

Evolutionary history of the genus *Listeria* and its virulence genes

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Abstract

The genus *Listeria* contains the two pathogenic species *Listeria monocytogenes* and *Listeria ivanovii* and the four apparently apathogenic species *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri*, and *Listeria grayi*. Pathogenicity of the former two species is enabled by an approximately 9 kb virulence gene cluster which is also present in a modified form in *L. seeligeri*. For all *Listeria* species, the sequence of the virulence gene cluster locus and its flanking regions was either determined in this study or assembled from public databases. Furthermore, some virulence-associated internalin loci were compared among the six species. Phylogenetic analyses were performed on a data set containing the sequences of *prs*, *ldh*, *vclA*, and *vclB* (all directly flanking the virulence gene cluster), as well as the *iap* gene and the 16S and 23S-rRNA coding genes which are located at different sites in the listerial chromosomes. *L. grayi* represents the deepest branch within the genus. The remaining five species form two groupings which have a high bootstrap support and which are consistently found by using different treeing methods. One lineage represents *L. monocytogenes* and *L. innocua*, while the other contains *L. welshimeri*, *L. ivanovii* and *L. seeligeri*, with *L. welshimeri* forming the deepest branch. Based on this perception, we tried to reconstruct the evolution of the virulence gene cluster. Since no traces of lateral gene transfer events could be detected the most parsimonious scenario is that the virulence gene cluster was present in the common ancestor of *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri* and *L. welshimeri* and that the pathogenic capability has been lost in two separate events represented by *L. innocua* and *L. welshimeri*. This hypothesis is also supported by the location of the putative deletion breakpoints of the virulence gene cluster within *L. innocua* and *L. welshimeri*.

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Introduction

Listeriae are non-spore-forming, motile, rod shaped, facultative-anaerobic, Gram-positive bacteria. The six

species of this genus include *Listeria monocytogenes*, *Listeria ivanovii*, *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri*, and *Listeria grayi* [31]. Because *L. monocytogenes* is an intracellular, foodborne pathogen potentially lethal for humans and animals, its virulence mechanisms have been intensively studied since the mid-1980s (for a recent review see Vazquez-Boland et al. [57]). *L. ivanovii* is also of great economic importance because it is pathogenic in livestock. *Listeriae* are widely distributed in the environment. With the exception of clinical specimens, they are physiologically quite similar and are found to inhabit the same environments [31,50].

L. monocytogenes represents a pathogenic species while *L. innocua* is a non-pathogenic relative. Since the genome sequences of both organisms are available [24,45], it would be helpful to interpret their genomic contents and differences in the context of the phylogenetic relationship and the evolution of the genus *Listeria*. Based on different approaches, several schemes of relatedness among the six *Listeria* species have been proposed. All schemes consistently support that *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. ivanovii* form a cluster excluding *L. grayi* but differ regarding the relationships within this five-species cluster. Using multilocus enzyme electrophoresis (MLEE) on 18 enzyme loci, Boerlin et al. [2] proposed that *L. seeligeri* and *L. ivanovii* form one group while *L. innocua*, *L. welshimeri* and *L. monocytogenes* form another. In this scheme, *L. welshimeri* and *L. innocua* are the most recently derived sister species of the latter group. In contrast, the 16S-rRNA phylogeny reported by Vaneechoutee et al. [55] placed *L. monocytogenes* and *L. innocua* as sister branches, with *L. welshimeri* forming a deeper branching within the group. However, it must be noted that rRNA phylogeny provides only very little resolution within the five species cluster of the genus *Listeria* since the 16S-rRNA or 23S-rRNA sequences of the respective species are almost identical [10,11,49,51,55]. In addition, phylogenetic analysis of the 16S–23S rDNA intergenic spacer regions (ISR) of all *Listeria* species were performed. These studies were complicated by the fact that all recognized members of this species possess a large and a small 16S–23S-rDNA ISR. Comparative sequence analyses of both ISR types indicate that *L. monocytogenes* and *L. innocua* form a monophyletic group in the genus *Listeria* but inconsistent affiliations were observed for *L. innocua*, *L. welshimeri*, and *L. seeligeri* [27].

The major virulence functions of *L. monocytogenes* are encoded on a cluster of six genes, 9 kb in length, which we will refer to as the virulence gene cluster in this paper. This cluster is now also called *Listeria* pathogenicity island 1 [33,57]. Three members of the *Listeria* genus possess some form of this virulence gene cluster. While *L. ivanovii* and *L. monocytogenes* have a very similar virulence gene cluster, the non-pathogenic

species *L. seeligeri* contains a more elaborate cluster with five additional genes. The virulence gene clusters of *L. monocytogenes* and *L. ivanovii* contain (i) *prfA*, which encodes the positive master-regulator of most of the known virulence genes, (ii) *hly*, which encodes the sulfhydryl-activated pore-forming listeriolysin necessary for bacterial escape from the phagosomes of host cells into the host cytosol, and (iii) *plcA* and *plcB* coding for two phospholipases facilitating the lysis of host cell membranes. The gene *plcA* encodes a phosphatidylinositol-specific phospholipase C, while *plcB* encodes a phosphatidylcholine phospholipase C. The latter lecithinase requires a metalloprotease encoded by *mpl* (also found on the virulence gene cluster) for proper maturation. In addition, the *actA* gene, endowing *Listeriae* with an actin assembly capability to drive intracellular movement within host cells, is part of the virulence gene cluster. ActA, together with the gene products of *mpl* and *plcB*, enables the phenomenon of cell-to-cell spread [17,21,53].

In addition to the virulence gene cluster, other virulence genes have been identified to be scattered elsewhere in the genome of *L. monocytogenes* and *L. ivanovii*. Most of these genes constitute a multigene family termed internalins and encode extracellular proteins containing varying numbers of 22 amino acid long leucine-rich repeats (LRRs). Multiple internalins have been identified in both *L. monocytogenes* [18,23,39,47] and *L. ivanovii* [19,20] and, by sequence analysis, also in *L. innocua* [24]. Some but not all of these internalins are necessary for the invasion of mammalian host cells [15]. In addition, the “invasion-associated protein” (IAP), encoded by the *iap* gene, has been implicated to be important in maintaining the invasive phenotype in mouse fibroblasts, hepatocytes and macrophages. However, IAP, also termed P60 reflecting its molecular size of 60 kDa, has another important function by acting as a murein hydrolase necessary for proper cell division [6,30,34,62].

It was the goal of this study to infer the phylogeny of the genus *Listeria* and to trace the evolution of the pathogenic lifestyle within this genus using sequence data from multiple loci of all six species. For each of the six species, DNA sequences of the virulence cluster locus and its flanking regions were either determined or assembled from existing sources. Aside from the virulence gene cluster region, other chromosomal regions are known to encode internalins in some listerial species. Some were PCR amplified and sequenced. An extended phylogenetic analysis of the genus *Listeria* was performed based on a data set including (i) house-keeping genes flanking the virulence gene cluster, (ii) 16S and 23S-rDNA, and (iii) the *iap* gene. This collection represents the entire currently available set of DNA sequence information common to all six listerial species.

Materials and methods

Bacterial strains

The following *Listeria* strains were used in this study: *L. monocytogenes* strains EGD (Special *Listeria* Culture Collection—SLCC 5835) and LO28, of serotypes 1/2a and 1/2c, respectively; *L. innocua* serotype Sv6b, *L. welshimeri* SLCC 5334, *L. ivanovii* American Type Culture Collection (ATCC 19119) (SLCC 2379), *L. seeligeri* SLCC 3954, and *L. grayi*. Species identity of all strains has been confirmed using species-specific primers for the *iap* gene [5]. All *Listeria* strains used were obtained from the strain collection maintained at the University of Würzburg (Würzburg; Germany); some of the strains are also deposited in the SLCC at the Institut Pasteur (Paris, France) and the American Type Culture Collection (ATCC). In addition, *E. coli* strain TOP10 used for cloning was provided in the TOPO TA Cloning Kit (Invitrogen; Groningen, Netherlands).

Media

All *Listeria* strains were grown aerobically in Brain Heart Infusion (BHI; Difco, Franklin Lakes, NY) at 37 °C and maintained on BHI agar plates. *E. coli* strains used for cloning were grown on Luria–Bertani (LB) medium. For antibiotic selection during the cloning procedure, 100 µg/ml Ampicillin was added to the LB medium.

DNA extraction methods

Chromosomal DNA was isolated from all *Listeria* strains using the following procedure. A single colony isolate was inoculated into 10 ml BHI and grown overnight under aerobic conditions at 37 °C. The cells were harvested 16 h after inoculation by centrifugation. The pellet was washed with 5 ml of 0.1 × SSC (1 × SSC: 0.15 M NaCl, 0.15 M trisodium citrate, pH7.0). The cells were treated at 37 °C for 1–2 h with 0.5 ml of 25 mg/ml lysozyme (Sigma, Deisenhofen, Germany) dissolved in TES (30 mM Tris/HCl pH8.0, 50 mM NaCl, 5 mM EDTA pH 8.0, 20% sucrose). Lysis was completed by the addition of 4.5 ml lysis buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA pH 8.0, 1% SDS, 0.5 mg/ml Proteinase K) and a further incubation at 37 °C for 1 h. DNA was extracted by gentle inversion using one or two extractions with phenol, followed twice with phenol/CHCl₃ (1:1) and once with CHCl₃. DNA was precipitated by the addition of 500 µl of 3 M Na(OAc) and 10 ml ethanol, and collected by spooling with a glass rod. Subsequently, the DNA was briefly washed in 70% ethanol and finally resuspended in 400 µl of sterile distilled water.

PCR and cloning

The primers applied for PCR amplification of DNA fragments are listed in Table 1. DNA templates used for all PCRs were chromosomal DNAs extracted from the

Table 1. PCR primers used in this study

Region of interest	Primer name	Primer sequence 5' to 3'
Virulence gene cluster	prs1 >	GCGCCGATTGCTATTATTGA
	ldh1 <	GAATCCCAGCATGGAGCCA
	ivan-plcb1 >	AAATGCGAAACAGACCTGCG
	see-plcb1 >	ACAAGGGCTTTCAGATTCTC
	see-vcfY3 <	TCATATGTAAAGCTGGATGATC
	see-vcfY1 >	GGTCTATTTAGTTAGAGGAGA
Extending 5' of <i>prs</i>	con-prs1 >	GTGGTTGTCATGTATATGTTATTCAA
	see-prs1 <	GTGGTGCTACAGACAGCTGT
	see-prs2 <	GAGCAATGGAGTTAGTAACAAC
	ivan-prs1 <	ACAGATGCATTTTCACGTACA
	ivan-prs2 <	ACGATTGCTTCACCTAGCAGT
<i>Loci</i> corresponding to <i>inlC</i>	rpls2 >	TCGAAGGCGCTGCAGTCAAACG
	infC1 <	GTCTTCGCACGCTTTTGA
<i>Loci</i> corresponding to <i>inlGHE</i>	pGluco2 >	GTAAGTGCCTGCAGAAGCGAAATGTCC
	PGluco1 >	AGTAAGTGCCTCCACAAGCG
	desuc1 <	TGTAAACATCTACCATCTCCAA
<i>Loci</i> corresponding to <i>i-inlDC</i>	li-inlD4 >	GAGAGAGCAATCTTTCAAC
	li-emr5 <	TTTCACTAAAGCATTTCAT
	li-emr6 >	GAGGTGTTTTTTTGAAGGAGAA
	li-emr1 <	GTGTATCCATCGTTAAGAACAT

respective *Listeriae*. Generally, the PCR reactions were performed using (i) 2 mM MgCl₂ for Taq DNA Polymerase (Promega, Madison) and 2 mM MgSO₄ for Deep Vent DNA polymerase (New England Biolabs, Beverly, MA), (ii) the respective buffer provided by the manufacturer, (iii) 200 μM of each dNTP, (iv) 30–50 pM of each primer, and (v) 1 U of Deep Vent or Taq DNA polymerase in 100 μl reaction volumes. Standard cycling parameters were 30 cycles with denaturation for 30 s at 94 °C, annealing temperatures as specified in Table 2 for each primer pair used, and extension times dependent on the expected length of the amplicate (1 min per kb, at 72 °C). For products larger than 5 kb, the GeneAmp XL PCR kit (Perkin Elmer, Wellesley, MA) was employed. The reagents used for PCR amplification of the virulence gene clusters were XL Buffer II, 200 μM of each dNTP, 15 μM of each primer, 2 mM Mg(OAc)₂, 1 U rTth DNA Polymerase XL in a 100 μl final volume. In total 25 cycles were conducted. The first 12 cycles consisted of 30 s denaturation at 94 °C, 30 s annealing at 53 °C, and 11 min extension at 68 °C. The following 13 cycles were run under identical conditions except an increase of 15 s of extension time for each proceeding cycle. Cycling was completed by a 60 min final extension at 68 °C. For all protocols listed, Techne (Cambridge, USA) and Perkin Elmer GeneAmp 2400PCR programmable thermo cyclers, respectively, were used.

In addition to conventional cloning in pUC18, the TOPO TA cloning kit (Invitrogen) was predominantly employed in this study for rapid cloning of PCR products. Because successful ligation with this kit depends on the presence of a 3' dATP overhang, characteristically generated by Taq DNA polymerase, blunt-end amplicates, produced, e.g. by the Deep Vent- and rTth-DNA polymerase (applied to achieve higher amplification accuracy and/or amplification of longer fragments), had to be treated prior to cloning. Initially, the respective PCR products were cleaned using a PCR purification kit (Qiagen, Hilden, Germany), and subsequently the 3' A extension was created by a 15 min incubation at 72° in a 25 μl reaction mix containing cleaned PCR product, 1 × Taq DNA polymerase buffer, 200 μM dATP, and 1 U Taq DNA polymerase. After this treatment, TA cloning was performed as suggested by the manufacturer.

DNA sequencing

Sequencing of either PCR products or cloned PCR products was done using the ABI 310 system (Applied Biosystems, Foster City, CA) and the Li-Cor system (Lincoln, Nebraska), respectively, following the instructions of the manufacturers. All DNA sequences determined in this study were obtained for both strands

and often confirmed by templates obtained from multiple clones and/or independent PCR reactions.

Computer analyses

Sequence information was managed using GCG v.10.0-UNIX (the Wisconsin Package). Homology searches with public nucleic acid or protein banks were performed with BlastX2, BlastP2 and BlastN2 in the NCBI, GenomeNet at University of Kyoto, TIGR or Prodom databases. (“<http://www.ncbi.nlm.nih.gov/BLAST/>”, “<http://www.blast.genome.ad.jp/>”, “<http://www.tigr.org>” and “http://www.toulouse.inra.fr/prodom/doc/blast_form.html”). Signal peptide predictions were performed using Signal P v.1.1 as provided by the Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark (“<http://www.cbs.dtu.dk/services/SignalP/>”). Blocks searches were done at the Blocks database maintained by the Fred Hutchinson Cancer Research Center (“http://blocks.fhrc.org/blocks/blocks_search.html”).

Phylogeny inference

Deduced amino acid sequences of the ORFs under investigation were aligned manually with the GDE2.2 sequence editor [54]. The corresponding nucleic acid sequences were then aligned according to the amino acid alignment. For all phylogenetic analyses performed, the ARB software package (www.arb-home.de, [40]) was used. Phylogenetic trees based on nucleic acids were calculated using “Maximum Parsimony” (PAR) (PHY-LIP version 3.57c, [22]), “Maximum Likelihood” (ML) (fast DNAmI program; [46]) and “Neighbor-Joining” (NJ) methods. Amino acid sequence-based trees were calculated using NJ, ML (ProtML 2.2 with the JTT-f amino acid replacement model; [1], and Protein Parsimony (PHYLIP version 3.57c; [22]) methods. In addition, amino acid trees were inferred from distances using FITCH with global rearrangements (PHYLIP version 3.57c, [22]). Bootstrap analysis (1000 resamplings) was performed for nucleic acid parsimony trees using the PHYLIP version 3.57c package.

The following section lists the accession numbers of previously published DNA sequences used for phylogenetic analysis in this study. Sequence data for 16S-rDNA were from X56153 (*L. monocytogenes*), X56149 (*L. welshimeri*), X56151 (*L. ivanovii*), X56148 (*L. seeligeri*), X56150 (*L. grayi*) [10], X55473 (*L. innocua*) [11]. 23S-rRNA sequences were derived from X92951 (*L. monocytogenes*), X92949 (*L. innocua*), X92954 (*L. welshimeri*), X92950 (*L. ivanovii*), X92953 (*L. seeligeri*), and X92948 (*L. grayi*) [51]. *Iap* gene sequences were derived from X52268 (*L. monocytogenes* EGD), M80351 (*L. monocytogenes* Mackness), M80347

Table 2. PCR results

Chromosomal region amplified	Organism	Product obtained	Product contains additional genes	Accession number (designation in Fig. 1)	Primer pair	DNA polymerase	Optimal annealing temp. (°C)
Virulence gene cluster	<i>L. monocytogenes</i> EGDe	11 Kb	Yes	NA	prs1 > <ldh1	rTth, XL	50
	<i>L. innocua</i> Sv6b	2.8 Kb	Yes	AJ249804 (a)	prs1 > <ldh1	Deep vent	54
	<i>L. ivanovii</i> ATCC 19119	3.4 Kb	Yes	AJ249805 (c)	ivan-plcb1 > <ldh1	rTth, XL	53
	<i>L. seeligeri</i> SLCC 3954	4.5 Kb	Yes	AJ249738 (e)	see-vcIH1 > <ldh1	rTth, XL	50
	<i>L. seeligeri</i> SLCC 3954	0.8 Kb	No	AJ249738 (e)	see-plcb1 > <see-vcIH3	Deep vent	54
	<i>L. welshimeri</i> SLCC 5334	2.9 Kb	Yes	AJ249808 (f)	prs1 > <ldh1	rTth, XL	53
	<i>L. grayi</i>	6.2 Kb	Yes	AJ249739 (g)	prs1 > <ldh1	rTth, XL	53
Extending 5' of <i>prs</i>	<i>L. ivanovii</i> ATCC 19119	0.8 Kb	NA	AJ249806 (b)	con-prs1 > <ivan-prs1	Taq	52
	<i>L. seeligeri</i> SLCC 3954	0.8 Kb	NA	AJ249807 (d)	con-prs1 > <ivan-prs1	Taq	52
<i>inlC</i> locus	<i>L. monocytogenes</i> EGDe	2.4 Kb	NA	NA	rpls2 > <infC1	Taq	54
	<i>L. innocua</i> Sv6b	2.7 Kb	Yes	AJ249401 (i)	rpls2 > <infC1	Taq	54
	<i>L. ivanovii</i> ATCC 19119	2.1 Kb	Yes	AJ249400 (h)	rpls2 > <infC1	Taq	54
	<i>L. seeligeri</i> SLCC 3954	Non-specific	NA	NA	rpls2 > <infC1	Taq	None
	<i>L. welshimeri</i> SLCC 5334	2.4 Kb	Yes	AJ249399 (j)	rpls2 > <infC1	Taq	53
	<i>L. grayi</i>	Non-specific	NA	NA	rpls2 > <infC1	Taq	None
<i>i-inlDC</i> locus	<i>L. monocytogenes</i> EGDe	1.2 Kb	No	AJ010599 (k)	li-inlD4 > <li-emr5	Taq	45
	<i>L. innocua</i> Sv6b	2.8 Kb	Yes	AJ249398 (l)	li-inlD4 > <li-emr5	Taq	45
	<i>L. ivanovii</i> ATCC 19119	3.9 Kb	NA	NA	li-inlD4 > <li-emr5	Taq	50
3' of <i>i-inlDC</i> locus	<i>L. monocytogenes</i> EGDe	2 Kb	No	AJ1010600	li-emr6 > <li-emr1	Taq	45
	<i>L. innocua</i> Sv6b	2 Kb	No	NA	li-emr6 > <li-emr1	Taq	45
	<i>L. ivanovii</i> ATCC 19119	2 Kb	NA	NA	li-emr6 > <li-emr1	Taq	45
	<i>L. murrayi</i>	0.8 Kb?	No	NA	pGluco2 > <desuc1	rTth, XL	54
<i>inlGHE</i> locus (<i>inlC₂DE</i>)	<i>L. monocytogenes</i> EGDe	6.7 Kb	NA	NA	pGluco2 > <desuc1	rTth, XL	54
	<i>L. innocua</i> Sv6b	1.4 Kb	No	AJ249403 (n)	pGluco1 > <desuc1	Taq	45
	<i>L. ivanovii</i> ATCC 19119	1.4 Kb	No	AJ249402 (m)	pGluco1 > <desuc1	Taq	54
	<i>L. seeligeri</i> SLCC 3954	1.5 Kb	No	NA	pGluco2 > <desuc1	Taq	47
	<i>L. welshimeri</i> SLCC 5334	1.6 Kb	No	NA	pGluco2 > <desuc1	Taq	None
	<i>L. grayi</i>	0.8 Kb?	No	NA	pGluco2 > <desuc1	Taq	None
	<i>L. murrayi</i>	0.8 Kb?	No	NA	pGluco2 > <desuc1	Taq	None

NA = not applicable.

(*L. innocua* Sv6a), M80349 (*L. innocua* Sv6b), M80348 (*L. welshimeri*), M80350 (*L. ivanovii*), M80353 (*L. seeligeri*) and M95579 (*L. grayi*) [6]. For phylogenetic analyses of the *ldh* genes the following sequences were used as outgroups: *Bacillus caldolyticus* (M19394), *B. caldotenax* (M19386), *B. stearothermophilus* (AB033627); *Bifidobacterium longum* (M33585), *Deinococcus radiodurans* (AB005539), *Lactobacillus casei* (M76708), *L. sakei* (U26688), *L. lactis* (M88490), *Mycoplasma genitalium* (U39733), *M. hyopneumonia* (X67286), *Streptococcus mutans* (M72545), *S. pneumonia* (AJ005815), *Thermotoga maritima* (X74302), *Thermus aquaticus* (D00858). For phylogenetic analyses of *vclB* (Lmo0209/Lin0289) genes homologous sequences of *E. coli* (AE000188) and *B. anthracis* (AF188935) were used as outgroups. The accession numbers of those listerial sequences determined in this study are listed in Table 2.

Results

Organization of the virulence gene cluster locus in the six *Listeria* species

In order to obtain the organization of the complete virulence gene cluster locus for all listerial species, all previously deposited virulence cluster sequences were assembled from public databases. The data set was then completed by sequencing the region between the house-keeping genes *prs* and *ldh*, flanking upstream and downstream of the virulence cluster locus, respectively. For this purpose, conserved primers were designed complementary to signature regions of these house-keeping genes. The subsequently obtained PCR products were cloned and sequenced. The sequences of the virulence cluster loci of *L. innocua*, *L. welshimeri*, *L. grayi*, the 5' and 3' fragments of *L. seeligeri*, and the 5' and 3' fragments of *L. ivanovii* are compiled in Fig. 1. Table 2 lists the PCR conditions, PCR product information, and the accession number for each of the sequenced fragments.

To avoid confusion in the nomenclature found among the previously published descriptions of various putative gene sequences within the virulence cluster locus, and to comply with current journal rules, we renamed the previously designated “*orf*” here as “*vcl*” (virulence cluster locus). Lmo/Lin numbers refer to the nomenclature from the published sequences [24]. Thus, the previously known *orfA* (Lmo0208/Lin0240) and *orfB* (Lmo0209/Lin0241) of *L. monocytogenes* (accession number M82881; [56] are designated here as *vclA* and *vclB*, while *orfA*, *orfB* and *orfC* of *L. seeligeri* (X97014; [35], which are unrelated to the *orfA* and *orfB* of *L. monocytogenes*, are renamed here as *vclC*, *vclD* and *vclE*, respectively. Highly conserved homologues of *vclB* are

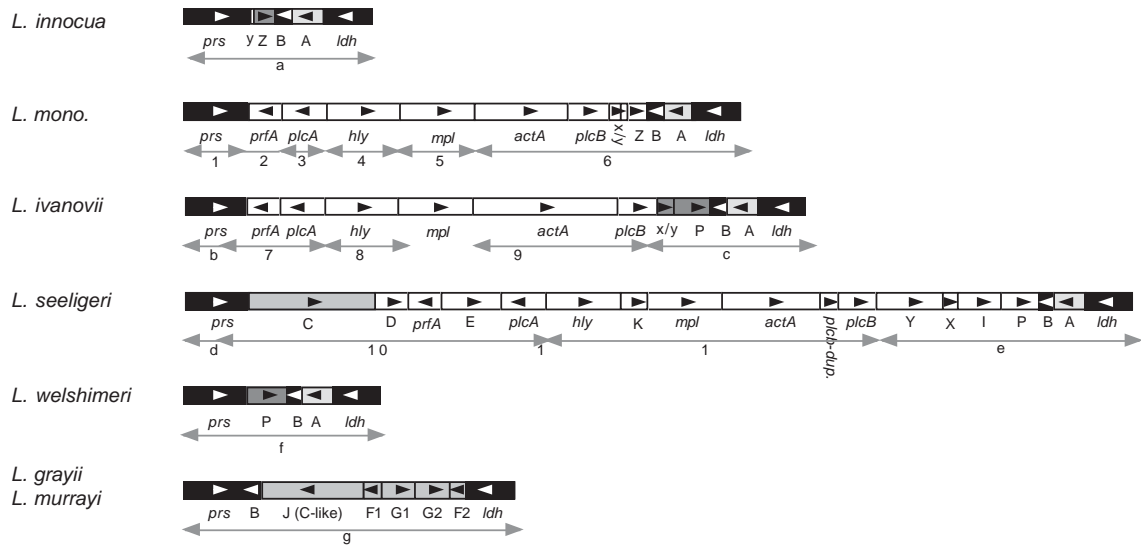
present in all *Listeriae* (70–92% nucleic acid sequence similarity; 79–100% amino acid identity). A homologue of *vclA* is only missing in *L. grayi*.

Fig. 1A shows a comparison of the organization of the virulence cluster locus in the six *Listeria* species. The sequences derived from this study are represented by letter codes, while information assembled from existing sources are noted by numbers. Table 3 shows the functional assignments of these open reading frames. As expected, no recognized virulence genes were detected between *prs* and *ldh* in *L. innocua*, *L. welshimeri* and *L. grayi*, confirming previously reported results from Southern hybridizations [56]. Within this chromosomal region, the invariable elements are *prs*, *vclB* and *ldh*, while the regions between them appear rather variable, by accommodating the virulence gene cluster and/or other genes in the different species.

The map of *L. grayi* differs from the other listerial species in that *vclB* is adjacent to *prs*, while the five additional coding sequences *vclJ*, *vclF1*, *vclG1*, *vclG2*, and *vclF2* not present in the other species precede *ldh*. *VclA*, whose function is unknown, is present downstream and adjacent to *vclB* in all *Listeriae* except *L. grayi*, although remnants of *vclA* sequence appear between *vclF2* and *ldh* of *L. grayi*. *vclP*, encoding a putative phosphate transfer enzyme, is specific to *L. seeligeri*, *L. welshimeri* and *L. ivanovii*. However, traces of *vclP* intragenic sequences could be detected between *vclJ* and *vclF1* of *L. grayi* indicating that *vclP* already was present in the ancestor of the genus. *vclZ* (Lmo0207/Lin0239), coding for a putative membrane protein with similarity to a hypothetical *E. coli* protein, is specific to *L. monocytogenes* and *L. innocua*. Database searches revealed no known proteins similar to *VclX*, which is present in *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*.

Three primes to the established virulence genes, the *L. seeligeri* virulence gene cluster contains a few intriguing coding sequences, *vclY*, *vclX*, *vclI* and *vclP*. Analysis of *vclY* of *L. seeligeri* revealed that this is the entire gene corresponding to the truncated sequences seen in *L. monocytogenes* and *L. innocua*, and potentially encodes a cell-wall anchored surface protein because of the presence of the Gram-positive cell-wall anchor signal sequence LPNTG [52]. The inverted order of *vclX* and *vclY* in *L. seeligeri* with respect to the *L. monocytogenes* arrangement probably reflects a genetic inversion event in at least one of the virulence cluster carrying species or ancestral species. The deduced *VclI* protein of *L. seeligeri* exhibits a remarkable resemblance in amino acid sequence and structure to the internalins of *L. monocytogenes*, particularly to *inlB*. *VclI* contains a predicted signal sequence, four–five LRRs, but is terminated within the inter-repeat region at a place similar to the terminations of the small internalins of *L. ivanovii*.

A. Region between *prs* and *ldh* among *Listeria* species



B. Chromosomal context of some internalins and the corresponding regions among *Listeriae*

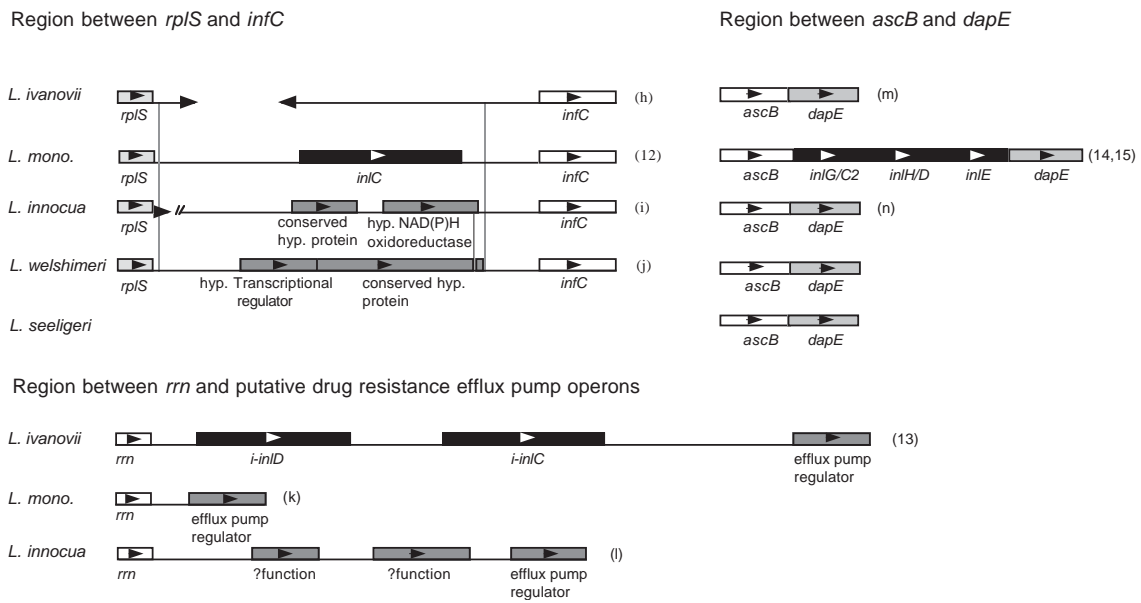


Fig. 1. (A) A schematic diagram of the virulence cluster locus (*vcl*). If appropriate, the nomenclature from the published sequences [24] is given in parentheses. This cluster is flanked by the house-keeping genes (black boxes) *prs*, *vclB* (Lmo0209/Lin0241) and *ldh* in the six species of the genus *Listeria*. Genes that are de facto, or potentially controlled by the master virulence regulator PrfA are shown as white boxes. *vclA* (Lmo0208, Lin0240) is present in all species except *L. grayi*. *vclP* is present in *L. welshimeri*, *L. seeligeri* and *L. ivanovii*. *vclZ* (Lmo0207/Lin0239) is present in *L. monocytogenes* and *L. innocua*. *vclY* and *X* are inverted in *L. seeligeri* with respect to all other *loci* carrying *vclXY*. Species-specific genes (medium gray boxes) not under PrfA control include *vclJ*, *vclF1*, *vclG1*, *vclG2*, *vclF2* of *L. seeligeri*; and *vclC* of *L. seeligeri*. (B) A schematic diagram of some chromosomal regions carrying internalin genes (black boxes) and the corresponding *loci* in other *Listeriae*. Regions of sequence divergence are defined by vertical dashed-lines spanning the alignments. Note the diversity of genetic content bordered by invariable house-keeping genes. Sources: sequences presented in this study are defined as (letter code, accession number). (A): (a) AJ249804; (b) AJ249806; (c) AJ249805; (d) AJ249807; (e) AJ249738; (f) AJ249808; (g) AJ249739. (B): (h) AJ249400; (i) AJ249401; (j) AJ249399; (k) AJ010599; (l) AJ249398; (m) AJ249402; (n) AJ249403. Sequences assembled from public sources are defined as (number code, accession number (reference)). (A): (1) M92842 [26]; (2) M55160 [38]; (3) X54618 [37,41]; (4) X15127 [12]; (5) X54619 [13]; (6) M82881 [56]; (7) X72685 [35]; (8) X60462 [29]; (9) U19035 [25,32]; (10) X97014 [35]; (11) 97014, pending update [35]; (B): (12) Y07640 [18,19]; (13) Y07639 [19]; (14) AJ007319 [47]; (15) U77368 [15].

Table 3. Coding sequences (CDS) identified in the listerial chromosomal regions of the virulence gene cluster, between *rpls* and *infC*, and between the *rrn* and drug efflux pump operons

Location	CDS	Present in	Size	Features	Greatest similarity	Organism with best matches	Blast score	<i>P(N)</i>	
Virulence gene cluster	<i>vcl A</i>	all except <i>grayi</i>	223–224aa	—	—	—	—	—	
	<i>vcl B</i>	All	110aa	—	Conserved hyp. protein	<i>E. coli</i> (pir:B64825)	BlastP 265	1.60E-31	
	<i>vcl X</i>	<i>monocytogenes</i> <i>ivanovii</i>	107aa 32+77aa	SP	—	—	—	—	
	<i>vcl Y^a</i>	<i>seeligeri</i>	115aa	SP, membrane anchor	(not similar to VclC)	—	—	—	
		<i>monocytogenes</i>	59aa						
	<i>vcl Z</i>	<i>innocua</i>	15aa	PrfA box (in <i>L. see</i>)	SP	hyp. lipoprotein	<i>E. coli</i> (sp:P33354)	BlastP 255	2.40E-35
		<i>seeligeri</i>	469aa						
	<i>vcl P</i>	<i>monocytogenes</i>	153aa	—	—	Probable phosphoesterase	<i>M. jannaschii</i> (sp:Y912_METJA)	BlastX 120	1.80E-11
		<i>innocua</i>	51aa						
	<i>vcl C</i>	<i>welshimeri</i>	264–287aa	SP, 5x 78–80aa repeats, membrane anchor	C alpha antigen precursor (Bca)	<i>Strep. agalactiae</i> (sp:Q02192)	BlastP1298	E-144	
		<i>ivanovii</i>	902aa						
	<i>vcl D</i>	<i>seeligeri</i>	228–248aa	PrfA box	Probable sugar isomerase. (AraD)	<i>E. coli</i> (prf:1303258C)	BlastP 364	3.00E-68	
	<i>vcl E</i>	<i>seeligeri</i>	423aa	PrfA box	—	—	—	—	
<i>vcl K</i>	<i>seeligeri</i>	187aa	—	—	<i>L. seeligeri</i>	—	—		
<i>vcl I</i>	<i>seeligeri</i>	304aa–284aa	SP, 5 LRR ^b ,	Internalin (InlB)	<i>L. monocytogenes</i> (M67471)	BlastP 311	5.00E-28		
			no anchor.	Also VclC and VclJ	<i>L. seeligeri</i> and <i>L. grayi</i>	—	—		
<i>vcl J</i>	<i>grayi</i>	716aa	SP, 1x LRR ^b , 3x 78–79aa repeats, Membrane anchor	L. <i>seeligeri</i> VclC, C alpha antigen	<i>L. seeligeri</i> <i>Strep. agalactiae</i> (PD018579)	BlastP 147 BlastP 165	2.30E-18 E-11		

	<i>vcl F1</i>	<i>grayi</i>	125–127aa	—	Transcription regulator of the MerR family	<i>H. influenzae</i>	BlastP100	8.50E-17
	<i>vcl F2</i>	<i>grayi</i>	126aa	—	Transcription regulator of the MerR family	(sp:Y186_HAEIN) <i>Archaeoglobus fulgidus</i>	BlastP 88	2.50E-19
	<i>vcl G1</i>	<i>grayi</i>	241aa	—	hyp. oxyacyl(acyl carrier protein) reductase	(pir:A69334) <i>Agrobacterium tumefaciens</i>	BlastP 279	4.70E-44
	<i>vcl G2</i>	<i>grayi</i>	253aa	—	hyp. oxyacyl(acyl carrier protein) reductase	(AtsC, gpu:U59485_33) <i>Agrobacterium tumefaciens</i>	BlastP 360	1.90E-44
Between <i>rplS</i> and <i>infC</i>	—	<i>innocua</i>	119aa	—	hyp. protein (YtcD)	<i>B. subtilis</i> (pir:B69989)	BlastP 334	3.00E-31
	—	<i>innocua</i>	177aa	—	Putative NAD(P)H oxidoreductase (YdeQ)	<i>B. subtilis</i> (pir:C69779)	BlastP 420	4.00E-41
	—	<i>welshimeri</i>	142aa	—	hyp. transcriptional regulator	<i>B. firmus</i> (BFU89914_7)	BlastP 133	1.00E-07
	—	<i>welshimeri</i>	287aa	—	Conserved hyp. protein (YesF)	<i>B. subtilis</i> (pir:H69795)	BlastP 237	2.00E-19
Between <i>rrn</i> and efflux pump regulator	<i>orf1</i>	<i>innocua</i>	109aa	—	—	—	—	—
	<i>orf2</i>	<i>innocua</i>	215aa	—	—	—	—	—

SP is signal peptide predicted.

^a*vclY* in *L. monocytogenes* and *L. innocua* is present only as a truncated sequence.

^bLRR stands for leucine-rich repeat, a 22amino acid unit (xLxxLxLxxNxLxDIxxLxxLx), which is characteristically present in the internalin proteins of *L. monocytogenes* and *L. ivanovii*.

Since PrfA is the positive master-regulator of a majority of the known virulence genes, the presence of a PrfA box [4,61] may implicate a potential virulence function of a particular gene. *L. seeligeri's* *vcl Y*, *vclX*, *vclI* and *vclP* which are potentially under PrfA control since a PrfA box is present in the promotor region 5' of *vclY* are thus possibly important for virulence. In *L. monocytogenes*, *vcl X* and *vclY*, along with *vclZ* can be transcribed via the PrfA controlled promoters of *mpl* and possibly *actA* [42,56]. Unlike in *L. seeligeri*, the *vclP* of *L. ivanovii* and *L. welshimeri*, and *vclX* of *L. ivanovii* are probably not controlled by PrfA as no PrfA boxes are present in their immediate upstream promoters.

Evolutionary history of the genus *Listeria* and the virulence gene cluster locus

The current organization of the virulence gene cluster loci of the six *Listeria* species raises questions about the evolutionary history of this chromosomal region. Were the virulence genes (*prfA-plcB*) of *L. monocytogenes*, *L. ivanovii* and *L. seeligeri* acquired by lateral gene transfer or are they orthologous and were lost by *L. innocua* and *L. welshimeri*? To address this question, we inferred the phylogeny of the genus *Listeria* and analyzed the virulence gene cluster for atypical sequence characteristics originating from previous lateral transfer events.

16S and 23S-rRNA have been used in earlier attempts to decipher the phylogenetic relationships of the genus *Listeria*, which forms a monophyletic grouping within the Gram-positive bacteria with a low DNA G+C content [10,51,55]. *L. grayi* consistently appeared as the most ancestral branch of the genus. However, due to the high sequence similarities of 16S (98.7–99.6%) and 23S-rRNA genes (99.5–99.7%) between the other members of the genus, the divergence of the other species could not be resolved using these molecules. The resolution cannot be significantly enhanced by including the 16S-23S-rDNA ISR of the *Listeria* species [27]. Phylogenetic trees based on the spacer sequences support the deep branching of *L. grayi* and the monophyly of *L. monocytogenes* and *L. innocua* but do provide conflicting results regarding the other listerial species if different treeing methods are applied. In order to enhance the resolution capacity of phylogenetic analysis for the genus *Listeria*, we combined all available genetic information for the six listerial species in a concatenated data set. This data set included, in addition to the 16S and 23S-rDNA, the house-keeping genes *prs* and *ldh* as well as the hypothetical conserved *vclB* flanking the virulence cluster, and the *iap* gene sequences located elsewhere in the listerial genome. Table 4 lists the nucleic acid similarities and amino acid identities of these genes and gene products, respectively, among the six listerial species.

Due to the high similarity of the deduced amino acid sequences of *Prs*, *VclB* and *Ldh*, most probably reflecting a very recent radiation among the members of the genus *Listeria*, phylogenetic inference was based on comparative analysis of nucleic acid sequences of *ldh*, *prs*, *vclB*, *iap*, 16S and 23S-rDNA.

Ldh codes for lactate dehydrogenase (~310 amino acids). The region encoding the last 134 amino acids, representing the last two of the six conserved amino acid blocks, was available for all six listerial species. The *ldh* genes of *B. caldolyticus*, *B. caldotenax*, *B. stearothermophilus*, *B. longum*, *D. radiodurans*, *L. casei*, *L. sakei*, *L. lactis*, *M. genitalium*, *M. hyopneumonia*, *S. mutans*, *S. pneumoniae*, *T. maritima*, and *T. aquaticus* were obtained from GenBank, aligned and used as outgroups. Phylogenetic trees for the *ldh* genes were estimated from the nucleotide data set by distance, parsimony, and ML methods. For all methods, the listerial *ldh* genes formed a monophyletic cluster with *L. grayi* as the deepest branch. The branch lengths of the other listerial species were extremely short and with the exception of a consistent grouping of *L. monocytogenes* and *L. innocua*, no common branching patterns could be observed using the different treeing methods (data not shown).

Prs encodes phosphoribosyl pyrophosphate synthetase (318 amino acids in *L. monocytogenes*). The *prs* data set used for the six listerial species encompasses the sequences for amino acid residues 191–318, containing the last two of five conserved amino acid blocks as determined by the “Blocks” database. Phylogenetic trees were estimated from the nucleotide data set by distance, parsimony, and ML methods. In distance and ML methods *L. grayi* had the longest branch. Considering *L. grayi* as outgroup, two stable groupings, (i) *L. monocytogenes* and *L. innocua* and (ii) *L. ivanovii*, *L. welshimeri* and *L. seeligeri*, were supported by all methods (data not shown).

VclB (Lmo0209/Lin0289) is a conserved protein of unknown function found in all six listerial species, *E. coli* (closest known homologue), other Gram-positive bacteria and Archaea. Phylogenetic trees obtained as described above were almost identical to those for the *prs* genes. However, the branching order within the *L. ivanovii*, *L. welshimeri* and *L. seeligeri* grouping differed depending on the treeing method used (data not shown). If the respective *vclB* homologues of *E. coli* and *B. anthracis* were included in the analysis, *L. grayi* always represented the deepest branch within the monophyletic *Listeria* cluster.

The *iap* gene encodes the “IAP” involved in host cell invasion by pathogenic *Listeriae* and acting in all *Listeria* species as a murein hydrolase necessary for proper cell division [6,30,34,62]. Both 16S-rRNA and *iap* (gene and mRNA) have been exploited as target molecules for the detection and identification of

block (alignment positions 1–1342), the 3' conserved block (pos. 1429–1755), and the 5' and 3' conserved blocks together were applied. In an additional analysis, the entire *iap* genes were omitted from the concatenated data set. None of the above-mentioned permutations significantly affected the composite tree topology.

To resolve the branching order within the *L. welshimeri*, *L. ivanovii* and *L. seeligeri* group, we performed comparative sequence analysis of the *vclA* genes which are present in all *Listeriae* except *L. grayi*. Unlike *prs*, *ldh*, *vclB* and *iap*, the nucleic acid similarity (73.7–90.5%) and amino acid identity values (75.8–94.5%) of *vclA* are significantly lower between the five listerial species (Table 4) thus allowing the use of both nucleic acid and amino acid based phylogenetic analyses. The results confirmed the phylogenetic position of *L. welshimeri* in the composite tree described above. Using both amino acid and nucleic acid data, *L. welshimeri* appeared almost equi-distant to both *L. innocua*/*L. monocytogenes* and the *L. ivanovii*/*L. seeligeri* branches (data not shown).

In addition to the phylogenetic analysis of the genus *Listeria*, we analyzed the virulence gene cluster for atypical sequence characteristics as signposts for lateral gene transfer events. Initially, the GC content of the virulence cluster *loci* genes was examined. The average GC contents of the virulence genes of the cluster under PrfA control (*prfA-plcB*, note that *mpl* was not available for *L. ivanovii*) were 36%, 36% and 34% for *L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*, respectively. The average GC contents of the virulence cluster *loci* including all open reading frames between *prs* and *ldh* were 36%, 36% and 35% for *L. monocytogenes*, *L. ivanovii* and *L. seeligeri*, respectively. There were no large discrepancies seen in the individual virulence genes from the reported, total genomic GC contents of 37–39% for *L. monocytogenes*, 37–38% for *L. ivanovii*, and 36% for *L. seeligeri* [31]. Likewise, the GC contents of the virulence cluster *loci* of *L. innocua* (37%), *L. welshimeri* (37%), and *L. grayi* (41%) resemble their genome averages of 36–38%, 39% and 45%, respectively. The only difference of note is *vclF2* of *L. grayi* with 34% GC vs. the genome content of 45% GC. In addition, the nucleotide composition at each codon position of the virulence genes was analyzed using the approach of Lawrence and Ochman [36]. The obtained results showed no obvious deviations of the virulence genes (data not shown). Furthermore, no insertion sequences (IS), obvious transposon, phage, or plasmid genes were detected. No direct repeats, “59-base elements” of integron gene capture systems, or partial identities there of flanking ORFs were identified. Repeat sequences present within the virulence gene cluster correspond to various transcriptional termination signals.

Genetic organization of some of the known internalin genes

Besides the main virulence cluster, an ever-growing family of internalin genes are discovered to contribute to virulence in *L. monocytogenes* and *L. ivanovii*. These genes are known to be scattered in different sections of the genomes of both species [7,14,24]. Using PCR with primers targeting house-keeping genes flanking some of these loci, we attempted to examine the corresponding chromosomal regions in the other *Listeriae*. Although many primer pair combinations were tested using different annealing temperatures for all six species, only some of these yielded specific products. Primers that amplified successfully the correct gene fragment are listed in Table 1, and the applied PCR conditions and obtained results are summarized in Table 2. This approach allowed to sequence (i) the region between *rplS* and *infC* (containing *inlC* in *L. monocytogenes*) of *L. ivanovii*, *L. welshimeri* and *L. innocua*, (ii) the region between *ascB* (Lmo0261/Lin0288) and *dapE* (Lmo0265/Lin0289, containing *inlGHE* in *L. monocytogenes* EGDe) of *L. innocua*, *L. ivanovii*, *L. welshimeri*, and *L. seeligeri*, and (iii) the region in *L. monocytogenes*, and *L. innocua* corresponding to the *i-inlDC* locus of *L. ivanovii*. Fig. 1B shows a schematic illustration of these regions.

Although the *inlC* gene of *L. monocytogenes* encodes the closest homologue of *i-inlC* of *L. ivanovii*, it is situated in a different chromosomal location between the *rplS* and the *infC* genes which encodes ribosomal protein L19 and translation initiation factor IF3, respectively [19]. The cloned and sequenced PCR products from PCR reactions using primers to *rplS* and *infC* revealed, as in the case of *i-inlDC* described below, remarkable heterogeneity of the genetic content between these extremely conserved house-keeping genes. In *L. innocua*, two genes exist in place of *inlC*, one resembles an NAD(P)H oxidoreductase and the other encodes a conserved hypothetical protein. *L. welshimeri* contains two genes entirely different from the above, a potential transcriptional regulator and another conserved hypothetical protein. Interestingly, downstream of the latter gene remains a fragment of sequence resembling the 3' end of the NAD(P)H oxidoreductase found in *L. innocua*, indicating that the *L. innocua* genetic arrangement is the ancestral state and the *L. welshimeri* version is a more recent replacement. *L. ivanovii*, on the other hand, contains a stretch of DNA that has no apparent coding sequences.

The *inlGHE* genes of *L. monocytogenes* EGDe are located between the *ascB* (or *bglH*) gene encoding 6 phospho-beta-glucosidase, and the *dapE* (or *msgB*) gene encoding succinyl-diaminopimelate desuccinylase. The fragment size of the products resulting from PCR using primers targeting regions within the *ascB* and *dapE*

genes showed that both genes are located directly adjacent to each other within the genome of *L. innocua*, *L. ivanovii*, *L. welshimeri* and *L. seeligeri*, indicating that *inlGHE* or *inlC₂DE* [15,47] are unique insertions in *L. monocytogenes*. This finding was confirmed by sequence analysis of the *L. ivanovii* and *L. innocua* fragments.

The *i-inlDC* genes of *L. ivanovii* are located between a ribosomal RNA (*rrn*) operon and a multidrug efflux pump operon [19]. Using specific PCR primers that hybridize within this *rrn* operon and within the putative transcriptional regulator gene (Lmo2589/Lin2734) of the efflux pump operon, we identified two potential open reading frames encoding proteins of unknown function in *L. innocua*, but found no additional putative genes in *L. monocytogenes*.

Discussion

Evolution of the genus *Listeria*

In this study, the phylogeny of the genus *Listeria* was inferred by analysing a concatenated data set containing the 16S and 23S-rDNA, as well as the *iap*-, *ldh*-, *prs*-, and *vclB*-genes. This collection encompasses all currently sequenced genes which are shared by all members of the genus. According to these analyses, *L. grayi* represents the oldest branch of the genus while the remaining five species radiated recently into two lineages from a common ancestor. One lineage contains *L. monocytogenes* and *L. innocua*, while the other harbors *L. welshimeri*, *L. ivanovii* and *L. seeligeri* (Fig. 2). In the latter group, *L. welshimeri* occupies the deepest branch, a finding also corroborated by phylogenetic analyses of the *vclP* and *vclA* genes, and the VclA protein. This bifurcation in the *L. monocytogenes*/*L. innocua* lineage and the *L. welshimeri*/*L. seeligeri*/*L. ivanovii* group is independently supported by the presence or absence of the genomic markers *vclP* and *vclZ* in the respective groups. *L. welshimeri*, *L. ivanovii* and *L. seeligeri* form one group containing the intact *vclP* gene. Neither *L. monocytogenes* nor *L. innocua*, which form the other group, contain any *vclP* sequence. Likewise, *vclZ* is present only in the *L. monocytogenes*/*L. innocua* lineage.

The phylogenetic tree presented in this study shows one topological conflict with a cluster analysis of the genus *Listeria* calculated from the results of MLEE [2]. The MLEE analysis placed *L. innocua* and *L. welshimeri* as sister species in the *L. monocytogenes* group while *L. seeligeri* and *L. ivanovii* formed the other group. This scenario is inconsistent with the phylogenetic analyses performed in this study and is also not supported by the distribution of the *vclP* and *vclZ* genes in the respective

species (see above). The observed discrepancy probably reflects that inference of evolutionary history is difficult to perform using MLEE data since neither lateral transfer events nor paralogues can be identified. Furthermore, the proposed phylogenetic tree of the genus *Listeria* also differs from the neighbor joining 16S-rDNA tree calculated by Vaneechoutte et al. [55]. This tree placed *L. welshimeri* as deepest branch within the *L. monocytogenes*/*L. innocua* group. However, verifying this result we found out that this tree topology is not supported if other treeing methods are applied. These inconsistencies could be expected since with the exception of *L. grayi*, all members of the genus *Listeria* possess highly similar 16S-rDNA genes thereby limiting the number of informative positions for phylogenetic analyses within this genus.

In essence, the phylogenetic tree presented in this study is the most parsimonious scenario for the evolutionary history of the genus *Listeria*, since (i) it is based on a data set containing all currently available gene sequences shared by the members of the genus, (ii) it is supported by different treeing methods, and (iii) it is consistent with the distribution of *vclP* and *vclZ* in the different species. However, the perception of the evolutionary history of the genus presented here is still only based on analyses of three different chromosomal loci (rRNA operon; flanking genes of the virulence gene cluster, *iap*-gene) and thus should be re-evaluated as soon as additional sequence informations of other chromosomal loci (or whole genome sequences of all *Listeria* species, respectively) become available.

The evolutionary history of the virulence gene cluster

The generally consistent tree topologies calculated from gene sequences of different chromosomal loci implies that the analyzed chromosomal regions including the genes flanking the virulence cluster have been shared by the common ancestor of the genus *Listeria*. Based on this knowledge we tried to infer the evolutionary history of the virulence gene cluster.

Since the natural ecological niches of all *Listeriae* overlap—the six species are, e.g. found in soil, rotting vegetation, sewage, contaminated waters of rivers, and estuaries and could also be detected in the intestinal tract of healthy animals [13,50]—genetic exchange among the species is theoretically possible. Keeping in mind the phylogeny of the genus *Listeria*, one plausible scenario would be that one of the three respective *Listeria* species acquired the virulence gene cluster laterally [9] and subsequently transferred it horizontally directly or indirectly to the other two species. However, we could not find indications that the virulence gene cluster had actually been transferred horizontally. In contrast to the classic definition of a “pathogenicity

Table 5. Amino acid identities of virulence genes within the virulence gene cluster

	PrfA	PlcA	Hly	Mpl	PlcB
<i>L. monocytogenes/L. seeligeri</i>	73	67	82	62	61
<i>L. monocytogenes/L. ivanovii</i>	77	71	80	ND	66
<i>L. seeligeri/L. ivanovii</i>	72	63	76	ND	55

ActA was excluded from the analysis since no unambiguous alignment could be achieved. ND=not determined since the *mpl* sequence of *L. ivanovii* is not available. Alignment columns with deletions and insertions were excluded from the identity calculations.

island” acquired en bloc from a foreign donor, the virulence gene cluster of the different listerial species does not show an atypical GC content compared to house-keeping genes or the total genomic GC values of the respective species. However, this does not formally exclude that the virulence cluster was transferred laterally from a donor species similar in GC content and codon usage. Furthermore, the existing methods to detect atypical sequence characteristics of DNA fragments as signposts for lateral acquisition have recently been demonstrated to provide inconsistent results [48]. Since no traces of lateral transfer were detected and the virulence gene cluster is “inserted” at the identical chromosomal location in all carrying species, it is more likely that at least a common ancestor of all present *Listeria* species, except *L. grayi*, already possessed the virulence gene cluster and inherited it vertically to its descendants. This scenario is also supported by the low sequence similarities of the virulence proteins (and their gene sequences, data not shown) encoded by genes within the cluster (Table 5) between *L. monocytogenes*, *L. seeligeri*, and *L. ivanovii*. More significant sequence similarities of these proteins between these species would be expected if they would have recently (after the divergence of *L. monocytogenes* from *L. innocua* and *L. ivanovii/L. seeligeri* from *L. welshimeri*) been either laterally transferred between the three species or acquired from the same donor. A more detailed phylogenetic interpretation of the sequence similarities of these virulence genes is complicated by the fact that they might not have evolved under an identical selective pressure since *L. ivanovii* and *L. monocytogenes* infect man and animals while *L. seeligeri* has been implicated with infection of protozoa.

Furthermore, our data suggests that this cluster has been lost in two independent events, presently represented by *L. innocua* and by *L. welshimeri*. There were likely multiple deletion events that led up to *L. innocua*'s present state as similarity searches of the intergenic region between *prs* and *vclY* of this species showed some short matches to the putatively deleted virulence genes (data not shown). These assumptions are supported by

other, recently published data [7,8,14,24]. Recently, atypical isolates of *L. innocua* have been characterized which surprisingly contained the entire *vcl* locus embedded into an otherwise typical *L. innocua* genetic background [30a]. The authors suggested that these isolates are intermediates in the evolution to typical *L. innocua*, supporting our notion described above. The latter authors speculated that the cluster has originally been acquired from another microorganism by a transposition-like event. This was based on the finding of a 16 bp Tn1545 integration consensus sequence flanking the *vcl* locus [8,30a]. However, our pattern search on the whole *L. monocytogenes* EGDe genome (<http://genolist.pasteur.fr/ListiList/>) revealed that this sequence is present at 182 positions, making the above-mentioned speculation very unlikely. Furthermore, the *vcl* locus does not contain any traces of mobile elements (transposases, integrases). Virulence factors related to the proteins encoded by the *vcl* locus (e.g. phospholipases, cytolysins) have been found in many other pathogens. However, there they never occur in such a compact gene cluster [33]. Therefore, we favor the idea that the *vcl* cluster has been evolved within a common ancestor of the present *Listeria* species.

The ancestral virulence gene cluster

Although we cannot know what exactly constituted the “ancestral” virulence gene cluster and how it differs from its present day manifestation, it must have included at least *prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*, *vclX*, and *vclY*; the latter present completely in *L. seeligeri* but only as relict sequences in *L. monocytogenes* and *L. ivanovii*. Since *vclP* is linked to PrfA control only in *L. seeligeri*, we cannot decide whether *vclP* is “ancestral” to the virulence gene cassette or a specific adaptation in *L. seeligeri*. *VclI*, the first internalin-like gene to be reported in *L. seeligeri*, appears potentially PrfA regulated. This linkage of PrfA control with an internalin-like gene to the virulence gene cluster may represent an ancestral arrangement that gave rise to the PrfA-controlled, internalin genes found widely dispersed in the present day genomes of *L. monocytogenes* and *L. ivanovii*. Whether potentially PrfA-controlled *vclD* and *vclE*, as well as the non-PrfA-controlled *vclC*, represent recent insertions into *L. seeligeri* or were deleted from the “ancestral” cluster in *L. ivanovii* and *L. monocytogenes* cannot be determined. The content differences between these virulence gene clusters, the genetic inversion event(s) of *vclX* and *vclY*, and the presence of the partially duplicated *plcB* gene in *L. seeligeri* are documents to the dynamic history of these loci as they underwent adaptations in their resident species.

Evolution of the internalin-like proteins

The characterized internalins of *L. monocytogenes*, with the exception of InlC, are larger than *L. ivanovii*'s and are covalently bound or associated to the cell wall via their additional C-terminus [44]. The smaller, secreted internalins known so far are all under strict PrfA control [18–20], whereas only two of the larger internalins of *L. monocytogenes* (InlAB) are partially controlled by PrfA [16,39,47]. Most of the known internalin genes of *L. monocytogenes* and *L. ivanovii* reside in numerous and diverse locations in their respective genomes. Many of these genes are present in multiple, divergent, tandem copies: *inlAB* [23], *inlGHE* [47] or *inlC₂DE* [15], *i-inlDC* [19], *i-inlFE* [20]. Some of these insertion sites are shown here to be unique for the species described as their corresponding chromosomal locations in the other species invariably contain either nothing or something else bordered by the same highly conserved house-keeping genes, which no doubt mark genomic locations less tolerant of change. In addition to frequent duplications, recombination was evidently the mechanism that generated *inlH* from *inlC₂* and *D* [47]. As suggested by Engelbrecht et al. [19], interspecific gene transfer may also have played a role. In this context, *L. monocytogenes* could have acquired the small, secreted, PrfA-controlled *inlC* from the *L. ivanovii* homologue *i-inlC*. Further research is required to elucidate the mechanism(s) that account for the apparent mobility of these genes within and between genomes.

The LRR motif, which is especially important in defining the biological activities of internalins [3,43], was found to be present also in proteins of non-pathogenic members of the genus. As mentioned earlier, *vclI* likely represents a *L. seeligeri* internalin gene. If it could be expressed, VclI would likely be secreted as it possesses a predicted signal peptide, but lacks the C-terminal cell-wall anchor sequences. The 4–5 LRRs of the deduced VclI most closely resemble the 5 LRRs of InlB from *L. monocytogenes*, while VclI protein ends at a similar sequence location shared among all the small internalins of *L. ivanovii*. One LRR motif was also observed in *L. grayi*'s deduced VclJ, which represents a large, anchored, surface protein with similarity to VclC of *L. seeligeri*. Both VclC and VclJ contain multiple 78–79 amino acid repeat units very similar to those observed in the C-alpha antigen encoded by the *bca* gene of *Streptococcus agalactiae*; five units are present in VclC while three units are found in VclJ. It is likely that the listerial-specific LRR motif is a widespread entity in the genus. It might exist in a variety of rapidly evolving surface molecules, each characterized by varying numbers of LRR units, variable N-terminal and C-terminal amino acid sequence contexts as is exemplified by the small internalins, the large internalins, and now VclJ

with its C-alpha antigen repeats. These different combinations presumably perform different functions while sharing the listerial LRR's mode of action.

Recently, the whole genome sequences of three *L. monocytogenes* Sv4b strains have been published by Nelson et al. [45]. The comparison with our data revealed that the gene content as well as the flanking genes of the *vcl* locus and of *inlC* are identical to *L. monocytogenes* EGDe, Sv1/2a. One interesting difference was found for the *inlGHE/C2DE* cluster. *L. monocytogenes* Sv4b F2365, similar to *L. monocytogenes* Sv1/2c LO28 [15], contains *inlC2DE*, but not in between *ascB/dapE*. Rather, here the *inl* cluster is flanked by an ABC transporter operon on the one side and a putative peptidase gene on the other side. Both *ascB* and *dapE* are present in the Sv4b F2365 genome, but at different positions and not adjacent to each other. This underscores the findings from other whole genome comparisons [7,14,24] that the genomes of *Listeria* species and isolates exhibit a high synergy but differ by numerous, specific small gene clusters which are interspersed all over the chromosome.

Nucleotide sequence data reported in this paper are available in the EMBL database under accession numbers: AJ249804, AJ249805, AJ249806, AJ249807, AJ249738, AJ249808, AJ249739, AJ249398, AJ249399, AJ249400, AJ249401, AJ249402, AJ249403, AJ010599.

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