Research Note

Description of a Simple Detection Assay for In Situ Production of Bacteriocin on Meat

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ABSTRACT

Using a modification of the agar diffusion assay, in situ bacteriocin production on meat was analyzed using cooked meat medium (CMM) and sterile pork tissue (lean and fat) with Carnobacterium piscicola LV17 as the producer and Carnobacterium divergens LV13 as the indicator strains. Contrary to what is observed in APT broth, bacteriocin production by C. piscicola LV17 occurred with growth at low inoculum levels (≤104 CFU/cm² or g of meat) on disks (10 cm²) of pork fat tissue (pH 6.58) and on CMM particles (pH 7.0) but not on disks of lean tissue (pH 5.61). The assays described in this study do not required sophisticated equipment and would be useful to study bacteriocin production on meat products stored under various conditions.

Carnobacterium piscicola LV17 is a nonaciduric lactic acid bacteria isolated from vacuum-packaged pork. It produces three bacteriocins that may be of interest in food preservation (1,16,19). Bacteriocin production by C. piscicola LV17 is not constitutive and is dependent on the inoculum level in liquid commercial medium (18). Similar results have been reported for Lactobacillus plantarum C11 (9,10) and Lactobacillus sakei (11). Growth conditions also have been reported to influence bacteriocin production. In fact, de Vuyst et al. (8) demonstrated that bacteriocin production by Lactobacillus amylovorus was stimulated at low temperatures that were unfavorable for the growth of the organism. C. piscicola LV17 grew on liquid media at pH 5.6 but failed to produce bacteriocins (1). Furthermore, its growth on lean beef under vacuum at 2°C was reported to be unpredictable (15). Other investigators have reported that meat constituents and endogenous enzymatic activity can interfere with antimicrobial activity of bacteriocins used in foods. For example, nisin is not active in meat systems because it reacts with glutathione in raw meat to produce an inactive complex (17), it is insoluble at the pH of meat, and it is subject to proteolytic degradation (14).

To assess the role of bacteriocin-producing strains in the extension of meat storage life, it must be demonstrated that bacteriocins are produced in the product. Furthermore, for bacteriocins to be of value in meat preservation, the producer organism must produce bacteriocin when low inoculation levels are used to limit spoilage. The modified agar diffusion assay described in this study was used to demonstrate bacteriocin production on two meat models, cooked meat medium (CMM) and disks of pork meat (lean and fat), inoculated with C. piscicola LV17.

MATERIALS AND METHODS

Bacterial cultures and growth media. C. piscicola LV17 and Carnobacterium divergens LV13 were isolated from meat and are available from the National Collection of Food Bacteria (Institute of Food Research, Langford, Bristol, England) as strains 2852 and 2855, respectively. The nonbacteriocinogenic strain, C. piscicola LV17C, was obtained by curing the producer strain of its three plasmids (3).

Cultures were stored at −70°C in APT broth (All Purpose Tween, Difco Laboratories, Detroit, Mich.) containing 20% (vol/vol) glycerol. Before experimental use, cultures were subcultured daily (1% [vol/vol] inoculum) in APT broth for a minimum of 2 and a maximum of 7 consecutive days. Cultures and plates were incubated at 25°C, and plates were incubated in anaerobic jars (Gas Pak, BBL Microbiology Systems, Cockeysville, Md.) in an atmosphere containing 10% CO₂ and 90% N₂.

Meat systems. Meat particles from 1.25 g of dry CMM were prepared as recommended by the manufacturer (BBL), and the liquid phase was aseptically decanted. Sterile meat tissue (lean or fat disks of 10 cm²) was prepared from pork longissimus thoracis muscles as described by Greer and Jones (12) and was inoculated with the test organisms as described below. The pH of excised lean and fat tissue was measured with a flat glass combination electrode (Model 913600, Orion Research, Chicago, Ill.) and a digital pH meter (Model 671, Exttech, Boston, Mass.). Five random readings were taken for each loin, and the mean of all pH readings for all of the loins was calculated.

Assay for bacteriocin production. Bacteriocin production in APT broth was determined using the spot-on-lawn technique with C. divergens LV13 as the indicator strain. Results are ex-
pressed as arbitrary activity units of bacteriocin per ml of heat-treated (65°C for 30 min) supernatant (2). The heat treatment serves to inactivate the producer strain and to stop further bacteriocin production (1, 2). The bacteriocins produced by C. piscicola LV17 are resistant to such heat treatment (16, 19).

Bacteriocin production on CMM particles was tested by covering the particles with 30 ml of soft APT agar (containing 0.75% agar) at 50°C. The CMM particles and soft agar mixture was heated at 65°C for 30 min to inactivate the producer strain similarly to what was done in APT broth (2). The soft APT agar mixture with meat particles was cooled to 50°C before adding 1% (vol/vol) of the indicator strain. The mixture of agar, CMM particles, and the indicator strain was poured into a 150 by 15-mm petri dish and allowed to solidify. Two spots of 5 µl of pronase E solution (10 mg/ml, Sigma Chemical Co., St. Louis, Mo.) were placed on the surface of the agar and allowed to dry before adding a second layer of soft APT agar inoculated with 1% of the indicator strain to ensure that the CMM particles were completely covered.

Because a smaller quantity of soft agar is sufficient for the heat treatment of a disk of pork tissue compared with CMM particles, a similar procedure, based on the well diffusion assay, was used to detect bacteriocin production from the inoculated disks of lean and fat tissue (6, 13). A disk of meat was placed in a sterile 50-ml polypropylene tube (BBL) and was covered by the minimal amount of soft APT agar to avoid dilution of the bacteriocin in a large quantity of agar. The meat and soft agar mixture was heated at 65°C for 30 min and poured into a well (3.6 cm in diameter) made in 60 ml of APT agar presolidified in a sterile 400-ml beaker. After 30 min, 5 µl of pronase E solution was spotted beside the well and allowed to dry before an overlay of 7.5 ml of soft APT agar inoculated with the indicator strain (1% [vol/vol]) was poured onto the agar surface. All plate and beaker samples inoculated with the indicator strain to test for bacteriocin production were incubated overnight at 25°C in anaerobic jars as previously described.

Inoculation of CMM particles and lean and fat pork tissue. Meat particles from CMM and disks of lean and fat pork tissue were inoculated using a fully grown 24-h culture diluted in APT broth to achieve cell densities that ranged from 10² to 10⁸ CFU/g for CMM particles or CFU/cm² for pork disks. CMM particles were immersed in 6 ml of inoculum, and disks of pork tissue were immersed in 200 ml of inoculum for 30 min. Inoculated disks were allowed to drip dry for 15 min on a sterile rack in a laminar flow hood. Inoculated meat was incubated at 25°C for a maximum of 3 days and were tested for bacteriocin production daily. C. piscicola LV17C was used as a negative control. Cell counts were determined by plating serial dilutions of meat samples (equivalent to 1.25 g of dry CMM or one pork tissue disk) that had been homogenized in a stomacher (A. J. Seward, St. Edmonds, UK) for 2 min in 90 ml of 0.1% peptone water.

RESULTS

Bacteriocin production on CMM particles. Detection of bacteriocin produced in situ on CMM particles inoculated with C. piscicola LV17 was demonstrated by inhibition of the indicator strain present in the top soft agar (Fig. 1). As shown in Figure 1A, the indicator strain grew only where pronase E was spotted, whereas in Figure 1C, the indicator was not inhibited when the meat particles were inoculated with the nonproducing strain C. piscicola LV17C (negative control).

Bacteriocin production was detected at all three inoculation levels tested on CMM particles inoculated with C. piscicola LV17 (Table 1). With inoculation levels of 10² or 10⁸ CFU/g, bacteriocin production was detected within 2 days of incubation at 25°C (Fig. 1B); at an inoculation level of 10⁷ CFU/g, bacteriocin was detected after 1 day (Fig. 1A). Final population reached 10⁹ CFU/g for all inoculation levels tested (data not shown). In APT broth, bacteriocin
TABLE 1. Effect of growth medium on bacteriocin production inoculated with C. piscicola LV17 at various cell densities

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<thead>
<tr>
<th>Inoculum levels (log CFU/ml)</th>
<th>Bacteriocin productiona</th>
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<tr>
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<td>APT</td>
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<tr>
<td>$10^2$</td>
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<td>$10^4$</td>
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a Experiment was repeated three times. Results are expressed as follows: −, no inhibition of the indicator strain; +, distinct inhibition of the indicator strain.

Bacteriocin production was only detected at a high inoculum level ($10^7$ CFU/ml) as previously reported (18).

Bacteriocin production on disks of pork tissue (lean and fat). Bacteriocin production on fresh meat was investigated using disks of lean and fat tissue that were aseptically prepared from pork loins. The mean pH of the lean and the fat tissues were 5.61 ± 0.06 and 6.58 ± 0.12, respectively. Growth of C. piscicola LV17 was similar on fat and lean pork disks and reached a final count of $10^8$ CFU/g within 3 days at 25°C for all inoculation levels tested (data not shown). C. piscicola LV17 produced bacteriocin from a low inoculum on pork fat (Fig. 2). The indicator strain was inhibited on the whole surface of the well and for a width of 2 and 4.5 mm around the well for an inoculum of 3.01 (Fig. 2A) and 5.06 (Fig. 2B) log CFU/cm², respectively. No inhibition was observed with the negative control (Fig. 2C). Bacteriocin production was detected after 1 day of incubation at 25°C but not at subsequent sampling times. Bacteriocin production was not detected on pork lean inoculated with C. piscicola LV17.

DISCUSSION

C. piscicola LV17 is a bacteriocin-producing strain with the potential to serve as a protective culture to improve the shelf life and safety of meat products. Bacteriocin production on meat may constitute a competitive advantage for a protective culture, but the organism should be inoculated at low levels to limit spoilage. Similar to what was observed on agar media (18), bacteriocin production by C. piscicola LV17 occurred on meat inoculated at a low level ($\leq 10^4$ CFU/g or cm²). It was previously reported that C. piscicola LV17 grew in liquid culture at pH 5.6 but failed to produce bacteriocins (1). These observations suggest that the pH of pork lean meat (pH 5.61) is limiting for bacteriocin production by C. piscicola LV17 but more favorable in the case of pork fat (pH 6.58), CMM (pH 7), and APT (pH 6.5). These pH values are closer to the pH of certain types of processed meat (5). CMM is a good model for processed meat because salt and sugar are also added to the meat base. Consequently, C. piscicola LV17 might be better suited for use as a protective culture in meat product with a pH higher than 5.6. Variation of bacteriocin production in pork tissue is most probably not due to a limitation of certain nutrients compared with CMM (e.g., carbohydrate), since bacteriocin production was observed on pork fat, and growth was as good on lean as on fat pork tissue.

On disks of pork fat tissue, bacteriocin production was detected at inoculation levels of 3.01 and 5.06 log CFU/cm² after 1 day of incubation at 25°C, but it was not detected thereafter, whereas on CMM particles, production was detected up to 2 days after the initial detection. Better growth of C. piscicola LV17 on CMM particles compared

FIGURE 2. Bacteriocin production from disks of pork fat tissue (white T) inoculated with C. piscicola LV17 (A, B) or LV17C (C; negative control) was evaluated by inhibition of the indicator strain, C. divergens LV13. Disks of tissue were stored in a sterile petri dish placed in a plastic bag and were incubated at 25°C for 1 day. The initial cell concentrations (log of CFU/cm²) and the width of the zone of inhibition around the well (black vertical bar measured in millimeters; neg = negative) are shown below each panel. Arrows indicate where pronase E solution was spotted. Growth of the indicator appears in white, and inhibition zones in gray. Experiment was repeated two times.
with pork disks of tissue may account for this difference in production. Furthermore, enzymatic degradation and inactivation through binding to meat constituents, as observed for nisin in raw meat (14, 17), may also contribute to the shorter time of detection on storage. Hence, variation of bacteriocin production, inactivation, or complexing of bacteriocin in situ (17) and ability to recover bacteriocin from a sample (4, 7) are all important factors that will influence bacteriocin detection.

Using the modified agar diffusion assay, we demonstrated that bacteriocin was produced on meat inoculated with low levels of *C. piscicola* LV17 and that production varied with respect to the growth conditions used. Bacteriocin production with *L. amylovorus* varies also with growth conditions. Bacteriocin production was stimulated at low temperatures despite the fact that these conditions are unfavorable for the growth of the organism (8).

The data reported here describe a rapid method for detecting bacteriocin production in situ during the storage of an inoculated food. The detection method described in this study is a modification of a commonly used method for bacteriocin detection, the agar diffusion assay, and may be useful in testing for bacteriocin production in solid food stored under various conditions. The method is based on the inhibition of an indicator organism and, therefore, can only assess the presence of the bacteriocin. It gives no indication of the efficiency of the bacteriocin to control microorganisms in food if performed alone without selective enumeration of the targeted organisms. Although qualitative in nature, this simple assay allows for the detection of bacteriocin production with no tedious extraction of the bacteriocin from the food matrix. It is performed with equipment readily available in a laboratory, and many samples can be tested within a day.

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