C for 24h followed by subsequent incubation at 10C up to 20d failed to show any growth. At 2C the cells sporulated fairly well and spores germinated and outgrew. The cells formed acid from several carbohydrates but not from lactose, xylose, adonitol and several others.

Characteristics of pediocins.
Pediocins from all four strains, P. acidilactici H, E, F, M were studied for several characteristics (Table 3). They retained their activity after high heat treatment, drying, at wide pH range and after treatments with catalase and ethanol. However, treatment with several proteolytic enzymes, including trypsin and chymotrypsin inactivated these proteins. On SDS-PAGE gel the MW of the band associated with the antibacterial activity was calculated to be about 2700 Da. Pediocin AcH, from strain H, was nontoxic and nonimmunogenic to mice and rabbits.

Isolates of spoilage bacteria from meat and several food-borne pathogens were tested by disc assay against pediocins from all four strains (Table 4). They inhibited growth of gram-positive spoilage and pathogenic bacteria. They were either inactive or partially active against the gram-negative bacteria.

Inhibition of Clostridium by pediocin AcH.
The vegetative cells of the Clostridium spp. isolated from spoiled beef were sensitive to pediocin AcH as determined from the clear zone around the disc as well as inability of pediocin-treated cells to grow subsequently in a broth as compared to the untreated cells (Table 5). This suggested that the pediocin AcH is bactericidal to these cells. Spores also failed to outgrow in a broth containing pediocin AcH. Beef inoculated with the Clostridium cells along with pediocin AcH and vacuum-packaged did not show characteristic spoilage during storage at 2C up to 4wk as compared to control with Clostridium cells only.

CONCLUSION

The results of these studies showed that a Clostridium spp. is associated with spoilage of vacuum-packaged refrigerated fresh beef and produced extensive proteolysis, loss of texture, foul odor, bright red to reddish-green color of meat. The cells are gram-positive, large rods, motile, contain peritrichous flagella and form single, oval terminal spores. A pure culture of the Clostridium was obtained from the heat treated as well as ethanol treated spores. The cells of the isolate are able to grow between -3 to 21C, but not at 25C, they grow rapidly in a broth between 10 to 20C within 2d, sporulate and germinate at 2C, and are killed by treating at 50C for 24h. Comparison of growth and biochemical characteristics with psychrotrophic Clostridium botulinum types B and E and C. putrefaciens suggested that the isolate could be a new species. We tentatively named it C. laramie. This aspect is being studied further. Specific bacteriocins from P. acidilactici strains (pediocins) inhibited growth of vegetative cells, outgrowth of the spores and production of characteristic spoilage of vacuum-packaged refrigerated fresh beef by this Clostridium spp. The pediocins, which are small proteins, also inhibited growth of several other bacteria associated with spoilage of vacuum-packaged beef and several food-borne pathogens.

Pediocin AcH is nontoxic and nonimmunogenic to mice and rabbit, and destroyed by the gastric proteolytic enzymes. As pediocins are from food-grade and safe bacteria, they can be used to enhance shelf-life and safety of vacuum-packaged refrigerated fresh beef.

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Table 1. Characteristics of beef and predominant bacterial cells from spoiled samples.

a. Beef and Package

Gas: Large accumulation sometimes rupturing bags, foul odor, H_2S present.

Purge: Large quantity, cellular fragments, red to reddish-green, foul odor, large quanitites of microscopic muscle fragments, pH 5 to 6, aerobic plate count $1X10^6$; psychrotrophs: $5X10^6$; psychrotrophic lactic acid bacteria: $7X10^6$/ml.

Meat: Soft, bright red to green depending upon time of spoilage, foul odor. Types included: top round, chuck roll, tri tip, strip loin.

Spoilage time: As low as one to two wk at 2 to 3C.

b. Predominant bacteria in purge

Morphology: Large thick rods, motile, single or small chains, some cells with single terminal large oval spore.

Other features: Cells are gram-positive with peritrichous flagella.

Table 2. Characteristics of Clsotridium isolated from spoiled beef.

a. Growth conditions

Aerobiosis: Strict anaerobes in agar media, less in broth media.

Media: Pure culture is able to grow in many broth including tryptic soy broth.

: On streaking, cells form colonies on many types of agar media including tryptic soy agar.

: On pour plating of a cell suspension only 5% formed colonies, even in media recommended for Clostridium.

Temperature: Cell growth -3 to 21C, optimum 10 to 20C, no growth at 25C, killed at 50C in 24h (with 10^7 cells no growth at 10C in 20d).

Sporulation: 2C in 2wk, 10C in 1wk, in 6 to 8wk about 50% cells sporulate, germination/outgrowth at 2C in 2wk.

b. Biochemical characteristics

Acid formation: Fructose, galactose, glucose, sucrose, raffinose.

Other traits: Hydrolyzed gelatin, starch; reduced nitrate; digested meat; beta-hemolysin and lipase-positive.

Table 3. Characteristics of Pediocins.

Parameter	Activity from strains H	E	F	M
Heat (121C, 15 min)	+	+	+	+
Dried (air and freeze, stored 6 mo)	+	+	+	+
pH 4-7	+	+	+	+
Catalase treated	+	+	+	+
Ethanol treated	+	+	+	+
Proteolytic enzyme[a]	-	-	-	-
Molecular weight (Da)[b]	2700	2700	2700	2700
Toxicity/immunogenic[c]	-	NT	NT	NT

[a]Treated with trypsin, chymotrypsin, papin, ficin (200 g/ml).
[b]From SDS-PAGE (approximate MW).
[c]Against mice and rabbit. NT:Not tested; +, activity retained; -, activity lost.

Table 4. Inhibitory Spectrum of Pediocins.

Test strain	Inhibitory zone from strains H	E	F	M
Lactobacillus spp[a]	+	+	+	+
Leuconostoc spp[a]	+	+	+	+
Brocothrix thermosphacta[a]	+	+	+	+
Streptococcus faecalis[a]	+	+	+	+
Pseudomonas fluorescens[a]	±	±	±	±
Clostridium perfringens	+	+	+	+
Staphylococcus aureus	+	+	+	+
Listeria monocytogenes	+	+	+	+
Campylobacter jejuni	-	+	-	-
Aeromonas hydrophilia	+	+	-	-
Salmonella typhimurium	-	-	-	-
Escherichia coli (enterotoxigenic)	-	-	-	-

[a]Isolates from spoiled meat; + inhibition, - no inhibition, ± weak.

Table 5. Inhibition of Clostridium Isolate by Pediocin AcH.

Test condition	Effect
Disc assay	Inhibited
Cells (10^5)) treated with Pediocin[a]	Failed to grow subsequently in broth
Cell (10^4) in broth + Pediocin	Failed to grow
Spores treated with Pediocin	Grew subsequently in broth
Spores in broth + Pediocin	Failed to outgrow
Vacuum-packaged meat + Clostridium cells (2C)	Spoiled in 1wk
Vacuum-packaged meat + Clostridium cells + Pediocin (2C)	Not spoiled (4wk)

[a]Pediocins from strains E, F, M also inhibited growth of treated cells.