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Rapid Identification of *Listeria* Species by Using Restriction Fragment Length Polymorphism of PCR-Amplified 23S rRNA Gene Fragments

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A molecular method based on restriction fragment length polymorphism (RFLP) of PCR-amplified fragments of the 23S rRNA gene was designed to rapidly identify *Listeria* strains to the species level. Two fragments (S1, 460 bp, and S2, 890 bp) were amplified from boiled DNA. S2 was cut with the restriction enzymes *Xmn*I or *Cfo*I and, if needed, S1 was digested by either *Alu*I or *Cla*I. This method was first optimized with six reference strains and then applied to 182 isolates collected from effluents of treatment plants. All isolates were also identified by the API *Listeria* kit, hemolysis, and phosphatidylinositol-specific phospholipase C production (PI-PLC) on ALOA medium. The PCR-RFLP method unambiguously identified 160 environmental strains, including 131 in concordance with the API system, and revealed that 22 isolates were mixed cultures of *Listeria monocytogenes* and *Listeria innocua*. Discrepant results were resolved by a multiplex PCR on the *iap* gene, which confirmed the PCR-RFLP data for 49 of the 51 discordances, including the 22 mixed cultures. Sequencing of the 16S rRNA gene for 12 selected strains and reconstruction of a phylogenetic tree validated the molecular methods, except for two unclassifiable strains. The 158 single identifiable isolates were 92 *L. monocytogenes* (including seven nonhemolytic and PI-PLC-negative strains), 61 *L. innocua*, 4 *Listeria seeligeri*, and 1 *Listeria welshimeri* strain. The PCR-RFLP method proposed here provides rapid, easy-to-use, inexpensive, and reliable identification of the six *Listeria* species. Moreover, it can detect mixtures of *Listeria* species and thus is particularly adapted to environmental and food microbiology.

The genus *Listeria* comprises six characterized species: *Listeria grayi*, *Listeria innocua*, *Listeria ivanovii*, *Listeria monocytogenes*, *Listeria seeligeri*, and *Listeria welshimeri* (42). All of these species are saprophytic bacteria that are ubiquitous in nature (42). Among them, only *L. monocytogenes* acts as an opportunistic pathogen for both humans and animals, mainly causing abortions in pregnant females, neonatal sepsis, and severe infections such as septicemia and meningoencephalitis in susceptible hosts (13, 27, 40). Indeed, from its soil-plant reservoir, this bacterium can infect animals, principally ruminants, and also humans via the ingestion of contaminated foodstuffs of vegetal or animal origin. In fact, *L. monocytogenes* is now recognized as an important food-borne pathogen (13, 40). *L. ivanovii* is principally responsible for abortions in sheep (27). Other *Listeria* species are considered nonpathogenic (42), although *L. ivanovii* and *L. seeligeri* have been implicated in human listeriosis and *L. innocua* has been implicated in animal disease (9, 26, 36, 47).

During a recent investigation on the possible enrichment of the environmental reservoir of *Listeria* spp. by the effluents of treatment plants (D. Paillard, V. Dubois, F. Nathier, E. Hoogland, P. Caumette, and C. Quentin, unpublished data), we isolated a series of *Listeria* strains from sludge and sewage waters and sought a rapid and accurate method for identifying them to the species level. Traditional methods such as the CAMP (Christie, Atkins, Munch-Petersen) test, sugar fermentation, and nitrate reduction are laborious and can take up to 1 to 2 weeks to complete (42). Easy-to-use and rapid miniaturized identification systems are now commercially available (2, 3, 15, 35). However, because of the high phenotypic similarity of the six *Listeria* species, their separation is based on a limited number of biochemical characteristics, and unreliable results for some critical tests have been reported, such as for atypical strains lacking critical features (3, 21, 25, 38). On the other hand, a number of molecular methods have been developed since the late 1980s for *Listeria* species characterization. However, most of them only aim at detecting the *Listeria* genus and/or *L. monocytogenes* in food specimens (32–34, 41, 42). The remaining methods fail to identify every *Listeria* species (18, 30, 43) and/or provide complex intraspecies discrimination (4, 7, 11, 12, 16, 19, 20, 43). Others may require complex reagents (5) or technology (46) or involve time-consuming extraction of chromosomal DNA (6).

In this paper, we describe a novel genotypic method based on restriction fragment length polymorphism (RFLP) of PCR-amplified fragments of the 23S rRNA gene. This method allows the identification of the six species of *Listeria*, without strain-to-strain variation, by use of a common DNA-based protocol not requiring preliminary DNA extraction. In addition, this method was able to identify mixed cultures of different species, especially *L. monocytogenes* and *L. innocua*, which are often found in combination in food and environmental samples (29, 42). The PCR-RFLP method was first validated on six reference strains of *Listeria* spp. and then was applied to

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182 isolates collected from sludge and sewage waters in comparison with biochemical identification.

MATERIALS AND METHODS

Bacterial strains. A total of 188 *Listeria* isolates were examined in this study, including six reference strains (*L. grayi* ATCC 19120^T, *L. innocua* ATCC 51742, *L. monocytogenes* ATCC 19115, *L. seeligeri* ATCC 35967^T, *L. ivanovii* ATCC 19119^T, and *L. welshimeri* ATCC 35897^T) and 182 isolates recovered over a 1-year period from sludge and sewage waters from six effluent sites in the Bordeaux area of France. All strains were routinely grown on Mueller Hinton (MH) agar (Bio Mérieux, Marcy l'Etoile, France) supplemented with 5% horse blood and were stored in 15% glycerol–Trypticase soy broth and 5% horse blood at −80°C.

Biochemical identification. Nonsporulating, regular, gram-positive rods occurring singly or in short chains that were catalase positive and oxidase negative and had growth after enrichment in Fraser broth on PALCAM selective medium (Bio-Rad, Marnes-La-Coquette, France) were presumptively considered *Listeria* spp. All isolates were identified to the species level by the API *Listeria* kit (Bio-Mérieux), according to the manufacturer's instructions. Hemolysis on horse blood MH agar and phenotype displayed on ALOA, a chromogenic medium for *L. monocytogenes* detection in food (Laboratoire AES, Combourg, France), were used as additional tests.

PCR-RFLP identification. For DNA fragment amplification and restriction endonuclease selection, 23S rRNA gene sequences of *Listeria* species available in the GenBank database (NC_003210 and Glaser 2001; accession numbers X92948, X92949, X92950, X92951, X92953, and X92954) were aligned and analyzed by use of the Infobiogen restriction program (http:\\www.infobiogen.fr). The theoretical banding patterns revealed a good discrimination between all six *Listeria* species for two fragments of the gene and four restriction enzymes, following a two- or three-digestion scheme (Fig. 1). Only the typical major bands were taken into account, the minor bands being often nondiscriminative and inconstantly visible at the bottom of the gels (Fig. 2).

For the cell lysis procedure, several colonies were suspended in 100 μl of sterile distilled water. This suspension was immersed in boiling water for 10 min and then placed in cold ice for 5 min. Next, it was centrifuged at 10,000 × *g* at ambient temperature for 5 min, and the supernatant was kept.

For DNA amplification, a final volume of 50 μl was used, containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM (each) deoxyribonucleoside triphosphates, 1.5 U of *Taq* polymerase (Applied Biosystems, Courtabœuf, France), 10 μl of total cell lysate, and 0.5 μM (each) designed laboratory primer for every reaction. Primers S1F (5′-AGTCGGATAGTATCCTTAC-3′) and S1R (5′-GGCTCTAACTACTTGTAGGC-3′) were chosen to amplify a 460-bp DNA fragment (S1) (Fig. 1), and primers S2F (5′-GCCTACAAGTAGTTAGAGCC-3′) and S2R (5′-ACTGGTACAGGAATCTCTAC-3′) were selected to obtain an 890-bp DNA fragment (S2) (Fig. 1). Primers Lis1B, MonoA, Ino2, and Sel1 (6) were used to amplify fragments of the *iap* gene to confirm the species identification when API and PCR-RFLP data were discordant. Primers hly-a and hly-b (22) were used to amplify the *L. monocytogenes* hemolysin gene (*hlyA*). All of these PCR experiments were performed under the following conditions: denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min and a final cycle at 72°C for 7 min. PCR products were visualized by gel electrophoresis using 1.5% agarose gels containing ethidium bromide.

Endonuclease digestion reactions were carried out separately for each enzyme by incubating 12.5 μl of the amplification products with 0.3 U of the endonuclease *Xmn*I, *Cfo*I, *Alu*I, or *Cla*I (Promega, Charbonnières, France) per μl (Fig. 1) in a final volume of 15 μl containing the corresponding buffer. The reaction mixture was incubated for 2 h at 37°C. Digestion products were visualized by gel electrophoresis using 2% agarose gels containing ethidium bromide. The molecular sizes of the obtained fragments were estimated by comparison with the 100-bp DNA ladder and pGEM DNA markers (Promega).

16S rRNA gene sequencing. For 12 strains, DNA amplification was achieved as described above, with 0.5 μM (each) universal primers 8F (5′-AGAGTTTGAT CCTGGCTCAG-3′) and 1489R (5′-TACCTTGTTACGACTTCA-3′), allowing amplification of the entire 16S rRNA gene. PCR experiments were conducted under the following conditions: denaturation at 95°C for 15 min, followed by 35 cycles at 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min 30 s and a final cycle at 72°C for 15 min. PCR products (≈1,400 bp) were visualized by electrophoresis using 1.5% agarose gels containing ethidium bromide.

Sequence reactions using the primers 8F, 1489R, 8F2 (5′-TAACTACGTGC CAGCAGCCG-3′), and 1489R2 (5′-GAAGGGAAAGCTCTGTCTC-3′) were performed by use of the DYEnamic ET Terminator cycle sequencing kit (Amersham, Orsay, France) according to the manufacturer's recommendations. Cycles were as follows: 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Sequences were analyzed on an automatic ABI 310 sequencer (Perkin Elmer, Courtabœuf, France), using Sequencing Analysis software, and were compared to homologous sequences with Sequence Navigator software.

The 16S ribosomal DNA (rDNA) sequences were aligned from positions 64 to 1459 (according to the *Escherichia coli* numbering), including gaps, with the CLUSTAL W program. Distance matrices were calculated with the DNADIST program using the algorithm of Jukes and Cantor (23) within the PHYLIP package (J. Felsenstein, University of Washington, Seattle). The phylogenetic tree was inferred from evolutionary distances with the FITCH program of the PHYLIP package (14). The confidence level of the phylogenetic tree topology was evaluated by performing 100 bootstrap replications with the programs SEQBOOT and CONSENSE of the same package.

Nucleotide sequence accession numbers. The tree sequences obtained in the present work were deposited with EMBL, under the following accession numbers: AJ535693 (strain L169), AJ535694 (L23), AJ535695 (L107), AJ535696 (L151), AJ535697 (L44), AJ535698 (L50), AJ535699 (L52), AJ535700 (L87), and AJ535701 (L97), AJ549928 (*L. innocua* ATCC 51742), AJ549929 (*L. monocytogenes* ATCC 19115), and AJ549930 (*L. ivanovii* subsp. *ivanovii* ATCC 19119).

RESULTS

Biochemical identification of *Listeria* species. The API *Listeria* system allows a 24-h identification of all *Listeria* species, based on 10 sugar fermentation reactions and enzymatic reactions in a microtube format, normally without any need for additional tests. All of the ATCC strains were accurately identified to the species level by the kit. Of the environmental strains, 95 were designated *L. monocytogenes*, 71 were *L. innocua*, and 1 was *L. seeligeri* (Table 1). For 15 isolates, no definitive identification was obtained, since several species were proposed for 13 isolates and none for 2 isolates. On horse blood MH agar plates, the ATCC strains of *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri* were hemolytic, while no hemolysis was observed with *L. grayi*, *L. innocua*, and *L. welshimeri*. Of the 182 environmental strains, 92 were hemolytic, 68 were nonhemolytic, and 22 showed a combination of both phenotypes. The ALOA selective medium differentially detects *Listeria* spp. by their β-glucosidase activity, which translates into bluish colonies, and the pathogenic species *L. monocytogenes* and *L. ivanovii* by their phosphatidylinositol-specific phospholipase C (PI-PLC) capacity, leading to an opaque halo around the colonies. PI-PLC production correlated with hemolysis except for four hemolytic, PI-PLC-negative strains.

PCR-RFLP identification of *Listeria* species. The Infobiogen program helped us to find two fragments of the 23S rRNA gene with species-specific sites for restriction by four enzymes. A large DNA fragment of 890 bp (S2) was used for *Xmn*I or *Cfo*I restriction, and a small DNA fragment of 460 bp (S1) was used for *Alu*I or *Cla*I digestion, according to the scheme presented in Fig. 1 and 2. This PCR-RFLP method was first optimized with the six reference strains of *Listeria*. A large amount of DNA was amplified and no nonspecific bands were obtained with the PCR conditions described here, using chromosomal DNA from boiled bacteria. Since several species of *Listeria*, principally *L. monocytogenes* and *L. innocua*, may coexist in food and environmental samples, an artificial mixture composed of both species was prepared and tested by the PCR-RFLP method. The *Xmn*I restriction enzyme created two bands, of 770 and 650 bp, respectively (Fig. 2E). Identical results were obtained when two species of each pattern were

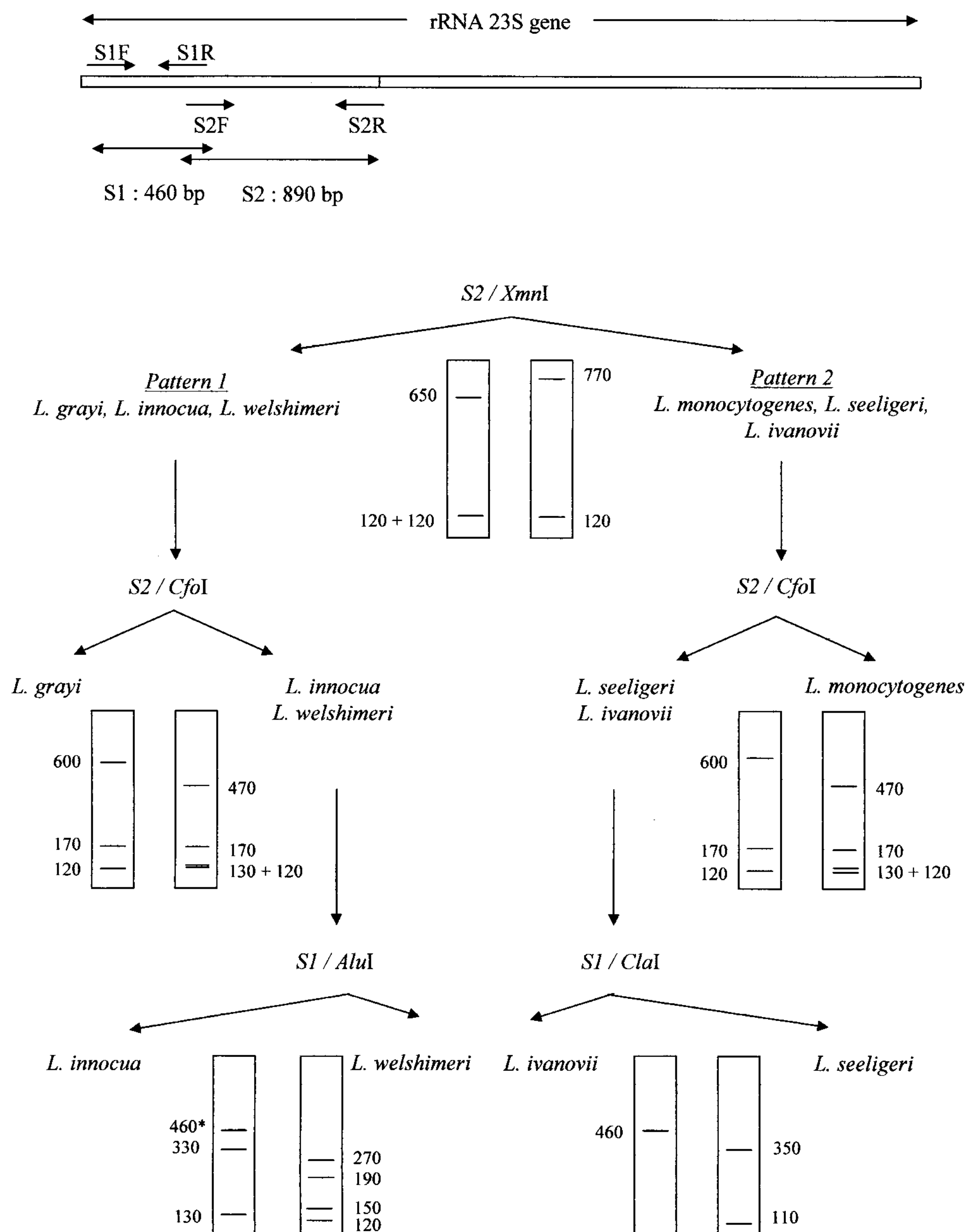

FIG. 1. Scheme used to identify *Listeria* species by PCR-RFLP on two fragments, S1 (460 bp) and S2 (890 bp), of the 23S rRNA gene, amplified by use of the primer pairs S1R-S1F and S2R-S2F, respectively. S2 was first cut with the restriction enzyme *Xmn*I, giving either pattern 1, for *L. grayi*, *L. innocua*, and *L. welshimeri*, or pattern 2, for *L. monocytogenes*, *L. seeligeri*, and *L. ivanovii*. With pattern 1, restriction of S2 by *Cfo*I individualized *L. grayi* and digestion of S1 by *Alu*I differentiated *L. innocua* and *L. welshimeri*. With pattern 2, restriction of S2 by *Cfo*I characterized *L. monocytogenes* and digestion of S1 by *Cla*I discriminated between *L. seeligeri* and *L. ivanovii*. The sizes of the bands are indicated in base pairs. *, unrestricted PCR fragment.

combined. When two species of the same pattern were pooled, the discrimination was achieved at the second or third step, and all mixtures of two species could be detected with the PCR-RFLP method (Fig. 2E). When more than two species were mixed together, many bands were present at the second and third steps and the results were not interpretable.

Of the 182 environmental isolates, 160 were unambiguously identified by PCR-RFLP as 94 *L. monocytogenes*, 61 *L. innocua*, 4 *L. seeligeri*, and 1 *L. welshimeri* (Table 1). When biochemical and molecular methods gave conflicting data (29 strains), a third method, multiplex PCR of the *iap* gene (6), was performed. For 27 isolates, the PCR-RFLP identification was confirmed, and for 2 isolates, the multiplex PCR agreed with the API system. The latter isolates (L87 and L151) were designated *L. monocytogenes* by the PCR-RFLP method and *L. innocua* by the kit and by amplification of the *iap* gene. The remaining 22 isolates (12.1%) gave an *Xmn*I PCR-RFLP pattern comprising two fragments, of 770 and 650 bp, consistent with the superimposition of the *L. monocytogenes* and *L. innocua* patterns. These data were confirmed by multiplex PCR

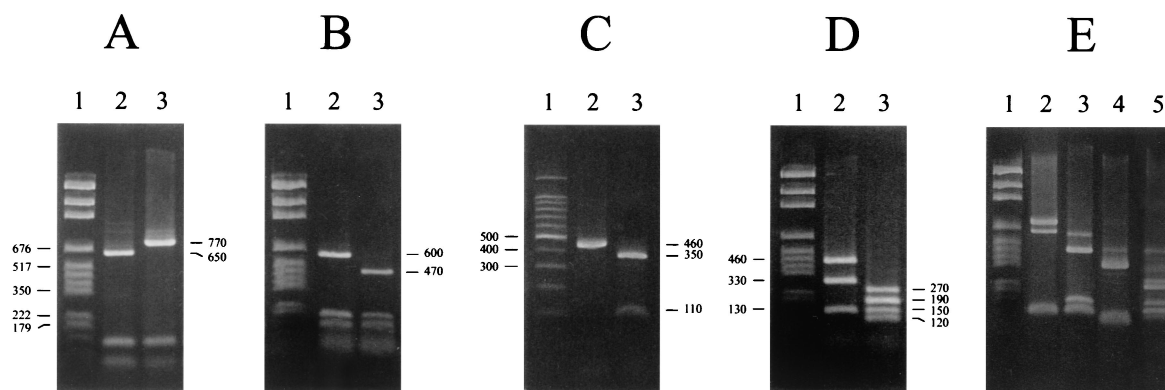

FIG. 2. DNA restriction patterns of PCR-amplified fragments S1 and S2. The sizes of the fragments are indicated in base pairs. (A) S2 digested by *Xmn*I. Lane 1, size marker; lane 2, pattern 1 (*L. grayi*, *L. innocua*, or *L. welshimeri*); lane 3, pattern 2 (*L. monocytogenes*, *L. seeligeri*, or *L. ivanovii*). (B) S2 digested by *Cfo*I. Lane 1, size marker; lane 2, profile 1 (*L. grayi*, *L. seeligeri*, or *L. ivanovii*); lane 3, profile 2 (*L. monocytogenes*, *L. innocua*, or *L. welshimeri*). (C) S1 digested by *Cla*I. Lane 1, size marker; lane 2, profile 1 (*L. ivanovii*); lane 3, profile 2 (*L. seeligeri*, *L. grayi*, *L. monocytogenes*, *L. innocua*, or *L. welshimeri*). (D) S1 digested by *Alu*I. Lane 1, size marker; lane 2, profile 1 (*L. innocua*, *L. grayi*, or *L. monocytogenes*); lane 3, profile 2 (*L. welshimeri*, *L. seeligeri*, or *L. ivanovii*). (E) Profiles obtained with mixed cultures. Lane 1, size marker; lane 2, S2 of a mixed culture of *L. monocytogenes* and *L. innocua* digested by *Xmn*I; lane 3, S2 of a mixed culture of *L. seeligeri* and *L. monocytogenes* digested by *Cfo*I; lane 4, S1 of a mixed culture of *L. ivanovii* and *L. seeligeri* digested by *Cla*I; lane 5, S1 of a mixed culture of *L. innocua* and *L. welshimeri* digested by *Alu*I.

of the *iap* gene (6), which showed amplification of both species-specific fragments (data not shown). Isolation on MH blood agar and/or ALOA medium was then performed to separate the strains, and a new PCR-RFLP was carried out. The 22 isolates actually consisted of a combination of the two most frequent species, *L. monocytogenes* and *L. innocua*. Therefore, the 182 initial isolates divided into 160 single strains and 22 mixtures.

16S rRNA gene sequences. A phylogenetic tree was reconstructed using the 16S rRNA sequences of the seven type strains of *Listeria* spp. available in GenBank, and the sequences were determined for three reference and nine environmental strains examined in this study (Fig. 3). This analysis showed that all strains exhibited a high level of similarity. Nevertheless, the *L. grayi* representative strain clearly formed a subline distinct from the other highly interrelated species, in particular from *L. monocytogenes* and *L. innocua*. The randomly selected environmental strains (L23, L44, L50, L52, L97, L107, and L169) were very similar to the four corresponding reference strains. According to the 16S rRNA gene sequence analysis, strain L87 was more closely related to the *L. monocytogenes* cluster than to the *L. innocua* cluster, consistent with classification by our PCR-RFLP method. However, this strain did not harbor the *hlyA* gene (data not shown). In contrast, strain L151 possessed this gene (data not shown) but was distant from both clusters. Sequencing of the 23S rRNA region encompassing the *Xmn*I restriction site in L87 and L151 revealed complete homology with the ATCC *L. monocytogenes* sequences.

Comparison of the biochemical and PCR-RFLP methods. Based on the concordant identifications obtained by at least two methods (except for the two unclassifiable strains L87 and L151), the API *Listeria* kit provided correct identification for 131 of the 158 single and identifiable isolates (82.9%), but the 22 mixed cultures remained mostly unsuspected since they did not yield any species name in one instance and they were positively identified as *L. innocua* or *L. monocytogenes* in the 21 other cases (Table 1). Of the 180 environmental isolates, 88 *L. monocytogenes* strains were hemolytic and PI-PLC positive and 66 strains (59 *L. innocua*, 6 *L. monocytogenes*, and 1 *L. welshimeri*) were nonhemolytic and PI-PLC negative, while the 22 mixtures produced both types of colonies; the 4 *L. seeligeri* isolates were hemolytic and PI-PLC negative. PCR-RFLP correctly identified all of the single isolates and the mixtures. From a practical point of view, the PCR-RFLP method took about 6 h, owing to the use of DNA from boiled bacteria,

TABLE 1. Correlation between RFLP-PCR method and API *Listeria* kit for identifying 182 environmental strains of *Listeria*

Listeria species	No. identified by API *Listeria* kit	No. identified by RFLP-PCR[a]				
		L. monocytogenes	*L. innocua*	*L. seeligeri*	*L. welshimeri*	Mixture
L. monocytogenes	95	**84**	5			6
L. innocua	71	9	**46**		1	15
L. seeligeri	1			**1**		
L. welshimeri						
Not identified	15	1	10	3		1
Total	182	94	61	4	1	22

[a] Concordant identifications between the two methods are indicated in bold.

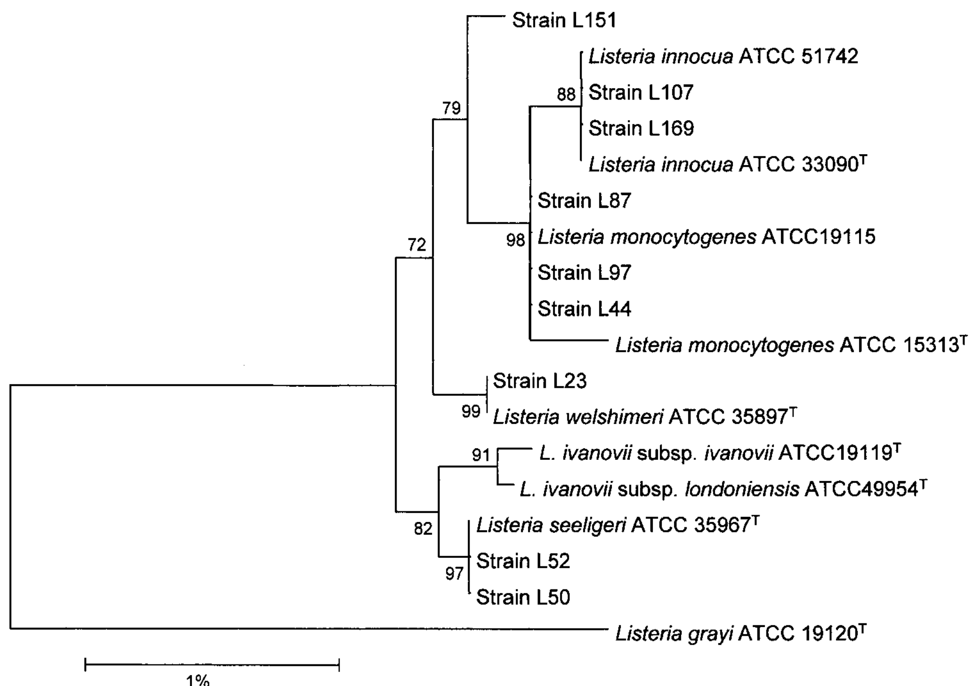

FIG. 3. Phylogenetic tree reconstructed with 16 rDNA sequences of *Listeria* strains. The bar indicates 1% difference in nucleotide sequence. The numbers in the tree indicate the significance (percent of outcomes) of the branches (bootstrap analysis).

compared to more than one working day for multiplex PCR of the *iap* gene because of the DNA extraction step required.

DISCUSSION

Polymorphism of rRNA genes is commonly used to characterize bacterial species (28). However, for the *Listeria* genus, such differentiation is difficult because of the highly conserved nature of these genes. Indeed, in the 16S rRNA sequence, only two domains appear to vary among *Listeria* species (8, 10), while only four signature regions have been identified (39) in the 23S rRNA, although it is twice as large (ca. 2.9 kb). Nevertheless, like DNA-DNA hybridization results (17, 37), 16S and 23S rRNA sequence data have provided evidence that the originally monospecific genus *Listeria* is composed of six very closely related species, divided into two lines of descent as follows: (i) *L. grayi* and (ii) the other *Listeria* spp., the latter subgroup being in turn formed by two distinct evolutionary branches, the first including *L. monocytogenes* and *L. innocua* and the second encompassing *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* (8, 39). Since most phylogenetic investigations have been done at the 16S rDNA level, during preliminary studies we performed computer-assisted alignment and restriction analysis of the corresponding available sequences in *Listeria* spp. to select fragments for amplification and enzymes for their species-specific restriction. However, large amounts of extracted DNA were necessary and many nonspecific bands were obtained (data not shown). In contrast, similar comparison of the 23S rDNA sequences led to a PCR-RFLP assay on boiled DNA, which allowed the precise identification of each *Listeria* species.

The PCR-RFLP method was first validated on six reference strains, representative of each *Listeria* species, and then applied to 182 *Listeria* isolates collected from effluents of treatment plants, in comparison with the API *Listeria* system. The PCR-RFLP method unequivocally identified 160 environmental strains, including 131 in conformity with the kit, and revealed that 22 isolates were mixed cultures of *L. monocytogenes* and *L. innocua*, mostly undetected by the API kit. Discordances between the PCR-RFLP method and the kit were resolved by a multiplex PCR on the invasion-associated protein p60 (*iap*) gene common to all *Listeria* species but with internal species-specific portions (6). This method differentiates *L. monocytogenes*, *L. innocua*, and *L. grayi* at the species level, but the *L. ivanovii*-*L. seeligeri*-*L. welshimeri* group has to be further differentiated by additional PCRs. This method also enables coidentification of two *Listeria* species if they belong to the four different groups (6). Multiplex PCR on the *iap* gene confirmed the PCR-RFLP data for 49 of the 51 discordances, including the 22 mixtures of *L. monocytogenes* and *L. innocua*. In contrast, the multiplex PCR agreed with the API system for two strains of *L. innocua*, designated *L. monocytogenes* by the PCR-RFLP method. Sequencing of the 16S rRNA gene for 12 selected strains further supported these findings. Indeed, the phylogenetic tree reconstructed with nine collection strains had a topology consistent with those previously published (17, 39, 44). Moreover, the seven randomly selected environmental strains shared the highest homology with the corresponding reference strains, thus confirming the validity of the PCR-RFLP method. Of the two strains with conflicting identification, strain L87 was more closely related to the *L. monocytogenes* than to the *L. innocua* cluster but did not carry the *hlyA* gene as *L. monocytogenes* strains do. Strain L151 contained this

gene, but could not definitively be identified at the species level on a phylogenetic basis. Other investigations, such as a DNA-DNA hybridization, would be necessary to determine the species affiliation of both strains. Apart from these two atypical strains, the 158 single isolates were 92 *L. monocytogenes*, 61 *L. innocua*, 4 *L. seeligeri*, and 1 *L. welshimeri*. In the absence of a "gold standard" method, concordant identification obtained by at least two methods (L87 and L151 being omitted) was taken into account. On this basis, the PCR-RFLP exhibited 100% accuracy. Other molecular methods claiming to identify *Listeria* strains to the species level either target differently conserved chromosomal regions (11, 16, 30, 43), including the *iap* gene (6, 7, 46), or belong to the epidemiological typing armamentarium, i.e., are usually utilized to type individual strains belonging to a single species (ribotyping, randomly amplified polymorphic DNA, REP- and ERIC-PCR, pulsed-field gel electrophoresis, and multilocus enzyme electrophoresis) (4, 5, 12, 18–20). However, depending on their discriminatory power, either they fail to distinguish all species (18, 30, 43) or they give puzzling intraspecies (often serotype-specific) differentiation (4, 7, 11, 12, 16, 19, 20, 43). Multilocus enzyme electrophoresis involves species-specific reagents (5), and the microarray-based assay using multiple oligoprobes for virulence factor genes is intended for industrial use (46). The multiplex *iap* PCR method (6) escapes these pitfalls but requires chromosomal DNA extraction and thus more than one working day for completion.

The API *Listeria* system is the most routinely used biochemical method for identifying *Listeria* species. In this study, the kit correctly identified 82.9% of the single isolates, gave 8.2% misidentifications, and gave no definitive identification for 8.9% of the strains. In its earliest evaluation (3), the kit accurately identified 85% of 646 *Listeria* strains: there was one misidentification of an *L. ivanovii* strain, three unidentified isolates (one *L. ivanovii* and two *L. welshimeri*), and 94 isolates needing additional tests to be precisely identified. In another work (21), the API system properly identified to the species level 88.6% of 44 *Listeria* strains; 3 *L. monocytogenes*, 1 *L. innocua*, and 1 *L. welshimeri* strain gave multiple-species, ambiguous, or no identification. For other investigators (31), the gallery exactly characterized 97% of 350 *Listeria* strains, with no misidentifications, but provided equivocal profiles for 6 *L. monocytogenes* and 3 *L. innocua* isolates. In our study, the high proportion of confusions between *L. monocytogenes* and *L. innocua* with the API *Listeria* system was related in 12 of 14 cases to the arylamidase DIM differentiation innocua-monocytogenes) reaction. Indeed, differentiation between both species relies on this unique characteristic (3), and a positive or negative result depends on the visual appreciation of a color shade. As a consequence, the kit misidentified nine strains of the most pathogenic species *L. monocytogenes* as the nonpathogenic *L. innocua* and did the reverse for three strains. In addition, the color strip missed the presence of *L. monocytogenes* in 16 mixtures, which may be frequent in polymicrobial food and environmental samples (24, 29, 42).

Hemolysis and PI-PLC activity are distinctive criteria for *L. monocytogenes* and *L. innocua* identification and might represent supplementary tests when the commercial kits give inconclusive data. Indeed, hemolysin is recognized as a major virulence factor of *L. monocytogenes* (44). However, other *Listeria* species (i.e., *L. ivanovii* and *L. seeligeri*) are hemolytic, and hemolysin expression in *L. monocytogenes* is highly variable depending on the strain and the technique employed (13, 15, 21, 25, 31). Accordingly, in our study, seven *L. monocytogenes* strains were nonhemolytic. ALOA medium differentiates *L. monocytogenes* and *L. ivanovii* from the remainder of the genus by their PI-PLC production (1, 24, 35, 45), like other recently proposed chromogenic media (24). Although easier to visualize, the resulting opaque halo was, as expected (24, 44), correlated with hemolysis except for the *L. seeligeri* strains.

In conclusion, the present PCR-RFLP method allows rapid and accurate identification of the six species of the *Listeria* genus. Furthermore, in contrast with the API *Listeria* kit, it can detect different *Listeria* species contaminating the same sample. Thus, it is particularly useful for identifying *Listeria* spp. isolated from food products and environment samples but might also be of benefit in clinical and veterinary microbiology for confirming identification of rare species or atypical strains of *Listeria* spp.

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