APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2004, p. 4256–4266 Vol. 70, No. 7
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.7.4256–4266.2004

Natural Atypical *Listeria innocua* Strains with *Listeria monocytogenes* Pathogenicity Island 1 Genes

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Received 11 September 2003/Accepted 5 April 2004

Identification of bona fide *Listeria* isolates into the six species of the genus normally requires only a few tests. Aberrant isolates do occur, but even then only one or two extra confirmatory tests are generally needed for identification to species level. We have discovered a hemolytic-positive, rhamnose and xylose fermentation-negative *Listeria* strain with surprising recalcitrance to identification to the species level due to contradictory results in standard confirmatory tests. The issue had to be resolved by using total DNA-DNA hybridization testing and then confirmed by further specific PCR-based tests including a *Listeria* microarray assay. The results show that this isolate is indeed a novel one. Its discovery provides the first fully documented instance of a hemolytic *Listeria innocua* strain. This species, by definition, is typically nonhemolytic. The *L. innocua* isolate contains all the members of the PrfA-regulated virulence gene cluster (*Listeria* pathogenicity island 1) of *L. monocytogenes*. It is avirulent in the mouse pathogenicity test. Avirulence is likely at least partly due to the absence of the *L. monocytogenes*-specific allele of *iap*, as well as the absence of *inlA*, *inlB*, *inlC*, and *daaA*. At least two of the virulence cluster genes, *hly* and *plcA*, which encode the *L. monocytogenes* hemolysin (listeriolysin O) and inositol-specific phospholipase C, respectively, are phenotypically expressed in this *L. innocua* strain. The detection by PCR assays of specific *L. innocua* genes (*lin0198*, *lin0372*, *lin0419*, *lin0558*, *lin1068*, *lin1073*, *lin1074*, *lin2454*, and *lin2693*) and noncoding intergenic regions (*lin0454-lin0455* and *nadA-lin2134*) in the strain is consistent with its *L. innocua* DNA-DNA hybridization identity. Additional distinctly different hemolytic *L. innocua* strains were also studied.

The bacterial genus *Listeria* is currently taxonomically subdivided into six species: *L. monocytogenes*, *L. seeligeri*, *L. ivanovii*, *L. innocua*, *L. grayi*, and *L. welshimeri*. Two of the species are pathogenic, causing the disease listeriosis. One, *L. monocytogenes*, is a human pathogen which infects in particular the fetuses of women in their third trimester of pregnancy, the aged, and immunocompromised subjects (18). *L. monocytogenes* is also pathogenic for a variety of animals. In contrast, the other pathogenic species, *L. ivanovii*, is virtually only pathogenic for animals and very rarely has been reported to cause human listeriosis. Although listeriosis is not common in humans, it is a clinically significant disease because of its high mortality and severity (1). Most cases of human listeriosis are now attributable to ingestion of *L. monocytogenes*-contaminated foods, especially ready-to-eat foods (1) that do not require heating before consumption. Control of human foodborne listeriosis involves reduction or elimination of food contamination by *L. monocytogenes*, which is complicated by the ability of *L. monocytogenes* to grow slowly at refrigeration temperatures. Evaluation of the effectiveness of control measures involves analyzing foods for the presence of *L. monocytogenes* by selective cultural enrichments and isolation on selective agar growth media. Since these methods are generic for *Listeria* spp., a crucial step in contamination analysis is the identification of *Listeria* isolates to species level. Conveniently, speciation of a bona fide *Listeria* isolate involves only a few simple tests (14, 27, 32). These are production, or not, of acid (without gas) from L-rhamnose, D-xylose, or mannitol and the lysis of sheep or horse red blood cells. The reaction patterns of isolates of the six *Listeria* species with these few reagents are generally sufficient for their differentiation (Table 1). Animal testing of isolates is not routinely required because clinical, food, and environmental isolates of *L. monocytogenes* are typically virulent in the mouse pathogenesis test. Although all *L. monocytogenes* isolates are thus assumed to be virulent, at least one study has reported the occurrence of naturally occurring virulence-attenuated *L. monocytogenes* strains (5).

A conveniently minimal number of tests can thus be used to identify known *Listeria* isolates to species level. This can lead to problems when a test reaction is atypical in an aberrant strain, for example, nonhemolytic strains of *L. monocytogenes* (13), because the small number of remaining tests is no longer sufficient for identification to the species level. As a result, auxiliary tests are needed. The occurrence of Rha$^-$ strains of

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TABLE 1. Reactions differentiating *Listeria* species[a]

Species	Acid production from:			
	L-rhamnose	D-xylose	D-mannitol	Hemolysis[b]
L. monocytogenes	+	−	−	+
L. seeligeri	−	+	−	+
L. ivanovii[c]	−	+	−	+++
L. innocua	v[d]	−	−	−
L. welshimeri	v	+	−	−
L. grayi[c]	v	−	+	−

[a] *Listeria* are small gram-positive rods which typically are motile and catalase positive and produce acid from esculin and glucose. +, positive reaction; −, negative reaction.

[b] *L. ivanovii* is strongly β-hemolytic (+++), while *L. monocytogenes* and *L. seeligeri* are weakly β-hemolytic (+). When occurrence of hemolysis is equivocal, it can be enhanced using the CAMP test. CAMP allows differentiation of *L. ivanovii* from the other two hemolytic species.

[c] *L. ivanovii* is comprised of subsp. *ivanovii* and subsp. *londiniensis.* The subspecies of *L. grayi* are subsp. *grayi* and subsp. *murrayi.*

[d] v, strain variable reaction.

L. monocytogenes (31, 36) is relevant to the present study. In such a case, is the isolate a hemolytic Rha$^-$ *L. monocytogenes* or is it a hemolytic Xyl$^-$ *L. seeligeri*? A cumbersome way to answer this question is to do animal pathogenicity testing, since one species (*L. monocytogenes*) is pathogenic and the other (*L. seeligeri*) is not. Fortunately quicker ways are available. One way is to test for *L. monocytogenes*-specific 16S rRNA with DNA probe kits. Alternatively, colorimetrically testing for naphthylamidase activity can distinguish *L. monocytogenes* from the other species of *Listeria* (api Listeria; bioMérieux, Marcy-l'Etoile, France). Thus, in most cases, aberrant strains can be identified to the species level with only some extra effort.

This study reports a novel hemolytic strain of *Listeria* that was unusually difficult to identify to the species level by the conventional methods (J. Johnson, K. Jinneman, G. Stelma, B. G. Smith, D. Lye, J. Messer, J. Ulaszek, L. Evsen, S. Gendel, R. W. Bennett, and A. D. Hitchins, Abstr. AOAC Int. Annu. Mtg., p. 24, 2000). Resolution of its identity as a hemolytic variant of *L. innocua*, which is regarded by definition as nonhemolytic, required an extended study (J. Johnson, K. Jinneman, G. Stelma, B. G. Smith, D. Lye, J. Messer, J. Ulaszek, L. Evsen, S. Gendel, R. W. Bennett, B. Swaminathan, J. Pruckler, A. Steigerwalt, S. Kathariou, S. Yildirim, D. Volokhov, A. Rasooly, V. Chizikov, M. Wiedmann, E. Fortes, R. E. Duvall, and A. D. Hitchins, Abstr. 9th Annu. FDA Sci. Forum Program Abstr., abstr. P-PO-01, 2003). The purpose of this study was to characterize and identify to species level this strain as well as three similar strains that were discovered during the study.

MATERIALS AND METHODS

Bacterial strains. *Listeria* sp. strain PRL/NW 15B95, the main subject of this study, was isolated from imported Korean clams by using selective enrichment and isolation (14). *L. innocua* type strain F4078 and *L. monocytogenes* type strain KC1778 are Centers for Disease Control and Prevention strains. Other *Listeria* strains (including J1-023, J1-155, and J1-156) were from the Cornell *Listeria* strain collection (Department of Food Science, Cornell University).

Culturing strains. Strain stock cultures were preserved at −80°C in Trypticase soy broth with 0.6% (wt/vol) yeast extract and 15% (vol/vol) glycerol. Working cultures were maintained on slants of Trypticase soy agar with 0.6% (wt/vol) yeast extract and stored at 5°C. Cultures for DNA array studies were grown in brain heart infusion broth.

Conventional *Listeria* species-level identification tests. Identification of strains was done according to the procedures in the Food and Drug Administration Bacteriological Analytical Manual (14). The main procedures were the Gram stain reaction, catalase test, motility tests, carbohydrate fermentation, and hemolytic reactions on sheep blood agar.

Auxiliary identification tests. The presence of *L. monocytogenes*-specific 16S rRNA, and hence the corresponding 16S rDNA, was determined with an AccuProbe DNA probe kit (GenProbe Inc., San Diego, Calif.) according to the manufacturer's instructions. Expression of naphthylamidase activity was determined with the DIM (differentiation of *L. innocua* and *L. monocytogenes*) reaction of the api *Listeria* identification kit (bioMérieux). No reports of variant negative strains of the positive species have been published, though the kit manufacturer's package insert indicates that such strains do exist.

Other tests. Alpha-methyl-D-mannosidase activity was determined by using alpha-methyl-D-mannoside (Sigma Chemical Co., St. Louis, Mo.) as an alternate substrate in the conventional sugar fermentation test (14). Phosphatidylinositol-specific phospholipase C (PlcA) activity was deduced from the appearances of colonies grown on BCM (R & F Laboratories, West Chicago, Ill.) (24) and ALOA (Microbiology International, Frederick, Md.) (21) *L. monocytogenes* plates.

Testing for the listeriolysin O gene (*hly*). The *L. monocytogenes*-specific *hly* gene was detected by using the PCR with specific probes and primers (20). The amplicons were separated and concentrated by electrophoresis and detected by Southern blotting with the specific DNA probe (20).

***Listeria* pathogenicity island 1 (LIPI-1) and flanking genes.** The gene order and potential insertions were assessed by PCR methodology. Primers were designed using Primer3 software (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) (Table 2) and were purchased from Bio-synthesis, Inc. (Lewisville, Tex.) or from QIAGEN (Foster City, Calif.). PCR employed X-*Taq* DNA polymerase (Fisher, Pittsburgh, Pa.). The reaction mixtures were subjected to a hot start (95°C for 5 min) prior to 30 cycles of amplification (95°C for 1 min, 50°C for 1 min, 72°C for 2 min, with a final extension at 72°C for 20 min) in a Progene thermocycler. Amplified products were separated by electrophoresis through a 1.0% (wt/vol) agarose gel with 1× Tris-borate-EDTA running buffer.

Species-level identification by total DNA-DNA hybridization. Total DNA-DNA hybridization was studied under high-stringency conditions (3, 28). DNA preparations from strains F4078 and KC1778 represented the species *L. innocua* and *L. monocytogenes*, respectively. Cells were grown in brain heart infusion broth at 36 ± 1°C, and DNA was extracted by sodium dodecyl sulfate lysis and purified as described previously (3). Purified DNA was labeled with [^{32}P]dCTP enzymatically (28) by using a nick translation kit (Invitrogen Life Technologies, Carlsbad, Calif.). Hybridization was carried out at 65°C. Relatedness between pairs of DNA samples was determined by the hydroxyapatite method with thermal elution (3).

Strain typing. Serotyping was done by agglutination with type 1 and type 4 polyclonal antisera (Difco, Detroit, Mich.). Tests for O antigens 5, 6, 7, 8, and 9 were performed as described by Bennett and Weaver (2). The reactivity pattern with monoclonal antibodies specific for serotypes 4b, 4d, and 4e was determined using c74.22 and c74.33 as described previously (16). Serotype-specific marker genes (*gltA*, *gltB*, *ami*, *gtcA*, and *mtrA*) for enzymes of cell wall teichoate metabolism and autolysis were studied using PCR. The primers used (Table 2) were based on published sequences (19). The *ami143* primer sequences were as described by Herd and Kocks (13). The *mtrA* primer sequences were listed under GenBank accession number AF288455.

Ribotyping was performed with a Qualicon (Wilmington, Del.) automated Riboprinter microbial characterization system. The riboprint pattern for this isolate was compared to the reference library of patterns by using the comparison software included in the Riboprinter system.

Pathogenicity testing. The immunocompromised mouse assay of Stelma et al. was used (32). Carrageenan is used to weaken the murine immune response. There were five mice in each control group and test group. The negative control strain was *L. innocua* ATCC 33090, and the positive control strain was *L. monocytogenes* ATCC 19115. The doses per mouse were 10,000 CFU. The bioassays were performed in accordance with a protocol approved by the Animal Care and Use Committee of the Andrew W. Breidenbach Research Center, U.S. Environmental Protection Agency, Cincinnati, Ohio.

Species-level identification by DNA microarray analysis. DNA was prepared for the microarray hybridization assay as follows. Freshly grown bacteria were resuspended in 0.5 ml of water (circa 10^8 cells/ml) and lysed with lysozyme (50 mg/ml) for 2 h at 37°C. Lysates were deproteinized by two sequential phenol-chloroform (1:1 by vol) extractions, and the DNA was precipitated with 3 volumes of anhydrous ethanol. The DNA precipitates were dried in vacuo and resuspended in 300 μl of water. The presence and the quality of genomic DNA

TABLE 2. Primers and oligonucleotide probes used in study 1[a]

Target region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Amplicon size (bp)
prs-F to T7-ISO-R	TTACAAAYTCCATYGCTCTTCCAGAAGAMAA ATGGA	*TAATACGACTCACTATAGGG*AACCGTTCTCCAC CATTCCCA	3,837
ISO-F to T7-*mpl*-LMR	GTTAATGAACCTACAAGHCCTTCC[b]	*TAATACGACTCACTATAGGG*AGTGGAAGCGATT CGTAATCTTTCATATGAG	2,424
mpl-LMF-T7-PLCB-R	CTCATATGAAAGATTACGAATCGCTTCCACTC	*TAATACGACTCACTATAGGG*TCATCTGAGCAAA ATCTTTTTGCTACCATGTC	3,102
actA2 to T7-*ldh*-R	CTAGCTGATTTAAGAGATAGAGGAACAG	*TAATACGACTCACTATAGGG*TTGTTGAAATGAA CTTAAAYGACAAAGAAAAAGAACAAATGAA	3,845
gltA	GGTTCAAACTCTTAACGGC	GTGCCATTACTACAGGTGCA	500
gltB	TTCAAATAAGCCGTTCCAAA	AAAGGCAGAGATTACGCA	450
gtcA-serotype 4	TGGGTTACTACAAGAAGAG	AGTACTGATGCGATAAAAGCA	244
gtcA-serotype 1/2	CCGGATCCGGGTTGTACAATTGGA	ACGGTACTCAGGATGAATTC	1,000
ami143-serotype 4	GATGCTGAATTCCGCAAATAC	ACCAACCAATTGCTTTCCCA	220
mtrA	GCAAAAGGCGATGCTGTG	CCCGCCTAAGAACAGTATTG	600
lin2693	TTGCTGGTGCGTTTGTTATA	CCCTCATCCACAAAATAAACA	661
lin1074	CCGAAGTATGTTGATTTGTTTG	TTCATTTCAACAAGTGGTCCT	836
lin1068	GTGAATCGCAATCATTAACG	TGTAGCTAATATCACTTGGTT	613
lin0558	GGAGTGTGGATTCTATTGTA	AAAGTAGACACCAGCAACTG	866
lin1073	GAAATTTGCGATTATAATTCCT	TCATTTCTTTTACAACAAGCG	952
prs-prfA	CGAAACTGCTGGTGCAACTA	AACCAATGGGATCCACAAGA	1,206
prs-hly	CGAAACTGCTGGTGCAACTA	GCTTTTACGAGAGCACCTGG	3,156
plcB-ldh	AAAGCGGACTACCCGAAAAT	GGTGCAATGGATACTATTT	2,691
prfA	AACCAATGGGATCCACAAG	ATTCTGCTAACAGCTGAGC	479
actA	TAGCGTATCACGAGGAGG	TTTTGAATTTCATATCATTCACC	1,994
hly	CAAACTGAAGCAAAGGATGCA	CTAATGTATTTACTGCGTTGTTA	496
plcA	CAGCATACTGACGAGGTGTG	GATGTCCGCTCTACCTGA	798
plcB	GCATGATATTGACAGCAAATTA	TGAAATACTTTGCTCCTGTT	320
mpl	TGTATCATCATGGTAATAGCT	TGGATCCGTAAACATATTCGT	674
inlA-inlB	CTACACCACCTTCCGCAAAT	AAAATTCCACTCATGCCCAC	1,423
inlB	AAGCACAACCCAAGAAGGAA	AAAATTCCACTCATGCCCAC	1,107

[a] Italicized base sequences are the T7 RNA polymerase promoter sequences.
[b] H is A, C, or T (due to ambiguity).

in samples were confirmed by 0.8% agarose gel electrophoresis followed by staining with ethidium bromide.

The 11-kb LIPI-1 gene cluster from the DNA was segmentally amplified by PCR. The segments consisted of four partially overlapping regions (I to IV) of the cluster. Table 3 lists the segments and their respective forward and reverse primers. Each of the reverse primers contains a T7 promoter at the 5′ end for transcription of the cluster segments into the single-stranded RNA (ssRNA) sequences needed for the microarray hybridizations. The standard PCR mixture (50 μl) contained 5 U of HotStarTaq DNA polymerase in the recommended buffer supplemented with 2.5 mM $MgCl_2$ (QIAGEN), 600 nM each forward and reverse primer, 300 μM each dATP, dGTP, dCTP, and dTTP, and 1 to 2 μl of DNA template (ca. 0.3 μg). Amplification was performed in a Gene Amp PCR system 9600 thermocycler (PE Applied Biosystems, Foster City, Calif.). The time-temperature conditions were as follows: initial activation at 95°C for 15 min; 35 cycles of the sequence at 94°C for 1 min, 57°C for 1 min, and 72°C for 4 min; and final extension at 72°C for 15 min. The presence of amplified PCR products was detected by 1% agarose gel electrophoresis.

ssRNA samples for microarray analysis were synthesized by in vitro transcription from the promoter-tagged PCR amplicons using a MEGAscript T7 high-yield transcription kit (Ambion, Austin, Tex.). After 4 h of incubation at 37°C, the unincorporated nucleotide triphosphates were removed using Centrisep spin columns (Princeton Separations, Adelphia, N.J.) according to the manufacturer's protocol.

A MICROMAX ASAP RNA labeling kit (Perkin-Elmer, Boston, Mass.) was used to incorporate Cy5 fluorophore into RNA molecules. Fluorescent-labeled ssRNA samples were purified from unincorporated dye by using Centrisep spin columns, dried in vacuo, and solubilized in the MICROMAX hybridization buffer III at a final concentration of 0.3 to 0.5 μM.

Each oligoprobe was spiked with a control non-bacterial-derived oligonucleotide to ensure the uniformity of array printing and hybridization conditions. Each hybridization experiment was conducted in the presence of Cy3-labeled oligonucleotide complementary to the internal control oligoprobe. The control probe and the complementary target were made such that, ideally, they had similar melting temperatures and did not have consensus sequences that were the same as the sequence of the other probe. The hybridization signals for the control probes were used as hybridization controls.

Fluorescent images of the microarrays were obtained by laser scanning the slides with wavelengths of 632 nm (for the Cy5 dye moiety) and 543 nm (for the Cy3 dye moiety) by using a ScanArray 5000 (Perkin-Elmer). The fluorescent signals from each spot were measured and compared using QuantArray software (Perkin-Elmer). Fluorescent signals that differed from the average background at a statistically significant level ($P < 0.01$) were considered positive.

Detection of *L. innocua*-specific genes. By using PCR in two different studies, the sizes of amplicons were compared, between test strains and control strains, for genes (*lin2693*, *lin1074*, *lin1068*, *lin0558*, and *lin1073* for study 1 and *lin0198*, *lin2454*, *lin0372*, and *lin0419* for study 2) and noncoding intergenic regions (*lin0454-lin0455* and *nadA-lin2134* for study 2) of *L. innocua* with no orthologues in *L. monocytogenes* EGD (11). The primers used for these studies are listed in Table 2 (study 1) and in Table 3 (study 2).

Sequencing. In some cases, sequences of the genes from some *Listeria* species were determined experimentally. The PCR-amplified DNA fragments were purified by electrophoresis in agarose gel, extracted using a QIAquick gel extraction kit (QIAGEN) according to the manufacturer's protocol, and sequenced using an ABI Prism 310 genetic analyzer system (PE Applied Biosystems).

RESULTS

Generic testing. The isolate PRL/NW 15B95 was obtained from a food sample by *Listeria* selective enrichment and selective isolation. It had a typical *Listeria* colonial appearance on the esculin-containing PALCAM and Oxford agars. It was a gram-positive bacillus. It was motile, exhibiting tumbling motility in wet mounts and producing an umbrella pattern in motility-stab agar. It was positive in the catalase, methyl red, and Voges-Proskauer tests as well as in the esculin, maltose, and glucose fermentation tests. The isolate was negative in the oxidase and indole tests. The triple sugar iron agar reactions were an acid slant and butt with no gas or hydrogen sulfide. It was concluded that the isolate is a *Listeria* species.

TABLE 3. Primers and oligonucleotide probes used in study 2[a]

Target	Primer	Nucleotide sequence (5′ to 3′)	Temp (°C)
prs-hly	PRSF	TTACAAAYTCCATYGCTCTTCCAGAAGAMAAATGGA	60–63
	T7-ISOR	TAATACGACTCACTATAGGGAACCGTTCTCCACCATTCCCA	68
hly-mpl	ISOF	GTTAATGAACCTACAAGHCCTTCC[b]	54–56
	T7-MPL-LMR	TAATACGACTCACTATAGGGAGTGGAAGCGATTCGTAATCTTTCATATGAG	68
mpl-plcB	MPL-LMF	CTCATATGAAAGATTACGAATCGCTTCCACTC	61
	T7-PLCBR	TAATACGACTCACTATAGGGTCATCTGAGCAAAATCTTTTTGCTACCATGTC	68
actA-ldh	ActA2F	CTAGCTGATTTAAGAGATAGAGGAACAG	57
	T7-LDHR	TAATACGACTCACTATAGGGTTGTTGAAATGAACTTAAAYGACAAAGAAAA AGAACAAATGAA	66–67
gcaD	GcaDF	CATGGGAGATGCGGAAATTGGTAAAAAC	58
ctc	Ctc-R	GGAAGATATCGCAAACAAATGCGAATC	57
lin0372	Lin0372F	CGTTGTTTTGGCGTTGTCGATTGTTATTG	59
	Lin0372R	TACACGATGATCTCCTGTTGCTGGTAG	60
Plasmid pLI100	LinPNCR1F	GTACATCATGCAAACGATTTTCTCAAGTATC	58
	LinPNCR1R	CTGTCTACTTTATTGACATCTATCCAACCT	58
	Lin0198F	ATGAACAAATTAGTTAGTCAAAGTAATG	51
lin0198	Lin0198R	TATCGATGTCTTGAGGTCACACAAAGTTC	59
	Lin2454F	TGGAAGTACTTATACGCCAGGAATTATTC	57
lin2454	Lin2454R	CAAAATGCTTTATTTTCAATAATCATTTCATC	53
	Lin0419F	CATTAAATTAGCTCTAAAACAACTACTATC	53
lin0419	Lin0419R	TACACCAAACACAATTCCAATAAACATC	54
lin0454-lin0455	LinNCR1F	GGATTTGGTAAATTATACAAAGGTTTTAAG	53
	LinNCR1R	TGCTTCTTGGCATTTTAGTAATCTTTC	54
nadA-lin2134	LinNCR3F	TGCGTTAAAAGCATTAGAAAGAATGCTC	56
	LinNCR3R	AGTGAAGGTATTATCGACTGGTGGTAG	58
prfA	prfAR	GTATTTTTCTATATGATGGTATCACAAAGCTC	57
	prfAF	CATATCTTTTGAGATAATCAAGATTTTGTAC	54
plcA	PlcAF	AAGTTGAGTACGAAYTGCTCTACTTTGTTG	58–59
	PlcAR	AACACAAACGATGTCATTGTACCAACAACTAG	59
actA	ActAR	GCTTATTTTCGATACCTTTGGACTTAACGAAG	59
plcB	PlcBF	GATAACCCGACAAATACTGACGTAAATAC	57
prfA	PrfA1probe	CATATCTTTTGAGATAATCAAGATTTTGTACAT	54
	PrfA2probe	TAAATTATCCAGTGTAATCTTGATGCCATC	56
	PrfA3probe	ACTCGTGAGCTTTGTGATACCATCATAT	57
plcA	PlcA1probe	TACTGACGAGGTGTGAATGTTAATGAAGT	57
	PlcA2probe	TCGTCTTTTAAACGCATAATAATGGTTTCTTTTGG	59
	PlcA3probe	ACATCCATTGTTTTGTAGTTACAGAGTTCTT	56
hly	HlyM1probe	ACTGGAGCGAAAACAATAAAAGCAAGCTAGC	60
	HlyM2probe	AACTCAGAATATATTGAAACAACTTCAAAAG	54
	HlyM3probe	ATGATCCTGAAGGTAACGAAATTGTTCAAC	58
mpl	Mpl1probe	ATTCTTGATATGACAAAAGATAAGCTAGGC	56
	Mpl2probe	ACGCTTAGTTTATAATATAAAATTGATTTTTG	52
	Mpl3probe	TGATTCGTTTGTCCATTATGGCTTGAATTG	58
actA	ActA1probe	ATATTTGCAGCGACAGATAGCGAAGATTC	59
	ActA2probe	GATAGTGAGCTTGAAAGCCTTACTTATCC	59
	ActA3probe	AGTAAAGAAAGCGATTGTTGATAAAAGTGC	56
plcB	PlcB1probe	AGTACATTTTTATCTCATTTTTATAATCCTGATAG	55
	PlcB2probe	ATGCGGATCATAAAAATCCATATTATGATACT	55

[a] Underlined sequences are the T7 RNA polymerase promoter sequences.
[b] H is A, C, or T (due to ambiguity).

Identification to species level. The isolate was weakly β-hemolytic. The hemolysis was enhanced by *Staphylococcus aureus* (S^+ reaction) but not by *Rhodococcus equi* (R^- reaction) in the Christie, Atkins, and Munch-Peterson (CAMP) test (7, 14). These hemolysis tests suggested that the isolate was not *L. ivanovii* but was either *L. monocytogenes* or *L. seeligeri*. The isolate did not produce acid from L-rhamnose, D-xylose, or mannitol. Together, the hemolysis and sugar reactions suggested that the strain was either an *L. monocytogenes* Rha^- or an *L. seeligeri* Xyl^- strain and not an *L. ivanovii* Xyl^- strain. To decide between these two species, the AccuProbe test for *L. monocytogenes* 16S rRNA and the DIM test for naphthylamidase were used. The isolate tested *L. monocytogenes* negative and DIM positive, suggesting that it was not an *L. monocytogenes* Rha^- variant strain but was an *L. seeligeri* Xyl^- variant. An *L. seeligeri* Xyl^- result would have been regarded as conclusive for routine identification purposes had other tests not given confounding or equivocal results.

Subtyping. Serotyping of the isolate with polyclonal antibodies showed that it was serotype 4, and thus the strain was not *L. ivanovii* (serotype 5) or *L. welshimeri* (serotype 6). More detailed typing showed that it contained O antigen 5 but not antigens 6, 7, 8, and 9, thus ruling out seroidentities *L. monocytogenes* 4a, 4ab, 4b, 4c, 4d, and 4e. Also, monoclonal antibody (16) tests, with antibodies c74.22 and c74.33 that recognize serotype 4b (and the relatively rare serotypes 4d and 4e), were negative. Thus, the isolate seems to be of a different serotype 4 designation. By default, it is presumably a serotype 4f, which contains O antigens 5 and 15 (the latter was not tested). *L. innocua* can be serotype 4f (30), but it has some undefined serotypes, and so the 4f designation is somewhat tentative. Also, *L. grayi* and *L. seeligeri* have some undefined serotypes,

and so they could not be definitively excluded from consideration on a serotypic basis alone. In contrast, tests for serotype-specific alleles of genes coding for products involved in cell wall metabolism and autolysis suggested that the GtcA (teichoic acid glycosylation protein) of the strain was serotype 1/2-related and not serotype 4-related (data not shown). This apparent anomaly is consistent with the emerging picture of an interspecific mosaic-like gene content of *Listeria* with respect to serotype-related genes (13). This phenomenon may be due to lateral gene transfer (13).

The ribotyping pattern for this strain did not closely match any of the library patterns for either *L. monocytogenes* (40 patterns) or *L. innocua* (17 patterns), although it did contain several bands that are common in patterns from *Listeria* species. The two library patterns with the closest matches were for *L. innocua* at a similarity of 0.75 (pattern DUP-1005 in the Riboprinter library) and *L. monocytogenes* at a similarity of 0.78 (pattern DUP-1033). These similarity values are not significantly different. For comparison, a similarity value of 0.86 is the minimum for coidentification.

Species confirmation tests. The putative *L. seeligeri* Xyl$^-$ identity of the strain was not confirmable by a variety of other tests. The Micro-ID *Listeria* code for the isolate was 44041, consistent with an *L. innocua* identity except for the positive hemolysis and CAMP test results. The API *Listeria* code was 7110, which is also consistent with an *L. innocua* identity. In the API battery of tests, the hemolysis test is replaced by the DIM test. The isolate was alpha-methyl-D-mannoside positive, and since only 1 in 10 of the small number of *L. seeligeri* strains examined so far are reported to be positive (29), it was probably not an *L. seeligeri* strain. Other carbohydrates metabolized by *Listeria* spp. (29) were tested in a tentative numerical analysis approach to species-level identification, but the results (data not shown) were not informative. Thus, the auxiliary tests suggested that the isolate was an *L. innocua* Rha$^-$ biotype strain and not an *L. monocytogenes* Rha$^-$ variant strain. If so, it must contain at least one non-*L. innocua* gene, *hly*, which is phenotypically expressed.

Additional Rha$^-$ Hly$^+$ isolates. The inability of hemolytic PRL/NW 15B95 to ferment L-rhamnose was superficially reminiscent of the Rha$^-$ or slow rhamnose-fermenting strains (36) of *L. monocytogenes* described by Wiedmann et al. (lineage III, subset E/G 5.8 H 7.1). However, Wiedmann et al. had proposed that this subset of *L. monocytogenes* should not be classified as *L. innocua*, even though the latter also contains the E/G 5.8 H 7.1 fragment. Some other strains in the lineage III group (36) pattern type, fragment subset G 8.1 H 7.1, are also rhamnose negative or slow rhamnose-fermenting strains. Strain ATCC 19114 also resembles our isolate, but by DNA-DNA homology (36), it is only 72% related to the type strain of *L. monocytogenes* and only 54% related to the type strain of *L. innocua*. It is avirulent in the immunocompromised mouse test (32). Although this strain has a unique V2 region sequence in the 16S rRNA (9, 36), it is AccuProbe positive (15), consistent with an *L. monocytogenes* designation. Thus, PRL/NW 15B95 did not appear to be generally related to this group, and it was specifically unrelated to strain 19114. However, a representative selection of the lineage III strains was screened with the AccuProbe test, because some of them had unusual 16S rRNA gene sequences, and therefore unusual 16S rRNA sequences. Most of them, including six strains of G 5.8 H 7.1, one of which was a Bruce et al. strain (3a), were *L. monocytogenes* strains as expected. However, three isolates in a different ribotype subset (G 5.8 H 12.0) were not *L. monocytogenes* by the AccuProbe test. Two of these (J1-155 and J1-156) were *hly*$^+$ Rha$^-$ and *lma*$^+$, and they are probably closely related if not identical. One (J1-023) was *hly*$^+$ Rha$^-$ and *lma* negative. The product of the *lma* locus is an *L. monocytogenes*-specific, delayed-type hypersensitivity-inducing protein (27). The presence of *hly*$^+$, in an apparently non-*L. monocytogenes* genotypic background, was suggestively similar to the genotype of PRL/NW 15B95, so these strains were further studied.

DNA-DNA homology. To resolve the uncertainties in the species designation of PRL/NW 15B95, a total DNA-DNA hybridization experiment was performed. Under high-stringency conditions (3, 28), the index of hybridization similarity of the strain's DNA with *L. innocua* type strain F4078 DNA was 86%, well above the 70% level considered to indicate the threshold of species relationships. In contrast, only 40% similarity was seen between this strain and the *L. monocytogenes* type strain KC1778. *L. innocua* is in the same phylogenetic group as *L. monocytogenes*. Since the strain is so closely related to *L. innocua*, it is not likely to be closely related to *L. seeligeri*, which is a member of another distinct phylogenetic group, the *L. ivanovii-L. welshimeri* (9, 26) group. Thus, the results showed that the aberrant strain was an *L. innocua*-like strain and not a strain of *L. seeligeri* or *L. monocytogenes*.

***L. innocua* gene tests.** This conclusion was supported by PCR data (not shown), which showed that five *L. innocua*-specific (11) genes (*lin2693*, *lin1074*, *lin1068*, *lin0558*, and *lin1073*) were present in the isolate and the type strain of *L. innocua* but not in *L. monocytogenes*. These genes had the approximate expected sizes, but accurate sizing by sequence analysis was not done. A second PCR study showed that additional *L. innocua* genes (*lin0198*, *lin2454*, *lin0372*, and *lin0419*) as well as noncoding intergenic regions (*lin0454-lin0455* and *nadA-lin2134*) were present. Thus, in PRL/NW 15B95, an *hly* gene is present in a largely *L. innocua* genomic background.

DNA array-based identificaton to species level. Data obtained using the oligonucleotide microarray assay system confirmed the presence of an *L. monocytogenes hly* gene in an *L. innocua* genomic background. The system identifies the species of *Listeria* isolates (35) on the basis of the presence (or absence) of species-specific forms (homologues or alleles) of 12 genes: *prfA*, *hly*, *plcA*, *plcB*, *mpl*, *actA*, *inlA*, *inlB*, *inlC*, *clpE*, *iap*, and *daaA*. Three to 10 individual oligonucleotide probes represented each gene. The isolate contained several genes characteristic for *L. monocytogenes* (*prfA*, *hly*, *plcA*, *plcB*, *mpl*, and *actA*) and *L. innocua* (*iap*, *clpE*, and *daaA*). The *L. monocytogenes*-specific *inlA*, *inlB*, and *inlC* genes and the *daaA* allele were shown to be absent in the isolate. Direct sequencing of the 16S rRNA gene and of the housekeeping genes, *ldh* and *prs*, further specified the isolate as an *L. innocua* strain. Thus, this strain contains the genes (*prfA*, *hly*, *plcA*, *plcB*, *mpl*, and *actA*) of the LIPI-1 pathogenicity island of *L. monocytogenes*. The J1-023, J1-155, and J1-156 isolates were also confirmed as being *hly*$^+$ *L. innocua* strains by this method.

Sequence analysis of the large 16S-23S IGS region and the complete 16S rRNA gene of the atypical hemolytic *L. innocua* strains. The sequences of the complete 16S rRNA gene and

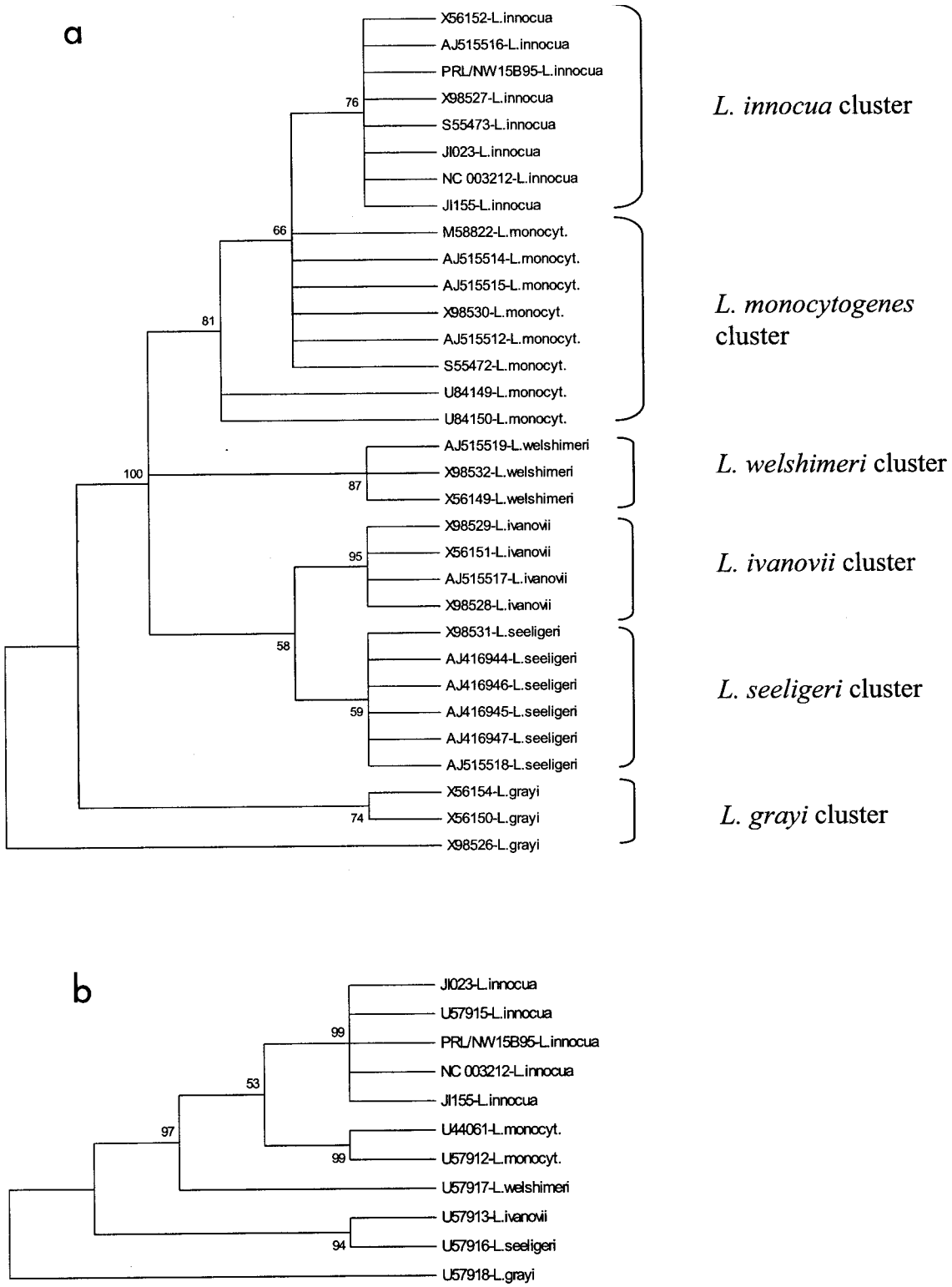

FIG. 1. (a) Dendrogram showing the phylogenetic relationship among the six *Listeria* species and the atypical hemolytic *L. innocua* strains based on nucleotide sequence data of the partial 16S rRNA gene. This tree was constructed by the maximum parsimony method in the MEGA 2.1 package. The bootstrap values presented at corresponding branches were evaluated from 500 replications. GenBank accession numbers are indicated for each strain used in creating this dendrogram. (b) Dendrogram showing the phylogenetic relationship among the six *Listeria* species and the atypical hemolytic *L. innocua* strains based on nucleotide sequence data of the large 16S-23S rRNA IGS region. This tree was constructed by the maximum parsimony method in the MEGA 2.1 package. The bootstrap values presented at corresponding branches were evaluated from 500 replications. GenBank accession numbers are indicated for each strain used in creating this dendrogram.

the large 16S-23S rRNA intergenic spacer (IGS) region (12) of the atypical hemolytic *L. innocua* strain and three similar strains (JI155, JI156, and JI023) were determined in order to classify them phylogenetically. Dendrograms representing the nucleotide sequence homologies for the partial 16S rRNA gene (Fig. 1a) and the large 16S-23S rRNA IGS regions (Fig. 1b) of the test strains and of strains of other *Listeria* species from the GenBank database were constructed. These analyses consistently grouped the atypical strains into the *L. innocua* cluster. BLAST alignments of the complete 16S rRNA gene (1,555 bp) of PRL/NW 15B95 showed 100 and 99% homologies with the corresponding genes from *L. innocua* CLIP11262 (AL596173) and *L. monocytogenes* strain EGD (AL591983). The other strains (JI155, JI156, and JI023) showed a 99% homology value with both reference sequences. The large 16S-23S rRNA IGS regions for PRL/NW 15B95 and the JI155, JI156, and JI023 strains were 585 and 586 bp, respectively, with 99% homology to the *L. innocua* sequences (NC_003212 and U57915), 95% homology to the *L. monocytogenes* sequences (U44061 and U57912), 94% homology to the *L. welshimeri* sequence (U57917), and 92% homology to both the *L. ivanovii* and *L. seeligeri* sequences (U57913 and U57916), exhibiting only a 78% match with the *L. grayi* sequence (U57918). The phylogenetic results based on sequences of the 16S rRNA gene and the large 16S-23S rRNA IGS region showed that JI155, JI156, and JI023 are *L. innocua* strains. The results also confirmed the conclusion from the total DNA hybridization study that PRL/NW 15B95 is an *L. innocua* strain.

LIPI-1 tests. The PRL/NW 15B95 strain was *hly* positive by Southern blotting and PCR, with *L. monocytogenes hly*-specific DNA probes and primers. The strain had phosphatidylinositol-specific phospholipase C (PlcA) activity by the BCM (24) and the ALOA (21) *L. monocytogenes* plating medium tests. Thus, the strain appeared to contain at least two of the five nonregulatory genes in the PrfA-regulated virulence gene cluster of *L. monocytogenes* (LIPI-1) (33), based on the expression of the associated phenotypic functions. Since these genes are actively expressed, the strain can be reasonably assumed to contain their expression-controlling gene, *prfA* (positive regulatory factor), which is part of the six-gene cluster, and to be able to produce its biologically active product, PrfA.

The other genes in LIPI-1 include those coding for phosphatidylcholine-specific phospholipase C (lecithinase), PlcB (10), a zinc metallo-protease (Mpl), which is required for maturation of prolecithinase (23), and the ActA protein involved in polymerization of host cell actin. Even though the presence of these proteins was not determined, molecular evidence suggests that all these LIPI-1 genes are present in the atypical isolate. The genes were detected both by PCR with primers derived from sequences of the genes in *L. monocytogenes* and by independent PCR amplifications and hybridization of resulting products with gene-specific oligoprobes by using the DNA microarray assay. In addition, PCR-based data (not shown) suggested that the organization of the genes in the virulence gene cluster (indicated in Table 4) and the expected approximate sizes were the same in this isolate as in *L. monocytogenes* and clearly different from *L. seeligeri*, where several genes have been inserted in the virulence gene cluster (6, 33). No evidence was obtained for gene size insertions or deletions in the virulence gene cluster of the atypical isolate. The contiguous flanking genes of the LIPI-1, in all the atypical strains studied here, were *gcaD-prs* (5′ end) and *orfX-orfZ-orfB-orfA-ldh-ctc* (3′ end) as expected from the *L. monocytogenes* genome. Nevertheless, two of the open reading frames (ORFs) (*orfX-orfZ*) appear to be *L. monocytogenes* specific, and the other genes and ORFs (*gcaD-prs* and *orfB-orfA-ldh-ctc*) were *L. innocua* specific (D. Volokhov, unpublished results).

TABLE 4. Allele specificities and expected chromosome map loci of genes in the aberrant *Listeria* PRL/NW 15B95, J1-023, J1-155, and J1-156 isolates

Gene[a]	Locus (kb)[b]	Specificity[c]
Map origin	0 (0)	-
lin0198	. (189)	Lin
prs	203 (235)	Lin
prfA	204	Lmo
hly	206	Lmo
plcA	206	Lmo
mpl	208	Lmo
actA	209	Lmo
plcB	211	Lmo
ldh	215 (238)	Lin
rRNA-16S, 2 operons[d]	237 (261), 244 (267)	Lin
lin0372	. (384)	Lin
lin0419	. (429)	Lin
inlA	455	Not present
inlB	457	Not present
lin0454-lin0455 intergenic region	. (~470)	Lin
lin0558	. (586)	Lin
iap	620 (619)	Lin
clpE	1029 (1,021)	Lin
lin1068	. (1,092)	Lin
lin1073	. (1,098)	Lin
lin1074	. (1,100)	Lin
daaA	1662 (1,671)	Lin
rRNA-16S, 2 operons[d]	1748 (1,798), 1854 (1,908)	Lin
inlC	1861	Not present
nadA-lin2134 intergenic region	~2159	Lin
lin2454	. (2,473)	Lin
rRNA-16S, 2 operons[d]	2443 (2,493), 2678 (2,764)	Lin
lin2693	. (2,711)	Lin
lin2979-82 (*lmo2847-50*)[e]	2936–2939 (3,003–3,006)	-
Map terminus	2944 (3,011)	-

[a] LIPI-1 gene abbreviations in bold type. Associated ORFs are not listed.
[b] Approximate expected distances from map start point. Values for *L. monocytogenes* (*L. innocua*) loci are rounded to the nearest kilobase. A period (·) indicates that no locus is present in *L. monocytogenes*.
[c] Lmo or Lin, *L. monocytogenes*- or *L. innocua*-specific allele; -, not tested.
[d] Presence of six rDNA operons is assumed. Negative AccuProbe *L. monocytogenes* test and partial sequencing.
[e] Expected locations of four L-rhamnose catabolism genes; one or more presumably defective or absent in the Rha^- phenotype.

The general conclusions drawn from the PCR results about the LIPI-1 gene cluster organization have been additionally confirmed by sequencing analysis of this cluster in PRL/NW 15B95 as well as in the other atypical isolates (JI155, JI156, and JI023). All isolates exhibited high nucleotide sequence homology with the corresponding genes of *L. monocytogenes*.

Sequence analysis of the 5′ and 3′ junction of the LIPI-1 virulence gene island in hemolytic *L. innocua* strains. Sequence alignments of the noncoding sequences between *prs* and *prfA* and the sequences between *plcB* and *ldh* clearly showed that the LIPI-1 island was inserted in the atypical *L. innocua* strains' genomes, and this probably resulted from transpositions. Alignments of the strains' *prs-prfA* intergenic regions revealed repeat sequences (AAAACAGGATTYC

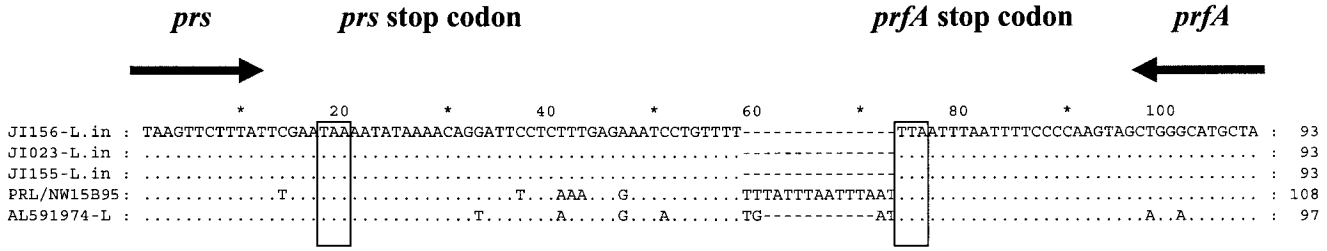

FIG. 2. Alignment of *prs-prfA* intergenic regions of the atypical hemolytic *L. innocua* strains with putative insertion junctions. *prs* and *prfA* stop codons are boxed (for details see the text). Asterisks indicate the precise halfway points between the sequence numbers.

TCW) (Fig. 2). Thus, there are putative transposon insertion junctions in all of the strains. The repeat sequences were immediately downstream of the *prs* and the *prfA* stop codons. In addition, PRL/NW 15B95 had a 15-bp insertion (TTTATTTA ATTTAAT) in its *prs-prfA* junction region, which distinguishes it from the other *hly*-positive *L. innocua* strains. Horizontal transfer of LIPI-1 in the *Listeria* genus was hypothesized (4, 11, 17, 33, 34) on the basis of sequence analysis of a potential LIPI-1 insertion site between *prs* and *ldh* in nonpathogenic *Listeria* species. The present study provides strong evidence for the hypothesis. Similarly, transposon-related repeat structures are present between *L. monocytogenes*-specific *orfZ* and *L. innocua*-derived *orfB* (D. Volokhov, personal communication).

Determination of invasion-associated protein gene (*iap*) origin. Even though the LIPI-1 genes were all found to be present in the atypical isolate, PRL/NW 15B95, PCR results with *iap L. monocytogenes*-specific primers (20) were negative. Some strains of the 4a and 4c serotypes of *L. monocytogenes* are negative with these primers (20), but this isolate was not 4a or 4c. Other *Listeria* species DNAs react negatively with these *L. monocytogenes*-specific primers, since they have their own different allelomorphs of *iap*. The *iap* gene, which is regarded as virulence associated in *L. monocytogenes*, codes for a gene product that has murein-lytic activity and is involved in cell division (22, 37).

Direct sequencing and phylogenetic analysis of whole *iap* genes from the four atypical hemolytic *L. innocua* strains was carried out to identify their species of origin by comparison with the *iap* sequences of 47 different *Listeria* strains available in GenBank. The results clearly show that the *iap* genes from these strains are of *L. innocua* origin (Fig. 3). The *iap* genes from strains JI155, JI156, and JI023 were 1,410 bp long. The *iap* gene of PRL/NW 15B95 was only 1,407 bp long, due to deletion of codon +291 (CAA), which suggests the absence of 291Gln in Iap. The *iap* gene nucleotide sequences were identical for strains JI155 and JI156 and were 97 and 98% homologous with the *iap* sequences of PRL/NW 15B95 and JI023, respectively. There was 98% nucleotide sequence homology between the *iap* genes of the latter two strains.

Avirulence of PRL/NW 15B95. Strain PRL/NW 15B95 was avirulent for artificially immunocompromised mice. This suggested that the strain was an animal-pathogenic *Listeria* species in which some virulence genes were apparently absent and others may have been altered so they may not be able to fulfill the same functions as in *L. monocytogenes*. Alternatively, it could be a strain of one of the nonpathogenic *Listeria* species. The other results suggested a third situation, that the strain was a partial hybrid of an avirulent species (*L. innocua*) and a virulent species (*L. monocytogenes*). Although *L. innocua* predominated, the possibility of full virulence expression still existed. However, while some of the *L. monocytogenes* virulence genes were present (the LIPI-1 gene cluster) and at least some of them were expressed, others were absent (*inlA*, *inlB*, *inlC*, *daaA*, and *iap*). Thus, the avirulent hybrid's complement of virulence genes was incomplete.

DISCUSSION

Species-level identification tests. Only a few classical tests are needed to identify members of the genus *Listeria* to the species level, and although aberrant strains may require extra tests, in general, species-level identification is not onerous. The strains studied here did require considerable extra effort in order to identify them to the species level. Thus, definitive species-level identification of PRL/NW 15B95 was multifaceted, being based on the combined results of different sophisticated tests: DNA-DNA hybridization; detection of genes indicating an *L. innocua* background genotype; sequencing of the 16S and 23S intergenic regions, the 16S rRNA and *iap* genes, and the junctions of LIPI-1 with its neighboring genes; and genotyping with a oligonucleotide microarray. Nevertheless, in the absence of any information on the frequency of occurrence of such strains, it is not yet clear how widespread is the problem they potentially pose with respect to complicating the species-level identification methodology for *Listeria* isolates. Pragmatically, if such isolates are more common than is apparent so far, they may justify a separate taxon. Otherwise, they can be regarded as taxon-transitional strains. Aberrant *Listeria* strains, in general, are not that rare in nature, and the addition to them of the unique strains studied here emphasizes the importance of not dismissing the unique strains as unreflective of nature.

Origins. Two possibilities can be invoked to explain how this unique strain, PRL/NW 15B95, of *L. innocua* contains the PrfA-regulated gene cluster of *L. monocytogenes*. One is that a common ancestor of both *L. monocytogenes* and *L. innocua* containing virulence-associated genes gave rise to the currently prevailing typical strains of *L. innocua* and *L. monocytogenes* and that the hemolytic *L. innocua* strain described here represents a rare atypical strain, which is a relic of that common ancestry. This possibility is consistent with the phylogenetic closeness of these two species as determined by their 16S and 23S rRNA relatedness (25, 26). The three species *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* form a more phylogenetically

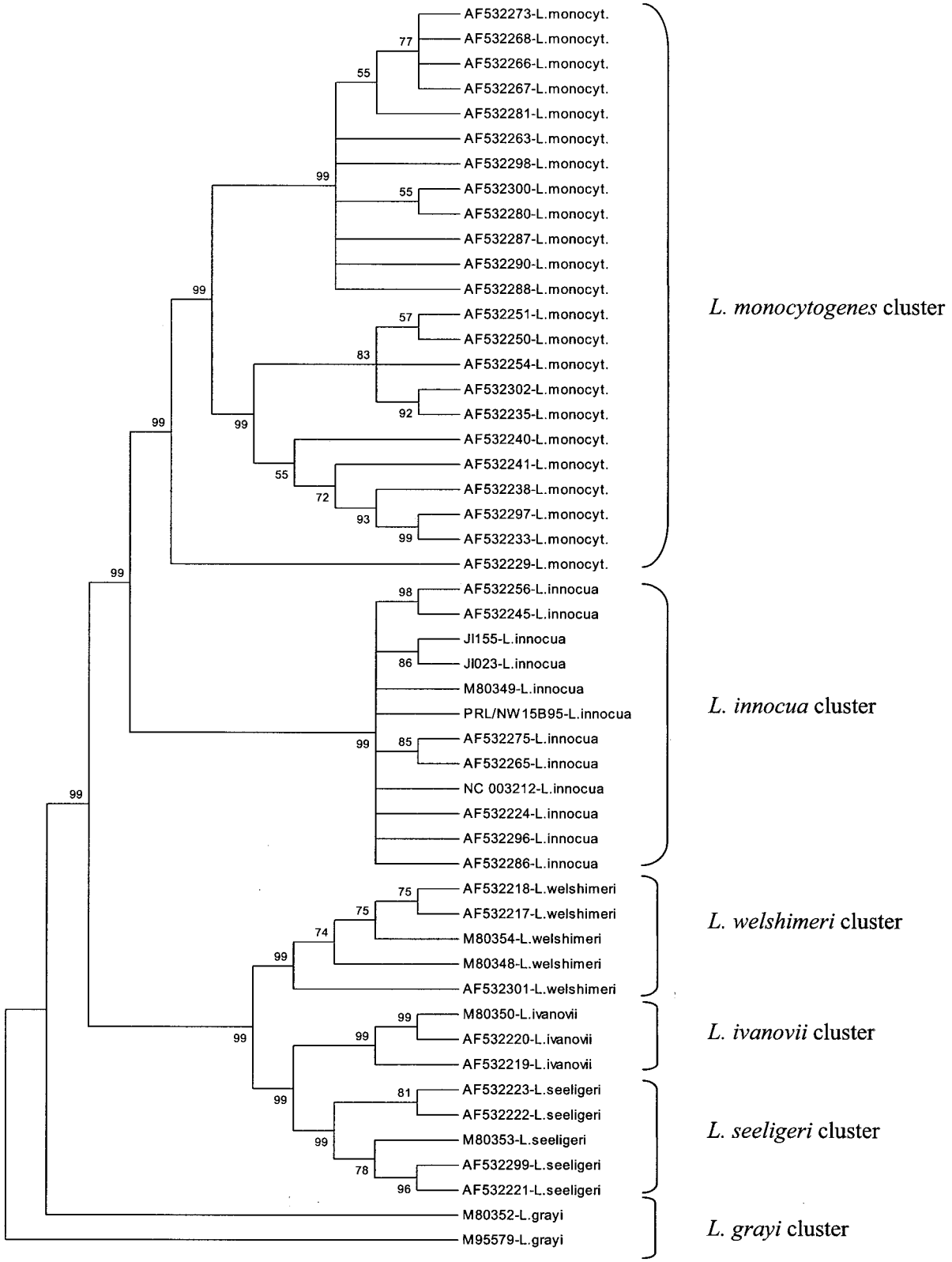

FIG. 3. Genetic relationships between the atypical hemolytic *L. innocua* strains and other *Listeria* species. The dendrogram is based on invasion-associated protein p60 (*iap*) gene sequence analysis, and it was constructed by the maximum parsimony method in the MEGA 2.1 package. The bootstrap values presented at corresponding branches were evaluated from 500 replications. GenBank accession numbers are indicated for each strain used in this analysis. To more clearly show the evolutionary relationships occurring among members of the *Listeria* species, the species subbranches of the phylogenetic tree were clustered.

distant grouping. The remaining species, *L. grayi*, is even more phylogenetically distant from all of the other five species. The other possibility is that a Rha$^-$ biotype strain of *L. innocua* received the virulence cluster genes by genetic transfer. LIPI-1 appears to be in a chromosomal location, being flanked by the chromosomal genes *prs* and *ldh*, which are within about a 2-kb distance from each other in the recently sequenced genome of *L. innocua* (4, 27). The virulence gene cluster may have been acquired by phage-mediated transfer or via transformation, the possibility of which has been suggested following identification of DNA uptake genes in the genome of both *L. monocytogenes* and *L. innocua* (11). Transfer of only part of the complement of virulence genes, i.e., just LIPI-l, by one of these mechanisms would be consistent with the avirulence of the strain. Horizontal transfer of genes from an *L. monocytogenes* lineage to a specific lineage of *L. innocua* has been proposed to explain the existence of *L. innocua* strains which express surface antigens and harbor genes unique to certain serogroup 4 strains of *L. monocytogenes* (19). Such horizontal transfer requires a mechanism for the observed site specificity of the virulence gene cluster, which is in the same chromosomal location (*prs-ldh* region) in this *L. innocua* strain and in *L. monocytogenes*. The genomic localization of LIPI-1 seems to be more compatible with vertical than horizontal transmission and supports the hypothesis that PRL/NW 15B95 represents an ancestral *L. innocua* lineage, which maintained the virulence gene cluster that was subsequently lost in descendant *L. innocua* strains. However, the sequence analysis of the 5′ and 3′ junctions of the LIPI-1 virulence gene island in the hemolytic *L. innocua* strains strongly suggests that horizontal transmission did occur at some undetermined time in the evolution of the *L. innocua* and *L. monocytogenes* clade.

Conclusion. The species-level identification-refractory strain PRL/NW 15B95 was a hemolytic *L. innocua* Rha$^-$ biotype, as were the other aberrant isolates studied. Table 4 lists the genes studied in all of the aberrant isolates. Their approximate expected locations on the chromosome map (11), relative to its origin and terminus, are indicated along with their allele specificities when determined. The putative locations provide a rational way to list the loci involved in this study. Also indicated are the loci of other relevant genes: the six rDNA operons of *L. monocytogenes* and *L. innocua* and the putative loci of the genes coding for L-rhamnose catabolism inferred from their sequence similarity to the corresponding L-rhamnose catabolism genes of *Bacillus subtilis* (11).

This study provides the first substantial documentation of naturally occurring hemolytic strains of *L. innocua*. The discovery of such strains suggests that caution is needed when detecting rhamnose-negative, hemolytic *Listeria* in foods: such isolates may sometimes be avirulent *L. innocua* strains, as was the case here. Apparently, strains like PRL/NW 15B95 are natural, as contrasted to artificial, species intermediates, where the term species is used in a pragmatic, phenotypic sense. This definition recognizes that in the bacteria, the species of systematics is probably not consonant with the concept of the species as a fundamental unit of biological diversity (8). Among the bacteria, examples of natural species intermediates at the chromosomal level seem to be undocumented. While DNA sequence similarities of particular genes may imply evolutionary relationships between different genera, for example, the already mentioned L-rhamnose catabolism genes in *Listeria* and *Bacillus*, strains like PRL/NW 15B95 seem to be tangible examples of intermediates in the evolution of the *L. innocua-L. monocytogenes* clade. Further study of these strains may contribute to our understanding of evolution in the genus *Listeria*, especially in terms of the evolution of its virulence.

ACKNOWLEDGMENT

We thank Jocelyne Rocourt for helpful advice.

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