Identification and Characterization of *Leuconostoc carnosum*, Associated with Production and Spoilage of Vacuum-Packaged, Sliced, Cooked Ham

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*L. carnosum* was shown to be the specific spoilage organism in vacuum-packaged, sliced, cooked ham showing spoilage during 3 weeks of shelf life. Identification of the specific spoilage organism was done by use of phenotypic data and *ClaI*, *EcoRI*, and *HindIII* reference strain ribopatterns. One hundred *L. carnosum* isolates associated with the production and spoilage of the ham were further characterized by pulsed-field gel electrophoresis (PFGE), together with some meat-associated *Leuconostoc* species: *L. citreum*, *L. gelidum*, *L. mesenteroides* subsp. *dextranicum*, and *L. mesenteroides* subsp. *mesenteroides*. *ApaI* and *SmaI* digests divided the industrial *L. carnosum* strains into 25 different PFGE types, *ApaI* and *SmaI* types being consistent. Only one specific PFGE type was associated with the spoiled packages. This type also was detected in air and raw-meat mass samples. The spoilage strain did not produce bacteriocins. Only seven isolates belonging to three different PFGE types produced bacteriocins. Similarity analysis of the industrial *L. carnosum* strains revealed a homogeneous cluster which could be divided into eight subclusters consisting of strains having at most three-fragment differences. The *L. carnosum* cluster was clearly distinguished from the other meat-associated *leuconostoc* clusters, with the exception of the *L. carnosum* type strain. Ribotyping can be very helpful in the identification of *L. carnosum*, but its discriminatory power is too weak for strain characterization. PFGE provides good discrimination for studies dealing with the properties of homogeneous *L. carnosum* strains.

Lactic acid bacteria (LAB) are the major spoilage bacteria in vacuum-packaged, cooked meat products (1, 2, 10, 13, 25, 27, 31, 38, 44, 47, 56). *Lactobacillus sake* and *Leuconostoc* have been the main genera associated with the spoilage of these products. *Lactobacillus sake* and *L. curvatus* being isolated commonly (12, 16, 18, 19, 24, 27, 30, 35, 39, 43–46). Compared to aerobic spoilage bacteria, spoilage LAB produce their typical sensory changes, such as souring, gas formation, and/or slime formation, later, at the stationary phase (29, 44), and a vacuum-packaged product is usually expected to maintain good sensory quality for at least 3 to 4 weeks. However, due to an increased level of LAB contamination or particularly active spoilage strains, spoilage may occur during the shelf-life period, subjecting the producer to recalls (30, 31, 33, 46).

In an LAB contamination study of vacuum-packaged, sliced, cooked ham, 982 LAB isolates from the spoiled product and production line were characterized in order to determine the underlying reasons for fluctuations in product quality (4, 6). Many lots had been showing spoilage changes, i.e., sour odor and taste, before the sell-by date. In that study, ribotyping (21) was used as a tool for contamination analysis. Based on *EcoRI* and *HindIII* ribopatterns, two major spoilage LAB types, types G and A, were detected. Contamination with these spoilage LAB was shown to have occurred postcooking, and a probable site of air-mediated contamination from the macerated raw-meat mass to the cooked product was revealed. Because type G showed the typical *EcoRI* and *HindIII* ribopatterns of *L. sake* (5), no further identification or characterization studies were warranted. However, the most important specific spoilage organism, type A, was not identified to the species level. Type A had been detected as the dominant type in the macerated raw-meat mass and in the spoiled packages with the strongest changes in sensory characteristics (6). It had also persisted in the plant during the 1-year study period, consisting of two separate large-scale contamination experiments (4, 6).

In this study, we set out to identify type A LAB to the species level and characterize in more detail the 100 isolates possessing the type A *EcoRI* and *HindIII* ribopatterns. Since phenotypic characteristics alone are seldom sufficient for species identification of LAB (15), a reference strain library was created by ribotyping and was used with phenotypic data. Pulsed-field gel electrophoresis (PFGE) was applied in order to provide further strain-level characterization. Production of bacteriocins was determined for evaluation of the impact of this characteristic in a population associated with process contamination and product spoilage.

MATERIALS AND METHODS

**Bacterial strains.** One hundred type A LAB possessing the same *EcoRI* and *HindIII* ribopatterns had been isolated during a contamination study of a meat plant (6). All isolates were gram-positive, oval cocci isolated from a macerated raw-meat mass, air in the macerating room, surfaces and air in the cooking room, worker’s gloves, surfaces of the ham prior to slicing, and vacuum-packaged, sliced, cooked ham cultured on the sell-by date. Isolates originating from different sources are listed in Table 1.

TABLE 1. Division of the isolates into different types and certain phenotypic properties

<table>
<thead>
<tr>
<th>Type*</th>
<th>Isolates*</th>
<th>Restriction enzyme profile</th>
<th>Production of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ApaI</td>
<td>SmaI</td>
</tr>
<tr>
<td>A I-a</td>
<td>M2n, M2h</td>
<td>A1</td>
<td>S1</td>
</tr>
<tr>
<td>A I-b</td>
<td>M3h, M6f, M6h</td>
<td>A2</td>
<td>S2</td>
</tr>
<tr>
<td>A I-c</td>
<td>P31a, M4I, M4m</td>
<td>A3</td>
<td>S3</td>
</tr>
<tr>
<td>A I-d</td>
<td>I27e</td>
<td>A4</td>
<td>S4</td>
</tr>
<tr>
<td>A I-e</td>
<td>M5I, M5j</td>
<td>A5</td>
<td>S5</td>
</tr>
<tr>
<td>A I-f</td>
<td>P36b</td>
<td>A6</td>
<td>S6</td>
</tr>
<tr>
<td>A I-g</td>
<td>M6j</td>
<td>A7</td>
<td>S7</td>
</tr>
<tr>
<td>A I-h</td>
<td>V8a-o, V9a-o, V11a-m, V13a-o, I2b, I27a, M5o, M6a</td>
<td>A8</td>
<td>S8</td>
</tr>
<tr>
<td>A I-i</td>
<td>M6o</td>
<td>A9</td>
<td>S9</td>
</tr>
<tr>
<td>A I-j</td>
<td>M2c, M2l, M2o, M3f, M3l</td>
<td>A10</td>
<td>S10</td>
</tr>
<tr>
<td>A I-k</td>
<td>M6g</td>
<td>A11</td>
<td>S11</td>
</tr>
<tr>
<td>A II-a</td>
<td>IIb</td>
<td>A12</td>
<td>S12</td>
</tr>
<tr>
<td>A II-b</td>
<td>IIc, IIf</td>
<td>A13</td>
<td>S13</td>
</tr>
<tr>
<td>A II-c</td>
<td>I26b, I28b</td>
<td>A14</td>
<td>S14</td>
</tr>
<tr>
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<td>I2a</td>
<td>A15</td>
<td>S15</td>
</tr>
<tr>
<td>A III-a</td>
<td>M2j, M3c, M3m</td>
<td>A16</td>
<td>S16</td>
</tr>
<tr>
<td>A III-b</td>
<td>M1e</td>
<td>A17</td>
<td>S17</td>
</tr>
<tr>
<td>A III-c</td>
<td>M5k</td>
<td>A18</td>
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<td>S20</td>
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<tr>
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<td>M1f</td>
<td>A21</td>
<td>S21</td>
</tr>
<tr>
<td>A VI</td>
<td>M1j</td>
<td>A22</td>
<td>S22</td>
</tr>
<tr>
<td>A VII-a</td>
<td>I27f</td>
<td>A23</td>
<td>S23</td>
</tr>
<tr>
<td>A VII-b</td>
<td>M1i</td>
<td>A24</td>
<td>S24</td>
</tr>
<tr>
<td>A VIII</td>
<td>M1c</td>
<td>A25</td>
<td>S25</td>
</tr>
</tbody>
</table>

* Types sharing the same roman numeral differ by at most three bands in the restriction enzyme profiles.

† Sampling was described previously (6). Sources were as follows: M, raw-meat mass; P, surface; I, air; V, spoiled product. Groups of lowercase letters indicate a series of isolates; e.g., a–o indicates 15 isolates from V8a to V8o.

‡ +, production; –, no production.

descens ATCC 12706T, and Weissella paramesenteroides DSM 20288T. In addition, the previously established (5, 7) CloI, EcoRI, and HindIII Lactobacillus ribotypes were compared with the Leuconostoc and Weissella ribotypes characterized in this study.

The most-associated reference strains L. carnosum NCFB 2776T, L. citreum (L. amelobium) D1 (35), L. gelidum NCFB 2775T, L. mesenteroides subsp. dextranicum DSM 20484T, L. mesenteroides subsp. mesenteroides DSM 20343T, L. pseudomesenteroides DSM 20193T, and W. paramesenteroides DSM 20288T were characterized by PFGE along with the industrial isolates.

All strains were maintained in MRS broth (Difco, Detroit, Mich.) at −70°C and cultured with MRS broth or MRS agar (Oxoid, Basingstoke, United Kingdom) as previously described (28).

**Phenotypic characterization.** The anaerobic growth of all industrial isolates on Rogosa selective Lactobacillus agar (Orion Diagnostica, Espoo, Finland) was determined, and the scheme of Villiani et al. (55) was used for the presumptive identification of Leuconostoc spp. Gas production from glucose was tested with modified MRS broth in Durham tubes (51). Production of ammonia from arginine was observed by the method of Briggs (14), and dextran formation was tested for the cleavage of DNA of three strains (NCFB 2776T, M6f, and I27a). Apel and SmaI, which produced convenient numbers of fragments with discriminatory patterns, were chosen for the cleavage of all strains. The samples were electrophoresed through a 1.2% (wt/vol) agarose gel (SeaKem Gold; FMC BioProducts, Rockland, Maine) in 0.5× TBE (45 mM Tris, 4.5 mM boric acid [pH 8.3, 1 mM sodium EDTA] at 14°C by use of a Gene Navigator system with the hexagonal electrode (Pharmacia). Interpolation ranging from 0.5 to 15 s for 20 h at 200 V was used for both enzyme digests.

**PFGE data management.** Photographs of the PFGE banding patterns were scanned with a ScanJet 4Cf scanner (Hewlett-Packard Co., Boise, Idaho). Numerical analysis of macrorestriction patterns was performed with a GelCompar system (version 4.0; Applied Maths, Kortrijk, Belgium). The similarity between all pairs was expressed by Dice coefficient correlation, and clustering by the unweighted pair-group method with arithmetic averages was used for the construction of the dendrogram. Types were considered closely related (53) in the presence of at most a three-band difference (one genetic event). This relationship was indicated in the type nomination by a shared roman numeral.

**RESULTS**

The 100 isolates did not grow on Rogosa selective Lactobacillus agar; all produced gas from glucose but did not produce ammonia from arginine. Fifteen isolates (11 different PFGE...
types) produced slime from sucrose, and bacteriocins were produced by 7 isolates (Table 1). The five isolates tested produced only D-lactic acid and had similar fermentation patterns for the utilization of ribose, D-glucose, D-fructose, α-methyl-D-glucoside, N-acetylglucosamine, cellobiose, saccharose, trehalose, β-gentiobiose, D-turanose, and gluconate. Growth occurred at 8, 10, and 15°C but not at 37°C.

Previously determined oval cell morphology and the phenotypic characteristics typical of leuconostocs led to the comparison with the Leuconostoc and Weissella type strains. Figures 1, 2, and 3 show that the ClaI, EcoRI, and HindIII ribotypes, respectively, of the reference strains differed clearly from the Lactobacillus ribotypes obtained previously (5, 7). The ClaI, EcoRI, and HindIII ribopatterns of the industrial isolates were found to be identical to those of L. carnosum NCFB 2776T (Fig. 1, 2, and 3, lanes 10). All of the other type strains were distinct from L. carnosum NCFB 2776T. Based on the phenotypic data and the identical ribopatterns, the industrial isolates were classified as L. carnosum. HindIII and EcoRI generated the least distinguishing ribotypes for the Leuconostoc and Weissella species. ClaI was the only enzyme distinguishing L. mesenteroides subsp. mesenteroides from L. mesenteroides subsp. cremoris (Fig. 1, lanes 5 and 7).

Both ApaI and SmaI generated 25 different patterns for the meat plant isolates when one-band differences are noted. The ApaI types were consistent with the SmaI types (Table 1). All meat-associated reference strains, with the exception of L. carnosum NCFB 2776T, were clearly distinguished from the industrial isolates (Fig. 4 and 5). Both ApaI and SmaI resulted in convenient numbers of fragments for macrorestriction anal-

![FIG. 1. ClaI ribopatterns. Lanes 4 and 11, phage lambda DNA cleaved with HindIII as a fragment size marker; lane 1, Weissella viridescens ATCC 12706T; lane 2, Weissella halotolerans ATCC 35410T; lane 3, Weissella paramesenteroides DSM 20288T; lane 5, Leuconostoc mesenteroides subsp. mesenteroides DSM 20343T; lane 6, Leuconostoc mesenteroides subsp. cremoris CCUG 21965T; lane 7, Leuconostoc mesenteroides subsp. dextranicum DSM 20484T; lane 8, Leuconostoc pseudomesenteroides DSM 20193T; lane 9, Leuconostoc carnosum NCFB 2776T; lane 10, Leuconostoc gelidum NCFB 2775T; lane 12, Leuconostoc lactis CCUG 30064T; lane 13, Leuconostoc fallax CCUG 30061T; lane 14, Leuconostoc citreum (Leuconostoc amelobiosum) D1.](image1)

![FIG. 2. EcoRI ribopatterns. Lanes 4 and 11, phage lambda DNA cleaved with HindIII as a fragment size marker; lane 1, Weissella viridescens ATCC 12706T; lane 2, Weissella halotolerans ATCC 35410T; lane 3, Weissella paramesenteroides DSM 20288T; lane 5, Leuconostoc mesenteroides subsp. mesenteroides DSM 20343T; lane 6, Leuconostoc mesenteroides subsp. cremoris CCUG 21965T; lane 7, Leuconostoc mesenteroides subsp. dextranicum DSM 20484T; lane 8, Leuconostoc pseudomesenteroides DSM 20193T; lane 9, Leuconostoc carnosum NCFB 2776T; lane 10, Leuconostoc gelidum NCFB 2775T; lane 12, Leuconostoc lactis CCUG 30064T; lane 13, Leuconostoc fallax CCUG 30061T; lane 14, Leuconostoc citreum (Leuconostoc amelobiosum) D1.](image2)
ysis (Fig. 4). However, SmaI cleaved the DNA efficiently, whereas some partial digestion was occasionally noted with ApaI. Because of the better reproducibility, SmaI patterns were chosen for the numerical analysis.

Figure 5 shows the dendrogram of the industrial isolates and the reference strains. L. carnosum formed a homogeneous cluster, within which eight subclusters consisted of strains having at most three-band differences. Reference strains, with the exception of the L. carnosum type strain, clustered separately from the industrial isolates. Isolates associated with the sensorially spoiled products all showed the type A I-h pattern (Fig. 4, lanes 8 and 17) and belonged to the largest cluster, consisting of A I types (Fig. 5 and Table 1). Type A I-h was also detected in two raw-meat mass samples and two air samples, one from the macerating room and one from the postcooking form removal area.

FIG. 3. HindIII ribopatterns. Lanes 4 and 11, phage lambda DNA cleaved with HindIII as a fragment size marker; lane 1, Weissella viridescens ATCC 12706T; lane 2, Weissella halotolerans ATCC 35410T; lane 3, Weissella paramesenteroides DSM 20288T; lane 5, Leuconostoc mesenteroides subsp. mesenteroides DSM 20343T; lane 6, Leuconostoc mesenteroides subsp. cremoris CCUG 21965T; lane 7, Leuconostoc mesenteroides subsp. dextranicum DSM 20484T; lane 8, Leuconostoc pseudomesenteroides DSM 20193T; lane 9, Leuconostoc carnosum NCFB 2776T; lane 10, Leuconostoc gelidum NCFB 2775T; lane 12, Leuconostoc lactis CCUG 30064T; lane 13, Leuconostoc fallax CCUG 30061T; lane 14, Leuconostoc citreum (Leuconostoc amelobiosum) D1.

FIG. 4. SmaI (lanes 1 to 9) and ApaI (lanes 10 to 18) ribopatterns. Lanes 1, 9, 10, and 18, Leuconostoc carnosum NCFB 2776T; lanes 2 and 11, Leuconostoc mesenteroides subsp. mesenteroides DSM 20343T; lanes 3 and 12, Leuconostoc mesenteroides subsp. dextranicum DSM 20484T; lanes 4 and 13, Leuconostoc pseudomesenteroides DSM 20193T; lanes 5 and 14, Weissella paramesenteroides DSM 20288T; lanes 6 and 15, Leuconostoc gelidum NCFB 2775T; lanes 7 and 16, Leuconostoc citreum (Leuconostoc amelobiosum) D1; lanes 8 and 17, Leuconostoc carnosum V-8a.
DISCUSSION

*L. carnosum* was identified as the specific spoilage organism in the vacuum-packaged, cooked ham studied here. This species was described along with *L. gelidum* by Shaw and Harding in 1989 (50). It belongs to the main *Leuconostoc* cluster designated *Leuconostoc* sensu stricto and shares 97 to 99% rRNA homology with the other sensu stricto species: *L. citreum, L. gelidum, L. lac- 

tis, L. mesenteroides, and L. pseudomesenteroides* (15). Characterization studies of *L. carnosum* have been sparse and have been done with a limited number of strains (50, 58). Studies associated with *L. carnosum* have mainly focused on the production and purification of bacteriocins produced by this species (3, 20, 23, 26, 40, 41, 49, 52, 54).

*L. carnosum* seems to be strongly associated with ham products. In an earlier meat production plant contamination study (6), type A was found to dominate in the microflora of the raw-pork mass macerated overnight. In this plant, we noted that *L. carnosum* contamination occurred mainly in ham, whereas *L. sake* and *L. curvatus* have been detected in a variety of products (4, 6). Approximately 36% of the spoilage flora in Vienna sausages has been reported to consist of *leuconostocs* (17). When these *Leuconostoc* species were identified (18), the absence of *L. carnosum* contamination was emphasized. In another characterization study of the LAB causing spoilage in vacuum-packaged, processed meats, a high prevalence of bacteriocin-producing psychrotrophic *leuconostocs* was revealed (58). In that study, nine isolates were identified as *L. carnosum*; eight of these nine originated from different types of ham and one originated from sliced turkey. The strains forming the *L. carnosum* cluster (III) in the work describing this species (50) were from cold-stored, vacuum-packaged beef, pork, bacon, cooked ham, and luncheon meat. Compared with ham and other whole-meat products, emulsion sausages have more variable raw materials, such as different meat mixtures, pork skin emulsion, and spices, and undergo a different type of processing. The process and ingredients used for ham manufacturing may favor the survival and/or growth of *L. carnosum*. However, an adequate cooking process, considered to be the most important factor destroying LAB on products prior to packaging (1, 33, 34, 36), and the use of nitrite are similar in the production of emulsion sausages and whole-meat products.

PFGE characterization of *L. carnosum* confirmed the assumption that the raw-meat mass was the major source of contamination. The type of LAB contamination in a product has been considered to reflect the type of contamination in the processing facility (25, 38). Various LAB types were shown to contaminate the environment associated with the ham processing line studied here (4, 6). The greatest diversity in the different types of LAB was found in the environmental surface samples (4, 6). However, the majority of these LAB types have never been isolated from packaged products (4, 6). Only type A I-h isolates associated with the spoiled packages (V isolates), raw material (M50 and M6a isolates), and air of the macerating room (isolate I2b) and postcooking form removal area (isolate I27a) contaminated the products before they were transferred to the slicing-packaging department. The products were contaminated with a spoilage organism from the raw-meat mass before they entered the slicing line. In this case, the slicing line and the slicing room were not the main site and source of contamination, as is so often thought (25, 38). This route of contamination may be more common than is generally considered, also explaining the link between raw-meat mass and cooked ham.

Identification of species of the genus *Leuconostoc* is difficult
(15, 55), which apparently is the main reason for the sparse population characterizations published. Leuconostoc spp. are phenotypically related to Weissella spp., heterofermentative lactobacilli, and pediococci and form a natural phylogenetic group with Weissella confusa, W. halotolerans, Weissella kandleri, Weissella minor, and W. viridescens (15). Due to the variable results obtained, sugar fermentation patterns are of little value in the species identification and could lead to misclassification (15). For presumptive identification, the scheme proposed by Villiani et al. (55) was found practical. However, in this scheme L. carnosum is supposed to form dextran. Only 15 of the 100 isolates tested here (11 of the established 25 PFGE types) formed slime from sucrose, lessening the value of this characteristic in L. carnosum identification.

It has been stated that reliable differentiation between L. carnosum and L. gelidum is impossible without DNA-DNA hybridization (15). Our results indicate that ribotyping can be used to distinguish L. carnosum from the other phenotypically related leuconostocs. However, care must be taken when enzymes are selected for species identification by ribotyping. Using HindIII-based ribopatterns, Villiani et al. (55) could not distinguish L. mesenteroides subsp. mesenteroides from L. mesenteroides subsp. dextranicum and L. lactis. We found HindIII and EcoRI to be the least distinguishing enzymes and ClaI to be the only enzyme generating a clear one-band shift in the patterns of these two subspecies (Fig. 1, lanes 5 and 7). ClaI may thus provide better results for the discrimination of L. mesenteroides subspecies. However, the HindIII pattern of the L. lactis type strain was clearly distinguished from the patterns of the L. mesenteroides subspecies (Fig. 3, lanes 5, 7, and 12). Despite its value in species identification and LAB contamination studies dealing with a diversity of species, ribotyping cannot be used for strain characterization when such a homogeneous population, such as the population of L. carnosum isolated from the meat production plant studied here, is assessed.

Only one type, A I-h (Fig. 4, lanes 8 and 17), from the largest lineage, was associated with the sensorially spoiled packages; strains of this type were clearly distinguished from the patterns of the type strain characterized by ClaI (Fig. 4, lanes 8 and 17). This situation differs clearly from the situation in which some or one species in a contaminating flora gradually occupies a niche in a package and, finally, when reaching the stationary phase, spoils the product.

Molecular typing methods also provide valuable information for applied microbiology. They can contribute to knowledge of different bacterial populations associated with food processing and enable future research to be focused accurately on specific spoilage organisms and their specific characteristics. Such work will rely mainly on the reliable species identification and good strain characterization of specific spoilage organisms.

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