Comparative Analysis of 16S and 23S rRNA Sequences of *Listeria* Species

BRUNEHILD SALLEN, ALAIN RAJOHARISON, SABINE DESVARENNE,
FIONNA QUINN,[†] AND CLAUDE MABILAT*
bioMérieux S.A., 69280 Marcy l'Etoile, France

In order to establish the taxonomic value of 16S rRNA and 23S rRNA for distinguishing *Listeria* species, the complete 23S rRNA sequences for all *Listeria* species were determined by using the type strains. We designed and experimentally validated a universal 23S rRNA sequencing method, which included PCR amplification of the rDNA gene and direct cycle sequencing of the amplicon with eubacterial primers. The results of our sequence comparison indicated that the genus *Listeria* can be divided into two subgroups; one subgroup is composed of *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, and *Listeria welshimeri*, whereas the other subgroup includes *Listeria grayi* subsp. *grayi* and *Listeria grayi* subsp. *murrayi*. A phylogenetic analysis revealed that these species diverged recently. These results are consistent with 16S rRNA sequence analysis data. For application purposes, one 16S rRNA region that can be used to distinguish each *Listeria* species except *L. monocytogenes* and *L. innocua* has been described. In this study we found four 23S rRNA signature regions which, when used in combination, can be used to distinguish the species.

Traditionally, bacterial classifications are based on phenotypic characteristics and principally involve analyses of differential metabolic properties. Recently, however, molecular analyses of phylogenetic markers have resulted in a second type of classification based on data from nucleic acid sequence comparisons. The genes that encode these markers possess the features of molecular clocks, and the numbers of mutations that occur in their nucleotide sequences are proportional to elapsed time (7, 17).

Among such markers, rRNAs are particularly useful because these molecules are present in every living cell and their function is highly conserved. Moreover, rRNAs share a mosaic structure composed of a succession of several domains that are more or less conserved as a result of variable evolution rates. Thus, distant relationships can be determined by comparing highly conserved regions, whereas closely related species are studied by using more variable (i.e., polymorphic) regions (16).

Currently, 16S rRNAs are the principal source of phylogenetic information, and about 3,500 complete 16S rRNA sequences have been determined. A comparison of the sequences of members of each *Listeria* species (5) demonstrated that this genus is composed of closely related species. The lack of mutations suggested that these species diverged very recently.

Analysis of the corresponding 23S rRNA sequences may provide more information concerning phylogenetic relationships. 23S rRNA is about twice as big as 16S rRNA, so we assumed that 23S rRNA contains more variable regions and hence is more discriminative than the smaller 16S rRNA. However, analysis of 23S rRNA has been hampered because of its greater size (about 2.9 kb) and the presence of marked secondary structures absent in 16S rRNA. Recently, the 23S rRNA sequences of two strains of *Listeria monocytogenes* were deter-

mined (8, 15). We extended this work by determining and analyzing the complete 23S rRNA sequences of strains belonging to each *Listeria* species in order to compare the diversity of the 23S rRNA and 16S rRNA sequences in this genus.

MATERIALS AND METHODS

Bacterial strains. The strains which we tested included seven type strains of the six *Listeria* species (Table 1) and the following 10 clinical or environmental strains that were obtained from the bioMérieux collection: *L. monocytogenes* C1 to C3, *Listeria innocua* C4 to C6, *Listeria ivanovii* C7, and *Listeria seeligeri* C8 to C10. Cultures were grown at 37°C on Trypticase soya agar (bioMérieux, Marcy l'Etoile, France) or in brain heart broth (bioMérieux) for DNA extraction.

Preparation of total DNA. The cells in 1.5 ml of an overnight broth culture were obtained by centrifuging the preparation at 12,000 rpm for 2 min in a microtop centrifuge (model 5402; Eppendorf). The resulting bacterial pellet was resuspended in 100 µl of a solution containing 37.5 mM sucrose, 7.5 mM EDTA, 18.75 mM Tris-Cl (pH 8.0), and 0.25 mg of lysozyme per ml, and the preparation was incubated at 37°C for 30 min. Then 100 µl of Tris-buffered phenol-chloroform (1:1) was added, and the mixture was vortexed for 30 s. The tube was centrifuged for 5 min, and the aqueous phase was collected. The extracted nucleic acids were checked by gel electrophoresis by using 0.8% SeaKem LE agarose (FMC Bioproducts, Philadelphia, Pa.).

DNA amplification. A PCR was performed by using 10 ng of total DNA, 40 pmol of each amplification primer, 200 mM dATP, 200 mM dCTP, 200 mM dGTP, 200 mM dTTP (Pharmacia, Uppsala, Sweden), 1 U of *Taq* polymerase, and 10 µl of 10× Cetus buffer (500 mM KCl, 15 mM MgCl₂, 100 mM Tris-Cl [pH 8.3], 1% gelatin [Bio-Rad, Richmond, Calif.]); each mixture was brought to a final volume of 100 µl with distilled water. The three following primer pairs were

TABLE 1. Origins and EMBL accession numbers of complete 23S rDNA sequences of the *Listeria* type strains analyzed in this study

Species or subspecies	Strain ^a	EMBL accession no.	Length of sequence (bp)
<i>Listeria grayi</i> subsp. <i>grayi</i>	ATCC 19120 ^T	X92948	2,631
<i>Listeria grayi</i> subsp. <i>murrayi</i>	CCUG 4984 ^T	X92952	2,685
<i>Listeria innocua</i>	ATCC 33090 ^T	X92949	2,666
<i>Listeria ivanovii</i> subsp. <i>ivanovii</i>	CIP 7842 ^T	X92950	2,648
<i>Listeria monocytogenes</i>	ATCC 15313 ^T	X92951	2,660
<i>Listeria seeligeri</i>	CCUG 15530 ^T	X92953	2,684
<i>Listeria welshimeri</i>	CIP 8149 ^T	X92954	2,666

^a ATCC, American Type Culture Collection; CCUG, Culture Collection of the University of Göteborg; CIP, Collection de l'Institut Pasteur.

* Corresponding author. Mailing address: Laboratoire des Sondes Froides bioMérieux, Ecole Normale Supérieure de Lyon, 46, allée d'Italie, 69364 Lyon Cedex 07, France. Phone: (33) 72 72 85 10. Fax: (33) 72 72 85 33.

[†] Present address: Department of Applied Biology and Chemistry, Regional Technical College, Tallaght, Dublin 24, Ireland.

TABLE 2. Amplification and sequencing primers

Primer ^a	Sequence ^b	Position ^c	Length (bases)
Amplification primers			
1f	TCC GAA TGG GGA AAC CC	114-130	17
10r	GAC CAG TGA GCT RTT AC	1093-1109	17
9f	AAG AGG GAA ACA RCC CAG A	972-990	19
16r	GGA ACT TAC CCG ACA AGG	1941-1958	18
15f	GCA ACT GTT TAT YAA AAA CAC	1770-1790	21
23r	TTC GTG CTT AGA TGC YTT CA	2746-2765	20
Sequencing primers			
3f	GTA GYG GCG AGC GA	242-255	14
3r	CGC TCG CCR CTA CT	241-254	14
4f	AGG GAA WGG YGA AAA	466-480	15
4ra	TCA CGG TAC RAG TTC ACT	449-466	18
6fb	AGC GAA AGC GAG TCY K	627-642	16
6rb	CGC CCT ATT CRG ACT C	636-651	16
8f	GCT GGT TCT CYC CGA	805-819	15
8r	TTT CGG RGA GAA CCA	807-821	15
9ra	GGT CTG GGT TGT TTC	978-992	15
9rb	GAT CTG GGC TGT TTC	978-992	15
10fb	GAA AGA GTG CGT AAT AGC	1083-1100	18
11f	TGC TGA CAT RAG TAR CGA	1255-1272	18
12f	AAG GCG TAG TBG ATG G	1353-1381	16
12rb	TAA CCY GTT RTC CAT C	1377-1391	16
13f	CSY AAA CCG ACA CAG	1606-1620	15
13r	TGT CGG TTT RSG GTA	1602-1616	15
16f	GAA ATT CCT TGT CGG GT	1935-1951	17
17f	GAC GGA AAG ACC CC	2053-2066	14
17r	GGG GTC TTT CCG TC	2053-2066	14
18fa	ACT GGG GCG GTY KC	2247-2260	14
18fb	TTT RAC TGG GGC GG	2243-2256	14
18r	CCG CCC CAG TYA AA	2243-2256	14
19r	GRG TAC CTT TTA TCC GTT G	2424-2442	19
20f	ATC CTG GGG CTG RAG H	2518-2533	16
21fa	GTC GTG AGA CAG TTC GG	2592-2608	17
21fb	ACA GTT CGG TCC CTA TC	2600-2616	17
21r	ACT GTC TCA CGA CGT TCT	2587-2604	18

^a The numbers in the primer designations refer to the corresponding conserved regions of the 23S rRNA (see text), whereas f and r refer to forward (5' to 3') and reverse (3' to 5') polarity of DNA elongation, respectively. Distinct alternative primers are differentiated by the suffixes a and b.

^b Primer sequences are given from 5' to 3'. Nucleotide residues other than A, T, G, and C are indicated by the following corresponding International Union of Biochemistry ambiguity codes: R, A or G; Y, T or C; K, G or T; S, G or C; W, A or T; B, G, C or T; H, A, C, or T.

^c Primer positions correspond to positions in the *E. coli* 23S rRNA nucleotide sequence (3).

used for PCR amplification: primers 1f and 10r, primers 9f and 16r, and primers 15f and 23r (Table 2). Samples were overlaid with 100 µl of paraffin oil. Amplification was performed with a model 480 DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.) by using the following parameters for 30 cycles: 94°C for 30 s, 45°C for 1 min, and 72°C for 2 min. The amplification products were analyzed by agarose gel electrophoresis as described previously.

DNA sequencing. Nucleotide sequences were determined by using a fluorescence-based DNA sequencer. The template PCR products were purified and sequenced as described previously (13). A total of 33 sequencing primers, including the six amplification primers, were used for DNA sequencing (Table 2).

Alignment and phylogenetic methods. Partial sequences that were 200 to 300 bp long were aligned with the previously published 23S rRNA or ribosomal DNA (rDNA) sequence of *L. monocytogenes* (8, 15). The resulting overlapping sequences were concatenated to obtain a single complete sequence for each species. The whole procedure was performed with the WSM set of programs written by R. Christen (URA 671, CNRS-Université Paris VI, Station Zoologique Villefranche sur Mer, France).

Our phylogenetic analyses were restricted to comparisons of nucleotide positions that were aligned with no doubt. These positions were determined visually with the WSM set of programs. A neighbor-joining method (12) was to construct phylogenetic trees as described previously (11). The robustness of the branching pattern was tested by performing a maximum-parsimony analysis through 100

bootstrap replications (heuristic search) with the PAUP program for Macintosh computers (14).

Nucleotide sequence accession numbers. The sequences which we determined have been deposited in the EMBL data library under the accession numbers shown in Table 1.

RESULTS AND DISCUSSION

Rationale for 23S rDNA sequencing and primer design. Our strategy for determining the 23S rRNA nucleotide sequences of *Listeria* species included (i) eubacterial PCR amplification of DNAs extracted from pure cultures and (ii) direct cycle sequencing of each strand of an amplicon by using eubacterial primers. This strategy allowed us to avoid fastidious and time-consuming cloning steps. The high elongation temperature used in the PCRs allowed us to sequence G+C-rich regions and secondary structures of 23S rRNAs. We determined the nucleotide sequences of both strands of amplified 23S rDNAs. Amplification and sequencing primers were designed for 24 conserved regions, which were defined by aligning the available 23S rRNA sequences of 22 species that represented a cross section of the 16S rRNA eubacterial tree (data not shown). Three 1-kb overlapping PCR amplicons covering the whole 23S rDNA gene were generated. Since 250 to 300 nucleotide stretches were reproducibly readable with our cycle sequencing protocol, we designed 27 sequencing primers for both strands in order to sequence these amplicons (Table 2). As we observed some polymorphisms within some conserved regions, up to two degenerated positions per primer were allowed. A unique hybridization temperature was used in order to facilitate routine and parallel amplification (45°C) or sequencing

TABLE 3. Homology values for 23S rRNA sequences of *Listeria* strains^a

Strain	% Homology							
	<i>L. grayi</i> subsp. <i>murrayi</i> CCUG 4984 ^T	<i>L. innocua</i> ATCC 33090 ^T	<i>L. ivanovii</i> CIP 7842 ^T	<i>L. monocytogenes</i> ATCC 15313 ^T	<i>L. monocytogenes</i> ^b	<i>L. monocytogenes</i> ATCC 19115	<i>L. seeligeri</i> CCUG 15530 ^T	<i>L. welshimeri</i> CIP 8149 ^T
<i>L. grayi</i> subsp. <i>grayi</i> ATCC 19120 ^T	99.9	97.5	97.4	97.5	97.3	97.5	97.5	97.5
<i>L. grayi</i> subsp. <i>murrayi</i> CCUG 4984 ^T		97.6	97.5	97.6	97.4	97.6	97.5	97.6
<i>L. innocua</i> ATCC 33090 ^T			99.6	99.8	99.6	99.8	99.7	99.8
<i>L. ivanovii</i> CIP 7842 ^T				99.8	99.6	99.8	99.8	99.8
<i>L. monocytogenes</i> ATCC 15313 ^T					99.7	99.9	99.8	99.8
<i>L. monocytogenes</i> ^b						99.7	99.6	99.6
<i>L. monocytogenes</i> ATCC 19115							99.8	99.8
<i>L. seeligeri</i> CCUG 15530 ^T								99.8

^a Overall levels of similarity were calculated by using a neighbor-joining method corrected with the one-parameter model of Jukes and Cantor (6). This method was applied to a discontinuous DNA stretch that had the following boundaries according to *Escherichia coli* numbering (3) (positions that were aligned without any doubt): positions 142 to 1172, 1175 to 1693, 1694 to 2418, and 2468 to 2729.

^b Isolate whose sequence was determined by Thompson et al. (15).

FIG. 1. 23S rRNA unrooted phylogenetic tree showing the relationships of *Listeria* species and subspecies and other gram-positive bacteria that have low G+C contents. The tree was constructed by using a neighbor-joining method corrected as described by Jukes and Cantor (6). Our sequence comparison was restricted to nucleotide positions which were aligned without any doubt. The numbers in parentheses are nucleotide sequence accession numbers. The values above and below the horizontal lines (expressed as percentages) indicate the robustness of the corresponding branches, as determined by a bootstrap analysis (heuristic search). Bootstrap values are not indicated for the outgroup. Scale bar = accumulated number of changes per nucleotide.

(50°C). This strategy allowed us to determine the sequences of other bacterial taxa (data not shown).

Sequence comparisons and phylogenetic analysis. The nearly complete 23S rRNA sequences of seven type strains representing the *Listeria* species were determined and analyzed (Table 1). The levels of similarity between these sequences are shown in Table 3.

The 23S rRNA sequences of *Listeria* species were also compared with the 23S rRNA sequences of several other gram-positive bacteria that have low DNA G+C contents. In this analysis we included previously published sequences for *Bacillus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Staphylococcus*, and *Streptococcus* species. An unrooted phylogenetic tree is shown in Fig. 1.

The complete 23S rRNA sequences of two strains of *L. monocytogenes* (strain ATCC 19115 [8] and another isolate [15]) have been published previously. These sequences differed by eight nucleotides (level of homology, 99.7%) (Table 3). The sequence of a third strain, strain ATCC 15313^T (T = type strain), was determined in this study in order to assess the reliability of our sequencing strategy. The 23S rRNA sequences of this strain and isolate ATCC 19115 exhibited only two base differences (level of homology, 99.9%). This almost perfect match confirmed the high fidelity of our routine PCR sequencing protocol, since the ATCC 19115 rDNA was sequenced after a restriction fragment was cloned in a plasmid. On the other hand, there were eight base differences between the ribosomal sequences of our strain and the strain studied by

A

	626	636	646	656	666
<i>L. monocytogenes</i> ATCC15313	AGCGAAAGCG	AGTCTGAATA	GGGCG*CATA	AGTAACAGGT	CGTAGACCC
<i>L. monocytogenes</i> ATCC19115	-----	-----	-----	-----	-----
<i>L. monocytogenes</i> a	-----	-----	-----	-----	-----*
<i>L. monocytogenes</i> C1	-----	-----	-----	-----	-----
<i>L. monocytogenes</i> C2	-----	-----	-----	-----	-----
<i>L. monocytogenes</i> C3	-----	-----	-----	-----	-----
<i>L. innocua</i> ATCC33090	-----	-----	-----	-----	-----
<i>L. innocua</i> C4	-----	-----	-----	-----	-----
<i>L. innocua</i> C5	-----	-----	-----	-----	-----
<i>L. innocua</i> C6	-----	-----	-----	-----	-----
<i>L. ivanovii</i> CIP7842	-----	-----	-A-	-----	-----
<i>L. seeligeri</i> CCUG15530	-----	-----	-A-	-----	-----
<i>L. seeligeri</i> C9	-----	-----	-A-	-----	-----
<i>L. seeligeri</i> C10	-----	-----	-A-	-----	-----
<i>L. welshimeri</i> CIP8149	-----	-----	-----	-----	-----
<i>L. grayi</i> subsp. <i>grayi</i> ATCC19120	-----	-----	-TATG-	-----	-----
<i>L. grayi</i> subsp. <i>murrayi</i> CCUG4984	-----	-----	-TATG-	-----	-----

B

	1155	1165	1175	1185
<i>L. monocytogenes</i> ATCC15313	AAACTGTGGA	TGAACCTC*T	TTAGAGGTTTC	GTGGTAGGA
<i>L. monocytogenes</i> ATCC19115	-----	-----	-----	-----
<i>L. monocytogenes</i> a	-----	-----	-----	-----
<i>L. monocytogenes</i> C1	-----	-----	-----	-----
<i>L. monocytogenes</i> C2	-----	-----	-----	-----
<i>L. monocytogenes</i> C3	-----	-----	-----	-----
<i>L. innocua</i> ATCC33090	-----	-----	-CG-	-----
<i>L. innocua</i> C4	-----	-----	-CG-	-----
<i>L. innocua</i> C5	-----	-----	-CG-	-----
<i>L. innocua</i> C6	-----	-----	-CG-	-----
<i>L. ivanovii</i> CIP7842	-----	-----	-T- A-	-----
<i>L. ivanovii</i> C7	-----	-----	-T- A-	-----
<i>L. seeligeri</i> CCUG15530	-----	-----	-----	-----
<i>L. seeligeri</i> C8	-----	-----	-----	-----
<i>L. seeligeri</i> C9	-----	-----	-----	-----
<i>L. seeligeri</i> C10	-----	-----	-----	-----
<i>L. welshimeri</i> CIP8149	-----	-----	-CG-	-----
<i>L. grayi</i> subsp. <i>grayi</i> ATCC19120	-----	-----	-A- -CG- -T-	-----
<i>L. grayi</i> subsp. <i>murrayi</i> CCUG4984	-----	-----	-A- -CG- -T-	-----

FIG. 2. Signature regions which allowed us to distinguish the *Listeria* species. The sequence of *L. monocytogenes* ATCC 15313^T was used as the reference. Identical nucleotides are indicated by dashes, whereas deletions are indicated by asterisks. *L. monocytogenes* a was the isolate whose sequence was determined by Thompson et al. (15).

Thompson et al. (level of homology, 99.7%) (15). The differences in the sequences of the three *L. monocytogenes* strains which we examined may reflect sequencing artifacts or may be true differences. Indeed, it is commonly accepted that there is sequence polymorphism between different isolates of the same species. Minor differences between the sequences of different rRNA operons have been shown to occur in a single strain of *Escherichia coli* (4).

At the intrageneric level, we distinguished two subgroups within the genus *Listeria* (Fig. 1). The nucleotide sequences of the species belonging to these two clusters were at least 97% homologous (Table 3). The *L. monocytogenes* subgroup is composed of *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *Listeria welshimeri*, and these organisms exhibit levels of similarity between 99.6 and 99.9%, which correspond to a maximum of 13 nucleotide differences. The members of the second cluster, which is composed of *Listeria grayi* subsp. *grayi* and *Listeria grayi* subsp. *murrayi*, also exhibit a very high level

of relatedness (99.9%) and differ at only three nucleotides. This result is consistent with the assignment of the previously distinct species *L. grayi* and *Listeria murrayi* to a single species on the basis of DNA-DNA hybridization, multilocus enzyme electrophoresis, and rRNA gene restriction pattern data (9). The number of mutations in the ribosomal sequences was too low to establish a precise phylogeny within each *Listeria* subgroup, as shown by multifurcations on the phylogenetic tree (Fig. 1). This result suggests that the *Listeria* species diverged very recently, which is consistent with the low rate of fixed mutations observed in the bacterial population.

The same division of *Listeria* species into two subgroups was also observed when the 16S rRNA data were used (5). Surprisingly, the numbers of species-specific mutations found in the 16S rRNA and 23S rRNA sequences were almost identical, although the 23S rRNA sequences are twice as long as the 16S rRNA sequences. This is due principally to the presence in the 16S rRNA sequence of a highly variable domain, the V2 re-

C

	1490	1500	1510	1520	1530	1540
<i>L. monocytogenes</i> ATCC15313	AGGCAAATCC	GCTTCTGCG	AAGCATGAGC	TGTGATGGG	AAGG*AAAT	AAGTACGGAA
<i>L. monocytogenes</i> ATCC19115	-----	-----A--	-----	-----	-----	-----
<i>L. monocytogenes</i> a	-----	-----	-----	-----	-----	-----
<i>L. monocytogenes</i> C1	-----	-----	-----	-----	-----	-----
<i>L. monocytogenes</i> C2	-----	-----	-----	-----	-----	-----
<i>L. monocytogenes</i> C3	-----	-----	-----	-----	-----	-----
<i>L. innocua</i> ATCC33090	-----	-----T--	-----	-----	-----	-----
<i>L. innocua</i> C4	-----	-----T--	-----	-----	-----	-----
<i>L. innocua</i> C5	-----	-----T--	-----	-----	-----	-----
<i>L. innocua</i> C6	-----	-----T--	-----	-----	-----	-----
<i>L. ivanovii</i> CIP7842	-----	-----	-----	-----	-----G	-----
<i>L. ivanovii</i> C7	--N-----	-----	-----	-----	-----G	-----
<i>L. seeligeri</i> CCUG15530	-----	-----	-----	-----	-----	-----
<i>L. seeligeri</i> C8	-----	-----	-----	-----	-----	-----
<i>L. seeligeri</i> C9	-----	-----	-----	-----	-----	-----
<i>L. seeligeri</i> C10	-----	-----	-----	-----	-----	-----
<i>L. welshimeri</i> CIP8149	-----	-----	-----	-----	-----A	-----
<i>L. grayi</i> subsp. <i>grayi</i> ATCC19120	-----	-----	-----	-----	-----	-----
<i>L. grayi</i> subsp. <i>murrayi</i> CCUG4984	-----	-----	-----	-----	-----	-----

D

	2175	2185	2195	2205	2215	2225
<i>L. monocytogenes</i> ATCC15313	TACCCITGGCT	GT*ATGACCA	TTCTAACCG	CCACGCTTAG	CGCGTGGGGA	GACAGT
<i>L. monocytogenes</i> ATCC19115	-----	-----	-----	-----	-----	-----
<i>L. monocytogenes</i> a	-----	-----	-----	---*---	-----	-----
<i>L. monocytogenes</i> C1	-----	-----	-----	-----	-----	-----
<i>L. monocytogenes</i> C2	-----	-----	-----	-----	-----	-----
<i>L. monocytogenes</i> C3	-----	-----	-----	-----	-----	-----
<i>L. innocua</i> ATCC33090	-----	-----	-----	-----A	-----	-----
<i>L. innocua</i> C4	-----	-----	-----	-----A	-----	-----
<i>L. innocua</i> C5	-----	-----	-----	-----A	-----	-----
<i>L. innocua</i> C6	-----	-----	-----	-----A	-----	-----
<i>L. ivanovii</i> CIP7842	-----	-----	-----	-----	-----	-----
<i>L. seeligeri</i> CCUG15530	-----	-----	-----	-----A	-----	-----
<i>L. seeligeri</i> C8	-----	-----	-----	-----A	-----	-----
<i>L. seeligeri</i> C9	-----	-----	-----	-----A	-----	-----
<i>L. welshimeri</i> CIP8149	-----	-----	-----	-----	-----	-----
<i>L. grayi</i> subsp. <i>grayi</i> ATCC19120	-----	-----	-----	-----	-----	-----
<i>L. grayi</i> subsp. <i>murrayi</i> CCUG4984	-----	-----	-----	-----	-----	-----

FIG. 2—Continued.

gion, which has no equivalent in the 23S rRNA (5). At the intergeneric level, *Listeria* species grouped in a monophyletic cluster (Fig. 1) and exhibited the highest levels of sequence similarity with bacilli (levels of homology, 92 to 95%) and staphylococci (level of homology, 92%). The phylogenetic uncertainty associated with the branching pattern of the diverse *Bacillus* species (Fig. 1) has been reported and discussed previously in other studies involving 16S rRNA sequences as well as 23S rRNA sequences (1, 8, 10). Lactobacilli and streptococci are significantly more distantly related to the genus *Listeria* (Fig. 1). This topology is strongly supported by the maximum-parsimony analysis data, and the same phylogenetic relationships were obtained when 16S rRNA sequences were compared (5).

Determination of signature regions. The nucleotide differences between the 23S rRNA sequences of members of the *L. monocytogenes* subgroup were sometimes confined to particular locations, and when two *Listeria* species differed by at least one mismatch, the locus was called the signature region. On

the basis of these criteria, four signature regions (regions A to D) were identified (Fig. 2). In order to confirm that the loci were really species specific, we determined partial sequences of other clinical or environmental strains of *Listeria* species (strains C1 to C10). All of the sequences obtained from different strains of a single species were identical in the portion of the sequence examined.

The species specificity of the signature regions may be used to design oligonucleotide probes for bacterial identification. If used in combination, signature regions A to D have the potential to discriminate each *Listeria* species and, in particular, the human pathogen *L. monocytogenes* (2). On the other hand, Collins et al. (5) described a single domain in the 16S rRNA sequence (the V2 region) which allowed them to differentiate every *Listeria* species except *L. monocytogenes* and *L. innocua*.

ACKNOWLEDGMENTS

We thank R. Christen for helpful advice concerning the phylogenetic analyses and M. J. Storrs for critically reading the manuscript.

REFERENCES

1. Ash, C., J. A. E. Farrow, S. Wallbanks, and M. D. Collins. 1991. Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal sequences. *Lett. Appl. Microbiol.* **13**:202–206.
2. bioMérieux S.A. February 1995. French patent 95 01431.
3. Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller. 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J. Mol. Biol.* **148**:107–127.
4. Cilia, V., B. Lafay, and R. Christen. Sequence heterogeneity exists among the 16S ribosomal RNA sequences of the seven operons in *Escherichia coli* strain PK3 that can affect phylogenetic analyses at the species level. *Mol. Biol. Evol.*, in press.
5. Collins, M. D., S. Wallbanks, D. J. Lane, J. Shah, R. Nietupski, J. Smida, M. Dorsch, and E. Stackebrandt. 1991. Phylogenetic analysis of the genus *Listeria* based on reverse transcriptase sequencing of 16S rRNA. *Int. J. Syst. Bacteriol.* **41**:240–246.
6. Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21–132. In H. N. Munro (ed.), *Mammalian protein metabolism*. Academic Press, New York.
7. Kimura, M. 1968. Evolutionary rate at the molecular level. *Nature (London)* **217**:624–626.
8. Ludwig, W., G. Kirchhof, N. Klugbauer, M. Weizenegger, D. Betzl, M. Ehrmann, C. Hertel, S. Jilg, R. Tatzel, H. Zitzelsberger, S. Liebl, M. Hochberger, J. Shah, D. Lane, P. R. Wallnöfer, and K. H. Schleifer. 1992. Complete 23S ribosomal RNA sequences of Gram-positive bacteria with a low DNA G+C content. *Syst. Appl. Microbiol.* **15**:487–701.
9. Rocourt, J., P. Boerlin, F. Grimont, C. Jacquet, and J. C. Piffaretti. 1992. Assignment of *Listeria grayi* and *Listeria murrayi* to a single species, *Listeria grayi*, with a revised description of *Listeria grayi*. *Int. J. Syst. Bacteriol.* **42**:171–174.
10. Rössler, D., W. Ludwig, K. H. Schleifer, C. Lin, T. J. McGill, J. D. Wisotzkey, P. Jurtshuk, and G. E. Fox. 1991. Phylogenetic diversity in the genus *Bacillus* as seen by 16S RNA sequencing studies. *Syst. Appl. Microbiol.* **14**:266–269.
11. Ruimy, R., P. Riegel, P. Boiron, H. Monteil, and R. Christen. 1995. Phylogeny of the genus *Corynebacterium* deduced from analyses of small-subunit ribosomal DNA sequences. *Int. J. Syst. Bacteriol.* **45**:740–746.
12. Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
13. Sallen, B., A. Rajoharison, S. Desvarenne, and C. Mabilat. 1995. Molecular epidemiology of integron-associated antibiotic resistance genes in clinical isolates of *Enterobacteriaceae*. *Microb. Drug Resist.* **1**:195–202.
14. Swofford, D. 1990. PAUP: phylogenetic analysis using parsimony, version 3.0. Illinois Natural History Survey, Champaign.
15. Thompson, D. E., J. T. Balsdon, J. Cai, and M. D. Collins. 1992. Studies on the ribosomal RNA operons of *Listeria monocytogenes*. *FEMS Microbiol. Lett.* **75**:219–224.
16. Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* **51**:221–227.
17. Zuckerkandl, E., and L. Pauling. 1965. Molecules as documents of evolutionary history. *J. Theor. Biol.* **8**:357–366.