Characterization and Pathogenic Potential of *Listeria monocytogenes* Isolates from the Smoked Fish Industry

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This study was designed to evaluate the hypothesis that some of the *Listeria monocytogenes* subtypes associated with foods, specifically smoked fish, may have an attenuated ability to cause human disease. We tested this hypothesis by using two different approaches: (i) comparison of molecular subtypes found among 117 isolates from smoked fish, raw materials, fish in process, and processing environments with subtypes found among a collection of 275 human clinical isolates and (ii) the evaluation of the cytopathogenicity of industrial isolates. Ribotyping and PCR-restriction fragment length polymorphism typing of the *hlyA* and *actA* genes differentiated 23 subtypes among the industrial isolates and allowed classification of the isolates into three genetic lineages. A significantly higher proportion of human isolates (69.1%) than industrial isolates (36.8%) were classified as lineage I, which contains human sporadic isolates and all epidemic isolates. All other industrial isolates (63.2%) were classified as lineage II, which contains only human sporadic isolates. Lineage I ribotypes DUP-1038B and DUP-1042B represented a significantly higher proportion of the human isolates than industrial isolates (5.1%). Lineage II ribotypes DUP-1039C, DUP-1042C, and DUP-1045, shown previously to persist in the smoked fish processing environment, represented nearly 50% of the industrial isolates, compared to 7.6% of the human isolates. Representatives of each subtype were evaluated with a tissue culture plaque assay. Lineage I isolates formed plaques that were significantly larger than those formed by lineage II isolates. Isolates from the smoked fish industry representing three ribotypes formed no plaques or small plaques, indicating that they had an impaired ability to infect mammalian cells. While *L. monocytogenes* clonal groups linked to human listeriosis cases and outbreaks were isolated, our data also suggest that at least some *L. monocytogenes* subtypes present in ready-to-eat foods may have limited human-pathogenic potential.

Listeria monocytogenes is responsible for nearly one-fourth of all estimated food-borne-disease-related deaths caused by known pathogens in the United States each year, which highlights its significance as a public health concern (25). The majority of human listeriosis cases occur in pregnant women, neonates, immunosuppressed individuals, and the elderly (12). As a growing segment of our population falls into high-risk groups, improved methods for reducing the levels of *L. monocytogenes* in foods are essential. A better understanding of the ecology, transmission, and pathogenicity of this organism should facilitate development of effective strategies.

While *L. monocytogenes* causes relatively few human disease cases, particularly compared to many other food-borne pathogens (25), it appears to be commonly present in raw and ready-to-eat foods. U.S. Department of Agriculture data, for example, indicated a 2.5% prevalence of *L. monocytogenes* in 3,547 samples of ready-to-eat products surveyed in 1998 and a 4.6 and 2.7% prevalence in sliced ham and pork and in sliced roasted and corned beef, respectively, sampled in 1999 (http://www.fsis.usda.gov/oa/topics/lm_action.htm). A number of other studies (for a review, see reference 12) have cited a notable 2 to 10% prevalence of *L. monocytogenes* in a variety of foods surveyed (12). However, some studies report much a

higher prevalence. Surveys of raw chicken, for example, show contamination rates ranging from 12 to 60% (12). Interestingly, the prevalence of *L. monocytogenes* in cold-smoked salmon and cooked fish products has been reported to range from 6 to 36% to as high as 78% (2, 10, 20). Nevertheless, cold-smoked fish and other seafoods are infrequently associated with human listeriosis (11, 21, 29). Thus, we believe that the smoked fish industry represents a good model system to investigate the hypothesis that some of the *L. monocytogenes* subtypes present in foods and food-processing environments may have limited human-pathogenic potential. Alternatively, human listeriosis cases linked to smoked fish may occur as commonly as cases linked to other foods. Insufficient epidemiological data have been gathered, however, to track the sources of such cases.

There is already evidence that there are differences in the human-pathogenic potentials of *L. monocytogenes* subtypes. Wiedmann et al. previously reported classification of *L. monocytogenes* isolates into three genetic lineages based on (i) ribotyping, a subtyping method based on restriction polymorphisms in the rRNA operon of prokaryotes (7, 8, 18); and (ii) allelic polymorphisms in the virulence genes *hlyA*, *inlA*, and *actA* (37). In their initial analysis, which included 20 human clinical *L. monocytogenes* isolates, all human epidemic isolates were classified into lineage I, while sporadic case isolates were classified into lineages I and II. No human isolates were classified into lineage III (37). This and other studies (27, 28) also

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found evidence of a clonal population structure of *L. monocytogenes* and no evidence of horizontal gene transfer. Thus, ribotyping and virulence gene alleles represent reliable markers for subgroup and lineage classification. Rasmussen et al. classified *L. monocytogenes* strains into three comparable lineages, as shown by analysis of several identical strains and similar classification of clinical isolates based on DNA sequence analysis of the *hlyA*, *iap*, and *flaA* genes (28). In addition to evidence provided by these studies, only 3 of 13 known serotypes (serotypes 1/2a, 1/2b, and 4b) are responsible for the majority of human listeriosis cases (23, 34). A total of 144 human isolates from sporadic cases were serotyped by the Centers for Disease Control in 1986, for example, and the majority (66%) were serotype 1/2b and 4b isolates, which classified into lineage I (34; C. A. Nadon, D. Woodward, C. Young, F. Rodgers, and M. Wiedmann, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. p-96, p. 533, 2000). Of 1,363 human isolates collected in the United Kingdom, 74% were serotype 1/2b and 4b isolates (23), further supporting the hypothesis that lineage I strains have a unique pathogenic potential. Studies by different groups have found evidence that lineage I strains, particularly serotype 4b strains, are isolated less frequently from foods and animals with listeriosis than from humans (12, 13, 30, 31, 37). Thus, the high frequency of lineage I and serotype 4b strains among human listeriosis cases and outbreaks probably cannot be explained by more frequent exposure of susceptible individuals to such strains.

In an effort to better understand the genetic characteristics and virulence properties of *L. monocytogenes* strains associated with foods and the food-processing environment, we characterized a collection of *L. monocytogenes* isolates from the smoked fish industry and compared them to human isolates. Specifically, the objectives of this study were (i) to characterize *L. monocytogenes* isolates recovered from samples of cold-smoked fish, raw materials, fish in process, and the smoked-fish-processing environment by ribotyping and allelic typing of the virulence genes *hlyA* and *actA*, (ii) to compare the rates of recovery of lineage I, II, and III strains and specific ribotypes for industrial isolates and a collection of human clinical isolates, and (iii) to evaluate the cytopathogenicity of the industrial isolates by a cell culture plaque assay.

MATERIALS AND METHODS

***L. monocytogenes* strains and isolates.** A total of 117 isolates from cold-smoked fish, raw fish, fish in process, and the smoked-fish-processing environment were characterized in this study. Of these, 85 isolates were recovered from samples collected from three East Coast smoked fish processors during five visits to each facility (6-month sampling period) in 1998. Specifically, 44 isolates were recovered from environmental samples, 9 isolates were recovered from raw materials, 21 isolates were recovered from fish during the brining process, and 11 isolates were recovered from cold-smoked fish (26). Isolates from cold-smoked salmon ($n = 30$) and raw salmon ($n = 2$) recovered from samples from Washington, Oregon, Alaska, Florida, New York, California, and Canada were kindly provided by the Northwest Fisheries Science Center (NFSC), Seattle, Wash. The 275 human clinical isolates used for comparative analyses are maintained in the Cornell *Listeria* Strain Collection. This collection includes the World Health Organization *L. monocytogenes* strain collection (4). The majority of the other isolates in this collection were obtained from patients with listeriosis symptoms in New York, Connecticut, Ohio, South Carolina, Michigan, Massachusetts, Oregon, Vermont, Arizona, some other U.S. states, and Canada primarily during 1997 to 1999 (M. Wiedmann, B. Saunders, J. Hibbs, D. Morse, L. Kornstein, T. Bannerman, S. E. Dietrich, and J. Massey, unpublished data). Only one representative human isolate from single-source clusters (represented by multiple

isolates in our collection) was included in analyses in order to avoid overrepresentation of specific subtypes.

Ribotyping. Automated ribotyping with normalized data was performed by using the RiboPrinter Microbial Characterization System (DuPont Qualicon, Wilmington, Del.) as previously described (7, 8, 18) at the Laboratory for Molecular Typing at Cornell University. This automated typing method involves *EcoRI* digestion of *L. monocytogenes* chromosomal DNA, followed by Southern hybridization with an *Escherichia coli* *rmB* rRNA operon probe. Images are acquired with a charge-coupled device camera and are analyzed by using custom software that normalizes fragment pattern data for band intensity and relative band size compared to a molecular weight marker.

Characterization of virulence genes *hlyA* and *actA*. The genes encoding the virulence factors listeriolysin O (*hlyA*) and ActA (*actA*) were screened for allelic polymorphisms as described by Wiedmann et al. (37). Briefly, *hlyA* was characterized by PCR-restriction fragment length polymorphism analysis. Following amplification of *hlyA*, PCR products were digested separately with restriction endonucleases *HhaI* and *HpaI*. One of eight allelic types was assigned based on the restriction pattern. Following amplification of *actA*, one of two alleles was assigned based on a product size difference which corresponded to the presence or absence of a 105-nucleotide direct repeat encoding a proline-rich repeat structure. *hlyA* typing and ribotyping allowed classification of each isolate into genetic lineage I, II, or III (37).

Cell culture plaque assay. The cytopathogenicities of selected isolates from cold-smoked fish, raw fish, fish in process, and the smoked-fish-processing environment were evaluated with a plaque assay performed with mouse L cells as previously described (35, 37). All NFSC isolates were evaluated for cytopathogenicity. Of the 85 isolates previously recovered by our group (26), representatives of each *L. monocytogenes* subtype, as differentiated by ribotype, *hlyA* type, and *actA* type, recovered from different sources at each facility were selected for analysis ($n = 47$). If available, two environmental isolates from each of the three processing facilities, along with a raw material isolate and a cold-smoked fish isolate recovered from samples taken at each facility, were evaluated for each subtype. If more than two environmental isolates of a given subtype were available from a facility, isolates obtained from two temporally separated samples were selected.

After *L. monocytogenes* isolates were grown overnight at 30°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.), cells were pelleted and then resuspended and serially diluted in phosphate-buffered saline (pH 7.4). Five microliters of a 10^{-2} dilution and 15 μ l of a 10^{-3} dilution were used as inocula for two wells containing monolayers of L cells. Inocula were enumerated by plating serial dilutions onto brain heart infusion agar in duplicate and incubating at 37°C. For each assay the average diameter based on at least 25 plaques was determined and expressed as a percentage of the plaque size of *L. monocytogenes* 10403S, a commonly used laboratory strain (5) which was included as an internal standard for each assay and assigned a value of 100%. Assays in which the relative plaque size had standard deviations of $\geq 20\%$ were repeated. Assays were also repeated for isolates that did not form plaques or that formed plaques with relative sizes that were $\leq 75\%$ of the internal standard size in order to confirm results. Plaquing efficiency (CFU/PFU) was determined and expressed as a percentage of the internal control *L. monocytogenes* 10403S value.

Statistical analyses. The rates of recovery of lineage I, II, or III strains and specific ribotypes for the isolate sources (human clinical isolates versus isolates from the smoked fish industry) were compared by using Pearson's chi-square test. Since the expected values for some cells were less than five, exact *P* values were calculated. The analyses described above were performed by using the statistical software program StatXact-4 for Windows (CYTEL Software Corporation, Cambridge, Mass.). No analyses were attempted for ribotypes isolated less than four times overall. *P* values of ≤ 0.05 were considered significant. Since additional studies are needed to confirm and extend these observations, adjustments to the alpha level of significance were not made for the multiple comparisons, but rather the actual *P* values are provided here. Box and whisker plots, which were used to graphically present measurements of central tendency and the variability of plaque assay data for different genetic lineages, were generated by using the software program Statistix for Windows (Analytical Software, Tallahassee, Fla.). The rank sum test (Mann-Whitney test) was used to test for differences in relative plaque size and plaquing efficiency in different lineages. A dot plot, which was used to graphically present relative plaque size according to ribotype, was generated by using the software program Microsoft Excel (Microsoft Corporation, Redmond, Wash.).

TABLE 1. Genetic characterization of *L. monocytogenes* isolates from raw fish, fish in process, cold-smoked fish, and the smoked-fish-processing environment

Lineage	Ribotype ^a	<i>actA</i> allele	<i>hlyA</i> allele	No. of isolates (n = 117)
I	DUP-1038B	3	1	2
	DUP-1042B	4	1	4
	DUP-1042C	3	1	6
		4	1	8
	DUP-1042D	3	1	1
	DUP-1043	3	1	2
	DUP-1044A	4	1	14
	DUP-1052	3	1	5
	116-459-S-1	3	1	1
	DUP-1023	4	2	2
II	DUP-1030A	4	2	2
	DUP-1039A	3	2	2
		4	2	2
	DUP-1039C	3	2	1
		4	2	24
	DUP-1045	3	2	9
		4	2	7
	DUP-1046A	3	2	1
	DUP-1046B	4	2	4
	DUP-1048	4	2	1
	DUP-1053	4	2	7
	DUP-1062	3	2	5
		4	2b	7

^a Subtypes A, B, C, and D are assigned based on riboprint pattern variation within a given ribotype.

RESULTS

Genetic characterization of *L. monocytogenes* isolates. A total of 117 *L. monocytogenes* isolates from raw fish, fish in process, cold-smoked fish, and the smoked-fish-processing environment were genetically characterized by ribotyping and screening for allelic polymorphisms in the virulence genes *hlyA* and *actA* (Table 1). Eighteen ribotypes were represented by these isolates. *L. monocytogenes* ribotypes DUP-1039C and DUP-1045 were isolated with the highest frequency (21.4 and 13.7%, respectively). Ribotyping results, together with allelic typing of *actA* and *hlyA*, allowed differentiation of 23 strains among the isolates. The strains were classified into genetic lineage I, II, or III based on their *hlyA* alleles and ribotypes (37). As shown in Table 1, 43 and 74 isolates were classified into lineages I and II, respectively. No isolates were classified into lineage III.

Distribution of *L. monocytogenes* lineage I, II, and III strains among human and industrial isolates. The rates of recovery of specific *L. monocytogenes* ribotypes and lineage I, II, and III strains from the smoked fish industry were compared to the rates of recovery for a collection of 275 human sporadic and epidemic listeriosis case isolates. The industrial isolates were analyzed on the basis of the following three sample definitions. (i) The first grouping included all 117 isolates with no exclusions for isolates that may have been related (e.g., isolates originating from the same source). (ii) The second grouping consisted of 72 isolates. We removed multiple isolates of a given subtype that may have originated from a common source and, if not removed, might have resulted in overrepresentation of some ribotypes. In a previous study, we analyzed the distribution of specific ribotypes among the three processing facili-

ties from which samples were collected (26). Several ribotypes (DUP-1039C, DUP-1042C, and DUP-1045) were shown to persist in the environments of specific facilities for up to 6 months. Furthermore, the distributions of these ribotypes and ribotype DUP-1046B among the three facilities were shown to be significantly different. As it is possible that these strains were part of the resident microflora and could have represented isolates from a single source, only one representative of each ribotype for each plant was included in the second sample definition to avoid overrepresentation of the subtypes. For isolates obtained from the NFSC, only one representative of each ribotype isolated from samples with a high probability of originating from the same lot of cold-smoked fish was included in this sample (F. Poysky, NFSC, personal communication). (iii) The group based on the most conservative sample definition consisted of 60 isolates. As isolates of a given ribotype recovered from samples collected on a single visit to a processing facility may be related due to tracking or cross-contamination, each ribotype isolated during our previous study was counted only once per visit to each facility. The NFSC isolates were included as described above for the second sample definition. The data resulting from evaluation of all isolates and the second sample definition ($n = 72$) are presented in Tables 2 and 3 for comparison. We believe that the second sample size most accurately represents the number of independent observations, as multiple isolates of a given subtype isolated from different samples at a facility could have originated from different sources.

Table 2 summarizes the distribution of *L. monocytogenes* isolates from human clinical samples and samples from the smoked fish industry. The distribution of lineage I, II, and III strains varied significantly for human and industrial isolates regardless of the sample definition used for the industrial isolates ($P \leq 0.003$). To better assess the source of variation across the three lineages, the distribution of isolates in one lineage was compared to that in each of the others in a separate analysis. Lineage I strains were found to be significantly more common among human isolates (compared to industrial isolates) than lineage II strains regardless of the sample definition used in the analysis ($P \leq 0.0006$). There were no significant differences in the distributions of lineage I and III strains or lineage II and III strains between human and industrial isolates ($P \geq 0.05$).

Distribution of specific *L. monocytogenes* ribotypes among human and industrial isolates. We also compared the distri-

TABLE 2. Distribution of *L. monocytogenes* lineage I, II, and III strains among a collection of human clinical isolates and isolates from the smoked fish industry

Genetic lineage	No. (%) of human isolates (n = 275)	No. (%) of fish isolates (n = 117) ^a	No. (%) of fish isolates (n = 72) ^b
I	190 (69.1)	43 (36.8)	34 (47.2)
II	80 (29.1)	74 (63.2)	38 (52.8)
III	5 (1.8)	0 (0.0)	0 (0.0)

^a All smoked fish industry isolates were included in the analysis.

^b Isolates shown to persist in the environments of smoked-fish-processing facilities were counted only once for each of three facilities (26). One representative of NFSC ribotypes likely to be isolated from samples from a single lot was included in the analysis.

TABLE 3. Distribution of specific *L. monocytogenes* ribotypes among a collection of human clinical isolates and isolates from the smoked fish industry

Lineage	Ribotype	No. (%) of human isolates (<i>n</i> = 275)	All industrial isolates ^a		Independent isolates ^c	
			No. (%) of fish isolates (<i>n</i> = 117)	<i>P</i> value ^b	No. (%) of fish isolates (<i>n</i> = 72)	<i>P</i> value ^b
I	DUP-1024	4 (1.5)	0 (0.0)	NS ^d	0 (0.0)	NS
	DUP-1025	4 (1.5)	0 (0.0)	NS	0 (0.0)	NS
	DUP-1038B ^e	40 (14.5)	2 (1.7)	0.0001	2 (2.8)	0.0071
	DUP-1042A ^e	15 (5.5)	0 (0.0)	0.0175	0 (0.0)	0.0476
	DUP-1042B ^e	55 (20.0)	4 (3.4)	<0.0000	4 (5.6)	0.0042
	DUP-1042C ^e	0 (0.0)	14 (12.0)	<0.0000	6 (8.3)	0.0001
	DUP-1043	6 (2.2)	2 (1.7)	NS	2 (2.8)	NS
	DUP-1044A ^e	12 (4.4)	14 (12.0)	0.0080	14 (19.4)	0.0001
	DUP-1044B ^e	14 (5.1)	0 (0.0)	0.0133	0 (0.0)	NS
	DUP-1052	24 (8.7)	5 (4.3)	NS	4 (5.5)	NS
	116-363-S2	4 (1.5)	0 (0.0)	NS	0 (0.0)	NS
II	DUP-1030A	5 (1.8)	2 (1.7)	NS	2 (2.8)	NS
	DUP-1030B	4 (1.5)	0 (0.0)	NS	0 (0.0)	NS
	DUP-1030C	7 (2.5)	0 (0.0)	NS	0 (0.0)	NS
	DUP-1039A	9 (3.3)	4 (3.4)	NS	4 (5.6)	NS
	DUP-1039B	8 (2.9)	0 (0.0)	NS	0 (0.0)	NS
	DUP-1039C ^e	13 (4.7)	25 (21.4)	<0.0000	3 (4.2)	NS
	DUP-1045 ^e	8 (2.9)	16 (13.7)	<0.0000	6 (8.3)	0.0479
	DUP-1046A	3 (1.1)	1 (0.8)	NS	1 (1.4)	NS
	DUP-1046B ^e	0 (0.0)	4 (3.4)	0.0077	1 (1.4)	NS
	DUP-1053	13 (4.7)	7 (6.0)	NS	7 (9.7)	NS
	DUP-1062 ^e	6 (2.2)	12 (10.3)	0.0010	11 (15.3)	0.0001

^a All industrial isolates were included in the analysis.

^b An exact *P* value was calculated for Pearson's chi-square test since expected values in some cells were less than 5.

^c Isolates shown to persist in the environments of smoked-fish-processing facilities ($P \leq 0.05$) were counted only once for each of three facilities (26). One representative of NFSC ribotypes likely to be isolated from samples from a single lot was included in the analysis.

^d NS, not significant ($P \geq 0.05$).

^e Ribotype for which distributions were shown to vary significantly among sample sources ($P \leq 0.05$).

butions of *L. monocytogenes* ribotypes among human clinical and industrial isolates. Preliminary analyses revealed that the overall distributions of ribotypes among human clinical isolates and industrial isolates were statistically different ($P < 0.0000$ regardless of sample definition). A summary of specific ribotypes, their prevalence among human clinical isolates and industrial isolates, and the results of analyses comparing the prevalence of each ribotype for human and industrial isolates is presented in Table 3. Ribotypes DUP-1038B and DUP-1042B (lineage I strains) were isolated most frequently from human clinical samples and represented nearly 35% of this collection of isolates. In contrast, these ribotypes represented only 1.7 and 3.4%, respectively, of all isolates from the smoked fish industry. As shown in Table 3, these ribotypes were shown to be significantly less common among the industrial isolates ($P \leq 0.0071$ and $P \leq 0.0042$, respectively).

L. monocytogenes ribotypes DUP-1042A and DUP-1044B each represented approximately 5% of the human clinical isolates, while they were not isolated from smoked fish industry samples. Both of these ribotypes were also significantly less common among fish industry-related samples than among human isolates if all industrial isolates ($n = 117$) were included in the analysis. The distribution of ribotype DUP-1044B did not vary significantly, however, if the adjusted sample sizes ($n = 72$ and $n = 60$) were used in analyses. Lineage I ribotype DUP-1044A represented at least 12% of the industrial isolates, a proportion significantly higher than the proportion of human isolates that it represented (4.4%; $P \leq 0.0080$).

L. monocytogenes ribotypes DUP-1045, DUP-1042C, and

DUP-1039C, which persisted in the processing facilities from which they were recovered for 2.5, 5, and 6 months, respectively (26), were significantly more prevalent among industrial isolates than among human isolates (Table 3). No significant difference in the prevalence of DUP-1039C was found, however, if the adjusted sample definitions were used in analyses. *L. monocytogenes* ribotype DUP-1062 was also significantly more common (10%) among industrial isolates; in contrast, it represented only 2.2% of human clinical isolates ($P \leq 0.0010$ for all three sample definitions used for analysis).

Cytopathogenicity characterization of isolates. The cytopathogenicities of selected isolates from the smoked fish industry were evaluated by a cell culture plaque assay performed with mouse L cells. As *L. monocytogenes* spreads from cell to cell, plaques are formed in the L-cell monolayer, and these plaques can be enumerated and measured. The plaque size data, expressed as percentages of the internal control *L. monocytogenes* 10403S value, are summarized by lineage in Fig. 1. Lineage I isolates ($n = 35$) formed plaques ranging in relative size from 70.7 to 139.3%, and the median relative size was 101.4%. The median relative plaque size for lineage II isolates ($n = 44$) was 89.6%, and the range was 45.6 to 104.9%. The median plaque size of lineage I isolates was statistically significantly greater than that of lineage II isolates ($P < 0.0000$), regardless of whether outlier values were included in the analysis.

A more detailed presentation of relative plaque sizes, in which isolates are placed into subgroups according to ribotype, is shown in Fig. 2. All ribotype DUP-1044A strains ($n = 10$)

FIG. 1. Box and whisker plot of relative plaque sizes (expressed as percentages of the size of *L. monocytogenes* 10403S plaques) of *L. monocytogenes* lineage I and II isolates from the smoked fish industry. The middle of half of the data are enclosed in a box. Each box is bisected by a line representing the median value. The vertical lines (whiskers) represent the ranges of values. The asterisks indicate possible outliers (values outside the boundaries of a box by more than 1.5 times the size of the box). The median plaque sizes are significantly different.

formed plaques that were larger (median relative plaque size, 108.3%) than those formed by the internal control, *L. monocytogenes* 10403S. Similarly, three of four ribotype DUP-1042B isolates and both ribotype DUP-1038B isolates formed plaques that were larger than those of the internal control. In contrast, all but two ribotype DUP-1042C isolates ($n = 11$) formed plaques smaller than those of *L. monocytogenes* 10403S. Ribotype 116-459-S-1, classified as a lineage I strain on the basis of the *hlyA* allele (type 1), and all three ribotype DUP-1046B isolates evaluated did not form plaques in this assay. The majority of the ribotype DUP-1039C ($n = 8$) and DUP-1045 ($n = 12$) isolates, which represented strains which persisted in the processing environments of the facilities from which they were isolated, also formed plaques that were smaller than those of the control strain (median values, 91.1 and 60.6%, respectively). No statistical analyses were attempted due to the numerous ribotypes represented by the industrial isolates and due to the small number of observations for each ribotype.

Plaques efficiency is a measure of the CFU of *L. monocytogenes* required to form one plaque in an L-cell monolayer. The plaques efficiency data are expressed as percentages of an internal control CFU/PFU value. Higher values indicate that a higher number of *L. monocytogenes* cells are required to form one plaque in this assay. The relative efficiencies for lineage I strains ranged from 31.3 to 320.4%, and the median was 92.0%. The median relative plaques efficiency for lineage II strains was 110.0%, and the values ranged from 32.3 to 779.9%. A high degree of variability within the two lineages was observed for plaques efficiency. While the median relative plaques efficiency of lineage II strains was nearly 20% higher than that of lineage I strains, this was not a statistically significant difference.

DISCUSSION

This study was designed to evaluate the hypothesis that some of the *L. monocytogenes* subtypes present in foods may have no

ability or an attenuated ability to cause human disease. We tested this hypothesis by using the following approaches: (i) we compared the rates of recovery of specific subtypes for isolates from the smoked fish industry and a collection of human clinical isolates and (ii) we evaluated the cytopathogenicities of the industrial isolates.

Comparison of the proportions of *L. monocytogenes* subtypes among human and industrial isolates. In a previous study, we isolated *L. monocytogenes* from samples collected from three smoked-fish-processing facilities (26). Analysis of ribotyping data revealed that each facility had a unique contamination pattern and that different ribotypes persisted in the environments of specific facilities. Surprisingly, none of the 85 isolates were classified as lineage III isolates. We thus chose to char-

FIG. 2. Dot plot of relative plaque sizes (expressed as percentages of the size of *L. monocytogenes* 10403S plaques) of specific *L. monocytogenes* ribotypes isolated from smoked fish industry samples. The asterisks indicate ribotypes classified into genetic lineage I. All other ribotypes belong to lineage II.

acterize these and an additional set of isolates from the smoked fish industry in order to explore the pathogenic potentials of food and environmental *L. monocytogenes* isolates. Molecular subtyping of these isolates led to their classification into lineages I (36.8%) and II (63.2%). Statistical analysis, in which adjusted sample definitions were used for industrial isolates to compensate for oversampling of persistent isolates, indicated that lineage I strains were more likely to be isolated from human clinical samples than from samples collected from the smoked fish industry. While the isolates used in our study may not be truly representative of all human clinical isolates or the subtypes most prevalent in the smoked fish industry, our results correlate well with those of previous investigations and provide a basis for designing population-based studies in the future. Several groups have shown that strains classified into our lineage I, which includes serotypes 1/2b and 4b (Nadon et al., Abstr. 100th Gen. Meet. Am. Soc. Microbiol.), are more likely to cause human disease than isolates classified into lineages II and III (27, 28, 36, 37). Our findings are also consistent with the results of surveys of *L. monocytogenes* subtypes isolated from other foods. Serotype 1/2 strains appear to be the most common *L. monocytogenes* isolates obtained from foods, including vegetables, meats and raw milk (12, 13, 30, 31). Serotype 1/2c, which is classified along with serotype 1/2a into genetic lineage II (Nadon et al., Abstr. 100th Gen. Meet. Am. Soc. Microbiol.), is the serotype most frequently isolated from meat products and is rarely associated with human disease (12). Previous studies have indicated that serotype 4b strains, which are classified into genetic lineage I (Nadon et al., Abstr. 100th Gen. Meet. Am. Soc. Microbiol.), are not among the common food isolates (12, 13, 30, 31). Given the frequent association of serotype 4b with human listeriosis (12, 19, 23, 33, 34), these observations support the hypothesis that lineage I strains may have increased human-pathogenic potential compared to lineage II strains.

In a more detailed analysis, we compared the prevalence of specific ribotypes among human clinical isolates to the prevalence observed in the smoked fish industry. Our results show that ribotypes DUP-1038B and DUP-1042B, which represented almost 35% of the human isolates, are seven times more likely to be isolated from human clinical samples than from smoked fish industry samples. Ribotypes DUP-1038 and DUP-1042 represent the two major *L. monocytogenes* epidemic clones that have been implicated in multiple listeriosis outbreaks. Specifically, ribotype DUP-1038 strains were implicated in listeriosis epidemics in Nova Scotia (coleslaw), Los Angeles (Mexican style soft cheese), and Switzerland (soft smear cheese) (3, 22, 32). *L. monocytogenes* ribotype DUP-1042 strains were implicated in epidemics in Boston (raw vegetables), Massachusetts (pasteurized milk), and the United Kingdom (pate) (14, 17, 24). The low prevalence of these ribotypes among our industrial isolates is consistent with the findings of a survey of 72 fish products for *L. monocytogenes* conducted by Boerlin et al. (6). Epidemic-associated multilocus enzyme electrophoresis type 1 (27), which correlates with ribotype pattern DUP-1038 (37), was isolated only once during the study.

A significantly higher proportion (12%) of *L. monocytogenes* ribotype DUP-1044A isolates was observed among smoked fish industry samples than among human clinical samples. This

ribotype is closely related to the serotype 4b strain implicated in the 1998-1999 multistate listeriosis outbreak, which was linked to consumption of contaminated hot dogs and deli meats (1). It is particularly interesting that this ribotype, which has otherwise been implicated infrequently in human listeriosis cases, also represented a significant proportion of our industrial isolates. It may be that this subtype is characterized by an enhanced ability to survive or persist under environmental conditions common to smoked fish and hot dog-deli meat-processing plants (e.g., in the presence of high salt concentrations).

L. monocytogenes ribotypes DUP-1045, DUP-1042C, and DUP-1039C, which were shown in a previous study to persist in the smoked-fish-processing environment (26), represented a significantly higher proportion of the industrial isolates than the human isolates. For example, even when a more conservative sample definition was used, DUP-1045 was nearly three times as likely to be isolated from smoked fish industry samples than from human clinical samples. Interestingly, DUP-1042C was not represented among human isolates. These results suggest that some strains that have the ability to persist in a nonhost environment may have reduced transmissibility or reduced pathogenic potential for humans. Environmental adaptation and its possible effect on the virulence properties of this food-borne pathogen, however, are currently poorly understood.

Cytopathogenicity of smoked fish isolates. In a previous study evaluating a collection of predominantly animal clinical *L. monocytogenes* isolates, Wiedmann et al. demonstrated the utility of the plaque assay as a screening tool for virulence-associated phenotypes (37). Specifically, the mean plaque size for lineage I isolates ($n = 11$) was 121%, compared to a mean plaque size of 107% for lineage II isolates ($n = 10$) from animals with clinical listeriosis symptoms. Furthermore, strains previously shown to be virulence attenuated in a mouse model (9) formed plaques that were less than 65% of the size of the plaques formed by the control strain, while animal clinical isolates had plaque sizes between 81 and 139%. The virulence-attenuated strains also showed lower plaquing efficiency than virulent isolates, highlighting the utility of this assay to screen for phenotypes indicative of attenuated virulence. Thus, we employed this method to evaluate 47 representative industrial isolates in an effort to gain additional insight into their pathogenic potential. Similar to the findings of Wiedmann et al., we found a significant difference between the relative sizes of the plaques formed by lineage I and lineage II isolates (37). The median relative size of the plaques formed by lineage I industrial isolates was 11% larger than that of lineage II isolates, indicating that lineage I strains spread from cell to cell more efficiently. This observation may also be related to the hypothesized increased human-pathogenic potential of lineage I strains compared to that of lineage II strains.

Our evaluation of the cytopathogenicity data according to ribotype also supported the hypothesis that there are differences in pathogenic potential among strains of *L. monocytogenes*. We found that isolates representing 3 of the 18 of ribotypes (17%) recovered from the smoked fish industry samples had cytopathogenicity phenotypes indicative of avirulence or virulence attenuation, as indicated by a plaque size less than 65% of the relative size of plaques formed by the

internal control strain (37). Notably, the ribotype 116-459-S-1 isolate and all three ribotype DUP-1046B isolates did not form plaques (Fig. 2), indicating that the ability of these isolates to infect mammalian cells or to spread from cell to cell was impaired. In addition, these ribotypes were not isolated from human clinical samples.

The median plaquing efficiency of lineage I strains was nearly 20% higher than the plaquing efficiency of lineage II strains. While statistical analysis did not show that the observed difference was significant, probably because of the small sample sizes and the wide range of observations, we believe that these results may be biologically significant and warrant further investigation. Indeed, Wiedmann et al. observed a significant difference in the plaquing efficiencies of strains in different lineages (the mean PFU/CFU value for lineage II strains was more than 1.5 times greater than that for lineage I strains) (37). Pooling data from multiple assays of the same strain may decrease the level of variability observed in this study.

Conclusions. In summary, our results are consistent with the hypothesis that *L. monocytogenes* strains and clonal groups differ in their human-pathogenic potentials. For example, isolates representing 3 of the 18 ribotypes found among isolates from the smoked fish industry had phenotypes indicative of attenuated virulence. Additional population-based studies, combined with comprehensive comparative virulence characterization of different *L. monocytogenes* subtypes in studies that include tissue culture models (in which human and animal cell lines are used) and animal models, will be necessary to investigate differences in human-pathogenic potentials among *L. monocytogenes* subtypes further. The use of multiple typing methods (16), multiple enzymes for ribotyping (15), or other typing methods (e.g., pulsed-field gel electrophoresis) (16) may increase the discriminatory power and provide information useful for further classification of *L. monocytogenes* subtypes. The development of a significant body of data for *L. monocytogenes* subtypes and their pathogenic potentials will be necessary to develop science-based approaches to regulate the presence of this organism in ready-to-eat foods. Establishment of tolerance levels, for example, may be appropriate for virulence-attenuated strains. Furthermore, a better understanding of the characteristics of virulence-attenuated and avirulent *L. monocytogenes* subtypes and the ability to rapidly identify them could prevent product recalls due to subtypes that do not present a public health risk.

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