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Sets of *Eco*RI fragments containing ribosomal RNA sequences are conserved among different strains of *Listeria monocytogenes*

(bacteria/ribotyping/classification/identification)

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ABSTRACT **To classify *Listeria monocytogenes* using taxonomic characters derived from the rRNA operons and their flanking sequences, we studied a sample of 1346 strains within the taxon. DNA from each strain was digested with a restriction endonuclease, *Eco*RI. The fragments were separated by gel electrophoresis, immobilized on a membrane, and hybridized with a labeled rRNA operon from *Escherichia coli*. The pattern of bands, positions, and intensities of hybridized fragments were electronically captured. Software was used to normalize the band positions relative to standards, scale the signal intensity, and reduce the background so that each strain was reproducibly represented in a data base as a pattern. With these methods, *L. monocytogenes* was resolved into 50 pattern types differing in the length of at least one polymorphic fragment. Pattern types representing multiple strains were recorded as the mathematical average of the strain patterns. Pattern types were arranged by size polymorphisms of assigned rRNA regions into subsets, which revealed the branching genetic structure of the species. Subtracting the polymorphic variants of a specific assigned region from the pattern types and averaging the types within each subset resulted in reduced sets of conserved fragments that could be used to recognize strains of the species. Pattern types and reduced sets of conserved fragments were conserved among different strains of *L. monocytogenes* but were not observed in total among strains of other species.**

Strains of *Listeria monocytogenes* are classified into the taxon based on genotypic and phenotypic similarities (1, 2). A general method for classification and identification of strains by using DNA restriction fragments containing portions of rRNA operons has been described (3, 4). This method has been applied to the genus *Listeria* (5) and to *L. monocytogenes* (6), demonstrating its utility for classifying, identifying, and typing strains.

We have described a standard method for species description by using conserved sets of species-specific rRNA gene restriction endonuclease-derived fragments (7). In the present study, >1000 strains of *L. monocytogenes* were characterized by using *Eco*RI fragments containing sequences complementary to an rRNA operon from *Escherichia coli*. The pattern structure of the species was described in detail by the use of fixed electrophoretic conditions, fragment standards, electronic imaging, and software for mobility normalization. In addition, we introduced the use of continuous-scale relative intensity in recording patterns from ≈9000 strains of ≈200 species. The *L. monocytogenes* patterns were arranged into the taxonomic structure by the use of squared correlation values (8) and visual assessment. We assigned letter names to the rRNA regions, each containing a given part of a given operon, and differentiated strains by restriction fragment length polymorphisms of those regions. The structure of patterns revealed similarities, reduced sets of conserved fragments shared by multiple pattern types within subsets of the sample set, and the progression of strain variation through the classically described species.

MATERIALS AND METHODS

Classical Characterizations. The following attributes were determined as described (9): cytochrome oxidase activity, catalase activity, and hemolysin activity enhancement (CAMP test). Biochemical profiles were obtained with the Micro-ID Listeria System (Organon Teknika–Cappel). The strains, representing all serotypes, were isolated from a wide variety of animals, foods, and environmental niches.

Reagent Preparation. The *rrnB* rRNA operon from *E. coli* (10), inserted and replicated in pGEM, was digested with *Eco*RI before labeling. After digestion, the DNA was precipitated, dissolved in water to a concentration of 0.8–1.0 mg/ml, denatured by immersion in a boiling water bath, and chilled on ice. To label the DNA by sulfonation (11), a volume of 2.0 M sodium bisulfite solution (pH 5.6) equivalent to one-half of the DNA-solution volume and a volume of 1.0 M methoxylamine hydrochloride solution (pH 6.0) equivalent to one-eighth of the DNA-solution volume were added. The samples were mixed, and the pH of the solution was adjusted to 6.0 or less with HCl before incubating overnight at 30°C. Labeling reagents were removed by Sephadex G-25 (Pharmacia) column chromatography.

A conjugate of anti-sulfonated DNA monoclonal antibody (Orgenics, Yavne, Israel) and alkaline phosphatase (AP) (Boehringer Mannheim) (12, 13) was prepared by adding 15 times molar excess of *N*-succinimidyl-4-(*N*-maleimidemethyl)-cyclohexane-1-carboxylate (Pierce) at 10 mg/ml in dimethyl sulfoxide to 50 mg of dialyzed antibody in 10 mM sodium phosphate/300 mM NaCl, pH 7.0. The mixture was incubated in the dark in a 25°C water bath for 30 min and then placed on ice to stop the reaction. Unreacted cross-linking reagent was removed by Sephadex G-50 column chromatography (Pharmacia). Fractions containing activated monoclonal antibody were pooled, and the molar concentration was determined.

N-Succinimidyl-*S*-acetylthioacetate (Pierce) at 10 mg/ml in dimethyl sulfoxide was added to an AP solution (Boehringer Mannheim) at a 15 times molar excess. The amount of AP (10 mg/ml) used was that required to produce a 1:1.6 (wt/wt) ratio of monoclonal antibody to AP. The mixture was incubated in the dark in a 25°C water bath for 30 min, and the reaction was stopped by placing the mixture on ice. The sulfhydryl groups were deprotected by adding 500 μl of 1.0 M hydroxylamine for every 10 mg of AP and placing the solution in the dark in a 25°C water bath for 30 min. This reaction was also stopped by placing the mixture on ice and was followed by the removal of the unreacted cross-linking agent by Sephadex G-50 column

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Abbreviation: AP, alkaline phosphatase.

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chromatography. Fractions containing activated AP were pooled, and the molar concentration was determined.

AP containing sulfhydryl groups was conjugated to the maleimide-activated monoclonal antibody by mixing the two solutions at a ratio of 1 mg of monoclonal antibody:1.3 mg of AP and incubating the mixture in the dark in a 25°C water bath for 2 hr. This reaction was stopped by adding 18 μl of 0.1 M *N*-ethylmaleimide for every 10 mg of monoclonal antibody and incubating the mixture in a 25°C water bath for 30 min. The conjugate was concentrated by using an Amicon ultrafiltration stirred cell, model 8050, with a YM100 Diaflo membrane and purified by Sephacryl S300 column chromatography (Pharmacia) using 50 mM Tris/100 mM NaCl, pH 8.0. The purified conjugate was stored at −20°C after 1:1 (vol/vol) dilution with storage buffer (50 mM Tris·HCl, pH 8.0/150 mM NaCl/1% bovine serum albumin/2 mM $MgCl_2$/0.2 mM $ZnCl_2$).

Lysis of Bacteria and DNA Extraction. Strains grown overnight in 3 ml of brain heart infusion broth (Difco) were collected by centrifugation in a 1.5-ml tube, resuspended in 200 μl of 10 mM Tris·HCl, pH 8.0/10 mM NaCl/50 mM EDTA, pH 8.0 and heated at 75°C for 10 min. Cells were treated with 30 μl of *N*-acetylmuramidase at 1 mg/ml (Seikagaku America, Rockville, MD), 30 μl of lysozyme at 20 mg/ml (Sigma), 5 μl of lysostaphin at 5000 units/ml (Sigma), and 5 μl of RNase, DNase free at 2000 units/ml (Boehringer Mannheim) at 37°C for 15 min, followed by addition of 40 μl of crude achromopeptidase at 20 mg/ml (Wako Pure Chemical, Osaka) and 15 min of additional incubation at 37°C. After the addition of 100 μl of 10% SDS (Bio-Rad) and 126 μl of proteinase K at 10 mg/ml (Boehringer Mannheim), the solution was incubated at 65°C for 30 min. The cell lysate was transferred to a 1.5-ml phase-lock gel I light centrifuge tube (5 Prime → 3 Prime, Inc.), extracted with phenol/chloroform (Applied Biosystems), and the DNA was ethanol-precipitated with 3 M NaOAc. The precipitated DNA was collected by centrifugation, and the ethanol was removed. The pellet was washed with ethanol and air-dried for at least 15 min. The DNA was resuspended in 500 μl of 1× TE buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA, pH 8.0) and 5 μl of DNase-free RNase (Boehringer Mannheim), incubated at 37°C for 4 hr and stored at 4°C.

DNA Digestion and Electrophoresis. After determination of nucleic acid concentration by absorbance at 260 nm, 5 μg of DNA was diluted to 158 μl with water, 40 μl of 5× *Eco*RI buffer [1× *Eco*RI buffer is 100 mM Tris·HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 10 μg of bovine serum albumin], and 2 μl of *Eco*RI at 50 units/μl (Boehringer Mannheim) were added, and the DNA was digested overnight at 37°C. Loading solution was prepared by mixing 2.75 ml of a 0.25% bromophenol blue/0.25% xylene cyanol/25% Ficoll solution with 0.55 ml of 0.5 M EDTA (pH 8.0), and 2.5 μl was added to 17.5 μl of digested DNA. Four microliters of the resulting solution (87.5 ng) was applied per lane to a 0.8% agarose (SeaKem GTG, FMC) gel prepared with TTNE buffer (20 mM Tricine/50 mM Tris base/5 mM NaOAc/10 mM EDTA, pH 8.65) and electrophoresed at 40 V for 2.75 hr (Horizon 58 mini-gel, BRL). To ensure accurate data extraction, size standards consisting of pooled *Bgl* I, *Cla* I, and *Ssp* I digests of pKK3535 (10) were electrophoresed in lanes adjacent to samples yielding eight samples and five standards per gel.

Electrophoretic Transfer, Denaturation, and UV Cross-Linking. After electrophoresis, the DNA was transferred to a membrane (N04HYT, Micron Separations, Westboro, MA) with a Hoefer transfer unit (TE 22) via a 1-hr electrophoresis at 1.0 A in TTNE buffer at 4°C. After transfer, the membrane was rinsed in TTNE buffer to remove residual agarose and placed, DNA-side up, on a sheet of 3MM paper (Whatman) saturated with the denaturing solution (0.2 M NaOH/1.5 M NaCl). After 2.5 min, the membrane was transferred to a second blotter paper saturated with TTNE buffer for 15–20 s. The membrane was dried for 30 min under a heat lamp, DNA-side up, on a piece of 3MM paper. The DNA was cross-linked to the membrane with a Bios crosslinker 312T (Bios) at an automatically timed dose of 0.6 J/cm^2.

Hybridization and Detection. Each membrane was prehybridized with 5 ml of hybridization solution (denatured, sonicated, salmon-sperm DNA at 125 μg/ml/0.5 M NaCl/1% SDS) for 10 min at 66°C in a roller bottle apparatus. This solution was then replaced with 6 ml of probe solution (hybridization solution containing 1.5 μg of heat-denatured sulfonated DNA). After overnight hybridization at 66°C, the probe solution was decanted, and 20 ml of 66°C wash buffer (0.5 M NaCl/1% SDS) was added. The bottle was returned to the hybridization oven for 15 min. This treatment with wash buffer was repeated for a total of four washes. The membrane was removed from the bottle, placed on a blotter, DNA-side up, and dried for 10 min at 30°C.

Before application of the conjugate, each hybridized membrane was submerged in 20 ml of freshly prepared blocking buffer [30 g of skim milk powder (Difco) per 100 ml of 25 mM NaCl/50 mM Tris·HCl, pH 7.5/1 mM EDTA/0.3% Tween 20] and placed on a rocker in a 30°C incubator. After 30 min, the buffer was decanted and replaced with an amount of conjugate (based on titer, typically 1:100 to 1:300) in blocking buffer. The tray was returned to the rocker for an additional hour. This incubation was followed by three successive 5-min washes (20 ml of 0.5 M NaCl/0.3% Tween 20) at room temperature. The membrane was then washed three times for 5 min each with assay buffer (50 mM sodium bicarbonate/carbonate and 1 mM $MgCl_2$, pH 9.5). After decanting the final assay-buffer wash, 20 ml of assay buffer containing 220 μl of phosphate phenyl dioxetane (10 mg/ml) (Lumigen, Southfield, MI) was added for each membrane. The tray was covered and held at room

Table 1. Invariant fragment sizes for the base pattern type and for each subset of *L. monocytogenes*

Name	Strains, no.	Strains, %	Sizes of fragments containing rRNA operons, kbp							
			H	G	F	E	D	C	B	A
dd 0566 (base type)			9.0	6.2	5.5	5.2	5.0	4.0	2.3	2.1
E 5.2	438	32.5	9.0	v	5.5	5.2	5.0	4.0	2.3	2.1
E 5.3	10	0.7	9.0	v	5.5	5.3	5.0	4.0	2.3	2.1
E 9.2	499	37.1	9.0	v	5.5	9.2	5.0	4.0	2.3	2.1
E 11.2	227	16.7	9.0	v	5.5	11.2	5.0	4.0	2.3	2.1
E 11.2-C	2	0.1	9.0	v	5.5	11.2	5.0		2.3	2.1
E 5.2-D	14	1.0	9.0	v	5.5	5.2		4.0	2.3	2.1
G 6.2, H 9.0	127	9.4	9.0	6.2	v	5.2	5.0	4.0	2.3	2.1
G 8.1, H 7.1	11	0.8	7.1	8.1	v	5.2	5.0	4.0	2.3	2.1
G 5.8, H 7.1	3	0.2	7.1	5.8	v	5.2	5.0	4.0	2.3	2.1
E/G 5.8, H 7.1	2	0.1	7.1	5.8	v	5.8	5.0	4.0	2.3	2.1
Thermal control	13	1.0	9.0	6.2/3.2	5.5	5.2	5.0	4.0	2.3	2.1

v, Variable.

temperature for 5 min with rocking, followed by 10 min without rocking. The membrane was then taped DNA-side up on blotter paper and dried in a 30°C incubator for a minimum of 30 min. The membrane was then removed from the blotter paper and heated in a 700-W microwave oven on the high setting for 10 s. The chemiluminescent image was recorded electronically and analyzed as described (7). The initial results, electronically recorded images of membranes showing *Eco*RI fragments and standards separated by size and labeled by hybridization with the probe containing the rRNA operon, were processed to extract and normalize the pattern of fragment bands for each lane of data, and the patterns were entered into a data base.

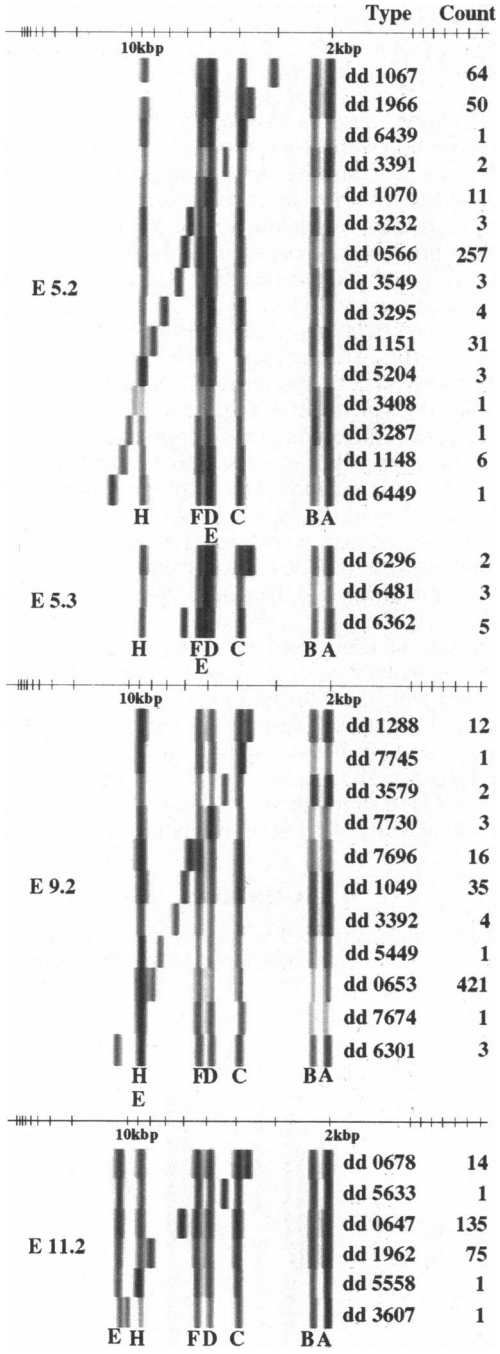

FIG. 1. *L. monocytogenes* types (*Eco*RI) arranged in subsets according to the indicated sizes (kbp) of E variants. The types within each subset were sorted with the G-fragment sizes increasing from small to large. The type name and the number of strains in each type are shown. DD 0566, ATCC 15313; nomenclatural type; DD 0647, ATCC 19118; and DD 0653, ATCC 19115.

RESULTS

Pattern Types. The patterns were arranged by similarity through the use of squared correlation values and visual assessment to determine nearest neighbors. These patterns, representing 1346 strains, revealed sets of hybridized fragments that were conserved among different strains of *L. monocytogenes* but were not observed as a set in any other of the >200 species tested. Patterns that were indistinguishable within the experimental error were averaged, and the average was stored as a pattern type. Any specific pattern representing a single strain also constituted a pattern type. With this approach the diversity within *L. monocytogenes* was resolved into 50 distinct types. Thirty-four types represented two or more strains. Each type that represented a single strain differed from its nearest neighbor by the position of a single band. Each type (prefix dd) was assigned the number of the strain first represented by the pattern type (prefix DD).

Formation of Coherent Subsets. Each hybridized fragment of a given size in the *L. monocytogenes* patterns was considered to be a taxonomic character, and the frequencies of occurrence of these fragments in the patterns of *L. monocytogenes* strains were determined. Type dd 0566 incorporated the hybridized fragments with the highest frequencies of occurrence and was designated as the base type for comparison of suggested polymorphic fragments. Table 1 includes the size of each of the fragments in dd 0566. These fragment bands were lettered sequentially on the basis of their relative positions in the base type.

Once the base type was established, other types were described by the size polymorphisms of the detected fragments. Accordingly, the types were grouped by squared correlation values and visual arrangement of the suggested size polymorphisms of a given fragment into coherent subsets. Within these subsets, the coherence is that all bands are fixed in position except one.

Fig. 1 shows four subsets. In each of these subsets, the E fragment was fixed at a different size, and G fragments were polymorphic. The bands with fixed positions in the subset are labeled; the G fragments are apparent by their different sizes. These four subsets represent the dominant structure of the species. The first subset, labeled E 5.2, included the base type (dd 0566) and other patterns that differed from the base pattern only in the restriction fragment length polymorphisms of the G region. The E 5.3, E 9.2, and E 11.2 subsets also represented E-variant subsets with some of the same multiplicity of G-fragment sizes. The two types in subset E 11.2-C (Fig. 2) differed from dd 0647 and dd 1962 by a 4.0-kbp C-fragment band deletion. Subset E 5.3-D included dd 6323, which differed from dd 6296 (Fig. 1, E 5.3 subset) by a 5.0-kbp D-fragment band deletion.

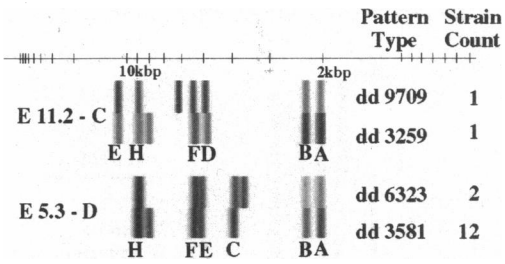

FIG. 2. Additional *L. monocytogenes* types (*Eco*RI) arranged in subsets according to the indicated sizes (kbp) of E variants and the noted deletions or unseen polymorphisms. The types within each subset were sorted with the G-fragment sizes increasing from small to large. The type name and the number of strains in each type are shown.

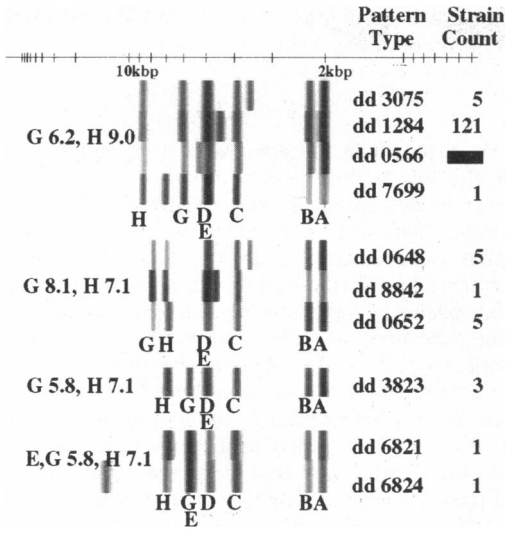

FIG. 3. Additional *L. monocytogenes* types (*Eco*RI) arranged in subsets according to the indicated sizes (kbp) of G, H, and E variants. The last three pattern types are considered as a single subset. The types within each subset were sorted with the F-fragment sizes increasing from small to large. The type name and the number of strains in each type are shown. DD 0566, ATCC 15313; DD 0648, ATCC 19114; and DD 0652, ATCC 19116.

Fig. 3 shows additional subsets. In the first two of these subsets, G and H fragments were of different fixed sizes. The two G-fragment sizes were the most frequently occurring, as seen in the strain counts in Fig. 1 (dd 0566, dd 6362, dd 1049, and dd 0647 for G 6.2; dd 1151, dd 0653, and dd 1962 for G 8.1). The bands with fixed positions in the subset are labeled, and the F fragments were apparent by their different sizes. Type dd 0566 fits into both the E 5.2 subset of Fig. 1 and the G 6.2, H 9.0 subset of Fig. 3 and links the subsets. The G 8.1, H 7.1 subset differed from the previous subset in the position of G and H. Because the H 7.1 polymorphism had not been observed in any other subsets, all strains in this subset were classically retested and verified as *L. monocytogenes* (cytochrome oxidase, negative; catalase, positive; CAMP, positive with *Staphylococcus aureus* ATCC 25923, and Micro-ID octal code, 44044).

The patterns labeled G 5.8, H 7.1 and E/G 5.8, H 7.1 (Fig. 3) differed from the G 6.2, H 9.0 subset in the variant positions of fragments G and H and of fragments E, G, and H, respectively. The G 5.8, H 7.1 and E/G 5.8, H 7.1 subsets had two (G and H) of six bands with a low frequency of occurrence for *L. monocytogenes.* The 5.8-kbp fragment (G) had a high frequency of occurrence in *Listeria innocua* (Fig. 4). However, these subsets shared the H 7.1 fragment with the G 8.1, H 7.1 subset, and this fragment is not present in the adjacent *L. innocua* patterns. Correlation analysis (data not shown) indicated that the represented strains were closer to *L. monocytogenes* than to *L. innocua.* To verify the identification, all strains in this subset were also retested classically. These strains were as follows: cytochrome oxidase, negative; catalase, positive; and CAMP, positive, as expected for *L. monocytogenes.* However, the rhamnose reaction in the Micro-ID system was negative and produced an octal code of 44040. This result is atypical, although rhamnose-negative strains of *L. monocytogenes* have been reported (14).

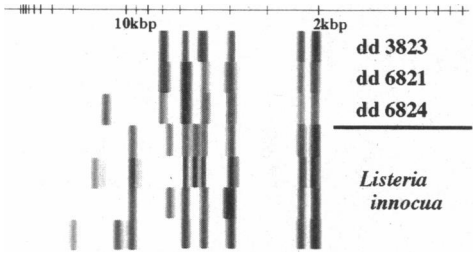

FIG. 4. Three distal pattern types of the structure of *L. monocytogenes*, shown with some of the adjacent edge of *L. innocua* pattern types.

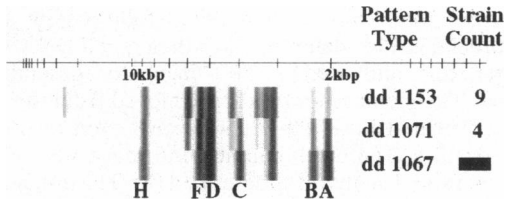

FIG. 5. Types affected by growth temperature. When the strains in type dd 1153 were grown either at 20°C or at 30°C, pure cultures presented at least two populations, as shown by the polymorphic duplication of some bands. Similar results were obtained with strains of type dd 1071, but the relative intensities of the bands differ, especially C, B, and A. When strains from either type were grown at 42°C, they produced patterns matching dd 1067. The type name and the number of strains in each type are shown.

The first two types in Fig. 5, dd 1153 and dd 1071, constitute the last subset; the patterns from strains of these types were affected by the growth temperature. These pattern types were similar to dd 1067 (included in Fig. 5 for comparison), except that at least two different genetic population types were present in the pure cultures, as shown by the same regions manifested in multiple bands. The different population types occurred when the cultures were grown either at 20°C or at 30°C. When the cultures were grown at 42°C, the resulting patterns matched the dd 1067 pattern type.

Table 1 summarizes the fragment sizes for each of the subsets.

Reduced Sets of Conserved Fragments. A reduced set of conserved fragments was constructed for each of the coherent subsets by removing the information of the highly polymorphic region from the pattern types and averaging the fragment bands occurring at 100% frequency in each subset. The G 5.8, H 7.1 and E/G 5.8, H 7.1 subsets were combined into one for this purpose. Fig. 6 shows the nine reduced sets of conserved fragments along with their descriptive names and strain counts.

DISCUSSION

Fragment Conservation. The survey of *L. monocytogenes* described here revealed similarities shared by strains of the

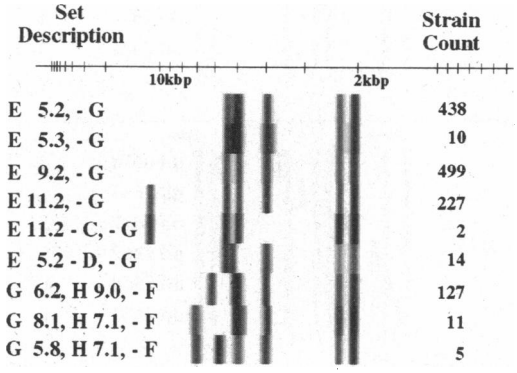

FIG. 6. Reduced sets of conserved fragments. By using the positions of the six or more bands that occurred at 100% frequency in each of the coherent subsets, nine reduced patterns were created. These reduced patterns were conserved among the strains of *L. monocytogenes* but were not observed as a set among the strains of the other species tested.

species and greater diversity than seen in classical serotyping (9). This description was made possible by use of a charge coupled-device camera detection of a light-emissive label (7), strict normalization of band positions relative to standards, and use of the full-scale normalized signal intensity in the computerized analysis. The conservation and strain variation permitted the data from 1346 strains, 50 pattern types, to be further reduced to nine minimal sets of fragments for identification of *L. monocytogenes*. Each minimal set was viewed as an ensemble of positive taxonomic characters with a related frequency of occurrence in the sample population (Fig. 6). The minimal sets, further sharing some of the fragment sizes, created a reduced definition of the species.

The cohesiveness of the total set of the 50 pattern types was verified by the observation that each pattern type differed from some other pattern types in the position of a single polymorphic fragment band. An exception was the step into the H-variant subsets, where a double polymorphic change was observed partitioning the strains branching toward *L. innocua*. A relationship of these strains was seemingly implied by the arrangement of pattern types, especially in the G-region polymorphisms shown in each subset of Fig. 1. However, the pattern types in these subsets could be rearranged into a number of different subsets, each explicitly showing the polymorphisms of the E region and G region conserved within each subset.

Fig. 5 includes two patterns types of strains that originally appeared to differ from the rest of the species. That the patterns in this subset could be recognized by using the reduced set of conserved fragments from subset E 5.2 and that patterns matching dd 1067 resulted when cultures were grown at higher temperatures suggested that this subset is intrinsically related to the E 5.2 subset.

Coherent Subsets. The similarities of the other 48 pattern types were made more apparent by the formation of coherent subsets. The E 5.2, E 5.3, E 9.2, and E 11.2 subsets (Fig. 1) differed from the base pattern, dd 0566, in the positions of both E variants and G-fragment sizes. Subsets E 5.3-D and E 11.2-C (Fig. 2) were viewed as low-frequency branches from the related E-variant subsets. Classical bacteriology recognizes that conserved attributes may not be present in all strains (15), and some minimal characters for identification may occur at a frequency of <100%. The base pattern was also a branch point for the F-variable subset (Fig. 3). The structure in Fig. 1 and the branches shown in Fig. 2 (all subsets) and 3 (subset G 6.2, H 9.0) include 1323 strains (98.3%).

The other subsets shown in Fig. 3 display greater divergence from the G 6.2, H 9.0 subset, with two and finally three regions with variant fragments. The decreasing number of variant fragments in common with the branching F-variable subset (G 6.2, H 9.0) seems to suggest divergence from the dominant structure of the species. However, the fragments do not appear randomly throughout the molecular size range but appear at discrete positions characteristic of the species. Correlation analysis indicated the G 8.1, H 7.1 subset was near the edge of the *L. monocytogenes* cluster. Classical methods confirmed the identification as *L. monocytogenes*. By DNA homology measurement, strain DD 0648 (ATCC 19114) is 72% related to the type strain of *L. monocytogenes* and 54% related to the type strain of *L. innocua* (1). In addition, several strains in type dd 0648 were associated with listeriosis in ruminants, a characteristic associated with *L. monocytogenes* rather than *L. innocua* (16).

The G 5.8, H 7.1 and E/G 5.8, H 7.1 types (Fig. 3) were considered together. Correlation analysis indicated that these subsets were clustered out from the branch point and in the direction of the *L. innocua* cluster. The negative rhamnose reactions and the octal codes from the Micro-ID kit were consistent with *L. innocua* (17). However, the positive CAMP results were consistent with *L. monocytogenes*. These separated branch subsets represented only a small percentage of the sample, and this last subset represented only five strains, apparently an edge of the species.

Reduced Sets of Conserved Fragments. Both the patterns and their embedded reduced sets of conserved fragments defined the species. On the basis of visual recognition of the E-variant band fragments, the first four patterns (representing reduced sets) in Fig. 6 could be further reduced to a single set by removing the information of the E polymorphic region (E 9.2, -G). The -C and -D patterns were viewed as branching strain variations with low frequencies of occurrence. This conservation progressed in stepwise fashion and branched at the F-variable patterns (Fig. 3) toward *L. innocua* (Fig. 4). The G 5.8, H 7.1 and E/G 5.8, H 7.1 conserved set was intermediate and was included provisionally within *L. monocytogenes* on the basis of the linkage to the previous set, the absence of an expected *L. innocua* fragment band and the positive CAMP reaction. The reduced sets of conserved fragments were recognized explicitly in the arrangement of patterns; this conservation was also recognized implicitly by similarity values.

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