Comparison of PCR-based DNA fingerprinting techniques for the identification of *Listeria* species and their use for atypical *Listeria* isolates

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Four PCR-based DNA fingerprinting techniques were compared for their ability to identify at the species level a heterogeneous collection of isolates belonging to the six valid *Listeria* species. 16S rDNA-RFLP analysis identified all species and 16S rDNA-SSCP analysis identified almost all species. Also, isolates with unusual biochemical characteristics and/or unusual antigenic composition could be identified correctly. rRNA-intracistronic length polymorphism analysis suffered from high intraspecific variability, a limited number of fragments per profile, and small length differences between the spacers of different species. tRNA-intergenic length polymorphism analysis resulted in identification of all isolates but one, when fluorescent DNA capillary electrophoresis was used such that fragment length differences of 1 bp could be resolved. The four techniques yielded comparable results relevant to the taxonomy of *Listeria*. They all indicate a high degree of genetic relatedness between *L. innocua* and *L. welshimeri*, homogeneity of *L. grayi*, distinct but clear relatedness of *L. grayi* to the other *Listeria* species, a clear distinction between the two subspecies of *L. ivanovii*, and a clear distinction between *Listeria* isolates and isolates from closely related taxa or from species which are phenotypically difficult to distinguish from *Listeria*. New sequence determination of the 16S rRNA gene was necessary to obtain sequences in accordance with the findings of 16S rDNA-RFLP analysis.

Keywords: *Listeria*, PCR-based DNA fingerprinting, 16S rDNA-RFLP, 16S rDNA-SSCP, spacer length polymorphism

INTRODUCTION

The genus *Listeria* is very homogeneous, except for *L. grayi* which is clearly distinct from the other species (4, 7, 22, 30, 34). The taxonomy of the genus *Listeria* has been difficult to assess in the past, but molecular methods (4, 30, 32) have clearly demonstrated the existence of six *Listeria* species (*L. monocytogenes*, *L.*

ivanovii, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi*). The former species '*L. murrayi*' has been assigned to the species *L. grayi* (33). Only *L. monocytogenes* and *L. ivanovii* are recognized as significant pathogens of humans and animals. The *Listeria* species differ in only a few phenotypic characteristics (12, 22, 31) and this high degree of similarity is the source of a lot of confusion in their identification. Furthermore, several other species, such as *Bacillus* species, *Enterococcus* species (particularly *Enterococcus faecalis*) and *Cellulomonas* (*Oerskovia*) *turbata*, are often misidentified as *Listeria* species by inexperienced microbiologists. Finally, the type strain of *L. monocytogenes* is phenotypically abnormal (23) and would be difficult to identify if it was encountered as a field isolate.

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Abbreviations: ARDRA, amplified rDNA restriction analysis; SSCP, single strand conformational polymorphism.

The EMBL accession numbers for the sequences reported in this paper are X98526–X98532.

Methods which combine the reliability of genotypic identification with the speed and simplicity of phenotypic methods such as biochemistry and serology, would be more than welcome, particularly in the presence of biochemically and serologically atypical isolates. Four PCR-based DNA fingerprinting methods have been described to enable identification of cultured bacterial organisms to the species level on the basis of genotype: rDNA-RFLP analysis (or ARDRA, amplified rDNA restriction analysis) and rDNA-single-strand conformational polymorphism (SSCP) analysis, which both make use of the species-specific sequence information in the 16S rRNA gene; and rDNA-spacer-PCR and tDNA-spacer-PCR, which have been reported to provide species-specific length polymorphism of rRNA intracistronic and tRNA-intergenic spacer regions, respectively. In this study we assessed the suitability of these four methods for the identification of the species of the genus *Listeria*.

ARDRA has been studied most thoroughly and has been used for the identification of species of different taxa, e.g. *Acinetobacter* (39), *Clostridium* (37), *Comamonadaceae* (41), *Corynebacterium* (40), *Mollicutes* (9), *Mycobacterium* (38), *Leptospira* (29) and *Streptococcus* (19), and to study phylogenetic relationships in *Bacillus* (16), *Clostridium* (13), *Moraxella* (18) and *Xanthomonas* (28).

Widjoatmodjo *et al.* (44, 45) used SSCP analysis of part of the 16S rDNA to identify a number of species, including all six *Listeria* species. Jensen *et al.* (20) showed that rDNA-spacer-PCR could be used to identify *Escherichia* species, some *Enterobacter* and *Staphylococcus* species and five of the six *Listeria* species (*L. seeligeri* was not studied). Others used this approach successfully for the identification of *Legionellaceae* (17).

tDNA-spacer-PCR has been used for the identification of *Staphylococcus* species (42, 43), *Streptococcus* species (26) and *Acinetobacter* species (10, 46).

METHODS

Bacteria. Table 1 lists the isolates which were used. Besides representatives of all the six valid *Listeria* species, including the two subspecies of *L. ivanovii* (3), isolates of the species *L. monocytogenes*, *L. innocua*, *L. welshimeri* and *L. ivanovii* representing unusual biotypes or atypical antigenic compositions were also included and tested blindly. The *Listeria* isolates representing atypical biochemical or serological reactions were also tested with API-*Listeria* galleries (BioMérieux), following the recommendations of the manufacturer. Isolates of genera phylogenetically closely related to *Listeria* were also included since these may be phenotypically misidentified as *Listeria*.

Sample preparation for PCR. Target DNA was prepared from cells grown overnight at 37 °C on Mueller-Hinton Agar II (MHAI; BBL Microbiology Systems) supplemented with 5% sheep blood. A 1 µl volume of cells was suspended in 300 µl 10 mM Tricine/1.5 mM MgCl₂ and heated at 100 °C for 10 min in a heating block. Five microlitres of the

supernatant of this boiled cell suspension was used as the target DNA and was added – using filter-protected tips – to 45 µl aliquots of PCR mix for all DNA fingerprinting techniques.

Amplification mixture. All reaction mixtures contained – at a final reaction volume of 50 µl – 0.005 U Goldstar polymerase µl⁻¹ (Eurogentec), 100 µM each dNTP (Pharmacia Biotech) and 0.2–0.5 µM each primer in reaction buffer [7.5 mM Tris/HCl, 2.5 mM MgCl₂, 20 mM (NH₄)₂SO₄, 0.1% (w/v) Tween 20, pH 9.0]. After aliquotting in 45 µl volumes, mixtures were overlaid with 40 µl mineral oil.

ARDRA. The 16S rRNA gene (16S rDNA) was amplified using 0.2 µM primers complementary to conserved regions present at the edges of the 16S rDNA. The sequences of the primers were 5'TGGCTCAGATTGAACGCTGGCGGC (*Escherichia coli* nucleotide positions 10–27) and 5'TACCTTGTTACGACTTCACCCCA (*E. coli* nucleotide positions 1507–1485). After initial denaturation at 95 °C for 5 min, the reaction mixtures were cycled 35 times for 45 s at 95 °C, 45 s at 55 °C, and 1 min at 72 °C. Finally, a 7 min extension period at 72 °C was carried out. The presence and yield of specific PCR products were determined by Multi-Purpose (Boehringer) agarose (1%, w/v) – ethidium bromide (50 ng ml⁻¹) gel electrophoresis for 30 min at 7 V cm⁻¹. Ten microlitre aliquots of the amplified DNA were used without further purification for restriction digestion. The following enzymes were used: *AluI*, *AvaI*, *BanI*, *BfaI*, *BspI*286I, *BstEII*, *BstUI*, *CfoI* (*HhaI* isoschizomer), *DraI*, *EcoT38I*, *HaeIII*, *HinfI*, *MboI*, *MspI*, *MvaI*, *NciI*, *RsaI*, *Sau96I*, *SerFI*, *StyI*, *TaqI* and *Tru9I*. Restriction digestion was carried out for 1 h at 37 °C, except for *TaqI* and *Tru9I*, which were incubated at 65 °C, in 20 µl volumes of commercial incubation buffer containing 1 U µl⁻¹ restriction enzyme and 5 µl amplified 16S rRNA gene product. Restriction fragments were separated by gel electrophoresis for 3 h at 7 V cm⁻¹ on 3% Metaphor agarose (FMC BioProducts Europe) containing 50 ng ethidium bromide ml⁻¹.

16S rDNA-SSCP analysis. rDNA-SSCP analysis was carried out essentially as described by Widjoatmodjo *et al.* (44). Primers P11P (5'GAG GAA GGT GGG GAT GAC GT) and P13P (5'AGG CCC GGG AAC GTA TTC AC) were used at concentrations of 0.2 µM to amplify a 216 bp fragment of the V6 region of the 16S rDNA (*E. coli* 16S rDNA positions 1175–1390). After initial denaturation at 95 °C for 5 min, the reaction mixtures were cycled 35 times for 20 s at 95 °C, 1 min at 55 °C and 1 min at 72 °C. Finally, a 7 min extension period at 72 °C was carried out: 0.3 µl volumes of the amplification products were mixed with 6 µl water and 3 µl sequencing buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The mixture was heated to 95 °C for 5 min and loaded on the gels. The amplification products were separated in non-denaturing 0.5 × MDE gels (FMC BioProducts) containing 5% glycerol. The gels were run overnight in a Protean II xi electrophoresis cell (Bio-Rad) in 0.5 × TBE at room temperature at 90 V. The gels were stained using the Silver Stain Plus kit (Bio-Rad) and the profiles were interpreted visually.

rRNA-intracistronic spacer length polymorphism analysis (rDNA-spacer-PCR). The intracistronic rDNA spacer regions were amplified by using primers – at concentrations of 0.5 µM – flanking the 16S–23S rDNA spacer region (forward, 5'GAA GTC GTA ACA AGG; reverse, 5'CAA GGC ATC CAC CGT) (20). After initial denaturation at 95 °C for 5 min, the reaction mixtures were cycled 25 times for 1 min

at 94 °C, 2 min at 55 °C, and 2 min at 72 °C, followed by a final 7 min extension period at 72 °C. Twenty microlitres of each amplification product was separated in 4% polyacrylamide gels using 1 × TBE at constant voltage in a Protean II xi electrophoresis cell. The gels were stained in a 1 µg ethidium bromide ml⁻¹ solution and photographed under short wavelength UV light. The migration profiles were compared visually.

tRNA-intergenic spacer length polymorphism analysis (tDNA-spacer-PCR). tDNA-spacer-PCR was carried out essentially as described by Welsh & McClelland (42). Primers T5A (5'AGT CCG GTG CTC TAA CCA ACT GAG) and T3B (5'AGG CCG CGG GTT CGA ATC C) were used at concentrations of 0.5 µM to amplify the spacer regions between the tRNA genes. For analysis by capillary electrophoresis the T3B primer was labelled at the 5' end with the fluorescent amidite TET (Applied Biosystems). After initial heating at 95 °C for 7 min, reaction mixtures were cycled 40 times for 30 s at 95 °C, 30 s at 50 °C and 2 min at 72 °C, followed by final extension for 7 min at 72 °C. Capillary electrophoresis of fluorescently labelled PCR products was done on an ABI 310 (Applied Biosystems) using the POP-4 polymer and capillaries and with the TAMRA-500 standard as the internal lane size standard.

16S rDNA sequence determination. An approximately 1.45 kb segment of the 16S rRNA gene was amplified using primer 41f (5'GCT CAG ATT GAA CGC TGG CG) and the biotin-labelled primer 1488r (5'biotin-CGG TTA CCT TGT TAC GAC TTC ACC). Forty microlitres of the PCR sample were added to Dynabeads M280 Streptavidin (Dyna), the DNA was denatured and the biotin-labelled single strand was immobilized onto the beads and used as one template for the sequencing reaction using fluorescent primers 358f (5' AGA CTC CTA CGG GAG GCA GCA GT), 536f [5' GTG CCA GC(AC) GCC GCG GTA ATA C] and 928f [5' TAA AAC T(CT)A AA(GT) GAA TTG ACG GGG]. The opposite strand remaining in solution was also used as template using fluorescent primers 336r [5' ACT GCT GCS (CT)CC CGT AGG AGT CT] and 515r [5' G(AT)A TTA CCG CGG C(GT)GCTG GCA C]. Sequencing reaction products obtained by using the T7 Autoread kit (Pharmacia Biotech) were processed using an ALF DNA sequencer (Pharmacia Biotech) and the ALF manager software. DNA sequence comparisons were done using the HIBIO DNASIS software package (Hitachi). Published *Listeria* sequences were obtained from the EMBL database and the database included in the ARB package (see below). Tree construction was done using the ARB software package developed by Oliver Strunk, Wolfgang Ludwig and others from the Microbiology Department of the Technical University, Munich, Germany. The software version for Linux and the database (6aug9.ascii.arb) were obtained by anonymous ftp from ftp.biol.chemie.tu-muenchen.de.

Computer-assisted restriction analysis of the 16S rRNA gene was carried out with HIBIO DNASIS on EMBL sequences X56148–X56154 (7) and on the sequences obtained in the present work. Distance values were calculated using the neighbour-joining method with Kimura correction.

RESULTS

ARDRA

Restriction of the 16S rRNA gene was carried out with a total of 22 enzymes. Only restriction with *AluI*,

Fig. 1. 16S rDNA-RFLP profiles obtained by 3% agarose gel electrophoresis of digests of the amplified 16S rDNA after restriction with *AluI*.

*Bst*UI, *Cfo*I, *Rsa*I and *Tru*9I enabled some differentiation (Table 1). Combination of the profiles observed after digestion with *AluI* (Fig. 1) and *Bst*UI enabled identification of all *Listeria* species, except for *L. innocua* and *L. welshimeri*, for which a constant difference could be shown only after restriction of the 16S rDNA with *Tru*9I (Table 1, Table 2). Also, only minor but constant differences could be observed for *Bst*UI restriction profiles of *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis*, and for *L. welshimeri* and *L. seeligeri* (Table 2). *L. grayi* was clearly different from the other five species, since restriction with *AluI* and *Rsa*I yielded unique restriction profiles not observed for any other *Listeria* species (Table 1, Table 2). One *L. innocua* isolate (TI6520) had a *Bst*UI profile (1c) with an additional 250 bp fragment. For *L. monocytogenes* isolate IP11 an extra fragment was observed repeatedly in the *Cfo*I profile. This could be resolved as the result of interference of minor non-specific amplification, not present in one of the collaborating laboratories. The isolates of the closely related non-*Listeria* species could easily be identified as such by restriction analysis of the 16S rDNA, although some of the restriction enzymes yielded identical profiles for some *Listeria* and non-*Listeria* species (Table 1).

16S rRNA gene sequence determination and phylogenetic tree construction for the genus *Listeria*

The sequences of the 16S rRNA genes were determined for seven isolates (indicated in Table 1). The distance

Table 1. Designation of isolates, serotype and biotype, and of profiles observed for different PCR-based DNA fingerprinting techniques for species identification

Code	Original no.	Species*	Serotype or unusual agglutination reactions†	ARDRA					rDNA-SSCP analysis‡	rDNA-spacer-PCR
				<i>AluI</i>	<i>CfoI</i>	<i>RsaI</i>	<i>BstUI</i>	<i>Tru9I</i>		
1	LL195§	<i>L. monocytogenes</i>	4b	1	1	1	1a	1a	1	6
2	ZH7415	<i>L. monocytogenes</i>	1/2a	1	1	1	1a	1a	1	4
3	SLCC2371	<i>L. monocytogenes</i>	1/2a	1	1	1	1a	ND	1	4
4	SLCC2755	<i>L. monocytogenes</i>	1/2b	1	1	1	1a	ND	1	5
5	SLCC2372	<i>L. monocytogenes</i>	1/2c	1	1	1	1a	ND	1	4
6	SLCC2373	<i>L. monocytogenes</i>	3a	1	1	1	1a	ND	1	4
7	SLCC2540	<i>L. monocytogenes</i>	3b	1	1	1	1a	ND	1	4
8	SLCC2479	<i>L. monocytogenes</i>	3c	1	1	1	1a	ND	1	4
9	SLCC2374	<i>L. monocytogenes</i>	4a	1	1	1	1a	1a	1	9
10	SLCC2375	<i>L. monocytogenes</i>	4b	1	1	1	1a	ND	1	7
11	SLCC2376	<i>L. monocytogenes</i>	4c	1	1	1	1a	ND	1	10
12	SLCC2377	<i>L. monocytogenes</i>	4d	1	1	1	1a	ND	1	4
13	SLCC2378	<i>L. monocytogenes</i>	4e	1	1	1	1a	ND	1	6
14	SLCC2482	<i>L. monocytogenes</i>	7	1	1	1	1a	ND	1	5
58	IP11	<i>L. monocytogenes</i>	V/VI; VI; VII; VIII; A; B; C	1	1	ND	1a	1a	1	8
59	IP15	<i>L. monocytogenes</i> ^a	4b	1	1	ND	1a	ND	1	ND
65	NV3953	<i>L. monocytogenes</i> ^b	1/2b	1	1	ND	1a	ND	1	ND
66	NV4172	<i>L. monocytogenes</i> ^b	1/2a	1	1	ND	1a	ND	1	ND
68	LU4526	<i>L. monocytogenes</i> ^c	4b	1	1	ND	1a	ND	1	ND
77	BS6179	<i>L. monocytogenes</i> ^c	4b	1	1	ND	1a	ND	1	ND
79	GE6366	<i>L. monocytogenes</i> ^c	1/2c	1	1	ND	1a	ND	1	ND
15	SLCC3379/ATCC 33090§	<i>L. innocua</i>	6a	2	1	1	1a	1a	2	13
16	LL271	<i>L. innocua</i>	6b	2	1	1	1a	1a	2	11
17	VD8304	<i>L. innocua</i>	6a	2	1	1	1a	1a	2	12
18	8716	<i>L. innocua</i>	6b	2	1	1	1a	1a	2	11
25	VD7945	<i>L. innocua</i>	6b	2	1	1	1a	1a	2	11
69	GE5432	<i>L. innocua</i>	1; I/II; A; B; C (1/2b)	2	1	ND	1a	1a	2	ND
70	GE5433	<i>L. innocua</i>	1; I/II; A; B; C (1/2b)	2	1	1	1a	1a	ND	ND
71	TI5796	<i>L. innocua</i>	V/VI; VII; XV; B; C; D	2	1	ND	1a	1a	2	ND
76	VD6140	<i>L. innocua</i>	V/VI; VII; XV; B; C; D	2	1	1	1a	1a	ND	ND
81	TI6520	<i>L. innocua</i>	XV; B; C; D	2	1	ND	1c	1a	2	ND
82	TI6551	<i>L. innocua</i>	V/VI; VII; XV; B; C; D	2	1	1	1a	1a	ND	ND
83	BL6621	<i>L. innocua</i>	No somatic factors A; B; C	2	1	1	1a	1a	ND	ND
87	BL6830	<i>L. innocua</i>	VII; VIII; A; B; C	2	1	ND	1a	1a	2	ND
23	SLCC5334§	<i>L. welshimeri</i>	6a	2	1	1	1a	1b	2	14
24	ZH7199	<i>L. welshimeri</i>	6b	2	1	1	1a	1b	2 + 3	14
60	LL244	<i>L. welshimeri</i>	V/VI; VI; VII; X; B; C; D	2	1	ND	1a	1b	2 + 3	ND
61	LL519	<i>L. welshimeri</i>	1; I/II; A; B; C (1/2b)	2	1	ND	1a	1b	2	ND
62	LL520	<i>L. welshimeri</i>	1; I/II; A; B; C (1/2b)	2	1	ND	1a	1b	2	ND
63	BS2094	<i>L. welshimeri</i>	V/VI; VI; VII; X; B; C; D	2	1	ND	1a	1b	2 + 3	ND
67	SO4352	<i>L. welshimeri</i>	V/VI; VI; VII; X; B; C; D	2	1	1	1a	1b	ND	ND
72	TI5800	<i>L. welshimeri</i>	V/VI; VI; VII; X; B; C; D	2	1	ND	1a	1b	2 + 3	ND
73	GE5877	<i>L. welshimeri</i>	V/VI; VI; VII; X; B; C; D	2	1	1	1a	1b	ND	ND
74	BE5963	<i>L. welshimeri</i>	V/VI; VI; VII; X; B; C; D	2	1	1	1a	1b	ND	ND
75	BE6108	<i>L. welshimeri</i>	V/VI; VI; VII; X; no H-factors	2	1	1	1a	1b	ND	ND
78	GE6254	<i>L. welshimeri</i>	V/VI; VI; VII; X; B; C; D	2	1	1	1a	1b	ND	ND
84	LU6662	<i>L. welshimeri</i>	V/VI; VI; VII; X; B; C; D	2	1	ND	1a	1b	2 + 3	ND
85	BE6112	<i>L. welshimeri</i>	V/VI; VI; VII; X; B; C; D	2	1	1	1a	1b	ND	ND
86	AG7166	<i>L. welshimeri</i>	V/VI; VI; VII; X; B; C; D	2	1	1	1a	1b	ND	ND
19	SLCC3954/ATCCC 35967§	<i>L. seeligeri</i>	1/2b	2	2	1	1b	1a	3	3
20	8375	<i>L. seeligeri</i>	4ab	2	2	1	1b	1a	3	1
21	BE8950	<i>L. seeligeri</i>	1/2b	2	2	1	1b	ND	3	1
22	BE8953	<i>L. seeligeri</i>	4d	2	2	1	1b	ND	3	2
64	NV2918	<i>L. seeligeri</i> ^a	1/2b	2	2	ND	1b	ND	3	ND
27	LL278	<i>L. ivanovii</i> subsp. <i>ivanovii</i>	5	2	2	1	2a	1a	6	ND
28	LL483	<i>L. ivanovii</i> subsp. <i>ivanovii</i>	5	2	2	1	2a	1a	6	21
29	BE5087	<i>L. ivanovii</i> subsp. <i>ivanovii</i>	5	2	2	1	2a	ND	6	21
46	CLIP12510T§	<i>L. ivanovii</i> subsp. <i>ivanovii</i>	5	2	2	1	2a	ND	6	21
51	SLCC4306	<i>L. ivanovii</i> subsp. <i>ivanovii</i>	5	2	2	1	2a	ND	6	20
55	SLCC2098	<i>L. ivanovii</i> subsp. <i>ivanovii</i>	5	2	2	1	2a	ND	6	21
30	BE1604	<i>L. ivanovii</i> subsp. <i>londoniensis</i>	5	2	2	1	2b	1a	4	19
31	BE5063	<i>L. ivanovii</i> subsp. <i>londoniensis</i>	5	2	2	1	2b	1a	ND	ND
32	BE1694	<i>L. ivanovii</i> subsp. <i>londoniensis</i>	5	2	2	1	2b	ND	4	ND
33	BE3728	<i>L. ivanovii</i> subsp. <i>londoniensis</i>	5	2	2	1	2b	ND	4	ND
47	CLIP12229T§	<i>L. ivanovii</i> subsp. <i>londoniensis</i>	5	2	2	1	2b	1a	4	18
48	BE5194	<i>L. ivanovii</i> subsp. <i>londoniensis</i>	I; I/II; A; B; C (1/2b)	2	2	1	2b	ND	4	ND
50	BE5195	<i>L. ivanovii</i> subsp. <i>londoniensis</i>	I/II; VII; no H-factors	2	2	1	2b	ND	ND	ND

continued opposite

Table 1 (cont.)

Code	Original no.	Species*	Serotype or unusual agglutination reactions†	ARDRA					rDNA-SSCP analysis‡	rDNA-spacer-PCR
				<i>AluI</i>	<i>CfoI</i>	<i>RsaI</i>	<i>BstUI</i>	<i>Tru9I</i>		
52	CLIP2737	<i>L. ivanovii</i> subsp. <i>londoniensis</i>	5	2	2	1	2b	ND	4	19
53	ZH7466	<i>L. ivanovii</i> subsp. <i>londoniensis</i>	I;1/II;A;B;C (1/2b)	2	2	1	2b	ND	4	ND
43	ATCC 25402	<i>L. grayi</i>		3	2	2	2a	2	5	17
44	ATCC 25403	<i>L. grayi</i>		3	2	2	2a	2	5	15
45	SLCC2080/ATCC 19120§	<i>L. grayi</i>		3	2	2	2a	ND	5	16
34	LA3056	<i>Brochothrix thermosphacta</i>		4	3	2	1	ND	ND	ND
35	LA3674	<i>Bacillus cereus</i>		5	2	3	2b	ND	ND	ND
39	CIP 103610T	<i>Carnobacterium gallinarum</i>		9	6	4	4	ND	ND	ND
42	LA3193	<i>Cellulomonas (Oerskovia) turbata</i>		11	8	6	6	ND	ND	ND
36	LA2365	<i>Enterococcus durans</i>		6	4	4	4	ND	ND	ND
37	LA2390	<i>Enterococcus faecalis</i>		7	4	4	4	ND	ND	ND
38	LA3225	<i>Listeria (Listeria) denitrificans</i>		8	5	5	5	ND	ND	ND
40	CIP 102976T	<i>Vagococcus fluvialis</i>		10	7	4	3	ND	ND	ND

ND, Not determined.

*Superscript letters indicate the following properties: a, arabinol-negative; b, L-rhamnose-negative; c, methyl α -D-mannoside-negative; d, methyl α -D-mannoside-positive.

†Serovar numbering and serological reactions are according to Seeliger & Hühne (35). Serotypes with flagellar factors B;C;D or somatic factors VII;VIII or I;I/II;VII have not been described previously.

‡rDNA-SSCP analysis was at room temperature.

§Isolates of which the 16S rRNA gene was sequenced in this study; their EMBL accession numbers are X98526–X98532.

|| These isolates were formerly '*L. murrayi*'.

values are given in Table 3 and a tree representation of this calculation is shown in Fig. 2. The sequences obtained in the present study differ from those obtained previously (7, 8). The number of differences (not counting undefined bases as differences and comparing with the best corresponding to previously published sequences for each species) was between 3 bp (with *L. seeligeri* sequence X56148) and 8 bp (with *L. grayi* sequence X56150). To construct a tree based on the 16S rRNA sequences of the *Listeria* species studied here, they were imported into the ARB package and aligned manually to match the alignment of the *Listeria* sequences determined previously (7, 8). A phylogenetic tree containing the published sequences and a combined tree containing also the sequences obtained in this study were constructed (data not shown). The clustering of the species in all three trees did not show significant differences. Thus, the sequence discrepancies found between published and new sequences had no influence on the determination of phylogenetic relationships, although there were less interspecific differences for the newly determined sequences. Computer-aided restriction digestion of the newly determined sequences corresponded perfectly with the observed ARDRA patterns, except for two minor discrepancies (Table 2).

rDNA-SSCP analysis

The results of the rDNA-SSCP analysis are reported in Table 1 and are illustrated in Fig. 3. Seven profiles were obtained for the six *Listeria* species examined. The profiles were composed of two or four bands. Five *Listeria* species and subspecies (*L. monocytogenes*, *L. seeligeri*, *L. ivanovii* subsp. *ivanovii*, *L. ivanovii* subsp.

londoniensis and *L. grayi*) had specific and homogeneous profiles. The eight *L. welshimeri* isolates presented two different profiles, and for three of these isolates this profile was identical to the unique *L. innocua* profile (Table 1). Electrophoresis was also performed under different conditions (four different buffer systems, electrophoresis at 6 °C or at room temperature, addition of 0 or 10% glycerol). The profiles obtained varied markedly, depending on the conditions used, but some or all *L. welshimeri* isolates remained indistinguishable from *L. innocua*, whereas differentiation of other species became difficult or impossible (data not shown).

rDNA-spacer-PCR

Analysis of the PCR products by PAGE showed that 21 profiles could be found among the 37 isolates tested (Fig. 4, Table 1). Several profiles were found within each species, except for *L. welshimeri* (for which only two isolates were tested). The profiles were composed of one main band and one or several minor bands. The variation within a species relied on both the minor and the main bands. Because of the variability within the species it was difficult to define reliable diagnostic criteria for species identification. rDNA-spacer-PCR was clearly reproducible. When repeating electrophoresis with new amplification products from the same strains, the same profiles were obtained and the same differences between profiles were reproducibly detectable.

tDNA-spacer-PCR

The discriminatory power of tDNA-spacer-PCR depended highly on the resolving power of the electro-

Table 2. Comparison of observed ARDRA patterns with restriction fragment sites and lengths as predicted by 16S rRNA sequence determination

Restriction with <i>AhaI</i> (AG/CT)	Restriction sites										Restriction fragment lengths										ARDRA			
	32/3	127	140	207	822/3	982/3	1029/30	1234/5	13	32/3	47	67	95	160	175	205	232/3	438	615/6					
<i>L. monocytogenes</i>	+			+	+	+	+	+		+	+	+	+	+	+	+	+	+		1				
<i>L. innocua</i>	+			+	+	+	+	+		+	+	+	+	+	+	+	+	+		2				
<i>L. welshimeri</i>	+			+	+	+	+	+		+	+	+	+	+	+	+	+	+		2				
<i>L. seeligeri</i>	+			+	+	+	+	+		+	+	+	+	+	+	+	+	+		2				
<i>L. ivanovii ivanovii</i>	+			+	+	+	+	+		+	+	+	+	+	+	+	+	+		2				
<i>L. ivanovii londoniensis</i>	+			+	+	+	+	+		+	+	+	+	+	+	+	+	+		2				
<i>L. grayi</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	3				
<i>CfoI</i> (GCG/C)	145	537/8/9/40 1/2	723/4	1069/70					2	145	182	346	392	395		537/8								
<i>L. monocytogenes</i>	+	++	+	+					++	+	+	+	+	+						1				
<i>L. innocua</i>	+	++	+	+					++	+	+	+	+	+						1				
<i>L. welshimeri</i>	+	++	+	+					++	+	+	+	+	+						1				
<i>L. seeligeri</i>	+	++	+	+					++	+	+	+	+	+	+					2				
<i>L. ivanovii ivanovii</i>	+	++	+	+					++	+	+	+	+	+	+					2				
<i>L. ivanovii londoniensis</i>	+	++	+	+					++	+	+	+	+	+	+					2				
<i>L. grayi</i>	+++	+	+	+					++	+	+	+	+	+	+					2				
<i>Bsu</i> UI (CG/CG)	196	362	487	537	539	723/4	932/3	1217/8	1224/ 5	1311/2	1326/ 7	15	50	87	94	125	140/1	166	184/5	196/7	209	242/3	285	292/3
<i>L. monocytogenes</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. innocua</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. welshimeri</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. seeligeri</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. ivanovii ivanovii</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. ivanovii londoniensis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. grayi</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Tth</i> 91 (T/TAA)	553	579/80	831	837	917	919	1051/2	1076	25/6/7	82/6	133/4	252	257	391	415	553								
<i>L. monocytogenes</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. innocua</i>	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. welshimeri</i>	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. seeligeri</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. ivanovii ivanovii</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. ivanovii londoniensis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. grayi</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. monocytogenes</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. innocua</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. welshimeri</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. seeligeri</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. ivanovii ivanovii</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. ivanovii londoniensis</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>L. grayi</i>	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* According to the sequences determined in this study, *L. welshimeri* has a deletion which destroys the *Bsu*UI restriction site at position 1217. This predicts ARDRA pattern 1b. However, for all *L. welshimeri* strains ARDRA pattern 1a was observed.

† For *L. grayi*, both the 252 and 257 bp fragments were observed on ARDRA patterns instead of the predicted 257 bp fragment only.

Table 3. Distance values data matrix of the *Listeria* 16S rRNA gene sequences determined in this work

Values were calculated using the neighbour-joining method (Kimura correction, vertical gaps compressed) provided by the ARB software package.

	<i>L. grayi</i>	<i>L. innocua</i>	<i>L. ivanovii</i> subsp.		<i>L. monocytogenes</i>	<i>L. seeligeri</i>	<i>L. welshimeri</i>
			<i>ivanovii</i>	<i>londoniensis</i>			
<i>L. grayi</i>	0.0000	0.0424	0.0348	0.0393	0.0386	0.0393	0.0401
<i>L. innocua</i>		0.0000	0.0100	0.0093	0.0057	0.0071	0.0057
<i>L. ivanovii</i> subsp. <i>ivanovii</i>			0.0000	0.0035	0.0122	0.0064	0.0100
<i>L. ivanovii</i> subsp. <i>londoniensis</i>				0.0000	0.0136	0.0043	0.0085
<i>L. monocytogenes</i>					0.0000	0.0114	0.0100
<i>L. seeligeri</i>						0.0000	0.0057
<i>L. welshimeri</i>							0.0000

Fig. 2. Tree representation of distances between the 16S rDNA sequences of *Listeria* species. Only the sequences as determined in this study were used for the above representation. *E. coli* K-12 was used as an outgroup. The tree was constructed with the built-in function of the ARB software package using the neighbour-joining method (Kimura correction, vertical gaps cpm-preserved). The value for the distance between *E. coli* and the *Listeria* cluster is indicated by the arrows. **Fig. 3.** SSCP profiles of an amplified 216 bp fragment of the 16S rDNA as observed after an overnight run in non-denaturing 0.5 × MDE gel in 0.5 × TBE + 5% glycerol at room temperature at 90 V and silver staining.

phoresis technique used. Agarose gel electrophoresis and 5% PAGE were not sufficiently discriminatory for interpretation of the tDNA-spacer-PCR profiles (data not presented).

Only capillary electrophoresis provided sufficient resolution to yield useful results. Although substantial

intraspecific differences were observed, several – inter-PCR and inter-electrophoresis run – reproducible differences between the species were present and enabled clustering into groups corresponding with established taxonomy (Table 4, Fig. 5). For instance, *L. monocytogenes* isolates were characterized by the presence of a 284, 285 or 286 bp peak, absent in all other isolates. Two *L. monocytogenes* isolates [SLCC 2374 (LIS9) and SLCC 2376 (LIS11)] lacked this peak, but could be differentiated from all other species by the presence of a 265 and a 267 bp peak (Fig. 5). *L. innocua* showed most intraspecific heterogeneity and had most peaks in common with *L. monocytogenes*, from which it could only be differentiated by the absence of the 284–286 and the 267 bp peaks. The profile of one *L. innocua* isolate (TI6520) showed combined characteristics of *L. innocua*, *L. welshimeri* and *L. monocytogenes* profiles (Table 4), and thus, despite repeated testing, could not be identified. This is the same isolate with a slightly aberrant *Bst*UI restriction pattern (Table 1). All isolates of *L. monocytogenes*, *L. innocua* and *L. welshimeri* had a tRNA spacer of 159 bp, while this spacer was 160 bp long for all *L. seeligeri* isolates and 162 bp (with sometimes an additional 161 bp peak) for all *L. ivanovii* isolates. The subspecies of the latter species could be differentiated by a single bp length difference of a tRNA spacer region of 85 bp for *L. ivanovii* subsp. *londoniensis* and 86 bp for *L. ivanovii* subsp. *ivanovii*. Isolates of species other than *Listeria* had clearly different tDNA PCR profiles.

Fig. 4. rDNA-spacer-PCR profiles as observed after 5% PAGE and ethidium bromide staining (negative image).

For tDNA spacer amplification, reproducibility problems of the amplification step were observed. Upon analysis with agarose gel electrophoresis and PAGE, sometimes additional peaks were observed depending on the PCR run and relative peak height was variable between PCR runs (data not shown). Similar problems have been observed with e.g. arbitrarily primed PCR (36). However, highly identical profiles for different PCR runs were observed using capillary electrophoresis.

Atypical isolates

The molecular identification of atypical *L. ivanovii* subsp. *londoniensis* and of all the atypical *L. monocytogenes* and *L. seeligeri* using ARDRA, rDNA-SSCP analysis (Table 1), and tDNA-spacer-PCR analysed on capillary electrophoresis was in full agreement with the results obtained with the API galleries (Table 1). The phenotypically atypical *L. innocua* and *L. welshimeri* isolates could only be definitely identified by ARDRA and tDNA-spacer-PCR.

DISCUSSION

Because of their high phenotypic similarity, only a limited number of biochemical tests can be used to differentiate the *Listeria* species from one another (31).

This is particularly true for *L. monocytogenes* and *L. innocua*, which must be clearly distinguished because of the health hazard represented by the former but not by the latter. However, both the haemolysis and the Christie-Atkins-Munch-Petersen (CAMP) test with *Staphylococcus aureus*, which are the distinctive criteria for these two species, are not always easy to interpret because some *L. monocytogenes* isolates produce only very faint haemolysis and/or weakly positive CAMP reaction. A positive CAMP reaction with *Rhodococcus equi* has recently been proposed as an additional identification criterion for *L. monocytogenes* (11). However, this test has long been controversial and has not been adopted by all laboratories. Its validity also heavily depends on the *R. equi* strain used for the reaction (11).

Several commercial galleries have recently been developed to make the identification of *Listeria* species easier (1, 2, 24). Nevertheless, since some identifications still rely on a unique characteristic (2) for which some isolates may show an ambiguous reaction, additional tests like haemolysis and CAMP may still be necessary for a correct identification (24). Furthermore, biochemically abnormal isolates are regularly encountered during routine identification of *Listeria* isolates. Thus, in spite of tremendous improvements in commercial kits, phenotypic identification still remains difficult for a proportion of the isolates (1,

Table 4. Summary of the lengths for different *Listeria* species of the characteristic fragments obtained by intergenic tDNA spacer amplification and determined by capillary electrophoresis

Species	No. of isolates	Observed length in bp amplified tRNA spacer regions																
		61	72	85	86	89	145-146	159	160	162	262	263	264	265	267	284-286	290	292
<i>L. monocytogenes</i> a	20	-	+	-	-	+	-	+	-	-	-	+	-	-	-	+	-	-
<i>L. monocytogenes</i> b	2	-	+	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-
<i>L. innocua</i>	12	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-
<i>L. innocua</i> TI 6250	1	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-
<i>L. seeligeri</i>	5	-	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-
<i>L. welshimeri</i>	14	-	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-
<i>L. ivanovii</i> subsp. <i>ivanovii</i>	6	-	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	-
<i>L. ivanovii</i> subsp. <i>londoniensis</i>	11	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-
<i>L. grayi</i>	3	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-

* All except the following isolates: *L. monocytogenes* isolate 68 (LU 4526) lacked a 89 bp fragment and isolate 1 (LL 195) had a 265 bp fragment; *L. innocua* isolate 15 (ATCC 33090) had a 72 bp peak; *L. seeligeri* isolate 64 (NV 2918) had a 292 bp peak instead of a 294 bp peak.

v, Variable presence of peak.

2). In such cases, rapid and reliable confirmation based on genetic criteria remains of great interest.

We evaluated the effectiveness of four of the PCR fingerprinting techniques for the identification of isolates of the genus *Listeria*. ARDRA enabled differentiation among all six species and between the two *L. ivanovii* subspecies, although only minor differences were detected between the non-haemolytic species *L. innocua* and *L. welshimeri* and between both *L. ivanovii* subspecies.

High discriminatory power was also obtained with rDNA-SSCP analysis (44), which identified all the *Listeria* species, except *L. innocua* and some *L. welshimeri* isolates, which shared indistinguishable profiles under several of the electrophoresis conditions tested. Since the sequences of the 16S rRNA gene regions amplified for SSCP analysis diverge at only one position for these two species, this lack of discrimination is not unexpected. A more recent study (45) showed that SSCP analysis of the rDNA required two different primer sets to differentiate all six *Listeria* species. Since six copies of the rRNA-operon are present in the genome of *Listeria* species (27), it is possible that the four bands observed in some of the *L. welshimeri* isolates rely on the simultaneous presence of two distinct alleles of the rRNA operon in the genome of these particular isolates.

Jensen *et al.* (20) reported that *Listeria* isolates can be identified on the basis of the sizes of the 16S-23S intracistronic rDNA spacers. The molecular sizes of the major fragments obtained by this method in the present work were approximately the same as those originally described. However, we found on several occasions differences in the banding profiles of isolates belonging to the same species, sometimes even in the major bands which have been suggested as diagnostic markers for species identification (Fig. 4). In our hands, this technique was therefore not useful for easy identification of the species of the genus *Listeria*, because of the lack of species-specific bands within the profiles of some species and because sometimes only minor electrophoretic migration differences were observed between profiles of different species. The reason for the discrepancy between both studies is not known, but the differences may be due to the use of different populations of *Listeria* isolates. Although we examined less isolates than Jensen *et al.* (20), the isolates used here were selected to cover a large spectrum of serotypes and may represent a more heterogeneous population than that used by these authors. Our results suggest that the sequence and length variability of the rDNA spacers may be greater than expected in the genus *Listeria* and that, as in other species (5, 14, 15, 25), rDNA-spacer-PCR may be used as a typing tool rather than an identification tool. Jensen & Straus (21) pointed to the need for using special amplification conditions – i.e. low concentration of polymerase and polymerase buffer – to avoid multiple banding patterns. However, we obtained identical profiles when using these special conditions (data not shown).

Fig. 5. Clustering with Ward algorithm of tDNA-spacer-PCR profiles obtained by capillary electrophoresis on an ABI 310 apparatus. Calculation of similarity matrix based on band-matching using area-sensitive coefficient (tolerance band position 0.1%, minimal surface bands: 0% of total profile). Similarity indicated as a percentage on top of the dendrogram; fragment length of amplified tRNA spacers indicated in bp on top of fingerprints. Abbreviations of strain designations: inn, *L. innocua*; iviv, *L. ivanovii* subsp. *ivanovii*; ivlo, *L. ivanovii* subsp. *londoniensis*; mon, *L. monocytogenes*; see, *L. seeligeri*; wel, *L. welshimeri*. Numbers in the strain designation abbreviations refer to the study code as indicated in Table 1.

Capillary electrophoresis could reproducibly separate fragments differing by only a single base pair in length and only this high resolution enabled sufficient discrimination between the tDNA PCR profiles. Enhanced tDNA-PCR reproducibility by the use of capillary electrophoresis may be explained by the fact that the high resolution offered by this electrophoresis technique enables the detailed analysis of smaller sized fragments (basically in the range 60–300 bp), which may be more reproducibly amplified.

However, the subtlety of the observed differences, the peak height variability, the possible interference of background amplification products and the huge intraspecific variability (as was also observed with 5% PAGE) require careful evaluation of the practical applicability of tDNA-spacer-PCR. It can be concluded that thorough standardization of PCR conditions, high-resolution electrophoresis, and powerful pattern recognition and clustering software are needed to automate species identification based on tDNA-spacer-PCR.

In addition to biochemical characterization, *Listeria* isolates may also be differentiated on the basis of their antigenic composition. Thirteen somatic antigens and five flagellar antigens have been described in the *Listeria* species and are currently used for serotyping (35). However, undescribed combinations of antigens are regularly encountered during serotyping of field isolates, several of which are listed in the present work. Some but not all serovars are species-specific. On one

hand, *L. ivanovii* and *L. grayi* have species-specific antigenic combinations and the biochemically very close species *L. monocytogenes* and *L. innocua* do not share the same serovars and can therefore be differentiated serologically. On the other hand, *L. seeligeri* cannot be serologically distinguished from *L. monocytogenes*, nor can *L. welshimeri* be distinguished from *L. innocua*.

The species identification of 13 antigenically abnormal *Listeria* isolates (Table 1), as obtained with standard biochemical tests and with the API *Listeria* galleries, was confirmed by ARDRA, rDNA-SSCP analysis and tDNA spacer analysis. Among others, these data show that serovar 1/2b isolates can also be found in *Listeria* species other than *L. monocytogenes* and *L. seeligeri* (i.e. in *L. innocua*, *L. ivanovii* subsp. *londoniensis* and *L. welshimeri*) and consequently, that serotyping may not be as trustworthy, as is generally accepted, as a confirmatory tool for species identification. In addition, PCR-based DNA fingerprinting enabled the identification of four rarely encountered biochemical reaction patterns in *L. monocytogenes* and *L. seeligeri* (indicated as a–d in Table 1).

Because of discrepancies between 16S ARDRA results and computer-assisted restriction analysis of the published sequences (7, 8), the sequences of the 16S rRNA genes of seven representative isolates were determined again. Computer-assisted restriction analysis of the sequences as determined in this study was almost in complete agreement with ARDRA results (Table 2),

thus confirming the validity of the new updated rRNA gene sequences.

Besides possible sequencing errors, other reasons have been discussed previously (6) and may explain the discrepancies between the three independent 16S rRNA gene sequence determinations carried out thus far (7, 8, this study). Furthermore, application of reverse transcription prior to sequencing may be a major source of sequence errors.

The results obtained here with different PCR-based DNA fingerprinting techniques reflect the previously established high intragenic relatedness of the *Listeria* species. For example, for a total of 17 restriction enzymes out of 22 tested, not a single restriction site difference could be established in the 16S rDNA of the six species. Correspondingly, the new sequences presented here indicate that the dissimilarity estimates between the 16S rRNA genes of the different *Listeria* species are even lower than previously reported (7), although this does not influence the phylogenetic relatedness as has been established previously.

Subtle, but reproducible, differences between *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* could be established by several techniques. Sequence comparison of the first 1200 bp of the 16S rDNA revealed only two base pair substitutions (at positions 46 and 1096) between both subspecies, and single bp length differences were observed between tDNA spacers of isolates of both subspecies. These data fit the classification of the two corresponding genomic groups at the subspecies level (3) and not as different species. Only ARDRA and tRNA-spacer-PCR could establish some differences between *L. innocua* and *L. welshimeri*. Interestingly, *L. innocua* and *L. welshimeri* also share common serotypes. 16S rDNA sequence similarity distances as calculated here for the species pairs *L. monocytogenes* – *L. innocua* and *L. welshimeri* – *L. innocua* are comparable (Table 3). Finally, the fingerprints obtained for *L. grayi* differed most from those of all the other *Listeria* species, thus confirming its distance from the rest of the genus (4, 7, 12, 31). *L. grayi* was found to be homogeneous with all techniques (except with rDNA-spacer-PCR), which is in agreement with the recent grouping of *L. grayi* and '*L. murrayi*' into one unique species (33).

Conclusions

In conclusion, the previously published 16S rRNA gene sequences of all six *Listeria* species have been updated and now fit the restriction profiles obtained by ARDRA. In our hands, ARDRA performed best with respect to both discriminatory power and practical applicability. Satisfactory results were obtained with rDNA-SSCP analysis and tDNA-spacer-PCR, although the former requires thorough standardization of electrophoresis conditions and the use of additional primer sets, and the latter requires high-resolution electrophoresis. All of these techniques also allowed

the reliable identification of biochemically and serologically atypical *Listeria* isolates. The data reported here confirm a high genetic relatedness between most *Listeria* species and suggest a closer relationship than expected between the two non-haemolytic species *L. innocua* and *L. welshimeri*, which also share common serotypes. The combination of the 16S rDNA sequence data and the data gathered with the different PCR fingerprinting methods suggests that *L. innocua* is as closely related to *L. welshimeri* as it is to *L. monocytogenes* and confirms that there are subtle but consistent genetic differences between the two *L. ivanovii* subspecies and that *L. grayi* is homogeneous and is validly included in the genus *Listeria*.

ACKNOWLEDGEMENTS

We are very grateful to C. L. Gyles for checking the manuscript.

REFERENCES

- Bannerman, E., Yersin, M. N. & Bille, J. (1992). Evaluation of the Organon-Technika MICRO-ID LISTERIA system. *Appl Environ Microbiol* **58**, 2011–2015.
- Bille, J., Catimel, B., Bannerman, E., Jacquet, C., Yersin, M. N., Caniaux, I., Monget, D. & Rocourt, J. (1992). API *Listeria*, a new and promising one-day system to identify *Listeria* isolates. *Appl Environ Microbiol* **58**, 1857–1860.
- Boerlin, P., Rocourt, J., Grimont, F., Grimont, P. A. D., Jacquet, C. & Piffaretti, J.-C. (1992). *Listeria ivanovii* subsp. *londoniensis* subsp. nov. *Int J Syst Bacteriol* **42**, 69–73.
- Boerlin, P., Rocourt, J. & Piffaretti, J.-C. (1991). Taxonomy of the genus *Listeria* by using multilocus enzyme electrophoresis. *Int J Syst Bacteriol* **41**, 59–64.
- Cartwright, C. P., Stock, F., Beekmann, S. E., Williams, E. C. & Gill, V. J. (1995). PCR amplification of rRNA intergenic spacer regions as a method for epidemiological typing of *Clostridium difficile*. *J Clin Microbiol* **33**, 184–187.
- Clayton, R. A., Sutton, G., Hinkle, P. S., Jr, Bult, C. & Fields, C. (1995). Intraspecific variation in small-subunit rRNA sequences in GenBank. Why single sequences may not adequately represent prokaryotic taxa. *Int J Syst Bacteriol* **45**, 595–599.
- Collins, M. D., Wallbanks, S., Lane, D. J., Shah, J., Nietupski, R., Smida, J., Dorsch, M. & Stackebrandt, E. (1991). Phylogenetic analysis of the genus *Listeria* based on reverse transcriptase sequencing of 16S rRNA. *Int J Syst Bacteriol* **41**, 240–246.
- Czajka, J., Bsat, N., Piani, M., Russ, W., Sultana, K., Wiedmann, M., Whitaker, R. & Batt, C. A. (1993). Differentiation of *Listeria monocytogenes* and *Listeria innocua* by 16S rRNA genes and intraspecies discrimination of *Listeria monocytogenes* strains by random amplified polymorphic DNA polymorphisms. *Appl Environ Microbiol* **59**, 304–308.
- Deng, S., Hiruki, C., Robertson, J. A. & Stemke, G. W. (1992). Detection by PCR and differentiation by restriction fragment length polymorphism of *Acholeplasma*, *Spiroplasma*, *Mycoplasma*, and *Ureaplasma*, based upon 16S rRNA genes. *PCR Methods Appl* **1**, 202–204.
- Ehrenstein, B., Bernards, A. T., Dijkshoorn, L., Gerner-Smidt, P., Towner, K. J., Bouvet, P. J. M., Daschner, F. D. & Grund-

- mann, H. (1996). *Acinetobacter* species identification by using tRNA spacer fingerprinting. *J Clin Microbiol* **34**, 2414–2420.
11. Fernandez-Garayzabal, J. F., Suarez, G., Blanco, M. M., Gibello, A. & Dominguez, L. (1996). Taxonomic note: a proposal for reviewing the interpretation of the CAMP reaction between *Listeria monocytogenes* and *Rhodococcus equi*. *Int J Syst Bacteriol* **46**, 832–834.
12. Feresu, S. B. & Jones, D. (1988). Taxonomic studies on *Brochothrix*, *Erysipelothrix*, *Listeria*, and atypical lactobacilli. *J Gen Microbiol* **134**, 1165–1183.
13. Gürtler, V., Wilson, V. A. & Mayall, B. C. (1991). Classification of medically important clostridia using restriction endonuclease site differences of PCR-amplified 16S rDNA. *J Gen Microbiol* **137**, 2673–2679.
14. Gürtler, V. (1993). Typing of *Clostridium difficile* strains by PCR-amplification of variable length 16S-23S rDNA spacer regions. *J Gen Microbiol* **139**, 3089–3097.
15. Gürtler, V. & Stanisch, V. A. (1996). New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. *Microbiology* **142**, 3–16.
16. Heyndrickx, M., Vandemeulebroecke, K., Scheldeman, P., Hoste, B., Kersters, K., De Vos, P., Logan, N. A., Aziz, A. M., Ali, N. & Berkeley, R. C. W. (1995). *Paenibacillus* (formerly *Bacillus*) *gordonae* (Pichinoty et al. 1986) Ash et al. 1994 is a later subjective synonym for *Paenibacillus* (formerly *Bacillus*) *validus* (Nakamura 1984) Ash et al. 1994: emended description of *P. validus*. *Int J Syst Bacteriol* **45**, 661–669.
17. Hookey, J. V., Birtles, R. J. & Saunders, N. A. (1995). Intergenic 16S rRNA gene (rDNA)-23S rDNA sequence length polymorphisms in members of the family *Legionellaceae*. *J Clin Microbiol* **33**, 2377–2381.
18. Jannes, G., Vaneechoutte, M., Lannoo, M., Gillis, M., Van Canneyt, M., Vandamme, P., Verschraegen, G., Van Heuverswyn, H. & Rossau, R. (1993). Polyphasic taxonomy leading to the proposal of *Moraxella canis* sp. nov. for *Moraxella catarrhalis*-like strains. *Int J Syst Bacteriol* **43**, 438–449.
19. Jayarao, B. M., Doré, J. J. E., Jr, Baumbach, G. A., Matthews, K. R. & Oliver, S. P. (1992). Restriction fragment length polymorphism analysis of 16S ribosomal DNA of *Streptococcus* and *Enterococcus* species of bovine origin. *J Clin Microbiol* **30**, 2235–2240.
20. Jensen, M. A., Webster, J. A. & Straus, N. (1993). Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl Environ Microbiol* **59**, 945–952.
21. Jensen, M. A. & Straus, N. (1993). Effect of PCR conditions on the formation of heteroduplex and single-stranded DNA products in the amplification of bacterial ribosomal DNA spacer regions. *PCR Methods Appl* **3**, 186–194.
22. Kämpfer, P., Böttcher, S., Dott, W. & Rüdén, H. (1991). Physiological characterization and identification of *Listeria* species. *Int J Med Microbiol* **275**, 423–435.
23. Kathariou, S. & Pine, L. (1991). The type strain(s) of *Listeria monocytogenes*: a source of continuing difficulties. *Int J Syst Bacteriol* **41**, 328–330.
24. Kerr, K. G., Rotowa, N. A., Hankey, P. M. & Lacey, R. W. (1991). Evaluation of the ROSCO system for the identification of *Listeria* species. *J Med Microbiol* **35**, 193–196.
25. Kostman, J. R., Edlind, T. D., Lipuma, J. J. & Stull, T. L. (1992). Molecular epidemiology of *Pseudomonas cepacia* determined by polymerase chain reaction ribotyping. *J Clin Microbiol* **30**, 2084–2087.
26. McClelland, M., Petersen, C. & Welsh, J. (1992). Length polymorphisms in tRNA intergenic spacers detected by using the polymerase chain reaction can distinguish streptococcal strains and species. *J Clin Microbiol* **30**, 1499–1504.
27. Michel, E. & Cossart, P. (1992). Physical map of the *Listeria monocytogenes* chromosome. *J Bacteriol* **174**, 7098–7103.
28. Nesme, X., Vaneechoutte, M., Orso, S., Hoste, B. & Swings, J. (1995). Diversity and genetic relatedness within genera *Xanthomonas* and *Stenotrophomonas* using restriction endonuclease site differences of PCR-amplified 16S rDNA. *Syst Appl Microbiol* **18**, 127–135.
29. Ralph, D., McClelland, M., Welsh, J., Baranton, G. & Perolat, P. (1993). *Leptospira* species categorized by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction polymorphism in PCR-amplified rRNA genes. *J Bacteriol* **175**, 973–981.
30. Rocourt, J., Grimont, F., Grimont, P. A. D. & Seeliger, H. P. R. (1982). DNA relatedness among serovars of *Listeria monocytogenes* sensu lato. *Curr Microbiol* **7**, 383–388.
31. Rocourt, J., Schrettenbrunner, A. & Seeliger, H. P. (1983). Différenciation biochimique des groupes génomiques de *Listeria monocytogenes* (sensu lato). *Ann Microbiol Inst Pasteur* **134A**, 65–71.
32. Rocourt, J., Wehmeyer, U., Cossart, P. & Stackebrandt, E. (1987). Proposal to retain *Listeria murrayi* and *Listeria grayi* in the genus *Listeria*. *Int J Syst Bacteriol* **37**, 298–300.
33. Rocourt, J., Boerlin, P., Grimont, F., Jacquet, C. & Piffaretti, J.-C. (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.
34. Sallen, B., Rajoharison, A., Desvarenne, S., Quinn, F. & Mabilat, C. (1996). Comparative analysis of 16S and 23 rRNA sequences of *Listeria* species. *Int J Syst Bacteriol* **46**, 669–674.
35. Seeliger, H. P. R. & Höhne, K. (1979). Serotyping of *Listeria monocytogenes* and related species. *Methods Microbiol* **13**, 31–49.
36. van Belkum A., Kluytmans, J., van Leeuwen, W. & 13 other authors (1995). Multi-center evaluation of arbitrarily primed PCR for typing of *Staphylococcus aureus* strains. *J Clin Microbiol* **33**, 1537–1547.
37. Vaneechoutte, M., Cartwright, C. P., Williams, E. C., Jäger, B., Tichy, H.-V., De Baere, T., De Rouck, A. & Verschraegen, G. (1996). Evaluation of 16S rRNA gene restriction analysis for the identification of cultured organisms of clinically important *Clostridium* species. *Anaerobe* **2**, 249–256.
38. Vaneechoutte, M., De Beenhouwer, H., Claeys, G., Verschraegen, G., De Rouck, A., Paepe, N., Elaichouni, A. & Portaels, F. (1993). Identification of *Mycobacterium* species with amplified rDNA restriction analysis. *J Clin Microbiol* **31**, 2061–2065.
39. Vaneechoutte, M., Dijkshoorn, L., Tjernberg, I., Elaichouni, A., De Vos, P., Claeys, G. & Verschraegen, G. (1995). Identification of *Acinetobacter* genomic species by amplified ribosomal DNA restriction analysis. *J Clin Microbiol* **33**, 11–15.

40. Vaneechoutte, M., Riegel, P., de Briel, D., Monteil, H., Verschraegen, G., De Rouck, A. & Claeys, G. (1995). Evaluation of the applicability of amplified rDNA-restriction analysis to identification of species of the genus *Corynebacterium*. *Res Microbiol* **146**, 633–641.
41. Vaneechoutte, M., Rossau, R., De Vos, P., Gillis, M., Janssens, D., Paepe, N., De Rouck, A., Fiers, T., Claeys, G. & Kersters, K. (1992). Rapid identification of bacteria of the *Comamonadaceae* with amplified ribosomal DNA-restriction analysis (ARDRA). *FEMS Microbiol Lett* **93**, 227–234.
42. Welsh, J. & McClelland, M. (1991). Genomic fingerprints produced by PCR with consensus tRNA gene primers. *Nucleic Acids Res* **19**, 861–866.
43. Welsh, J. & McClelland, M. (1992). PCR-amplified length polymorphisms in tRNA intergenic spacers for categorizing staphylococci. *Mol Microbiol* **6**, 1673–1680.
44. Widjooadmotjo, M. N., Fluit, A. C. & Verhoef, J. (1994). Rapid identification of bacteria by PCR-single-strand conformation polymorphism. *J Clin Microbiol* **32**, 3002–3007.
45. Widjooadmotjo, M. N., Fluit, A. C. & Verhoef, J. (1995). Molecular identification of bacteria by fluorescence-based PCR-single-strand conformation polymorphism analysis of the 16S rRNA gene. *J Clin Microbiol* **33**, 2601–2606.
46. Wiedmann-Al-Ahmad, M., Tichy, H.-V. & Schön, G. (1994). Characterization of *Acinetobacter* type strains and isolates obtained from wastewater treatment plants by PCR fingerprinting. *Appl Environ Microbiol* **60**, 4066–4071.