Inter- and Intraspecies Comparison of the 16S-23S rRNA Operon Intergenic Spacer Regions of Six *Listeria* spp.

THOMAS A. GRAHAM,¹ ELIZABETH J. GOLSTEYN-THOMAS,¹
JAMES E. THOMAS,² AND VICTOR P. J. GANNON^{1*}

*Animal Diseases Research Institute, Agriculture and Agri-Food Canada¹ and
Department of Biological Sciences, University of Lethbridge,²
Lethbridge, Alberta, Canada*

The 16S-23S rRNA intergenic spacer (IGS) regions found in six *Listeria* species were characterized. PCR amplification of the 16S-23S IGS with a "generic primer" set generated products of about 340 bp (small) and 550 to 590 bp (large) with DNA from all *Listeria* strains tested. Seven *Listeria monocytogenes* serotype 4b strains and one *L. monocytogenes* serotype 4d strain also had an additional PCR product of ca. 360 bp. The 360-bp PCR product from one of these *L. monocytogenes* serotype 4b strains was identical in nucleotide sequence to the small 340-bp IGS, except that it contained an 18-bp tandem repeat. The small rRNA IGSs of *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* were 83 to 99% homologous to that of *L. monocytogenes*. The large rRNA IGS of *L. monocytogenes* was 81 to 96% homologous to those of the other *Listeria* species and agreed with current taxonomic division among these species. The nucleotide sequences of the central 274 bp of the large rRNA IGS of strains from seven different *L. monocytogenes* serotypes were highly homologous; however, serotype-specific differences were noted, and four groups were identified within *L. monocytogenes* based on this analysis.

The genus *Listeria* consists of six species: *Listeria monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*, and *L. grayi*. *L. monocytogenes* is pathogenic for humans and animals, *L. ivanovii* is an animal pathogen, and the remaining species in this genus are considered nonpathogenic.

A number of investigators have exploited rRNA and rRNA genes for identification and characterization of members of the genus *Listeria* (10, 15, 17, 24) and as a taxonomic tool in investigations of relationships among other pathogenic and nonpathogenic bacteria (6, 8, 18, 19). Differentiation among *Listeria* species has been accomplished through nucleotide sequence analysis of genes in the rRNA operon, such as the 16S (7, 10) and 23S rRNA genes (24). These sequence data have been used for the identification of *Listeria* with DNA probes that hybridize to genus-specific regions of *Listeria* 16S rRNA (5, 25, 26) and by exploitation of single-base-pair differences in these genes for species identification with the ligase chain reaction (27, 28). Unfortunately, the highly conserved nature of the rRNA genes in *Listeria* has made differentiation among species within the genus difficult. The conserved nature of the rRNA genes is not surprising given that their function in protein synthesis is essential to the bacterium. Barry et al. (1) hypothesized that the nucleotide sequences of the rRNA intergenic spacer (IGS) regions are not as essential as the rRNA genes themselves and are more subject to genetic drift and consequently not as highly conserved. The rRNA IGSs have, therefore, become areas of interest to be used in the detection of specific bacteria and in differentiation among several closely related bacterial species (1, 9, 16, 18).

In this study, nucleotide sequences of all six *Listeria* species 16S-23S rRNA IGSs are presented and nucleotide sequence differences among serotypes of *L. monocytogenes* are examined. This nucleotide sequence information allows differenti-

ation among members of the genus *Listeria* and among serotypes within *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and DNA extraction. Thirty-four strains of *L. monocytogenes*, representing 12 (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, and 4e) of the 13 serotypes, were used in this study (Table 1). The strains include clinical isolates from humans and animals and isolates from foods and the environment. Other *Listeria* strains used were *L. innocua* NCTC 11288^T, *L. ivanovii* ATCC 19119^T, *L. seeligeri* ATCC 35967^T, *L. welshimeri* ATCC 35897^T, and *L. grayi* ATCC 19120^T. Bacterial strains were kindly provided by J. Farber, Banting Research Centre, Health Canada (Ottawa, Ontario); A. Lammerding, Health of Animals Laboratory, Health Canada (Guelph, Ontario); S. Galsworthy, University of Western Ontario (London, Ontario); J. Lopez, Health of Animals Laboratory, Agriculture and Agri-Food Canada (Charlottetown, Prince Edward Island); and R. Gordon, Provincial Laboratory of Public Health, Alberta Health (Calgary, Alberta). Bacteria were grown and DNA was extracted as previously described (12).

PCR amplification, cloning, and DNA sequence analysis. Oligonucleotide primers were synthesized with a model 391 DNA synthesizer (PCR-Mate; Applied Biosystems Inc., Foster City, Calif.). Oligonucleotide primers A1 and B1 (1) are complementary to the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene of *Escherichia coli*, respectively. *Listeria* genus-specific oligonucleotide primers LA2 and LB2 have been described previously, as have the conditions for PCR amplification (13).

PCR products generated by amplification with the A1 and B1 primers were cloned into a pCR II DNA plasmid vector by using the TA Cloning System, version 2.2 (Invitrogen Corp., San Diego, Calif.) according to the manufacturer's instructions. Ligation mixtures were used to transform INVαF' *E. coli* One Shot competent cells (Invitrogen Corp.). Bacterial cells were lysed by heating to 94°C for 5 min and were tested for plasmid inserts by PCR using the M13/pUC forward and reverse sequencing primers (Gibco BRL, Burlington, Ontario).

Plasmids containing insert DNA were extracted from bacterial cells by using an alkaline lysis extraction procedure as described by Nicoletti and Condorelli (22). Nucleotide sequencing reaction mixtures consisted of a 1.0-μl (1 μg/μl) sample of purified plasmid DNA, 9.5 μl of Prism Ready Reaction DyeDeoxy Terminator Mix (Applied Biosystems Inc.), 5.0 μl (5 μg/ml) of either M13/pUC forward or reverse sequencing primer, and 4.5 μl of distilled water, as per the manufacturer's instructions. DNA cycle sequencing reactions were performed in 25 cycles, with each cycle consisting of three steps: 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, by using a 9600 Gene Amplification PCR thermal cycler (Perkin Elmer, Rexdale, Ontario). The nucleotide sequence of insert DNA was determined for five different plasmids, each containing DNA inserts derived from independent PCR amplification experiments. Each DNA insert was sequenced with both M13/pUC forward and reverse sequencing primers.

The nucleotide sequence of insert DNA was determined by using the 373A Automated DNA Sequencer (Applied Biosystems Inc.) according to the manufacturer's instructions. Sequencing data were collected by using the Applied

* Corresponding author. Mailing address: Animal Diseases Research Institute, Agriculture and Agri-Food Canada, P.O. Box 640, Gask Rd., Lethbridge, Alberta, Canada T1J 3Z4. Phone: (403) 382-5514. Fax: (403) 381-1202. E-mail: GANNONV@EM.AGR.CA.

TABLE 1. List of bacterial strains

Strain	Serotype	Source ^a
<i>L. monocytogenes</i> ATCC 15313 ^T	1/2a	Animal
<i>L. monocytogenes</i> NCTC 7973	1/2a	Animal
<i>L. monocytogenes</i> ATCC 19112	1/2b	Human clinical isolate
<i>L. monocytogenes</i> HBP307	1/2c	Food
<i>L. monocytogenes</i> NCTC 5105	3a	NA
<i>L. monocytogenes</i> AC 19	3b	Food
<i>L. monocytogenes</i> CIP 7836	3c	NA
<i>L. monocytogenes</i> ATCC 19114	4a	NA
<i>L. monocytogenes</i> HBP1	4ab	Food
<i>L. monocytogenes</i> HBP 3 (Scott A)	4b	Human clinical isolate
<i>L. monocytogenes</i> LI0521 (Murray B)	4b	Human clinical isolate
<i>L. monocytogenes</i> LI0549	4b	Food
<i>L. monocytogenes</i> ATCC 13932	4b	Human clinical isolate
<i>L. monocytogenes</i> ATCC 19115	4b	Human clinical isolate
<i>L. monocytogenes</i> CDC F9089	4b	Human clinical isolate
<i>L. monocytogenes</i> CIP 2698	4b	Human clinical isolate
<i>L. monocytogenes</i> LI0531	4b	NA
<i>L. monocytogenes</i> HPB 329	4b	NA
<i>L. monocytogenes</i> HPB 333	4b	NA
<i>L. monocytogenes</i> HPB 391	4b	Animal
<i>L. monocytogenes</i> HPB 468	4b	Food
<i>L. monocytogenes</i> HPB 520	4b	Environment
<i>L. monocytogenes</i> LCDC 15 μ	4b	Human
<i>L. monocytogenes</i> LI0597	4b	Food
<i>L. monocytogenes</i> HBP448	4b	Human
<i>L. monocytogenes</i> LI0532	4b	NA
<i>L. monocytogenes</i> LI0533	4b	Food
<i>L. monocytogenes</i> PLPH 44819	4b	Human clinical isolate
<i>L. monocytogenes</i> PLPH 44564	4b	Human clinical isolate
<i>L. monocytogenes</i> SLCC 5524	4b	NA
<i>L. monocytogenes</i> HBP17	4c	Animal
<i>L. monocytogenes</i> LI0600	4d	Food
<i>L. monocytogenes</i> ATCC 19117	4d	Animal
<i>L. monocytogenes</i> ATCC 19118	4e	Animal
<i>L. ivanovii</i> ATCC 19119 ^T	5	Animal
<i>L. innocua</i> NCTC 11288 ^T	6a	Environment
<i>L. seeligeri</i> ATCC 35967 ^T	1/2b	Environment
<i>L. welshimeri</i> ATCC 35897 ^T	6b	Environment
<i>L. grayi</i> ATCC 19120 ^T	NA	Animal

^a NA, not available.

Biosystems 373 Data Collection and Analysis software. Phylogenetic trees were created with CLUSTAL 4 (Higgins-Sharp algorithm), a component of the MacDNASIS, version 3.2 software program (HIBO; Hitachi Software, San Bruno, Calif.). Nucleotide sequence data were compared with the *Bacillus subtilis* 16S-23S rRNA operon DNA sequences found in GenBank, release 83 (accession no. J01551 and K00637).

RESULTS

Small 16S-23S rRNA IGS regions of *Listeria* spp. The small rRNA IGS PCR product of *L. monocytogenes* ATCC 15313^T serotype 1/2a consisted of 338 nucleotides, based on DNA sequence analysis of the cloned fragment. The first 53 nucleotides of the PCR product were homologous to the 3' end of the 16S rRNA gene, and the last 43 nucleotides of the PCR product were homologous to the sequence at the 5' end of the 23S rRNA gene. The portion representing the 16S-23S rRNA IGS region, therefore, consisted of 243 nucleotides (nucleotides 54 to 296 in *L. monocytogenes* ATCC 15313^T) (Fig. 1). The small 16S-23S rRNA IGS region of *L. monocytogenes* ATCC 15313^T (serotype 1/2a) was 99.7% homologous (a 1-nucleotide difference of A to T at position 239) (Fig. 1) to the nucleotide sequence of the *L. monocytogenes* ATCC 19118 (serotype 4e) small 16S-23S rRNA IGS presented by Emond et al. (10) (GenBank accession no. LO5172). The *L. monocyto-*

genes NCTC 7973 (serotype 1/2a) sequence (Table 2) was identical to the Emond sequence (data not shown).

We have previously reported that a PCR product of ca. 360 bp from *L. monocytogenes* Scott A (serotype 4b) is identical to the 338-bp small rRNA IGS of Scott A except that it contains an 18-bp tandem repeat (13) between nucleotides 135 and 136 (Fig. 1, sequence 2). Six other *L. monocytogenes* serotype 4b strains and one *L. monocytogenes* serotype 4d strain also had the ca. 360-bp PCR product in addition to the 338-bp band. Two of the serotype 4b strains (Scott A and Murray B) with the 360-bp product were isolated from patients involved in a milk-borne outbreak of listeriosis in Massachusetts in 1983. Four of the other serotype 4b strains with this product were isolated in sporadic cases of human infection, two from Germany (ATCC 19115 and ATCC 13932) and one each from France (Institut Pasteur 2698) and the United States (CDC F9089); one other serotype 4b strain with this product was isolated from cheese linked to a case of human infection (LI0549), and the serotype 4d strain with this product was isolated in a case of ovine listeriosis (ATCC 19117).

The number of nucleotides in the small 16S-23S IGS region ranged from 232 to 244 bp among members of the other *Lis-*

Sequence 1:	<i>L. monocytogenes</i> ATCC 15313 ^a	1 - 334 (334 bps)	Homology
Sequence 2:	<i>L. monocytogenes</i> Scott A	1 - 334 (334 bps)	100%
Sequence 3:	<i>L. innocua</i> NCTC 11288	1 - 335 (335 bps)	99%
Sequence 4:	<i>L. ivanovii</i> ATCC 19119	1 - 335 (335 bps)	96%
Sequence 5:	<i>L. welshimeri</i> ATCC 35897	1 - 334 (334 bps)	96%
Sequence 6:	<i>L. seeligeri</i> ATCC 35967	1 - 335 (335 bps)	95%
Sequence 7:	<i>L. grayi</i> ATCC 19120	1 - 323 (323 bps)	83%

1	(1)	AGTCGTAACAAGGTAGCGGTATCGGAAGGTGCGGCTGGATCACTCCTTTCTAAGGAAAA
2	(1)	AGTCGTAACAAGGTAGCGGTATCGGAAGGTGCGGCTGGATCACTCCTTTCTAAGGAAAA
3	(1)	AGTCGTAACAAGGTAGCGGTATCGGAAGGTGCGGCTGGATCACTCCTTTCTAAGGAAAA
4	(1)	AGTCGTAACAAGGTAGCGGTATCGGAAGGTGCGGCTGGATCACTCCTTTCTAAGGAAAA
5	(1)	AGTCGTAACAAGGTAGCGGTATCGGAAGGTGCGGCTGGATCACTCCTTTCTAAGGAAAA
6	(1)	AGTCGTAACAAGGTAGCGGTATCGGAAGGTGCGGCTGGATCACTCCTTTCTAAGGAAAA
7	(1)	AGTCGTAACAAGGTAGCGGTATCGGAAGGTGCGGCTGGATCACTCCTTTCTAAGGAAAA

1	(61)	GGAAACCTGTGAGTTTTCGTTCTTCTCTATTGTTTCAGTTTGTAGAGGTTAGTACTTCTC
2	(61)	GGAAACCTGTGAGTTTTCGTTCTTCTCTATTGTTTCAGTTTGTAGAGGTTAGTACTTCTC
3	(61)	GGAAACCTGTGAGTTTTCGTTCTTCTCTATTGTTTCAGTTTGTAGAGGTTAGTACTTCTC
4	(61)	GGAAACCTGTGAGTTTTCGTTCTTCTCTATTGTTTCAGTTTGTAGAGGTTAGTACTTCTC
5	(61)	GGAAACCTGTGAGTTTTCGTTCTTCTCTATTGTTTCAGTTTGTAGAGGTTAGTACTTCTC
6	(61)	GGAAACCTGTGAGTTTTCGTTCTTCTCTATTGTTTCAGTTTGTAGAGGTTAGTACTTCTC
7	(61)	GGAAACCTGTGAGTTTTCGTTCTTCTCTATTGTTTCAGTTTGTAGAGGTTAGTACTTCTC

1	(121)	A-GTA-TGT---TTGTTCTTTGAAAACCTAGATAAGAAAGTTAGTAAAGTTAGCATA---G
2	(121)	A-GTA-TGT---TTGTTCTTTGAAAACCTAGATAAGAAAGTTAGTAAAGTTAGCATA---G
3	(121)	A-GTA-TGT---TTGTTCTTTGAAAACCTAGATAAGAAAGTTAGTAAAGTTAGCATA---G
4	(121)	t-aTGTGT---TTGTTCTTTGAAAACCTAGATAAGAAAGTTAGTAAAGTTAGCATA---a
5	(121)	t-GTA-TGT---TTGTTCTTTGAAAACCTAGATAAGAAAGTTAGTAAAGTTAGCATA---a
6	(121)	t-GTA-TGT---TTGTTCTTTGAAAACCTAGATAAGAAAGTTAGTAAAGTTAGCATA---a
7	(115)	t-cTA-aGaaaTcGTTCTTTGAAAACCTAGATAAGAAAGTTAGTAAAGTTATCacAagTG

1	(173)	ATAATTAT-TATTTATGACACAAGTAACCGAGAAATCATCTGAAAGTGAATCTTTTCATCT
2	(173)	ATAATTAT-TATTTATGACACAAGTAACCGAGAAATCATCTGAAAGTGAATCTTTTCATCT
3	(174)	ATAATTAT-TATTTATGACACAAGTAACCGAGAAATCATCTGAAAGTGAATCTTTTCATCT
4	(174)	ATAAGTAAc-TATTTATGACACAAGTAACCGAGAAATCATCTGAAAGTGAATCTTTTCATCT
5	(173)	ATAAGTAAc-TATTTATGACACAAGTAACCGAGAAATCATCTGAAAGTGAATCTTTTCATCT
6	(173)	gTAgTgTAAc-TATTTATGACACAAGTAACCGAGAAATCATCTGAAAGTGAATCTTTTCATCT
7	(173)	AAaAcTTgT-AA-----AAgAAGTAACCGAGAAATCATCTGAAAGTGAaAcTTTCgCT

1	(232)	GATTGGAGTA-TCATCGCTGATACGGAATAACAGAAAACAACTTTTACTTCGTAGAAG
2	(232)	GATTGGAGTA-TCATCGCTGATACGGAATAACAGAAAACAACTTTTACTTCGTAGAAG
3	(233)	GATaAGaGTA-TCATCGCTGATACGGAATAACAGAAAACAACTTTTACTTCGTAGAAG
4	(233)	GATTGGAGTA-TCATCGCTGATACGGAATAACAGAAAACAACTTTTACTTCaTcGAAG
5	(232)	aATTGGaGTA-TCATCGCTGATAcGAcAAATAGAAAACAACTTTTACTTCGTcGAAG
6	(233)	aATTcGAcGTA-TCATCGCTGATAcGAcAAATAGAAAACAACTTTTACTTCGAcGAAG
7	(226)	--TTGGAAGTggtCATCGCTGA-cCGAGaAGaAGaAGaAGACC-TTACgcaATcG-cG

1	(291)	TAAATTGGTTAAGTTAGAAAGGGCGCACGGTGGATGCTTTGGCA
2	(291)	TAAATTGGTTAAGTTAGAAAGGGCGCACGGTGGATGCTTTGGCA
3	(292)	TAAATTGGTTAAGTTAGAAAGGGCGCACGGTGGATGCTTTGGCA
4	(292)	TAAATTGGTTAAGTTAGAAAGGGCGCACGGTGGATGCTTTGGCA
5	(291)	TAAATTGGTTAAGTTAGAAAGGGCGCACGGTGGATGCTTTGGCA
6	(292)	TAAATTGGTTAAGTTAGAAAGGGCGCACGGTGGATGCTTTGGCA
7	(281)	TAAa-TGGTTAAGTTAGaAGGGCGCACGGTGGATGCTTTGGCA

^aReference sequence

FIG. 1. Homology alignment of the small 16S-23S rRNA IGS regions of *Listeria* species. Sequencing data for the small 16S-23S rRNA IGS regions of *L. monocytogenes* ATCC 15313^T (GenBank accession no. U44062), *L. monocytogenes* Scott A (U78980), *L. innocua* NCTC 11288^T (U57914), *L. ivanovii* ATCC 19119^T (U78981), *L. welshimeri* ATCC 35897^T (U78982), *L. seeligeri* ATCC 35967^T (U78984), and *L. grayi* ATCC 19120^T (U78983) were determined in this study. Alignment of the sequences was performed with Align Plus, version 2 software (Scientific and Educational Software, State Line, Pa.). Restriction endonuclease *RsaI* sites are underlined. Differences in nucleotide sequence are indicated by lowercase lettering.

TABLE 2. *Listeria* strain 16S-23S rRNA IGS region sizes

Strain	A1-B1 PCR product (bp) ^a		16S-23S IGS size (bp) ^a	
	Small	Large	Small	Large
<i>L. monocytogenes</i> ATCC 15313 ^T	338	589	243	494
<i>L. monocytogenes</i> NCTC 7973	338	589	243	494
<i>L. monocytogenes</i> Scott A	338	589	243	494
<i>L. innocua</i> NCTC 11288 ^T	339	589	244	494
<i>L. ivanovii</i> ATCC 19119 ^T	339	585	244	490
<i>L. seeligeri</i> ATCC 35967 ^T	339	590	244	495
<i>L. welshimeri</i> ATCC 35897 ^T	338	592	243	497
<i>L. grayi</i> ATCC 19120 ^T	327	552	232	457

^a Determined from nucleotide sequencing information.

ter species examined (Fig. 1). *L. innocua* NCTC 11288^T, *L. ivanovii* ATCC 19119^T, and *L. seeligeri* ATCC 35967^T had IGSs of 244 bp, and the nucleotide sequence was 99, 96, and 95% homologous, respectively, to that of the *L. monocytogenes* ATCC 15313^T small rRNA IGS. *L. welshimeri* ATCC 35897^T had an IGS of 243 bp which was 96% homologous to that of *L. monocytogenes*. The small IGS of *Listeria grayi* ATCC 19120^T was 232 bp and was 83% homologous to that of *L. monocytogenes* (Fig. 1). No DNA sequences with homology to tRNAs were found in the nucleotide sequences of any of the small IGSs from the *Listeria* strains examined.

A dendrogram comparing the nucleotide sequences for the small 16S-23S rRNA IGS regions of the *Listeria* species examined is shown in Fig. 2. The small IGSs of *L. monocytogenes* and *L. innocua* were closely related, with a matching percentage of 96.2. The small rRNA IGSs of *L. welshimeri* and *L. seeligeri* were related to one another, with a match of 93.8%, and more distantly related to the *L. monocytogenes* and *L. innocua* small rRNA IGSs, with a 91% match (Fig. 2). The *L. ivanovii* small rRNA IGS had a matching percentage of 90.2 with the small IGSs of *L. monocytogenes* and *L. innocua*. In contrast, the small rRNA IGS of *L. grayi* stood out from those of other *Listeria* species, with only a 56.3% relatedness. The *B. subtilis* small 16S-23S rRNA IGS (GenBank accession no. K00637) was, as expected, distantly related, exhibiting only a 31.3% match with the *Listeria* species small rRNA IGSs.

Large 16S-23S rRNA IGS regions of *Listeria* spp. The large 16S-23S rRNA IGS PCR product of *L. monocytogenes* ATCC 15313^T was 589 bp (Fig. 3). As with the small 16S-23S rRNA IGS, the first 53 nucleotides were homologous to the 3' nucleotide sequence of the 16S rRNA gene and the last 42 nucleotides were homologous to the 5' nucleotide sequence of the 23S rRNA gene. Therefore, the length of the large IGS was 494 bp in *L. monocytogenes* (nucleotides 54 to 547 of *L. monocytogenes* ATCC 15313^T in Fig. 3); in *L. innocua* NCTC 11288^T, it was 494 bp, with 96% homology to the *L. monocytogenes* large 16S-23S rRNA IGS; in *L. welshimeri* ATCC 35897^T, 497 bp, with 95% homology; in *L. seeligeri* ATCC 35967^T, 495 bp, with 93% homology; in *L. ivanovii* ATCC 19119^T, 490 bp, with 93% homology; and in *L. grayi* ATCC 19120^T, 457 bp, with 78% homology to the *L. monocytogenes* sequence (Fig. 3). While small differences among the species were evident throughout the entire length of the large IGS, two areas of dramatic sequence heterogeneity were evident, between nucleotide positions 135 and 163 and between positions 268 and 300 of the *L. monocytogenes* ATCC 15313^T large IGS sequence (Fig. 3). The large 16S-23S rRNA IGSs of all the *Listeria* isolates examined also contained regions with nucleotide sequence homology to genes for isoleucine tRNA and alanine

tRNA. The isoleucine tRNA gene was present between positions 180 and 253, while the alanine tRNA gene was present between positions 300 and 375, of the large *L. monocytogenes* 16S-23S rRNA IGS (Fig. 3). The *L. monocytogenes* isoleucine and alanine tRNA genes are 97 and 100% homologous, respectively, to the tRNA genes of *B. subtilis* (GenBank accession no. K01986) (data not shown).

A dendrogram comparing the nucleotide sequence matching percentages for the large 16S-23S rRNA IGS regions of the *Listeria* species is shown in Fig. 4. The large IGSs of *L. monocytogenes* ATCC 15313^T and *L. innocua* NCTC 11288^T had the highest match percentage at 94.1. The large IGS of *L. welshimeri* ATCC 35897^T also was closely related to those of *L. monocytogenes* ATCC 15313^T and *L. innocua* NCTC 11288^T, at 91.2%. The large rRNA IGSs of *L. seeligeri* ATCC 35967^T and *L. ivanovii* ATCC 19119^T were related to each other with a match percentage of 93.0% but less closely related to the large rRNA IGSs of other *Listeria* species (89.0% matching). The large rRNA IGS of *L. grayi* ATCC 19120^T was distinct from those of other *Listeria* species tested, with a matching percentage of 63.0. The large IGS from *B. subtilis* (GenBank accession no. J01551) was distinct from those of the *Listeria* species tested, with only 55.6% relatedness.

Comparison of the large and small 16S-23S rRNA IGS regions of *L. monocytogenes*. The large and small 16S-23S rRNA IGSs of *L. monocytogenes* ATCC 15313^T have a high level of nucleotide sequence homology. The 5' ends of both IGSs were identical for the first 58 nucleotides, and the 3' ends were identical for 169 nucleotides. The primary differences among the small and large IGSs occurred in the 267-bp region containing the isoleucine and alanine tRNAs and the two regions of localized heterogeneity mentioned above. A 16-nucleotide antitermination Box A consensus sequence (5'-TGTTCTTTG AAAACTA-3') (2) was present in both the large and small 16S-23S rRNA IGS regions of the *L. monocytogenes* strain. In the large rRNA IGS, this was 5 nucleotides downstream of the alanine tRNA gene. A Box B sequence of 18 nucleotides (5'-GAGAGGTTAGTACTTCTC-3') (2) was found 9 bases upstream of the Box A sequence on the small IGS region. However, only the first 9 nucleotides of this Box B sequence (5'-GAGAGGTTA-3') were found in the large rRNA IGS sequences (Fig. 3).

A *RsaI* site was found in the small rRNA IGS of *L. monocytogenes* 57 bp from the 5' end of the sequence (Fig. 1 and 3). This site was also present in the large IGS of *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* and in both the large and small IGS sequences of *L. innocua*. No *RsaI* site was present in the large or small 16S-23S rRNA IGS of *L. grayi*.

FIG. 2. Dendrogram showing the genetic relationships among the six species of *Listeria* and *B. subtilis* based on the DNA sequence obtained of the small 16S-23S rRNA IGS region. The phylogenetic tree was created as described in Materials and Methods. DNA sequences (obtained from the PCR products generated with primers A1 and B1) for the small 16S-23S rRNA intergenic spacer regions of *L. monocytogenes* ATCC 15313^T, *L. ivanovii* ATCC 19119^T, *L. innocua* NCTC 11288^T, *L. seeligeri* ATCC 35967^T, *L. welshimeri* ATCC 35897^T, *L. grayi* ATCC 19120^T, and *B. subtilis* (GenBank accession no. K00637) were used in creating this dendrogram.

Sequence 1:	<i>L. monocytogenes</i>	ATCC 15313 ^a	1 - 585	(585 bps)	Homology
Sequence 2:	<i>L. monocytogenes</i>	Scott A	1 - 585	(585 bps)	99%
Sequence 3:	<i>L. innocua</i>	NCTC 11288	1 - 585	(585 bps)	96%
Sequence 4:	<i>L. ivanovii</i>	ATCC 19119	1 - 581	(581 bps)	93%
Sequence 5:	<i>L. welshimeri</i>	ATCC 35897	1 - 588	(588 bps)	95%
Sequence 6:	<i>L. seeligeri</i>	ATCC 35967	1 - 586	(586 bps)	93%
Sequence 7:	<i>L. grayi</i>	ATCC 19120	1 - 548	(548 bps)	81%

1	(	1)	AGTCGTAAACAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGAAAAGGAAACCTGTGAGTTTTCGTCTCTCTATTTGTTTCAGTT
2	(	1)	AGTCGTAAACAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGAAAAGGAAACCTGTGAGTTTTCGTCTCTCTATTTGTTTCAGTT
3	(	1)	AGTCGTAAACAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGAAAAGGAAACCTGTGAGTTTTCGTCTCTCTATTTGTTTCAGTT
4	(	1)	AGTCGTAAACAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGAAAAGGAAACCTGTGAGTTTTCGTCTCTCTATTTGTTTCAGTT
5	(	1)	AGTCGTAAACAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGAAAAGGAAACCTGTGAGTTTTCGTCTCTCTATTTGTTTCAGTT
6	(	1)	AGTCGTAAACAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGAAAAGGAAACCTGTGAGTTTTCGTCTCTCTATTTGTTTCAGTT
7	(	1)	AGTCGTAAACAGGTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTCTAAGGAAAAGGAAACCTGTGAGTTTTCGTCTCTCTATTTGTTTCAGTT

1	(101)	TTGAGAGGTTACTCTCTTTTATGTGATAGATAAAGTATGCAAGGCACATA--TGCT--TGAAGCATCGGCCACTACATTTTTCGCGGCCCTATAGCTCAGCTG
2	(101)	TTGAGAGGTTACTCTCTTTTATGTGATAGATAAAGTATGCAAGGCACATA--TGCT--TGAAGCATCGGCCACTACATTTTTCGCGGCCCTATAGCTCAGCTG
3	(101)	TTGAGAGGTTACTCTCTTTTATGTGATAGATAAAGTATGCAAGGCACATA--TGCT--TGAAGCATCGGCCACTACATTTTTCGCGGCCCTATAGCTCAGCTG
4	(101)	TTGAGAGGTTACTCTCTTTTATGTGATAGATAAAGTATGCAAGGCACATA--TGCT--TGAAGCATCGGCCACTACATTTTTCGCGGCCCTATAGCTCAGCTG
5	(101)	TTGAGAGGTTACTCTCTTTTATGTGATAGATAAAGTATGCAAGGCACATA--TGCT--TGAAGCATCGGCCACTACATTTTTCGCGGCCCTATAGCTCAGCTG
6	(101)	TTGAGAGGTTACTCTCTTTTATGTGATAGATAAAGTATGCAAGGCACATA--TGCT--TGAAGCATCGGCCACTACATTTTTCGCGGCCCTATAGCTCAGCTG
7	(97)	TTGAGAGGTT-----TTTTCCTCTCtaATaAgA-aATG---GGCTCTTatTCT-TtAA--ATGgGcagcCaAtcTgTaaatCGGCCCTATAGCTCAGCTG

1	(198)	GTTAGAGCGCAGCCTGATAAGCGTGAAGTTCGATGGTTTCGAGTCCATTTAGGCCCACTTTTTCCTTTCGACGCAAGAAATACAAATAATCATATCCT--TT
2	(198)	GTTAGAGCGCAGCCTGATAAGCGTGAAGTTCGATGGTTTCGAGTCCATTTAGGCCCACTTTTTCCTTTCGACGCAAGAAATACAAATAATCATATCCT--TT
3	(198)	GTTAGAGCGCAGCCTGATAAGCGTGAAGTTCGATGGTTTCGAGTCCATTTAGGCCCACTTTTTCCTTTCGACGCAAGAAATACAAATAATCATATCCT--TT
4	(198)	GTTAGAGCGCAGCCTGATAAGCGTGAAGTTCGATGGTTTCGAGTCCATTTAGGCCCACTTTTTCCTTTCGACGCAAGAAATACAAATAATCATATCCT--TT
5	(199)	GTTAGAGCGCAGCCTGATAAGCGTGAAGTTCGATGGTTTCGAGTCCATTTAGGCCCACTTTTTCCTTTCGACGCAAGAAATACAAATAATCATATCCT--TT
6	(198)	GTTAGAGCGCAGCCTGATAAGCGTGAAGTTCGATGGTTTCGAGTCCATTTAGGCCCACTTTTTCCTTTCGACGCAAGAAATACAAATAATCATATCCT--TT
7	(184)	GTTAGAGCGCAGCCTGATAAGCGTGAAGTTCGATGGTTTCGAGTCCATTTAGGCCCACTTTTTCCT-----ATcaatTtctTtTATatc--TT

1	(297)	T--AT--GGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTTGACGCGAGGAGGTGACGCGTTTCGATCCCGCTAGGCTCCACCAAAATTTGTTCTTTGAAA
2	(297)	T--Ac--GGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTTGACGCGAGGAGGTGACGCGTTTCGATCCCGCTAGGCTCCACCAAAATTTGTTCTTTGAAA
3	(297)	T--ta--GGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTTGACGCGAGGAGGTGACGCGTTTCGATCCCGCTAGGCTCCACCAAAATTTGTTCTTTGAAA
4	(293)	T--Aa--GGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTTGACGCGAGGAGGTGACGCGTTTCGATCCCGCTAGGCTCCACCAAAATTTGTTCTTTGAAA
5	(298)	ctaAg--GGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTTGACGCGAGGAGGTGACGCGTTTCGATCCCGCTAGGCTCCACCAAAATTTGTTCTTTGAAA
6	(294)	T--ATtaGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTTGACGCGAGGAGGTGACGCGTTTCGATCCCGCTAGGCTCCACCAAAATTTGTTCTTTGAAA
7	(269)	T--AT--GGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTTGACGCGAGGAGGTGACGCGTTTCGATCCCGCTAGGCTCCACCAAAATTTGTTCTTTGAAA

1	(392)	ACTAGATAAGAAAGTTAGTAAAGTTAGCATA---GATAATTAT--TATTTATGACACAAGTAACCGAGAATCATCTGAAAGTGAATCTTTTCATCTGATTG
2	(392)	ACTAGATAAGAAAGTTAGTAAAGTTAGCATA---GATAATTAT--TATTTATGACACAAGTAACCGAGAATCATCTGAAAGTGAATCTTTTCATCTGATTG
3	(392)	ACTAGATAAGAAAGTTAGTAAAGTTAGCATA---GATAATTAT--TATTTATGACACAAGTAACCGAGAATCATCTGAAAGTGAATCTTTTCATCTGATTG
4	(388)	ACTAGATAAGAAAGTTAGTAAAGTTAGCATA---aATAggTaAc--TATTTATGACACAAGTAACCGAGAATCATCTGAAAGTGAATCTTTTCATCTGATTG
5	(395)	ACTAGATAAGAAAGTTAGTAAAGTTAGCATA---GATAAGTaAT--TATTTATGACACAAGTAACCGAGAATCATCTGAAAGTGAATCTTTTCATCTGATTG
6	(392)	ACTAGATAAGAAAGTTAGTAAAGTTAGCATA---agTAgTgTaAc--TATTTATGACACAAGTAACCGAGAATCATCTGAAAGTGAATCTTTTCATCTGATTG
7	(363)	ACTAGATAAGAAAGTTAGTAAAGTTATCacAagtGaaAaCTTgT-aA-----AaAgAAGTAACCGAGAATCATCTGAAAGTGAATCTTTTCgCT--TTG

1	(488)	GATGTA--TCATCGCTGATACGGAATAACAGAAAAACAACCTTTTACTTCGTAGAGTAAATTTGGTTAAGTTAGAAAGGGCGCACGGTGGATGCCCTTGGCA
2	(488)	GaaGTA--TCATCGCTGATACGGAATAACAGAAAAACAACCTTTTACTTCGTAGAGTAAATTTGGTTAAGTTAGAAAGGGCGCACGGTGGATGCCCTTGGCA
3	(488)	GaaGTA--TCATCGCTGATACGGAATAACAGAAAAACAACCTTTTACTTCGTAGAGTAAATTTGGTTAAGTTAGAAAGGGCGCACGGTGGATGCCCTTGGCA
4	(484)	GaaGTA--TCATCGCTGATACGGAATAACAGAAAAACAACCTTTTACTTCGTAGAGTAAATTTGGTTAAGTTAGAAAGGGCGCACGGTGGATGCCCTTGGCA
5	(491)	GaaGTA--TCATCGCTGATACcAaGATAGAAAAACAACCTTTTACTTCGTGAGTAAATTTGGTTAAGTTAGAAAGGGCGCACGGTGGATGCCCTTGGCA
6	(489)	GAcGTA--TCATCGCTGATACaGAcAATAGAAAAACAACCTTTTACTTCGAcGAAGTAAATTTGGTTAAGTTAGAAAGGGCGCACGGTGGATGCCCTTGGCA
7	(454)	GaaGTTgTCATCGCTGA--cCGcAgAaAGaAGcAAAAAGACC--TTACGcaatTcG-cGTAAA--TGGTTAAGTTAGgAAGGGCGCACGGTGGATGCCCTTGGCA

^aReference sequence

FIG. 3. Homology alignment of the large IGS regions of *Listeria* species. Nucleotide sequence data for the large 16S-23S rRNA IGS regions of *L. monocytogenes* ATCC 15313^T (GenBank accession no. U44061), *L. monocytogenes* Scott A (U57912), *L. innocua* NCTC 11288^T (U57915), *L. ivanovii* ATCC 19119^T (U57913), *L. welshimeri* ATCC 35897^T (U57917), *L. seeligeri* ATCC 35967^T (U57916), and *L. grayi* ATCC 19120^T (U57918) were determined in this study. Alignment of the sequences was performed as described in the legend to Fig. 1. Restriction endonuclease *Rsa*I sites are underlined. Differences in nucleotide sequence are indicated by lowercase lettering.

Differentiation of serotypes within *L. monocytogenes* based on the large 16S-23S rRNA IGS sequence. The oligonucleotide primer set LA2-LB2 amplified a 274-bp PCR product from *L. monocytogenes* genomic DNA which contains the nucleotide sequence homologous to the two tRNA genes (isoleucine and alanine) and one of the two areas of heterogeneity (268 to 300 bp) in the large rRNA IGS region (Fig. 3). The LA2-LB2 PCR products from seven different *L. monocytogenes* serotypes (1/2a, 3a, 3b, 4a, 4b, 4d, and 4e) were amplified, and the DNA was sequenced. The nucleotide sequence of this portion of the large IGS was highly homologous among the different *L. monocytogenes* serotypes examined (Fig. 5). In contrast, the same area for the large IGS of *L. innocua* NCTC 11288^T was only 94.0% homologous to *L. monocytogenes* ATCC 15313^T.

The relationships among strains from different *L. monocytogenes* serotypes based on nucleotide sequence differences in

this 274-bp portion of the large 16S-23S rRNA IGS are presented in Fig. 6. These strains had highly homologous large IGSs which differed in at most six base-pair positions. However, four different sequence groups were identified. *L. monocytogenes* serotypes 1/2a and 3a were identical to each other but different from the other five *L. monocytogenes* serotypes examined. A second group included *L. monocytogenes* serotype 4a, 4b, and 4d strains, which also had sequences identical to each other, and two single *L. monocytogenes* strains of serotype 3b and 4e, which had unique sequences.

DISCUSSION

Barry et al. (1) and Jensen et al. (17) have described PCR amplification of the 16S-23S rRNA IGS region with oligonucleotide primers complementary to the 3' end of the 16S and

FIG. 4. Dendrogram showing the genetic relationships among the six species of *Listeria* and *B. subtilis* based on nucleotide sequence data obtained for the large 16S-23S rRNA IGS region. The phylogenetic tree was created as described in Materials and Methods. DNA sequences (obtained from the PCR products generated with primers A1 and B1) for the large 16S-23S rRNA IGS regions of *L. monocytogenes* ATCC 15313^T, *L. ivanovii* ATCC 19119^T, *L. innocua* NCTC 11288^T, *L. seeligeri* ATCC 35967^T, *L. welshimeri* ATCC 35897^T, *L. grayi* ATCC 19120^T, and *B. subtilis* (GenBank accession no. J01551) were used in creating this dendrogram.

the 5' end of the 23S rRNA structural genes of *E. coli*. These two primer sets are highly conserved and allow for the amplification of the 16S-23S rRNA IGS from a large number of bacterial species (1, 17). In this study, the "generic" oligonucleotide primer set described by Barry et al. (1) was used to amplify two 16S-23S rRNA IGS regions in representative strains of the genus *Listeria*.

The small rRNA IGS PCR product for all the *Listeria* strains tested contained 42 bp from the 3' end of the 16S rRNA gene and 52 bp from the 5' end of the 23S rRNA gene. The size of the small IGS was 242 bp for *L. monocytogenes* and 244 bp for *L. welshimeri*, *L. innocua*, and *L. ivanovii*. The nucleotide sequence presented for the small rRNA IGS of *L. monocytogenes* in this study was the same size and was also 99.7% homologous to the *L. monocytogenes* small rRNA IGS sequence presented by Emond et al. (10).

Seven *L. monocytogenes* serotype 4b strains and one *L. monocytogenes* serotype 4d strain had an additional small rRNA IGS band of ca. 360 bp following PCR amplification with the A1-B1 primer set. We have previously shown for one serotype 4b strain that this extra rRNA IGS band is identical to the 338-bp rRNA IGS except that it contains an 18-bp tandem repeat (13). An additional band of approximately this size also was recently noted by Drebot et al. (9) in 4 of 27 clinical isolates of *L. monocytogenes*. However, complete serotype data for the *L. monocytogenes* strains with this extra PCR band were not given in the latter report. One of the strains mentioned by Drebot et al. (9), *L. monocytogenes* Scott A (serotype 4b), was also found to have the anomalous band in the present study. *L. monocytogenes* Murray B (serotype 4b) and *L. monocytogenes* Scott A (serotype 4b) are independent isolates from patients involved in a 1983 milk-borne outbreak of listeriosis in Massachusetts (11), and both have the ca. 360-bp rRNA IGS PCR product. This suggests that the anomalous rRNA IGS is not a laboratory artifact but rather a characteristic of the outbreak strain. A second *L. monocytogenes* strain with this PCR product mentioned by Drebot et al. (9) was also a serotype 4b strain which was isolated from an outbreak of food-borne illness in Nova Scotia (23). All the *L. monocytogenes* serotype 4b strains with this anomalous PCR product examined in the present study were associated with cases of human infection and were not confined to one particular geographic region (two were from Germany, one from France, and four from the United States). It is possible that this band is a marker for a widespread pathogenic clone of *L. monocytogenes* serotype 4b. It would be interesting to compare the *L. monocytogenes* serotype 4b strains with this extra rRNA IGS PCR band by using techniques such

as multilocus enzyme electrophoresis (MEE) and phage typing to see if these serotype 4b strains are indeed closely related. This anomalous PCR band may be a result of chromosomal rearrangement through homologous recombination at rRNA IGS sites, as has been suggested for *Salmonella* (20) and other bacterial species (21), or simply some other form of recombination.

Differences also were found between the size estimates of Jensen et al. (17) for the large rRNA IGS DNA bands and the sizes determined in this study. Jensen et al. (17) used polyacrylamide gel electrophoresis to determine IGS sizes and suggested that *L. monocytogenes* and *L. ivanovii* had large rRNA IGS PCR products of 620 and 605 bp, respectively. However, in this study, sizes determined by nucleotide sequence analysis were 589 and 585 bp for *L. monocytogenes* and *L. ivanovii*, respectively. As with the small rRNA IGS, these PCR products contained 42 bp from the 3' end of the 16S rRNA gene and 52 bp from the 5' end of the 23S rRNA gene. The size of this large rRNA IGS is, therefore, 494 bp for *L. monocytogenes* and 490 bp for *L. ivanovii*.

Berg et al. (2) described Box B-Box A antitermination se-

Sequence	1:	<i>L. monocytogenes</i>	ATCC 15313 ^a	serotype	1/2a	Homology
Sequence	2:	<i>L. monocytogenes</i>	NCTC 5105	serotype	3a	100%
Sequence	3:	<i>L. monocytogenes</i>	AC 19	serotype	3b	99%
Sequence	4:	<i>L. monocytogenes</i>	ATCC 19114	serotype	4a	99%
Sequence	5:	<i>L. monocytogenes</i>	Scott A	serotype	4b	99%
Sequence	6:	<i>L. monocytogenes</i>	ATCC 19117	serotype	4d	99%
Sequence	7:	<i>L. monocytogenes</i>	ATCC 19118	serotype	4e	97%
Sequence	8:	<i>L. innocua</i>	NCTC 11288	serotype	6a	94%

1	(1)	CTATAGCTCAGCTGGTTAGAGCGCAGCGCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
2	(1)	CTATAGCTCAGCTGGTTAGAGCGCAGCGCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
3	(1)	CTATAGCTCAGCTGGTTAGAGCGCAGCGCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
4	(1)	CTATAGCTCAGCTGGTTAGAGCGCAGCGCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
5	(1)	CTATAGCTCAGCTGGTTAGAGCGCAGCGCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
6	(1)	CTATAGCTCAGCTGGTTAGAGCGCAGCGCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
7	(1)	CTATAGCTCAGCTGGTTAGAGCGCAGCGCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
8	(1)	CTATAGCTCAGCTGGTTAGAGCGCAGCGCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA

1	(61)	TTTAGGCCACCTTTTCTTTCTGACGCAAGAAATACAAATATCATATCTTTTATGGGG
2	(61)	TTTAGGCCACCTTTTCTTTCTGACGCAAGAAATACAAATATCATATCTTTTATGGGG
3	(61)	TTTAGGCCACCTTTTCTTTCTGACGCAAGAAATACAAATATCATATCTTTTATGGGG
4	(61)	TTTAGGCCACCTTTTCTTTCTGACGCAAGAAATACAAATATCATATCTTTTATGGGG
5	(61)	TTTAGGCCACCTTTTCTTTCTGACGCAAGAAATACAAATATCATATCTTTTATGGGG
6	(61)	TTTAGGCCACCTTTTCTTTCTGACGCAAGAAATACAAATATCATATCTTTTATGGGG
7	(61)	TTTAGGCCACCTTTTCTTTCTGACGCAAGAAATACAAATATCATATCTTTTATGGGG
8	(61)	TTTAGGCCACCTTTTCTTTCTGACGCAAGAAATACAAATATCATATCTTTTATGGGG

1	(121)	CCCTAGCTCAGCTGGGAGAGCGCGCTGCTTTGACCGCAGGAGGTGACGGTTCGATCCCG
2	(121)	CCCTAGCTCAGCTGGGAGAGCGCGCTGCTTTGACCGCAGGAGGTGACGGTTCGATCCCG
3	(121)	CCCTAGCTCAGCTGGGAGAGCGCGCTGCTTTGACCGCAGGAGGTGACGGTTCGATCCCG
4	(121)	CCCTAGCTCAGCTGGGAGAGCGCGCTGCTTTGACCGCAGGAGGTGACGGTTCGATCCCG
5	(121)	CCCTAGCTCAGCTGGGAGAGCGCGCTGCTTTGACCGCAGGAGGTGACGGTTCGATCCCG
6	(121)	CCCTAGCTCAGCTGGGAGAGCGCGCTGCTTTGACCGCAGGAGGTGACGGTTCGATCCCG
7	(121)	CCCTAGCTCAGCTGGGAGAGCGCGCTGCTTTGACCGCAGGAGGTGACGGTTCGATCCCG
8	(121)	CCCTAGCTCAGCTGGGAGAGCGCGCTGCTTTGACCGCAGGAGGTGACGGTTCGATCCCG

1	(181)	TAGGCTCCACCAAAATTTGTTCTTTGAAAACCTAGATAGAAAGTTAGTAAAGTTAGCATAG
2	(181)	TAGGCTCCACCAAAATTTGTTCTTTGAAAACCTAGATAGAAAGTTAGTAAAGTTAGCATAG
3	(181)	TAGGCTCCACCAAAATTTGTTCTTTGAAAACCTAGATAGAAAGTTAGTAAAGTTAGCATAG
4	(181)	TAGGCTCCACCAAAATTTGTTCTTTGAAAACCTAGATAGAAAGTTAGTAAAGTTAGCATAG
5	(181)	TAGGCTCCACCAAAATTTGTTCTTTGAAAACCTAGATAGAAAGTTAGTAAAGTTAGCATAG
6	(181)	TAGGCTCCACCAAAATTTGTTCTTTGAAAACCTAGATAGAAAGTTAGTAAAGTTAGCATAG
7	(181)	TAGGCTCCACCAAAATTTGTTCTTTGAAAACCTAGATAGAAAGTTAGTAAAGTTAGCATAG
8	(181)	TAGGCTCCACCAAAATTTGTTCTTTGAAAACCTAGATAGAAAGTTAGTAAAGTTAGCATAG

1	(241)	ATAATTTATTTATTTATGACACAAGTAACCGAGAA
2	(241)	ATAATTTATTTATTTATGACACAAGTAACCGAGAA
3	(241)	ATAATTTATTTATTTATGACACAAGTAACCGAGAA
4	(241)	ATAATTTATTTATTTATGACACAAGTAACCGAGAA
5	(241)	ATAATTTATTTATTTATGACACAAGTAACCGAGAA
6	(241)	ATAATTTATTTATTTATGACACAAGTAACCGAGAA
7	(241)	ATAATTTATTTATTTATGACACAAGTAACCGAGAA
8	(241)	ATAATTTATTTATTTATGACACAAGTAACCGAGAA

^aReference sequence

FIG. 5. Homology alignment of a 274-bp variable region of the large 16S-23S rRNA IGS region of strains of *L. monocytogenes* from different serotypes. Sequencing data for the variable region of the large 16S-23S rRNA IGS region of *L. monocytogenes* ATCC 15313^T serotype 1/2a (GenBank accession no. U44061), *L. monocytogenes* NCTC 5105 serotype 3a, *L. monocytogenes* AC 19 serotype 3b, *L. monocytogenes* ATCC 19114 serotype 4a, *L. monocytogenes* Scott A serotype 4b (GenBank accession no. U57912), *L. monocytogenes* ATCC 19117 serotype 4d, *L. monocytogenes* ATCC 19118 serotype 4e, and *L. innocua* NCTC 11288^T serotype 6a (GenBank accession no. U57915) were determined in this study. Alignment of the sequencing data was done as described in the legend to Fig. 1. Differences in nucleotide sequence are indicated by lowercase lettering.

FIG. 6. Dendrogram showing the relationship among seven serotypes of *L. monocytogenes* and *L. innocua* based on the DNA sequence obtained for a 274-bp fragment of the large 16S-23S rRNA IGS region. The phylogenetic tree was created as described in Materials and Methods. DNA sequences (obtained from the PCR products generated with primers LA2 and LB2) for the variable region of the large 16S-23S rRNA IGS regions of *L. monocytogenes* ATCC 15313^T (serotype 1/2a), *L. monocytogenes* NCTC 5105 (serotype 3a), *L. monocytogenes* AC 19 (serotype 3b), *L. monocytogenes* ATCC 19114 (serotype 4a), *L. monocytogenes* Scott A (serotype 4b), *L. monocytogenes* ATCC 19117 (serotype 4d), *L. monocytogenes* ATCC 19118 (serotype 4e), and *L. innocua* NCTC 11288^T (serotype 6a) were used in creating the dendrogram.

quences which are found in all the 16S-23S IGS regions of *E. coli*, as well as in a number of other bacterial species, including *B. subtilis*. The Box A sequence and the 8 nucleotides following the Box A region in two of the *B. subtilis* 16S-23S rRNA IGS regions consist of the nucleotide sequence 5'-TGTTCTTTGA AACTA-3'. These same 16 nucleotides are present in both the large and small IGS regions of *L. monocytogenes*. Berg et al. (2) also found that the Box A sequence in both the 16S-23S rRNA IGS region of the O ribosomal operon in *B. subtilis* and the 16S-23S rRNA IGS region of the A ribosomal operon in *Thermotoga maritima* followed an alanine tRNA gene. This arrangement also was evident in the large rRNA IGS region of *L. monocytogenes*. It is interesting that the Box A site is at the beginning of a 168-bp 3' region of homology between the large and small rRNA IGSs of *L. monocytogenes*. The conserved nature of the Box A sequence among these different bacterial species may indicate that it has an important functional role in bacterial survival.

One way of differentiating among various nucleotide sequences is by use of restriction enzymes. Examination of the *Listeria* sp. sequence data reveals that only *L. monocytogenes* and *L. innocua* contain a *RsaI* site (GTAC) within the small rRNA IGS. This site begins 1 nucleotide away from the 57-bp alignment at the 5' end of the small IGS region and 13 nucleotides upstream from the Box A sequence of the small rRNA IGS region. However, this site was also present in the large IGS region of *L. innocua*, as well as in the large IGS regions of *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*. It was not present in the small rRNA IGS sequences of *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*. This information is in agreement with previous observations we have made concerning the use of this restriction enzyme to digest PCR products of the 16S-23S rRNA IGS and indicates how digestion of the rRNA IGS PCR products with *RsaI* allows differentiation of *L. monocytogenes* from other species of *Listeria* (13).

To our knowledge, this is the first study in which nucleotide sequence data for the large 16S-23S rRNA IGS have been presented for all species within the genus *Listeria*. The relationships observed among the large 16S-23S rRNA IGS nucleotide sequences for the *Listeria* species (Fig. 4) correspond closely to the unrooted phylogenetic tree produced by Collins et al. (7), based on analysis of 16S rRNA for low-G+C-content gram-positive bacteria; i.e., in both studies *L. monocytogenes* and *L. innocua* show a high degree of sequence relatedness, and *L. welshimeri* is most closely related to the *L. monocyto-*

genes-L. innocua group. *L. seeligeri* and *L. ivanovii* are closely related to each other and show a lesser degree of sequence matching with *L. monocytogenes* and *L. innocua*. *L. grayi* is most distant from the other five *Listeria* species. Nucleotide sequence information from the small 16S-23S IGS region produced a similar phylogenetic tree (Fig. 2), except that *L. ivanovii* appeared to be more closely related to *L. monocytogenes* and *L. innocua* than to *L. welshimeri*. This is likely a result of the limited number of nucleotides used in creating the phylogenetic tree for the small rRNA IGS. Collins et al. (7) indicated that the next closest genus to *Listeria* was *Brochothrix*, and this genus is followed by *Bacillus*. Unfortunately, the DNA sequence for the 16S-23S IGS region was available only for *B. subtilis*. It would have been interesting to compare IGS regions of *Brochothrix* spp. with those of *Listeria*.

It has been shown, by using a number of typing techniques, that strains of *L. monocytogenes* fall into one of two major groups. This division is serologically evident from the flagellum type of the organism and from data from more modern typing techniques, such as ribotyping and MEE (3, 4, 14). The two ribotypes of *L. monocytogenes* are designated RT α and RT β , and the two MEE types have been designated ETGA and ETGB. Both ribotyping and MEE techniques separate strains of *L. monocytogenes* with AB (serotypes 1/2a and 3a) or BD (serotypes 1/2c and 3c) flagellar antigens into RT β or ETGB types and strains of *L. monocytogenes* with ABC flagellar antigens (serotypes 1/2b, 3b, and all 4's) into RT α or ETGA types. Examination of the dendrogram resulting from homology alignment of the DNA sequencing data for the 274-bp variable region of the large rRNA IGS region (Fig. 6) reveals that *L. monocytogenes* strains of serotypes 1/2a and 3a all group together. The dendrogram also shows that strains of serotype 4 (4a, 4b, and 4d) group together except for the single serotype 4e strain examined. In addition, *L. monocytogenes* serotype 3b also was grouped with group 4 (serotypes 4a, 4b, and 4d). It is anticipated that a better analysis could be performed if more strains of *L. monocytogenes*, representing all the serotypes of the organism, could be included in the analysis.

ACKNOWLEDGMENT

This project was supported by Agriculture and Agri-Food Canada.

REFERENCES

- Barry, T., G. Colleran, M. Glennon, L. K. Dunican, and F. Gannon. 1991. The 16S/23S ribosomal spacer region as a target for DNA probes to identify eubacteria. *PCR Methods Appl.* 1:51-56. (Erratum, 1:149.)
- Berg, K. L., C. Squires, and C. L. Squires. 1989. Ribosomal operon antitermination: function of leader and spacer region BoxB-BoxA sequences and their conservation in diverse microorganisms. *J. Mol. Biol.* 209:345-358.
- Bibb, W. F., B. G. Gellin, R. Weaver, B. Schwartz, B. D. Plikaytis, M. W. Reeves, R. W. Pinner, and C. V. Broome. 1990. Analysis of clinical and food-borne isolates of *Listeria monocytogenes* in the United States by multilocus enzyme electrophoresis and application of the method to epidemiologic investigations. *Appl. Environ. Microbiol.* 56:2133-2141.
- Bibb, W. F., B. Schwartz, B. G. Gellin, B. D. Plikaytis, and R. E. Weaver. 1989. Analysis of *Listeria monocytogenes* by multilocus enzyme electrophoresis and application of the method to epidemiologic investigations. *Int. J. Food Microbiol.* 8:233-239.
- Brooks, J. L., J. P. Back, and R. G. Kroll. 1992. Direct application to dairy foods of a *Listeria*-specific oligonucleotide probe to 16S rRNA. *Int. J. Food Microbiol.* 16:303-312.
- Burgess, J. G., R. Kawaguchi, T. Sakaguchi, R. H. Thornhill, and T. Matsunaga. 1993. Evolutionary relationships among *Magnetospirillum* strains inferred from phylogenetic analysis of 16S rDNA sequences. *J. Bacteriol.* 175:6689-6694.
- Collins, M. D., S. Wallbanks, D. J. Lane, J. Shah, R. Nietupski, J. Smida, M. Dorsch, and E. Stackebrandt. 1991. Phylogenetic analysis of the genus *Listeria* based on reverse transcriptase sequencing of 16S rRNA. *Int. J. Syst. Bacteriol.* 41:240-246.
- Collins-Thompson, D. L., P. J. Slade, and M. Goethals. 1991. Use of low molecular mass RNA profiles to identify lactic acid bacteria and related

- organisms associated with foods. *Int. J. Food Microbiol.* **14**:135–143.
9. **Drebót, M., S. Neal, W. Schlech, and K. Rozee.** 1996. Differentiation of *Listeria* isolates by PCR amplicon profiling and sequence analysis of 16S-23S rRNA internal transcribed spacer loci. *J. Appl. Bacteriol.* **80**:174–178.
  10. **Emond, E., I. Fliss, and S. Pandian.** 1993. A ribosomal DNA fragment of *Listeria monocytogenes* and its use as a genus-specific probe in an aqueous-phase hybridization assay. *Appl. Environ. Microbiol.* **59**:2690–2697.
  11. **Fleming, D. W., S. L. Cochi, K. L. MacDonald, J. Brondum, P. S. Hayes, B. D. Plikaytis, M. B. Holmes, A. Audurier, C. V. Broome, and A. L. Reinhold.** 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *N. Engl. J. Med.* **312**:404–407.
  12. **Golsteyn-Thomas, E. J., R. K. King, J. Burchak, and V. P. J. Gannon.** 1991. Sensitive and specific detection of *Listeria monocytogenes* in milk and ground beef with the polymerase chain reaction. *Appl. Environ. Microbiol.* **57**:2576–2580.
  13. **Graham, T. A., E. J. Golsteyn-Thomas, V. P. J. Gannon, and J. E. Thomas.** 1996. Genus- and species-specific detection of *Listeria monocytogenes* using polymerase chain reaction assays targeting the 16S/23S intergenic spacer region of the rRNA operon. *Can. J. Microbiol.* **42**:1155–1162.
  14. **Graves, L. M., B. Swaminathan, M. W. Reeves, S. B. Hunter, R. E. Weaver, B. D. Plikaytis, and A. Schuchat.** 1994. Comparison of ribotyping and multilocus enzyme electrophoresis for subtyping of *Listeria monocytogenes* isolates. *J. Clin. Microbiol.* **32**:2936–2943.
  15. **Greisen, K., M. Loeffelholz, A. Purohit, and D. Leong.** 1994. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. *J. Clin. Microbiol.* **32**:335–351.
  16. **Gurtler, V., and V. A. Stanisich.** 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology* **142**:3–16.
  17. **Jensen, M. A., J. A. Webster, and N. Straus.** 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl. Environ. Microbiol.* **59**:945–952.
  18. **Ji, Y. E., K. E. Kempell, M. J. Colston, and R. A. Cox.** 1994. Nucleotide sequences of the spacer-1, spacer-2 and trailer regions of the rrn operons and secondary structures of precursor 23S rRNAs and precursor 5S rRNAs of slow-growing mycobacteria. *Microbiology* **140**:1763–1773.
  19. **Kita-Tsakamoto, K., H. Oyaizu, K. Nanba, and U. Simidu.** 1993. Phylogenetic relationships of marine bacteria, mainly members of the family *Vibrionaceae*, determined on the basis of 16S rRNA sequences. *Int. J. Syst. Bacteriol.* **43**:8–19.
  20. **Liu, S. L., and K. E. Sanderson.** 1995. Rearrangements in the genome of the bacterium *Salmonella typhi*. *Proc. Natl. Acad. Sci. USA* **92**:1018–1022.
  21. **Moxon, E. R., P. B. Rainey, M. A. Nowak, and R. E. Lenski.** 1994. Adaptive evolution of highly mutable loci in pathogenic bacteria. *Curr. Biol.* **4**:24–33.
  22. **Nicoletti, V. G., and D. F. Condorelli.** 1993. Optimized PEG method for rapid plasmid DNA purification: high yield from "Midi-Prep." *BioTechniques* **14**:532–536.
  23. **Slech, W. F., P. M. Lavigne, R. A. Bortolussi, A. C. Allen, E. V. Haldane, A. J. Wort, W. A. Hightower, S. E. Johnson, S. H. King, E. S. Nicholls, and C. V. Broome.** 1983. Epidemic listeriosis—evidence for transmission by food. *N. Engl. J. Med.* **308**:203–206.
  24. **Thompson, D. E., J. T. Balsdon, J. Cai, and M. D. Collins.** 1992. Studies on the ribosomal RNA operons of *Listeria monocytogenes*. *FEMS Microbiol. Lett.* **75**:219–224.
  25. **Wang, R. F., W. W. Cao, and M. G. Johnson.** 1991. Development of a 16S rRNA-based oligomer probe specific for *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **57**:3666–3670.
  26. **Wang, R. F., W. W. Cao, H. Wang, and M. G. Johnson.** 1993. A 16S rRNA-based DNA probe and PCR method specific for *Listeria ivanovii*. *FEMS Microbiol. Lett.* **80**:85–92.
  27. **Wiedmann, M., F. Barany, and C. A. Batt.** 1993. Detection of *Listeria monocytogenes* with a nonisotopic polymerase chain reaction-coupled ligase chain reaction assay. *Appl. Environ. Microbiol.* **59**:2743–2745.
  28. **Wiedmann, M., J. Czajka, F. Barany, and C. A. Batt.** 1992. Discrimination of *Listeria monocytogenes* from other *Listeria* species by ligase chain reaction. *Appl. Environ. Microbiol.* **58**:3443–3447.