

## *Listeria marthii* sp. nov., isolated from the natural environment, Finger Lakes National Forest

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Four isolates (FSL S4-120<sup>T</sup>, FSL S4-696, FSL S4-710, and FSL S4-965) of Gram-positive, motile, facultatively anaerobic, non-spore-forming bacilli that were phenotypically similar to species of the genus *Listeria* were isolated from soil, standing water and flowing water samples obtained from the natural environment in the Finger Lakes National Forest, New York, USA. The four isolates were closely related to one another and were determined to be the same species by whole genome DNA–DNA hybridization studies (>82 % relatedness at 55 °C and >76 % relatedness at 70 °C with 0.0–0.5 % divergence). 16S rRNA gene sequence analysis confirmed their close phylogenetic relatedness to *Listeria monocytogenes* and *Listeria innocua* and more distant relatedness to *Listeria welshimeri*, *L. seeligeri*, *L. ivanovii* and *L. grayi*. Phylogenetic analysis of partial sequences for *sigB*, *gap*, and *prs* showed that these isolates form a well-supported sistergroup to *L. monocytogenes*. The four isolates were sufficiently different from *L. monocytogenes* and *L. innocua* by DNA–DNA hybridization to warrant their designation as a new species of the genus *Listeria*. The four isolates yielded positive reactions in the AccuProbe test that is purported to be specific for *L. monocytogenes*, did not ferment L-rhamnose, were non-haemolytic on blood agar media, and did not contain a homologue of the *L. monocytogenes* virulence gene island. On the basis of their phenotypic characteristics and their genotypic distinctiveness from *L. monocytogenes* and *L. innocua*, the four isolates should be classified as a new species within the genus *Listeria*, for which the name *Listeria marthii* sp. nov. is proposed. The type strain of *L. marthii* is FSL S4-120<sup>T</sup> (=ATCC BAA-1595<sup>T</sup> =BEIR NR 9579<sup>T</sup> =CCUG 56148<sup>T</sup>). *L. marthii* has not been associated with human or animal disease at this time.

## INTRODUCTION

The genus *Listeria* contains six species of low G + C content Gram-positive bacteria closely related to the genus *Bacillus* (Collins *et al.*, 1991; Sallen *et al.*, 1996). The species are *Listeria monocytogenes*, *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri* and *L. grayi* (Rocourt & Buchrieser, 2007). *L.*

*ivanovii* was the last species added to the genus in 1985 (Seeliger *et al.*, 1984) and was divided into two subspecies, *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* (Boerlin *et al.*, 1992). The genus *Listeria* includes three haemolytic species (*L. monocytogenes*, *L. ivanovii* and *L. seeligeri*), all of which contain variants of the main *Listeria* virulence gene cluster; this cluster includes the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of FSL S4-696, FSL S4-965, FSL S4-120<sup>T</sup> and FSL S4-710 are EU545980 to EU545983, respectively. The accession number for the sequence of the *ldh-prs* intergenic region of FSL S4-696 is FJ692340.

*EcoRI* ribotype and PFGE patterns, sources and geographical origin, AccuProbe test results and differentiating physiological characteristics, GenBank accession numbers of sequences used for the *sigB-gap-prs* phylogenetic analysis, and primer sequences used to screen for the presence/absence of *L. monocytogenes* virulence genes are available with the online version of this paper.

virulence genes *hly* (which encodes a haemolysin), *prfA*, *plcA*, *mpl*, *actA* and *plcB*. While *L. monocytogenes* is a human and animal pathogen, and *L. ivanovii* is an animal pathogen (predominantly linked to disease in sheep), *L. seeligeri*, despite the presence of the virulence gene homologues, has been rarely linked to disease in humans or animals (Gouin *et al.*, 1994; Rocourt *et al.*, 1983, 1986).

The phylogeny of the members of the genus *Listeria* suggests a common ancestor of a clade containing *L. monocytogenes* and *L. innocua*, as well as a common ancestor of a second clade containing *L. seeligeri*, *L. ivanovii* and *L. welshimeri*. Both of these clades contain species with and without the virulence gene cluster, which is found in the same genomic location in all three species with this cluster. It has been hypothesized that the evolution of the genus *Listeria* included an ancestor with the virulence gene cluster and two independent deletion events that led to a loss of the virulence gene cluster in members of both of these clades (Kuhn & Goebel, 2007; Nightingale *et al.*, 2006). The status of *L. grayi* is still uncertain, but current sequencing efforts will soon resolve this more distantly related species (Hain *et al.*, 2007; Kuhn & Goebel, 2007).

Although some recent reports have identified and described unusual *Listeria* isolates, including haemolytic *Listeria* isolates that contain the virulence gene cluster but are classified into the species *L. innocua* (Johnson *et al.*, 2004), no new species of the genus *Listeria* have been described since 1985. In this report, we describe polyphasic analysis of four *Listeria*-like bacilli that were isolated from the natural environment in the Finger Lakes region of New York and propose that they should be placed in a new species within the genus *Listeria*.

## METHODS

**Bacterial strains.** The strains studied, along with their sources, geographical origins and GenBank Accession numbers for 16S rRNA gene sequences, are presented in Supplementary Table S1 (available in IJSEM Online). Four *Listeria*-like isolates (FSL S4-120<sup>T</sup>, FSL S4-696, FSL S4-710 and FSL S4-965, hereafter referred to as the FSL S4-isolates) were characterized in this investigation. Isolates FSL S4-120<sup>T</sup>, FSL S4-696, FSL S4-710 and FSL S4-965 were cultured from soil in a forest area (42° 26' 36" N 76° 48' 53" W), a standing water puddle (42° 20' 38" N 76° 39' 41" W), a flowing water runoff (42° 20' 37" N 76° 39' 40" W) and a river/stream (42° 22' 59" N 76° 39' 21" W), respectively, via environmental samples collected in the Finger Lakes National Forest ([http://www.fs.fed.us/r9/forests/greenmountain/html/fingerlakes/f\\_home.htm](http://www.fs.fed.us/r9/forests/greenmountain/html/fingerlakes/f_home.htm)) and Connecticut Hill Wildlife Management Area, which is located in the Finger Lakes region of New York, USA.

The FSL S4-isolates were cultured on heart infusion agar supplemented with 5% rabbit blood (RBA) (BBL Microbiology Systems) for use in conventional biochemical tests and incubated at 35 °C in a candle jar. All strains were stored as suspensions in defibrinated rabbit blood in liquid nitrogen and in trypticase soy broth + 20% glycerol at -70 °C.

**AccuProbe, phenotypic tests and growth range.** The AccuProbe (Ninet *et al.*, 1992; Okwumabua *et al.*, 1992) test was performed according to the manufacturer's instructions (Gen-Probe). Liquid

broth, semi-solid media and other plating media were incubated aerobically at 1, 4, 25, 30, 37, 43 or 45 °C. Biochemical testing was done using previously described conventional methods (Weyant *et al.*, 1996). All biochemical tests were performed at 35 °C in an aerobic incubator except for the temperature growth range tests, which were performed at temperatures ranging from 1 °C to 45 °C. The oxidase, catalase and growth temperature tests were read after 1 day of incubation. All other biochemical tests were read after 1, 2, and 7 days of incubation (Supplementary Table S2). Temperature growth range tests were done using trypticase soy broth (Becton and Dickinson Company) and sampled up to 30 days. Isolates were also characterized using the API-*Listeria* test strips (Bille *et al.*, 1992).

**Cellular fatty acid analysis.** Cells from 2 day-old cultures were saponified, and the liberated fatty acids were methylated and analysed by capillary GLC (Weyant *et al.*, 1996). Profiles were identified using a commercial system (MIDI).

**DNA relatedness and G+C determination.** All bacteria were cultured on 20–30 RBA plates and incubated for 24 h at 35 °C. Cells were harvested and lysed, and the DNA was isolated and purified according to the method of Brenner *et al.* (1982). DNA from *L. monocytogenes* ATCC 15313<sup>T</sup> and FSL S4-120<sup>T</sup> was labelled with [<sup>32</sup>P]dCTP using a commercial nick translation kit (Bethesda Research Laboratories) and tested for reassociation to unlabelled DNA from the same strain (homologous reaction) as well as to other strains and to the type and reference strains studied (heterologous reactions). Relative binding ratios ([percentage heterologous DNA bound to hydroxyapatite/percentage homologous DNA bound to hydroxyapatite] x 100) and percentage divergence (the percentage of unpaired bases in related DNA sequences) were calculated as described previously (Brenner *et al.*, 1982). Divergence was calculated to the nearest 0.5%, with each decrease of 1 °C in thermal stability of a heterologous DNA duplex due to approximately 1.0% unpaired bases within related DNA (Bonner *et al.*, 1973). All reactions were done in duplicate at the optimal temperature of 55 °C for labelled DNA from the type strain (FSL S4-120<sup>T</sup>) and *L. monocytogenes* ATCC 15313<sup>T</sup>. The G+C content (mol%) was determined for strains FSL S4-120<sup>T</sup> and ATCC 15313<sup>T</sup> by thermal denaturation (Mandel *et al.*, 1970).

**16S rRNA gene sequencing and phylogenetic analysis.** 16S rRNA gene sequencing was performed as described previously (Morey *et al.*, 2006). DNA for 16S rRNA gene sequence determination was extracted and purified with a QIAamp DNA Mini kit (Qiagen) and amplified by using the Expand high-fidelity PCR system (Roche) with primers fD1 and rP2 (Weisburg *et al.*, 1991). Excess deoxynucleoside triphosphates and primers were removed with magnetic carboxylate beads (Agencourt Bioscience). Cycle sequencing was performed with Big Dye version 3.1 dye terminator chemistry (Applied Biosystems) using 16 primers by previously described methods (Morey *et al.*, 2006). Excess dyes were removed with magnetic carboxylate beads (Agencourt Bioscience), and reaction products were sequenced on an ABI 3100 sequencer (Applied Biosystems). The sequence data were edited and compiled using the Wisconsin Sequence Analysis Package (Genetics Computer Group). The novel and reference 16S rRNA gene sequences were aligned with sequences from GenBank using the pairwise alignment algorithm in MacClade version 4.08 (Maddison & Maddison, 2005). The multiple sequence alignment was edited manually to remove terminal regions that were not represented by all members. PHYML 3.0 (Guindon & Gascuel, 2003) was used to infer a maximum-likelihood tree and to perform an additional bootstrap analysis (500 replicates). The model of molecular evolution used in the analysis was the HKY+I model as inferred by the corrected Akaike Information Criterion in the jmodelTest package 0.1.1 (Posada, 2008). The 16S rRNA gene sequences of FSL S4-isolates were submitted to GenBank and assigned the accession numbers EU545980 to EU545983. The type strain FSL S4-120<sup>T</sup> was designated

ATCC BAA-1595<sup>T</sup> corresponding to GenBank accession number EU545982. Additionally, 16S rRNA gene sequences of five species of the genus *Listeria* were submitted to GenBank (Supplementary Table S1).

**Sequencing of *sigB*, *gap* and *prs* and sequence analysis.** In addition to phylogenetic analysis of 16S rRNA gene sequences, a phylogenetic analysis was performed based on partial sequences of three protein coding genes, including *sigB*, which encodes an alternative sigma factor involved in stress response, and the housekeeping genes *gap* and *prs*, which encode glyceraldehyde-3-phosphate dehydrogenase and phosphoribosylpyrophosphate synthase (see Nightingale *et al.*, 2005b for more information). Sequencing of part of these genes was performed as previously described (Nightingale *et al.*, 2005b); new primers were designed to amplify *prs* in *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. innocua* and the FSL S4-isolates (HdB40*prs*F 5'-CCAAATTAACATTGAAGAAAGTATCCGTGGTTGTC-3' and HdB41*prs*R 5'-GAACTTACAGAWGCATTYTCATGWAC-3'). To further probe the phylogenetic position of the FSL S4-isolates, a phylogenetic analysis was performed with the concatenated *sigB-gap-prs* sequences. The entire alignment was 1760 bp long (*sigB*: 653 bp, *gap*: 568 bp and *prs*: 539 bp). PHYML 3.0 was used to perform the maximum-likelihood analysis and an additional bootstrap analysis (500 replicates) to infer branch support. The model of molecular evolution used in the analysis was the GTR+G+I model as inferred with the corrected Akaike Information Criterion in the jmodelTest package 0.1.1. The *sigB*, *gap* and *prs* sequences of the FSL S4-isolates were submitted to GenBank (Supplementary Table S3).

**Automated ribotyping.** All four FSL S4-isolates were characterized by automated ribotyping using the restriction enzyme *EcoRI* and the RiboPrinter Microbial Characterization System (Qualicon) according to the manufacturer's instructions and as previously described in detail (Gray *et al.*, 2004).

**Restriction fragment length polymorphism analysis using pulsed-field gel electrophoresis.** The FSL S4-isolates and other species of the genus *Listeria* were characterized using *Ascl* and *Apal* restriction endonucleases in accordance with the PulseNet standardized protocol (Graves & Swaminathan, 2001). Agarose gel images obtained using Gel Doc 2000 were converted to TIFF image files and analysed using BioNumerics (Applied Maths) software version 4.0, PulseNet customized version (Graves *et al.*, 2005).

**Virulence gene PCR assays.** All FSL S4-isolates were characterized using PCR assays (Supplementary Table S4) to screen for the presence of the six virulence genes located in the *L. monocytogenes* *prfA* virulence gene island (i.e. *prfA*, *plcA*, *hly*, *mpl*, *actA* and *plcB*) (Nightingale *et al.*, 2005b; Norton *et al.*, 2001; Orsi *et al.*, 2008; Wiedmann *et al.*, 1997) and to screen for the presence of *inlA* (Nightingale *et al.*, 2005a).

**Sequencing of the *prs*-*ldh* intergenic region.** The *L. monocytogenes* virulence gene island is located between *prs* and *ldh*; to probe for presence of virulence genes in this region, the region between *prs* and *ldh* was sequenced in FSL S4-696. This region was PCR amplified with the primer pair LMVI-F (which anneals in *prs*) and LMVI-R (which anneals in *ldh*) (Cai & Wiedmann, 2001) and then sequenced with these primers as well as internal primers, using a primer walking strategy. The final sequence for this region was assembled in Sequencher 4.8 (Gene Codes). Alignments were made in MacClade 4.08 and nucleotide identities of the individual ORFs were calculated using PAUP\* (Swofford, 2002). The sequence of the *ldh-prs* intergenic region of FSL S4-696 is deposited under GenBank accession number FJ692340.

**Molecular serotyping.** Molecular serotyping was performed using a previously described multiplex PCR assay (Doumith *et al.*, 2004a), which detects serotype-specific marker genes. This assay differentiates five *L. monocytogenes* profiles and classifies the four most common disease-associated serotypes (i.e. 1/2a, 1/2b, 1/2c and 4b) into unique serogroups. Each multiplex PCR profile contains *L. monocytogenes* isolates belonging to more than one serotype, including profile IIa (serotypes 1/2a and 3a), IIb (serotypes 1/2b, 3b and 7), IIc (serotypes 1/2c and 3c), IVb (serotypes 4b, 4d and 4e) and L (serotypes 4a and 4c), as noted previously (Doumith *et al.*, 2005).

**Tissue culture invasion assays.** Invasiveness for human intestinal epithelial cells of all four FSL S4-isolates was determined using Caco-2 cells (ATCC HTB 37) as previously described (Nightingale *et al.*, 2005a). *L. monocytogenes* 10403S (Bishop & Hinrichs, 1987) and *L. innocua* FSL C2-008 were used as control strains in these assays. Briefly, bacterial cells were grown overnight (16–18 h) at 30 °C to stationary phase, and confluent Caco-2 monolayers grown in a 24-well plate were inoculated with approximately  $2 \times 10^7$  c.f.u. of the test isolates (3 wells per strain). After incubation for 30 min, Caco-2 cells were washed three times with PBS, and at 45 min post-inoculation, fresh medium containing gentamicin ( $150 \mu\text{g ml}^{-1}$ ) was added to kill extracellular bacteria. At 90 min post-inoculation, Caco-2 cells were washed three times with PBS; they were lysed by addition of ice-cold sterile distilled water and vigorous pipetting. Intracellular numbers of the bacteria were determined by plating lysed Caco-2 cell suspensions on BHI agar. Invasion efficiency was calculated as the number of intracellular bacteria recovered (in c.f.u.) relative to the bacterial numbers (in c.f.u.) used for the inoculation.

## RESULTS

### Phenotypic characteristics and temperature limits of FSL S4-isolates

The four FSL S4-isolates were identified by the API Identification System as *L. monocytogenes*/*L. innocua* (octal code 6110; rhamnose- and xylose-negative). By conventional biochemical profiles, all four isolates were positive for catalase activity, aesculin hydrolysis, hydrogen sulfide production, and the methyl red test; negative for oxidase activity; tolerant of sodium chloride; positive for assimilation of D-glucose, lactose and maltose; and negative for assimilation of D-xylose, D-mannitol, sucrose and L-rhamnose, nitrate reduction, urease activity, indole production and gelatin hydrolysis. The FSL S4-isolates were short, Gram-positive rods that were motile at 25–30 °C, were non-haemolytic on 5% sheep blood agar (SBA) and RBA at 37 °C, and did not grow on MacConkey, *Salmonella-Shigella*, citrate or cetrimide agars. The optimum growth temperature for FSL S4-120<sup>T</sup> was between 30 and 37 °C. The temperature limits of growth were 1–45 °C in trypticase soy broth. The organism did not survive heating at 60 °C for 30 min. Strain FSL S4-120<sup>T</sup> was streaked onto SBA and brain heart infusion agar plates, then stored at 4 °C inside a plastic zip lock bag; after 15 days of storage, tiny colonies were visible. The colonies continued to increase in size and were viable for >30 days.

## Molecular characterization methods classified the four FSL S4-isolates as either *L. monocytogenes* or *L. innocua*

Initial molecular characterization using the AccuProbe test identified the four FSL S4-isolates as *L. monocytogenes*. The four FSL S4-isolates were classified as profile L by the multiplex PCR molecular serogrouping method of Doumith *et al.* (2005). Profile L is characterized by amplification of the *prs* control amplicon, but absence of amplification of any of the serogroup specific genes; profile L has previously been found to be typical for *L. monocytogenes* isolates belonging to lineage III (Doumith *et al.*, 2004b; Wiedmann *et al.*, 1997). Strains in lineage III represent serotypes 4a and 4c as well as atypical serotype 4b isolates (Nightingale *et al.*, 2007; Roberts *et al.*, 2006). Since isolates representing species of the genus *Listeria* other than *L. monocytogenes* can also show profile L (Doumith *et al.*, 2004b), molecular serogrouping was not useful for obtaining species-level identification of the four FSL S4-isolates.

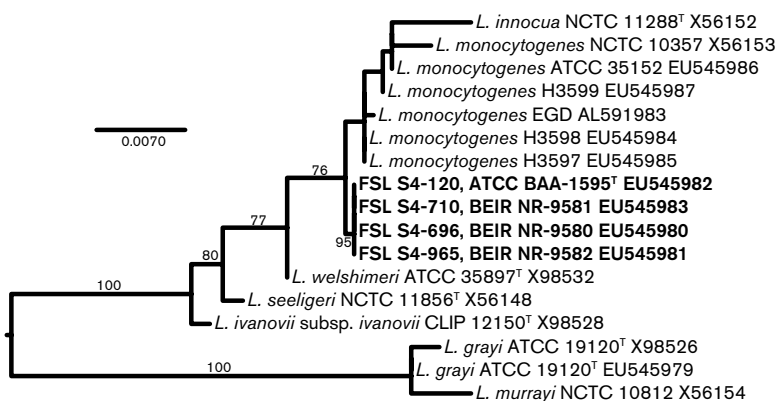
Characterization of the four FSL S4-isolates by automated *EcoRI* ribotyping yielded highly similar *EcoRI* ribotypes (Supplementary Table S1). Isolates FSL S4-696 and FSL S4-710 had the same DNA fragment pattern, characterized by the presence of an additional fragment of approximately 10 kb that was absent from the other two isolates. The DNA fragment pattern of FSL S4-120<sup>T</sup> had a unique DNA fragment of approximately 30–40 kb. None of the *EcoRI* ribotype patterns for these four isolates allowed for specific identification by the RiboPrinter database (ribotype patterns are identified to the species level if the test pattern matches a pattern in the DuPont database with a similarity score of >0.85 %). The closest *EcoRI* ribotype matches for all four isolates were *L. monocytogenes* ribotype patterns in the DuPont database, but the similarity scores for these matches were only between 0.69 % and 0.75 %.

The four FSL S4-isolates had unique PFGE patterns when their DNA was restricted with *Ascl* or *ApaI* restriction endonucleases. Although the individual PFGE patterns of the four isolates were different, a composite dendrogram generated using *Ascl* and *ApaI* restriction endonucleases

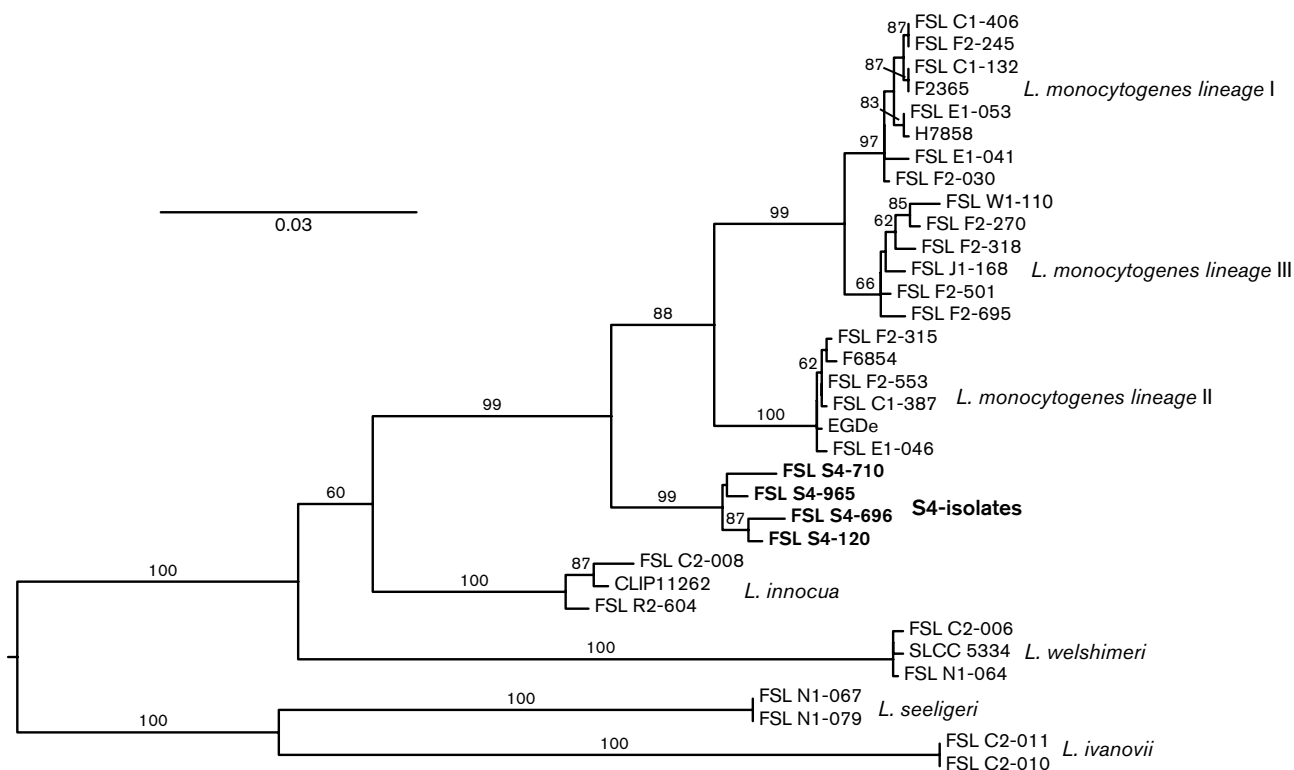
clustered all four of the isolates together when compared with a well-characterized *L. monocytogenes* strains set (Fugett *et al.*, 2006) and a representation of other species of the genus *Listeria* (Supplementary Fig. S2).

## Phylogenetic analysis of 16S rRNA gene and *sigB-gap-prs* sequence data consistently clustered FSL S4-isolates separately from *L. monocytogenes* and *L. innocua*

Initial 16S rRNA gene sequence analysis (Fig. 1) showed that all four isolates representing FSL S4-isolates grouped together with a high bootstrap support (95 %) and formed a clade separate from *L. monocytogenes* and *L. innocua*. The close phylogenetic relationship of the FSL S4-isolates with *L. monocytogenes* and *L. innocua* was demonstrated by the fact that they were found in a well-supported (76 % bootstrap support) clade together with these species. The phylogenetic relationships between the FSL S4-isolates, *L. innocua* and *L. monocytogenes* remained without significant bootstrap support and therefore remain unresolved by the 16S sequence analysis (Fig. 1). This may be explained by the high similarity between sequences of the FSL S4-isolates and *L. monocytogenes* (99.8–99.3 %) and *L. innocua* (99 %) and the low number of phylogenetically informative sites in the 16S rRNA gene fragment analysed. To further characterize the phylogenetic position of the FSL S4-isolates, partial *sigB*, *gap* and *prs* sequences were obtained for the four FSL S4-isolates and used together with partial *sigB*, *gap* and *prs* sequences for selected *L. monocytogenes* and other *Listeria* isolates to construct a maximum-likelihood (ML) tree. The concatenated *sigB*, *gap* and *prs* sequences proved to be more phylogenetically informative (148 parsimony informative characters within the *L. innocua*, *L. monocytogenes* and FSL S4-isolates clade, versus only 6 parsimony informative characters for the 16S rRNA gene). The ML tree based on the concatenated partial *sigB*, *gap* and *prs* sequences showed that the four FSL S4-isolates clustered together (bootstrap support of 99 %) forming a sistergroup of *L. monocytogenes* (Fig. 2).



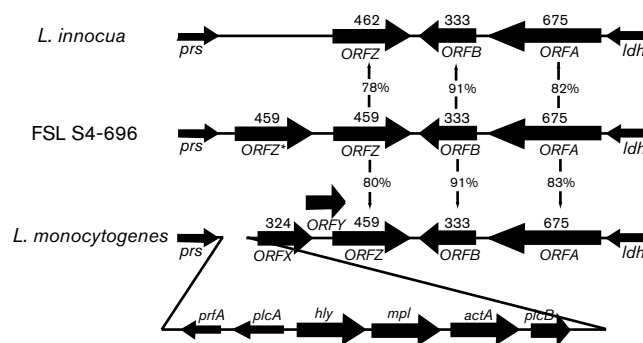
**Fig. 1.** Maximum-likelihood phylogram based on 16S rRNA gene sequences (1398 nucleotide positions) showing the phylogenetic position of FSL S4-isolates and other species of the genus *Listeria*. Bootstrap analysis was done with 500 replicates; bootstrap values are indicated above the clades and bootstrap values <60 % are not shown. Bar, number of inferred substitutions per nucleotide site. GenBank accession numbers for sequences are shown.



**Fig. 2.** Phylogram based on maximum-likelihood analysis of concatenated *sigB*, *gap* and *prs* sequences (1760 nucleotide positions total) from the four FSL S4-isolates as well as selected isolates representing other species of the genus *Listeria*. Values above the clades represent bootstrap values based on 500 replicates (values <60 % are not shown). Sequence data for *L. monocytogenes* isolates were obtained from Cai *et al.* (2002), Roberts *et al.* (2006) and Nightingale *et al.* (2005b); sequence data for *L. innocua*, *L. seeligeri*, *L. welshimeri* and *L. ivanovii* can be found in GenBank (accession numbers are shown in Supplementary Table S3). Bar, number of inferred substitutions per nucleotide site.

### FSL S4-isolates lacked the *prfA* virulence genes cluster and *inlA* and were non-invasive in Caco-2 cells

PCR-based screens found no evidence of the presence of the six *L. monocytogenes* virulence genes in the *prfA* virulence gene island (i.e. *prfA*, *plcA*, *hly*, *mpl*, *actA* and *plcB*) or for the presence of *inlA* in the four FSL S4-isolates. The absence of the *prfA* virulence gene island was further confirmed by sequencing the *ldh-prs* intergenic region. The intergenic region contained four open reading frames (Fig. 3), three of which are homologues to ORFA, ORFB and ORFZ that have been previously reported in *L. innocua* and *L. monocytogenes* (Cai & Wiedmann, 2001). The fourth ORF (designated ORFZ\*) had a high sequence similarity to the FSL S4-isolate ORFZ (74 % nucleotide identity). FSL S4-isolates thus contained a duplicated ORFZ, with ORFZ\* representing an ancient orthologue of ORFZ (Fig. 3), as ORFZ\* shared only 74 % and 73 % nucleotide identity with the ORFZ of *L. monocytogenes* and *L. innocua*, respectively, while FSL S4 ORFZ shared a higher homology with *L. monocytogenes* and *L. innocua* ORFZ (80 % and 78 %, respectively).



**Fig. 3.** Schematic overview and comparison of the genetic organization of the *ldh-prs* intergenic region in FSL S4-696, *L. innocua* and *L. monocytogenes*. Names and lengths in bp of the different ORFs are indicated. ORFZ\* represents an orthologue of ORFZ. Arrows between the ORFs in the different species indicate the percentage homology.

In addition, tissue culture invasion assays using the human intestinal epithelial cell line Caco-2 showed that all four FSL S4-isolates had extremely low levels of invasiveness (0.001–0.002 %), which was similar to the *L. innocua* control strain FSL C2-008 (invasion efficiency of 0.003 %) and >100-fold lower than the invasion efficiency for an *L. monocytogenes* control strain (1.44 %). These data provide phenotypic support for the absence of the main *L. monocytogenes* virulence genes in these isolates.

### The FSL S4-isolates shared a common cellular fatty acid (CFA) profile

The FSL S4-isolates shared a common CFA profile, characterized by major amounts of branched chain acids, including iso-C<sub>15:0</sub> (3–15 %), anteiso-C<sub>15:0</sub> (41–57 %), iso-C<sub>16:0</sub> (1–7 %), iso-C<sub>17:0</sub> (1–4 %) and anteiso-C<sub>17:0</sub> (13–36 %). They also contained C<sub>16:0</sub> (4–7 %), C<sub>18:1 $\omega$ 9c</sub> (1–3 %) and C<sub>18:0</sub> (1–3 %). This profile is essentially identical to other species of the genus *Listeria* tested and, therefore, FSL S4-isolates cannot be differentiated from other species of this genus by CFA analysis.

### DNA/DNA hybridization data indicate that FSL S4-isolates represent a new species separate from *L. monocytogenes* and *L. innocua*

Results from the DNA relatedness studies are shown in Table 1. DNA from FSL S4-120<sup>T</sup> was labelled with [<sup>32</sup>P]dCTP and tested for relatedness to the three other FSL S4 strains, two *L. monocytogenes* strains (ATCC 15313<sup>T</sup> and ATCC 35152), one *L. innocua* strain (ATCC 33090<sup>T</sup>), and one atypical strain of *L. monocytogenes* (H3597). Labelled DNA from the type strain showed an average of 82 % relatedness to the three other FSL S4 strains (range, 71–90 %) in reactions at 55 °C. The divergence in related

DNA sequences was between 0.0 % and 0.5 %. In reactions at 70 °C, labelled FSL S4 DNA showed an average of 76 % relatedness (range, 71–80 %). Labelled DNA from FSL S4-120<sup>T</sup> showed an average of 73 % relatedness to the two *L. monocytogenes* strains, *L. innocua* and strain H3597 (range, 69–75 %) in reactions at 55 °C, but the divergence in related DNA sequences was between 7.5 % and 9.5 %. In reactions at 70 °C, the labelled FSL S4-120<sup>T</sup> strain DNA showed an average of 45 % relatedness to the two *L. monocytogenes* strains, *L. innocua* and strain H3597 (range, 30–53 %). Labelled DNA from *L. monocytogenes* type strain ATCC 15313<sup>T</sup> showed an average of 64 % relatedness to the four FSL S4 strains (range, 60–67 %) in reactions at 55 °C. The divergence in related DNA sequences was between 7.0 % and 8.0 %. In reactions at 70 °C, labelled *L. monocytogenes* DNA, exhibited an average of 35 % relatedness to the four FSL S4 strains.

## DISCUSSION

We have characterized isolates from the natural environment that, by colony morphology, resembled *L. innocua* and other non-haemolytic species of the genus *Listeria*, but that represent a novel species that may be initially differentiated from other species of this genus based on a positive result with the AccuProbe test and the absence of the distinct haemolysis produced on blood agar by species other than *L. welshimeri*, *L. grayi* and *L. innocua*. Consequently, for definitive identification, a polyphasic approach is required. Molecular subtyping methods clustered the four isolates representing this novel species together when compared with *L. monocytogenes* and other species of the genus *Listeria*. Furthermore, phylogenetic analysis of 16S, *sigB*, *gap* and *prs* sequence data consistently clustered the FSL S4-isolates separately from *L. monocytogenes* and *L. innocua*. The phylogenetic position with

**Table 1.** DNA relatedness of FSL S4-isolates and *Listeria monocytogenes*, *Listeria innocua* and an atypical *Listeria monocytogenes* strain

RBR, Relative binding ratio; % D, percentage divergence.

Source of unlabelled DNA	Results of reaction with labelled DNA from strain:					
	FSL S4-120 <sup>T</sup> (ATCC BAA-1595 <sup>T</sup> )			<i>L. monocytogenes</i> (ATCC 15313 <sup>T</sup> )		
	RBR (%) 55 °C	% D	RBR (%) 70 °C	RBR (%) 55 °C	% D	RBR (%) 70 °C
FSL S4-120 <sup>T</sup> (ATCC BAA-1595 <sup>T</sup> )	100	0.0	100	67	8.0	42
FSL S4-696 (BEIR NR-9580)	85	0.5	78	65	7.5	30
FSL S4-965 (BEIR NR-9582)	90	0.0	80	60	8.0	36
FSL S4-710 (BEIR NR-9581)	71	0.0	71	62	7.0	32
<i>L. monocytogenes</i> (ATCC 15313 <sup>T</sup> )	73	7.5	51	100	0.0	100
<i>L. monocytogenes</i> (ATCC 35152)	69	7.5	30	100	0.0	92
<i>L. monocytogenes</i> (H3597)*	75	7.5	53			
<i>L. innocua</i> (ATCC 33090 <sup>T</sup> )	74	9.5	46			

\*Atypical *Listeria monocytogenes*; L-rhamnose-negative.

respect to *L. innocua* and *L. monocytogenes* of the FSL S4-isolates, however, could not be unambiguously resolved by 16S rRNA gene phylogenetic analysis, mainly because of the lack of phylogenetically informative sites in the 16S rRNA gene of members of the genus *Listeria* (Collins *et al.*, 1991; Czajka *et al.*, 1993). The *sigB*, *gap* and *prs* sequence data proved to be more phylogenetically informative. Analysis of the *sigB*, *gap* and *prs* sequence data placed the FSL S4-isolates in a well-supported sistergroup to *L. monocytogenes*. In addition, the PCR-based screens and sequencing of the *prs-ldh* intergenic region found no evidence for the presence of the six *L. monocytogenes* virulence genes in the *prfA* virulence gene island or for the presence of *inlA* in the four FSL S4-isolates. Tissue culture invasion assay data phenotypically confirm the absence of the main *L. monocytogenes* virulence genes in these isolates.

Johnson *et al.* (2004) investigated a novel haemolytic strain of *Listeria* that was unusually difficult to identify to the species level by the conventional methods. The issue was resolved by using total DNA–DNA hybridization testing and confirmed by specific PCR-based tests including a *Listeria* microarray assay. Their study documented the first naturally occurring haemolytic strains of *L. innocua*. Our DNA relatedness data indicate that the FSL S4-isolates constitute a new species of the genus *Listeria*. According to established molecular criteria for species-level relatedness, DNA data of strains that are 70% or more related at optimal conditions and DNA-related sequences that show 5% or less divergence are sufficient to identify bacteria to the species level, even in the absence of phenotypic data (Brenner *et al.*, 1982; Wayne *et al.*, 1987). In the DNA–DNA hybridization analysis reported here, FSL S4-120<sup>T</sup> exhibited 73%, 69%, 75% and 74% similarity under low stringency conditions and 51%, 30%, 53% and 46% similarity under high stringency conditions to *L. monocytogenes* (ATCC 15313<sup>T</sup>), *L. monocytogenes* (ATCC 35152), *L. monocytogenes* (H3597) and *L. innocua* (ATCC33090<sup>T</sup>), respectively. The values for hybridization reactions at optimal conditions were close to the recommended threshold value and the values at less-than-optimal condition were below the threshold value for defining a new species. However, the divergence in related nucleotide sequences was 7.5% for *L. monocytogenes* and 9.5% for *L. innocua* strains tested, well above the recommended 5% threshold value for divergence in related nucleotide sequences (Brenner *et al.*, 1982; Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987), hence supporting the distinct position of FSL S4-isolates within the genus *Listeria*. Conversely, labelled DNA from *L. monocytogenes* (ATCC 15313<sup>T</sup>) and DNA from the four FSL S4-isolates had average similarities of 63.5% under optimal and 35.0% under less-than-optimal conditions, respectively, while the average divergence within related sequences was greater than 7.6%. This novel species not only represents an important branching point in *Listeria* phylogeny that can help further our understanding of this group of closely related pathogenic and non-pathogenic species, but also

may present a diagnostic challenge for clinical, food and public health microbiologists who are seeking to identify *L. monocytogenes*, the human pathogen. On the basis of the phenotypic and genotypic properties presented, the FSL S4-isolates should be assigned to a novel species of the genus *Listeria*, for which the name *Listeria marthii* sp. nov. is proposed.

### Description of *Listeria marthii* sp. nov.

*Listeria marthii* (mar'thi.i. N.L. gen. masc. n. *marthii* after Emeritus Professor Elmer H. Marth, for his research and contributions on *Listeria monocytogenes*).

*L. marthii* exhibits characteristics of the genus *Listeria* as reported by Rocourt & Buchrieser (2007). Cells are small, regular, Gram-positive rods with rounded ends. Grows well on most commonly used bacteriological media. The temperature range for growth is 1–45 °C (optimal growth at 30–37 °C). Motile, non-sporing, aerobic and facultatively anaerobic. Form an umbrella-type growth appearing 3–5 mm below the surface of a stabbed semisolid motility medium incubated at 20–30 °C, but not at 37 °C. Colonies are non-haemolytic on agar surface and in stab on SBA and RBA after incubating for 18–24 h at 37 °C. On clear, solid media colonies are 0.2–0.8 mm in diameter, smooth, bluish grey, translucent and slightly raised with a fine surface texture and entire margin after incubating for 24 h at 37 °C. Colonies present a characteristic blue-green iridescence typical of *Listeria* on clear medium when viewed by oblique light. Positive for catalase activity, aesculin hydrolysis, hydrogen sulfide production and the methyl red test; negative for oxidase activity; tolerant of sodium chloride; positive for assimilation of D-glucose, lactose and maltose; and negative for assimilation of D-xylose, D-mannitol, sucrose and L-rhamnose, nitrate reduction, urease activity, indole production and gelatin hydrolysis.

The type strain is FSL S4-120<sup>T</sup> (=J3452<sup>T</sup> =H3507<sup>T</sup> =ATCC BAA-1595<sup>T</sup> =BEIR NR 9579<sup>T</sup> =CCUG 56148<sup>T</sup>) isolated from environmental samples collected in the Finger Lakes National Forest, New York, USA. Three additional *L. marthii* isolates are registered in the Biodefence and Emerging Infections Research Resources Repository (BEIR), Manassas, VA and the CCUG.

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