Tetracycline resistance in *Listeria monocytogenes* and *L. innocua* from wild black bears (*Ursus americanus*) in the United States is mediated by novel transposable elements

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ABSTRACT *Listeria monocytogenes* is a facultative intracellular foodborne pathogen and the causative agent of the severe disease listeriosis. It is found ubiquitously in the environment and exhibits innate resistance to certain antimicrobials, but acquired antimicrobial resistance remains relatively uncommon. Given the potentially dire health outcomes associated with listeriosis, acquisition of antimicrobial resistance (AMR) by this pathogen is of considerable public health concern. AMR in *L. monocytogenes* has been surveyed frequently in strains of clinical and food origin, but much less commonly in wildlife. We analyzed 158 strains of *L. monocytogenes* and 27 of non-pathogenic *Listeria* spp. isolated from wild black bears (*Ursus americanus*) for resistance to a panel of antimicrobials. AMR was uncommon and noted mostly for tetracycline. Tetracycline resistance was more common in *Listeria innocua* than in *L. monocytogenes*. All tetracycline-resistant *L. monocytogenes* strains belonged to sequence type ST1039 and harbored the Tn*916*-like *tet*(M) transposon Tn*916.1039* in a conserved chromosomal location. In contrast, three different tetracycline resistance elements, i.e., the *tet*(M) elements Tn*5801.UAM* and Tn*5801.551* and the *tet*(S) element Tn*6000.205*, were identified among tetracycline-resistant strains of *L. innocua*. The greater prevalence and diversity of tetracycline resistance elements among bear-derived non-pathogenic *Listeria* strains suggest the potential of the latter to serve as reservoirs for retention and diversification of AMR determinants in this wildlife host and warrant their further monitoring and study.

IMPORTANCE *Listeria monocytogenes* causes severe foodborne illness and is the only human pathogen in the genus *Listeria*. Previous surveys of AMR in *Listeria* focused on clinical sources and food or food processing environments, with AMR in strains from wildlife and other natural ecosystems remaining under-explored. We analyzed 185 sequenced strains from wild black bears (*Ursus americanus*) from the United States, including 158 and 27 *L. monocytogenes* and *L. innocua*, respectively. Tetracycline resistance was the most prevalent resistance trait. In *L. monocytogenes*, it was encountered exclusively in serotype 4b strains with the novel Tn*916*-like element Tn*916.1039*. In contrast, three distinct, novel tetracycline resistance elements (Tn*5801.UAM*, Tn*5801.551*, and Tn*6000.205*) were identified in *L. innocua*. Interestingly, Tn*5801.551* was identical to elements in *L. monocytogenes* from a major foodborne outbreak in the United States in 2011. The findings suggest the importance of wildlife and non-pathogenic *Listeria* species as reservoir for resistance elements in *Listeria*.

KEYWORDS *Listeria monocytogenes*, *Listeria*, foodborne pathogens, antibiotic resistance, tetracyclines, bear, genome analysis, environmental microbiology, transposons

*L*isteria monocytogenes is a facultative intracellular pathogen that is ubiquitous in the environment and responsible for the severe, invasive disease listeriosis in humans and other animals. At high risk are pregnant women and their fetuses, the elderly, and patients who are immunocompromised. Symptoms include septicemia, meningitis, abortions, and stillbirths, and case fatality rates are high, even with appropriate antimicrobial treatment (1–5). *L. monocytogenes* represents the sole human pathogen in the genus *Listeria* which includes a number of other species, with novel species being increasingly recognized (6–11). *L. monocytogenes* has been isolated from a wide array of both domesticated and wild animals (12–15).

With a growing human population, greater wildlife habitat fragmentation, and an increasingly porous human-wildlife interface, human-wildlife interactions are also increasing and with them the potential for zoonotic disease transmission (16). While antimicrobial resistance (AMR) determinants are ubiquitous among environmental microorganisms, several studies have detected an increasing correlation of the prevalence of AMR genes with human activities and anthroponotic sources (17, 18).

AMR remains relatively uncommon in *Listeria* spp., except for intrinsic resistance to certain antimicrobials including fosfomycin, many modern cephalosporins, oxacillin, and nalidixic acid (19–24). Given the severity and high case fatality rate of listeriosis, and the critical role of antibiotics for treatment, typically a beta-lactam paired with an aminoglycoside (21, 22), continued surveillance of the emergence and dissemination of AMR in *Listeria* is clearly needed. While many previous studies have surveyed AMR in *L. monocytogenes*, the majority examined strains from human illness, food products, and food-processing environments (19, 20, 23–26). There is a notable scarcity of data on AMR in *L. monocytogenes* and other *Listeria* species from natural ecosystems, particularly those that may serve as reservoirs for AMR genes (19, 27).

Especially noteworthy is the scarcity of studies investigating AMR in *L. monocytogenes* and other *Listeria* spp. from wildlife. Complicating matters further, *L. monocytogenes* and other *Listeria* spp. are often uncommonly recovered from wildlife (12, 27–29). The overall trend from investigations with more than 10 wildlife samples has been that *L. monocytogenes* and other *Listeria* spp. were largely susceptible to the antimicrobials that were tested, with the exception of occasional resistance to clindamycin and common resistance to cefuroxime and oxacillin (12, 24, 24), to which *Listeria* tends to exhibit innate resistance (19, 20, 23, 30). However, recent studies have demonstrated the potential for wildlife to harbor multidrug-resistant *Listeria* strains (31, 32).

Our laboratory has conducted a 3-year study of *L. monocytogenes* and other *Listeria* spp. from wild black bears (*Ursus americanus*) in the United States, with most samples collected from bears in urban and suburban habitats in North Carolina (33). A significant portion (105 of 231, 45%) of the black bears yielded *L. monocytogenes*, either alone or together with other *Listeria* spp. (33). Here we employed phenotypic assessments of resistance to a panel of antimicrobials and whole-genome sequence data to analyze AMR in a subset of 158 of the *L. monocytogenes* strains from the black bears for which whole-genome sequence data have become recently available (34). Since AMR has the potential to be disseminated from non-pathogenic *Listeria* spp. to *L. monocytogenes* (35), we furthermore analyzed AMR and the underlying determinants in a panel of non-pathogenic strains of *Listeria* spp., mostly *Listeria innocua*, which were also derived from the black bears in the same study. The above-mentioned increasing incidence of human-wildlife encounters due to animal mobility and changing human demographics, together with the important public health implications of AMR for treatment of listeriosis, mandates better understanding of current trends and enhanced surveillance of AMR in *Listeria* from wildlife.

MATERIALS AND METHODS

Bacterial strains and growth media

The *Listeria* panel analyzed in this study consisted of strains collected between 2014 and 2017 from black bears in the United States with the majority of the strains being isolated from bears in urban and suburban North Carolina (33). The genome sequences of 158 strains of *L. monocytogenes* analyzed in this study have been recently reported (34). These strains were selected for whole-genome sequencing from a larger collection of 537 isolates and were chosen to be representative of different animals and sample types (i.e., feces and rectal and nasal swabs) (33, 34). Serotypes of *L. monocytogenes* were originally determined by multiplex PCR (33). Analysis of whole-genome sequence data was used for confirmation of serotype designations for *L. monocytogenes* and for *in silico*-based multilocus sequence typing (MLST) designations (34). The non-pathogenic panel included 25 strains selected based on their cadmium or tetracycline resistance from a larger cohort of 240 bear-derived isolates of *Listeria* spp. other than *L. monocytogenes* from North Carolina (33), and the previously described *L. innocua* UAM003-1A from a wild bear in California (32).

Listeria strains were routinely grown in tryptic soy broth supplemented with 0.7% yeast extract or in brain heart infusion (BHI) broth (Becton, Dickinson and Co., Sparks, MD, USA). Agar cultures employed tryptic soy broth with 1.2% agar (Becton, Dickinson and Company, TSAYE) (33). Cultures were preserved at −80°C in BHI with 20% glycerol.

Antibiotic resistance determinations

Listeria strains were screened for resistance to a panel of antibiotics (Table 1) using previously published thresholds for resistance (24, 36). In addition, a subset of the strains was screened against cefotaxime (2 µg/mL), ceftriaxone (2 µg/mL), and linezolid (4 µg/mL) (37). The strains were grown in duplicate for 24 or 36 h at 37°C in 200µL BHI in individual wells of 96-well plates (Greiner Bio-One, Monroe, NC, USA). The cultures from each well were then transferred using a flame-sterilized 48-prong stainless-steel device onto Mueller-Hinton agar (MHA) (Becton, Dickinson and Co.) supplemented with the indicated levels of antimicrobials (Table 1), as well as onto antimicrobial-free MHA. Plates were examined following incubation for 48 h at 37°C. Resistance was indicated by confluent growth in both spots, and each strain was tested in at least two independent trials. *L. monocytogenes* strains F2365 (serotype 4b) and 2010L-1723 (serotype 1/2a) were previously known to be pan-sensitive and were included for quality assurance on all antimicrobial-supplemented plates. In addition, a panel of strains was included each time as positive controls for resistance to selected antimicrobials. These included *L. monocytogenes* strains 10403S and J2E3, previously known to be resistant to streptomycin and erythromycin, respectively (38, 39), as well as strain 2011L-2858, which was found in our laboratory to be resistant to tetracycline.

A subset of strains was tested for ampicillin and tetracycline minimum inhibitory concentrations (MICs) using MIC strips [Liofilchem, Roseto degli Abruzzi (TE), Italy]. The strips were overlaid on BHI agar lawns of the strains as suggested by the vendor, and the MIC levels were determined visually following incubation at 37°C for 24 h. Strains were tested in at least two independent trials, and results were averaged.

Bioinformatics analysis

Core-genome and whole-genome multilocus sequencing typing (MLST) was performed *in silico* as described (34) using the MLST package (40), which incorporates components of the PubMLST database (https://bigsdb.pasteur.fr/listeria/listeria.html) curated by the Pasteur Institute. Species determinations were based on average nucleotide identity analysis performed using the pyani software package (https://github.com/widdowquinn/pyani). Phylogenetic analysis using a single-nucleotide polymorphism (SNP) matrix among *L. innocua* strains was performed using the CFSAN SNP Pipeline v.2.2.1 (41) utilizing the complete genome of *L. innocua* CLIP 11262 (National Center for

TABLE 1 Resistance in *L. monocytogenes* from black bears to selected antimicrobials

Antibiotic (concentration)	Resistance prevalence (%)
Ampicillin (1 µg/mL)	<0.63[a]
Trimethoprim (1 µg/mL)	<0.63[a]
Vancomycin (2 µg/mL)	<0.63[a]
Erythromycin (2 µg/mL)	<0.63[a]
Rifampicin (0.5 µg/mL)	<0.63[a]
Gentamicin (2 µg/mL)	<0.63[a]
Penicillin (1 µg/mL)	<0.63[a]
Tetracycline (5 µg/mL)	3.77[a]
Chloramphenicol (10 µg/mL)	<0.63[a]
Kanamycin (10 µg/mL)	<0.63[a]
Streptomycin (20 µg/mL)	<0.63[a]
Cefotaxime (2 µg/mL)	100[b]
Ceftriaxone (2 µg/mL)	100[b]
Linezolid (4 µg/mL)	<4.3[b]

[a]Based on the 158 strains of *L. monocytogenes* that were screened.
[b]Based on 23 strains of *L. monocytogenes* that were screened.

Biotechnology Information (NCBI) Accession, NC_003212.1) as the reference. A maximum likelihood tree was constructed using this SNP matrix with Molecular Evolutionary Genetics Analysis X software (42) based on the Tamura-Nei model (43). Mobile genetic elements and their corresponding genomic localization sites were determined using the proteome comparison tool in the Bacterial and Viral Bioinformatics Resource Center (PATRIC) (44), confirmed using basic local alignment search tool (BLAST), and visualized using EasyFig (45). Isolates exhibiting antibiotic resistance were further analyzed via the NCBI pathogen detection pipeline (https://www.ncbi.nlm.nih.gov/pathogens/isolates/) and BIGSdb-*Lm* hosted by the Institut Pasteur (46). Nucleotide BLASTs were conducted with the NCBI BLAST using the nucleotide and whole-genome shotgun contig databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (47). Minimum spanning trees using the seven-locus MLST scheme were constructed with BioNumerics v.8.1 (https://www.applied-maths.com).

RESULTS AND DISCUSSION

Antimicrobial resistance is generally uncommon in *L. monocytogenes* from black bears

The 158 *L. monocytogenes* strains were chosen for sequencing from a previously described larger panel of 537 *L. monocytogenes* from the black bears (33, 34) to minimize redundancy by avoiding multiple isolates of the same serotype from the same animal, location, and date. The genome sequences of these 158 strains were recently reported (34). As previously described (34), the most prevalent serotypes were 1/2a (lineage II, 51.6%) and 4b (lineage I, 37.1%), followed by much lower prevalence of serotypes 1/2c (lineage II, 3.8%) and 1/2b (lineage I, 2.5%). The few ($n = 8$) remaining strains belonged to lineage III or IV, and their serotype was not determined. The lineage and serotype distributions were representative of the larger panel (33). Of the 59 strains of serotype 4b, 35 (59.3%) had the atypical multiplex PCR profile designated IVb-v1, which was also commonly encountered in the larger panel from the black bears (33, 34).

None of the 158 *L. monocytogenes* strains were resistant to ampicillin, penicillin, trimethoprim, vancomycin, erythromycin, rifampicin, gentamicin, chloramphenicol, streptomycin, or kanamycin. The only encountered resistance was to tetracycline, detected in six strains, i.e., a frequency of approx. 3.8% (Table 1). Previous studies identified frequent resistance of *L. monocytogenes* to cefotaxime, ceftriaxone, and linezolid (37). Screening of a subpanel of 23 *L. monocytogenes* strains representative of different serotypes revealed that all were resistant to both cefotaxime and ceftriaxone,

suggesting frequent intrinsic resistance to these two antibiotics, in agreement with previous studies (20, 37). However, none demonstrated resistance to linezolid (Table 1).

The panel of non-pathogenic *Listeria* isolates characterized here was chosen to primarily consist of those previously determined to be resistant to tetracycline (*n* = 19) or heavy metals, specifically cadmium (*n* = 8) (Table 2). Only one strain, *L. innocua* SKB205, was concurrently resistant to tetracycline and cadmium (Table 2). Furthermore, *L. innocua* strain UAM003-1A was previously found to be resistant to tetracycline and one additional antimicrobial, i.e., streptomycin (32). Resistance to ampicillin, penicillin, trimethoprim, vancomycin, erythromycin, rifampicin, gentamicin, chloramphenicol, or kanamycin was not encountered. Of note, *L. innocua* UAM003-1A was earlier shown to harbor *mphB* and thus predicted to be resistant to macrolides, but was found susceptible to erythromycin (32). Furthermore, it harbored *ant(6)-Ia_2* (aminoglycoside resistance) and, as noted above, was resistant to streptomycin but susceptible to the other tested aminoglycosides, i.e., gentamicin and kanamycin, as also reported previously (32).

Testing of a subpanel of 10 *L. innocua* strains for resistance to cefotaxime and ceftriaxone revealed that all were resistant to both of these antimicrobials, as also described above with *L. monocytogenes*. However, and similarly to the results described above with *L. monocytogenes*, resistance to linezolid was not encountered (data not shown).

Non-pathogenic *Listeria* strains from black bears are markedly more likely to be resistant to tetracycline than *L. monocytogenes*

Tetracycline-resistant *Listeria* strains belonging to species other than *L. monocytogenes* were isolated from 17 (32%) of the 53 bears that yielded non-pathogenic *Listeria* spp. (32, 33). This prevalence of tetracycline resistance among non-pathogenic *Listeria* spp. was noticeably higher than with *L. monocytogenes*, for which only 6 of the 105 *L. monocytogenes*-positive bears (5.7%) yielded strains with resistance to tetracycline. These six bears were captured in North Carolina in 2014 (*n* = 2), 2015 (*n* = 1), and 2016 (*n* = 3) (Table 2). All 19 of the non-pathogenic tetracycline-resistant *Listeria* strains that were sequenced were found to be *L. innocua* based on analysis of the whole-genome sequence data (Table 2). A higher prevalence of tetracycline resistance among *L. innocua* than *L. monocytogenes* from wildlife was also noted, albeit with lower numbers of isolates, from wild rodents in China, where tetracycline resistance was exhibited by 5 of 10 *L. innocua* isolates, but not among any of the 11 isolates of *L. monocytogenes* (27).

We did not detect any bears co-colonized by tetracycline-resistant strains of both *L. monocytogenes* and *L. innocua* (Table 2). However, the bears were often co-colonized with tetracycline-resistant and tetracycline-susceptible *Listeriae* (Table 2). Most of the tetracycline-resistant *L. innocua* strains were from feces or rectal swabs, but three bears yielded tetracycline-resistant *L. innocua* from nasal swabs (Table 2). For two bears (N061 and N141), tetracycline-resistant *L. innocua* of the same sequence type (ST) (ST637) was obtained from nasal swabs as well as feces or rectal swabs (Table 2), suggesting the possibility that tetracycline-resistant *L. innocua* strains had colonized multiple sites of the same animal.

L. monocytogenes and *L. innocua* from black bears harbor novel tetracycline resistance elements, with pronounced element diversity noted in *L. innocua*

Tn916.1039 in *L. monocytogenes* from black bears

All tetracycline-resistant *L. monocytogenes* strains harbored *tet*(M) based on PCR, and all six strains that were sequenced (one each from the six bears positive for tetracycline-resistant *L. monocytogenes*) belonged to ST1039 (Table 2). ST1039 has been identified relatively recently among human clinical isolates of *L. monocytogenes* (48), and the six bear-derived strains were closely related, with 0–28 core-genome MLST differences and 5–116 allele differences based on whole-genome MLST analysis. In the seven-locus MLST scheme (https://bigsdb.pasteur.fr/listeria/), ST1039 matches ST2 at four loci and is a

TABLE 2 *Listeria monocytogenes* and *L. innocua* strains investigated in this study

Strain[a]	Species[a]	Bear[b]	Isolation date	Location	Sample[c]	ST[d]	AMR, Tn[e]	MIC[f]
SKB111	L. monocytogenes	N044	7/30/2014	NC, USA	S	1039	tet(M) (Tn916.1039), lin, fosX, norB, sul, abc-F, mprF	32
SKB121	L. monocytogenes	N021*	8/20/2014	NC, USA	F	1039	tet(M) (Tn916.1039), lin, fosX, norB, sul, abc-F, mprF	48
SKB297	L. monocytogenes	N085*	6/12/2015	NC, USA	NS	1039	tet(M) (Tn916.1039), lin, fosX, norB, sul, abc-F, mprF	32
SKB461	L. monocytogenes	N117	5/12/2016	NC, USA	S	1039	tet(M) (Tn916.1039), lin, fosX, norB, sul, abc-F, mprF	32
SKB537	L. monocytogenes	N126*	6/14/2016	NC, USA	F	1039	tet(M) (Tn916.1039), lin, fosX, norB, sul, abc-F, mprF	28
SKB542	L. monocytogenes	N130*	7/6/2016	NC, USA	F	1039	tet(M) (Tn916.1039), lin, fosX, norB, sul, abc-F, mprF	48
SKB115	L. innocua	N049*	8/12/2014	NC, USA	F	**1481**	tet(M) (Tn5801.551), lin, fosX, norB, sul, abc-F	24
SKB205#	L. innocua	N065	3/20/2015	NC, USA	S	603	tet(S) (Tn6000.205), lin, fosX, sul, abc-F	40
SKB363	L. innocua	N095*	7/3/2015	NC, USA	S	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	28
SKB388	L. innocua	N0128	10/9/2015	VA, USA	F	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	40
SKB410	L. innocua	N108/109*	4/11/2016	NC, USA	F	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	24
SKB415	L. innocua	N109	4/11/2016	NC, USA	S	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	24
SKB453	L. innocua	N116*	5/12/2016	NC, USA	S	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	20
SKB551	L. innocua	N121*	7/12/2016	NC, USA	NS	**1481**	tet(M) Tn5801.551, lin, fosX, norB, sul, abc-F	28
SKB572	L. innocua	N132*	7/15/2016	NC, USA	F	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	24
SKB626	L. innocua	N061*	8/10/2016	NC, USA	S	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	16
SKB635	L. innocua	N061*	8/10/2016	NC, USA	NS	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	24
SKB638	L. innocua	N138*	8/10/2016	NC, USA	F	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	28
SKB650	L. innocua	N140*	8/15/2016	NC, USA	F	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	24
SKB663	L. innocua	N141*	8/15/2016	NC, USA	F	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	24
SKB673	L. innocua	N141*	8/16/2016	NC, USA	NS	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	28
SKB687	L. innocua	N143	9/15/2016	NC, USA	F	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	20
SKB713	L. innocua	N092*	9/30/2016	NC, USA	F	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	24
SKB716	L. innocua	N138*	9/30/2016	NC, USA	F	637	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F	24
UAM003-1A	L. innocua	UAM003	10/10/2017	CA, USA	F	1495	tet(M) (Tn5801.UAM), lin, fosX, norB, sul, abc-F, ant (6)-Ia, mphB	20
SKB154#	L. innocua	N060	2/15/2015	NC, USA	F	1086	lin, fosX, norB, sul, abc-F	ND
SKB476#	L. innocua	N119	5/20/2016	NC, USA	F	**1480**	lin, fosX, norB, sul, abc-F	ND
SKB640	L. innocua	N138*	8/10/2016	NC, USA	F	**1482**	lin, fosX, norB, sul, abc-F	ND
SKB651##	L. innocua	N140*	8/15/2016	NC, USA	F	**1482**	lin, fosX, norB, sul, abc-F	ND
SKB662##	L. innocua	N141*	8/15/2016	NC, USA	F	**1482**	lin, fosX, norB, sul, abc-F	ND
SKB674##	L. innocua	N141*	8/17/2016	NC, USA	NS	**1482**	lin, fosX, norB, sul, abc-F	ND
SKB766#	L. innocua	N158	5/23/2017	NC, USA	F	**1485**	lin, fosX, norB, sul, abc-F	ND

[a]Whole-genome sequence data were available for all strains. Species designations for non-pathogenic *Listeria* spp. were made utilizing the whole-genome sequence data as described in Materials and Methods. # Indicates strains were resistant to high levels of cadmium (70 µg/mL). ## Indicates strains were resistant to lower levels of cadmium (35 µg/mL). *L. innocua* UAM003-1A is also resistant to streptomycin (31).

[b]*Indicates bears co-colonized with both tetracycline-resistant and tetracycline-susceptible *Listeriae*.

[c]S, F, and NS are rectal swab, feces, and nasal swab samples, respectively.

[d]Novel STs are indicated in bold. ST1495 of *L. innocua* UAM003-1A was reported before (31). ST, sequence type.

[e]AMR determinants were derived from the analysis of the whole-genome sequence data. AMR determinants for *L. innocua* UAM003-1A were reported before (31).

[f]MIC values are in µg/mL. ND, not determined.

member of the ubiquitous hypervirulent clonal complex (CC) 2 (49). Besides *tet*(M), all six ST1039 strains harbored *fosX*, *lin*, *norB*, *sul*, *abc-F*, and *mprF* (Table 2), which are routinely detected in *L. monocytogenes* genomes with *fosX* mediating inherent fosfomycin resistance, at least during growth *in vitro* (19, 50).

All six strains harbored an identical Tn*916*-like, approx. 18-kb novel tetracycline resistance transposon designated Tn*916.1039*, in the same chromosomal insertion site in the intergenic region between *rpoZ* and *coaBC* (Fig. 1). Tn*916.1039* shared 99.82% nucleotide sequence identity to Tn*916* from *Enterococcus faecalis*. BLAST of Tn*916.1039* against the NCBI nucleotide database revealed matches with 100% coverage and 100% identity with elements in *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Selenomonas ruminantium*, and *E. faecalis*, as well as multiple matches (100% coverage, >99.8% identity) with other Gram-positive pathogens. Highly similar elements (100% coverage, >99.8% nucleotide identity) were detected, albeit infrequently, in other strains of *L. monocytogenes* of various serotypes and in *L. innocua*. Despite the relatively uncommon natural occurrence of these Tn*916*-like elements in *Listeria*, Tn*916* can be readily mobilized from *E. faecalis* via conjugation and has been used extensively for transposon mutagenesis in *L. monocytogenes* (37, 51, 52).

Tn*5801*-like and Tn*6000*-like elements in bear-derived *L. innocua*: Tn*5801.UAM,* Tn*5801.551,* and Tn*6000.205*

As indicated earlier, the non-pathogenic *Listeria* isolates characterized here were chosen based on their resistance to tetracycline and cadmium. The strains included several STs of *L. innocua*, of which ST637 (CC140) predominated (Fig. 2). The strains were from various types of samples, i.e., feces, rectal swabs and nasal swabs, with ST637 including strains from all three sample types (Table 2; Fig. 2A). With the exception of one strain (ST603) which exhibited resistance to both tetracycline and cadmium, strains of the other STs were resistant either only to tetracycline (*n* = 18, 3 STs) or only to cadmium (*n* = 6, 4 STs). One strain lacked resistance to either compound, and one was resistant to tetracycline as well as streptomycin, as indicated above (Fig. 2B). The latter strain, *L. innocua* UAM003-1A, was ST1495, with one-allele difference from ST637 and thus a member of the same CC, i.e., CC140 (Fig. 2B).
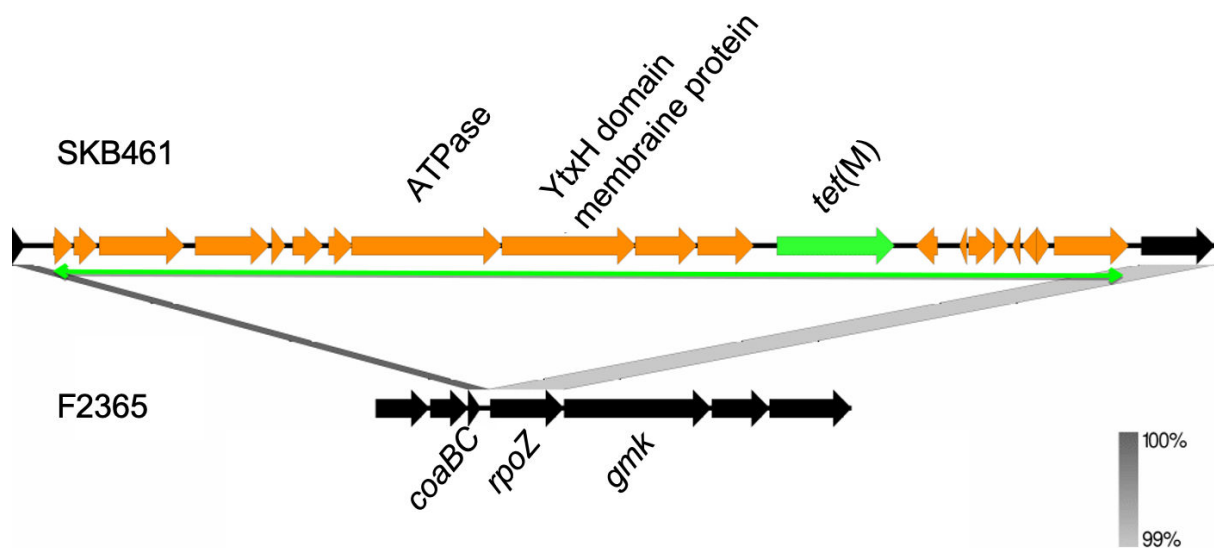

FIG 1 Genomic organization of the Tn*916.1039*-harboring region in the bear-derived *Listeria monocytogenes* strain SKB461. The Tn*916*-like element Tn*916.1039* is indicated with the green double-pointed arrow. Open reading frames (ORFs) conserved in the reference strain *L. monocytogenes* F2365 are in black. The tetracycline resistance determinant *tet*(M) is in green. The gray scale indicates nucleotide sequence identity (%) in the flanking sequences. Comparative genomic organization was determined as described in Materials and Methods and visualized using EasyFig.

Tet(M)-harboring element Tn5801.UAM

The genetic basis of tetracycline resistance proved to be noticeably more diverse in the sequenced *L. innocua* strains, being mediated by two distinct *tet*(M) elements as well as a *tet*(S)-harboring element (Table 2; Fig. 2B). As indicated above for *L. monocytogenes*, the ubiquitous determinants *lin*, *fosX*, *norB*, *sul*, and *abc-F* were also identified in all *L. innocua* sequenced genomes (Table 2).

The most commonly encountered tetracycline resistance element, Tn5801.UAM, was approx. 20-kb with Guanine-Cytosine (GC) content of 35% and was harbored with 100% conservation by 17 of the 19 tetracycline-resistant bear-derived *L. innocua* strains with sequenced genomes (Table 2; Fig. 3). In all 17 genomes, Tn5801.UAM was in the same integration site, i.e., the intergenic region adjacent to *guaA* (Fig. 3). The 17 strains included all 16 *L. innocua* strains of ST637 from different bears in North Carolina as well as the closely-related *L. innocua* UAM003-1A (ST1495) from a bear captured in 2017 in California, thousands of kilometers away (Table 2). Phylogenetic analysis indicated that these strains were closely related, with all but one of the ST637 strains partitioning in one highly conserved cluster, while, interestingly, the other cluster consisted of the ST637 strain SKB388 and the ST1495 strain UAM003-1A (Fig. 4). As indicated above, ST637 and ST1495 are members of CC140, which also includes the tetracycline-susceptible reference strain *L. innocua* CLIP 11262, one of the first *Listeria* strains to be sequenced (53). *L. innocua* CLIP 11262 had ST140, and even though it exhibited a single-allele difference from ST637, it was placed clearly apart from ST637 or ST1495 in the phylogenetic tree (Fig. 4).

It is remarkable that Tn5801.UAM was harbored by ST637 and 1495 strains from multiple bears and all three animal sample types (Table 2; Fig. 2). The predominance of this element among the tetracycline-resistant *L. innocua* strains in our panel may reflect possible fitness advantages that it may confer to *L. innocua* colonizing the bears, or possibly the overall higher fitness of *L. innocua* strains of ST637/ST1495 in this wildlife host. It is also possible that these strains may have higher fitness in harborage sites in the environment (e.g., soil, rhizosphere, or aquatic niches) and thus may be more likely to be acquired by the animals.
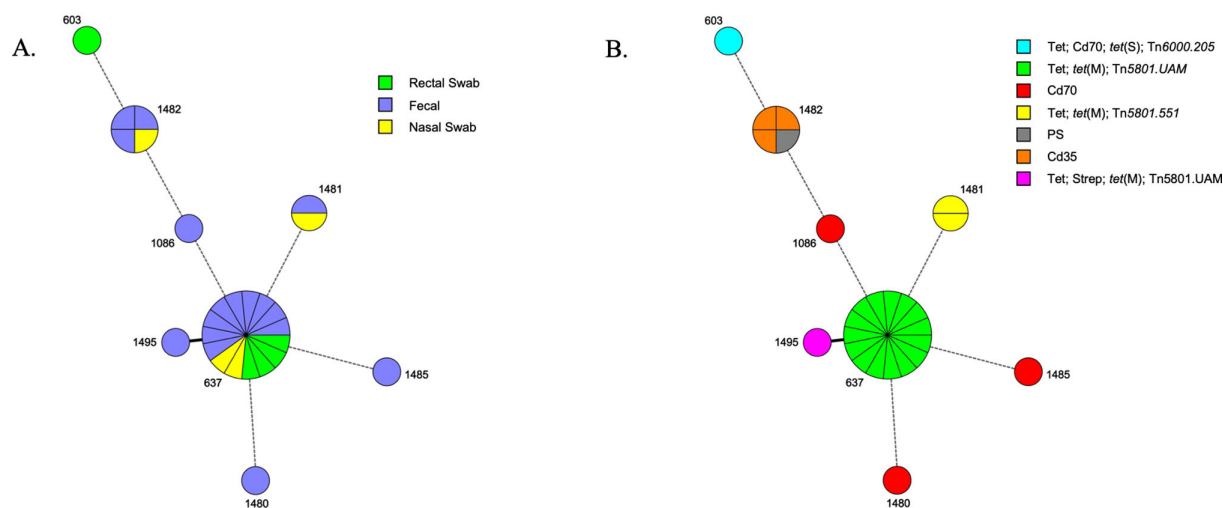
FIG 2 Minimum spanning trees (MSTs) of the bear-derived *Listeria innocua* strains investigated in this study. The MST was generated using BioNumerics version 8.1 as described in Materials and Methods. Strains of the same sequence type are grouped into circles, with each division in a circle indicating a separate strain. The circles are connected by lines that reflect relatedness using the seven-locus MLST scheme: thick black line (one allele difference) and thin dotted gray line (four or more allele differences). (A) Strains are color-coded depending on sample type; (B) strains are color-coded to indicate antimicrobial resistance profiles and tetracycline resistance elements that were encountered: Cd35 and Cd70, resistance to cadmium 35 and 70 µg/mL, respectively; Tet and Strep, resistance to tetracycline and streptomycin, respectively. Tetracycline resistance determinant *tet*(M) or *tet*(S) is indicated after the transposon designation. PS indicates a strain pan-sensitive to all tested antimicrobial compounds.
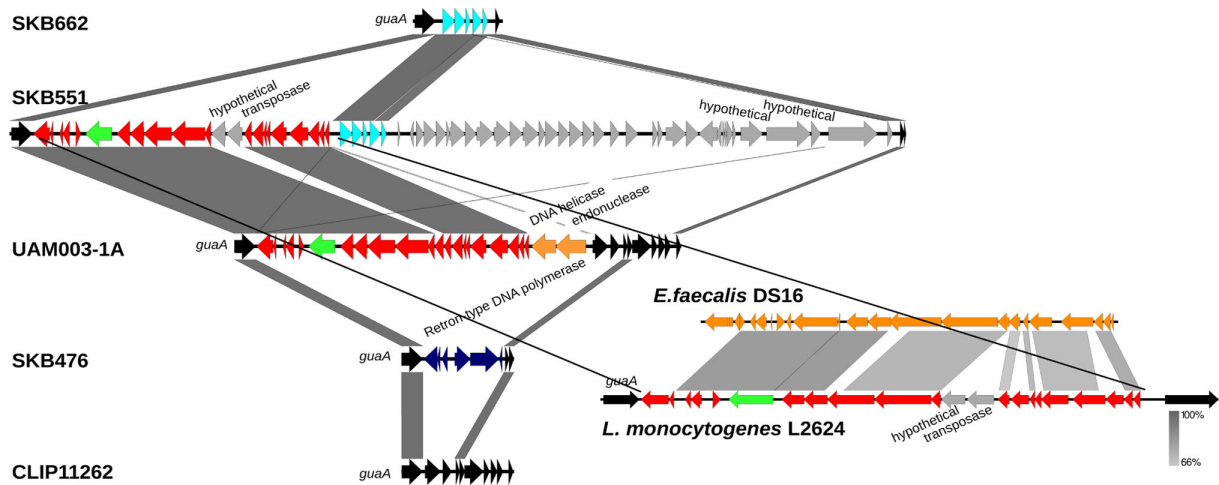
FIG 3 Genomic organization of the *lmo1096* (*guaA*) region in the bear-derived strains *Listeria innocua* UAM003-1a and SKB551. The corresponding region in the bear-derived *L. innocua* strains SKB662 and SKB476 is also shown, as is *L. innocua* CLIP11262 (53), used as reference strain. Flanking ORFs conserved in *L. innocua* CLIP11262 are in black, and the tetracycline resistance determinant *tet*(M) in the *Listeria* genomes is in green. Sequences shared specifically between SKB551 and SKB662 are in turquoise, and include a partial *Listeria* pathogenicity island 3 consisting of *llsAGHX*. Shared colors in the *Listeria* genomes indicate homologous ORFs. Dark gray shading across homologous regions is indicative of 99% or greater nucleotide sequence identity. The inset to the right demonstrates a comparison between Tn*5801.551* in *L. monocytogenes* L2624 (serotype 1/2b, ST5, 2011 cantaloupe outbreak) and the canonical Tn*916* in *E. faecalis*. Comparative genomic organizations were determined and visualized as in the legend of Fig. 1.

BLAST searches identified elements highly similar to Tn*5801.UAM* (100% coverage, >99.7% identity) in certain strains of *L. monocytogenes* serotypes 1/2a and 1/2b and in *L. innocua*, as well as multiple other Gram-positive bacteria including *Staphylococcus aureus*, *Staphylococcus pseudintermedius*, *Enterococcus faecalis*, *Enterococcus avium*, *Enterococcus faecium*, *Streptococcus pyogenes*, *Streptococcus mitis*, and *Mammaliicoccus sciuri*. A previous genomic analysis of over 5,000 genomes of *Firmicutes* identified Tn*5801*-like elements in many of the same genera and, interestingly, in the same genomic location (54). The *guaA* locus has been previously identified as a genomic hotspot both in bacteria and archaea (55). In *L. monocytogenes*, this hotspot can harbor a wide variety of gene cassettes, including *Listeria* pathogenicity island 3 (LIPI-3) (56, 57), and our findings suggest that it serves as a hotspot in *L. innocua* as well. All strains in our *L. innocua* panel except SKB205 appeared to harbor additional sequences adjacent to *guaA*, with several distinct cassettes identified in this location (Fig. 3). Indeed, analysis of *L. innocua* strains from black bears and water has revealed a partial, truncated form of LIPI-3 in many of the genomes in the *guaA* hotspot, while full-length LIPI-3 was detected in three strains, one of which, the tetracycline-susceptible strain SKB154 (ST1086), was from a bear (58) and was included in our panel (Fig. 4). None of the examined ST637 and ST1495 strains from the bears harbored LIPI-3 in this location (58). Collectively, the findings suggest that in *L. innocua*, the *guaA* hotspot harbors the tetracycline resistance element Tn*5801.UAM* in tetracycline-resistant strains of ST637/ST1495, while in most other strains, this genomic hotspot harbors LIPI-3 or other, unrelated genomic content.

It is noteworthy that even though Tn*5801.UAM* was repeatedly encountered in *L. innocua*, it was absent from *L. monocytogenes* from the black bears. It is possible that the element was only recently acquired by *L. innocua* of ST637 and closely related STs and has not yet disseminated to *L. monocytogenes*. The temporal distribution of Tn*5801.UAM*-harboring *L. innocua* strains in black bears from North Carolina was also intriguing, since all were recovered between April and September 2016, with the exception of one strain in 2015 (Table 2). The underlying reasons remain unknown and may reflect ecological conditions favoring ST637 during one of the years of the study. Alternatively, it is possible that tetracycline-resistant *L. innocua* strains of ST637 became introduced in the region in 2015 and began to proliferate subsequently. Further surveillance would be
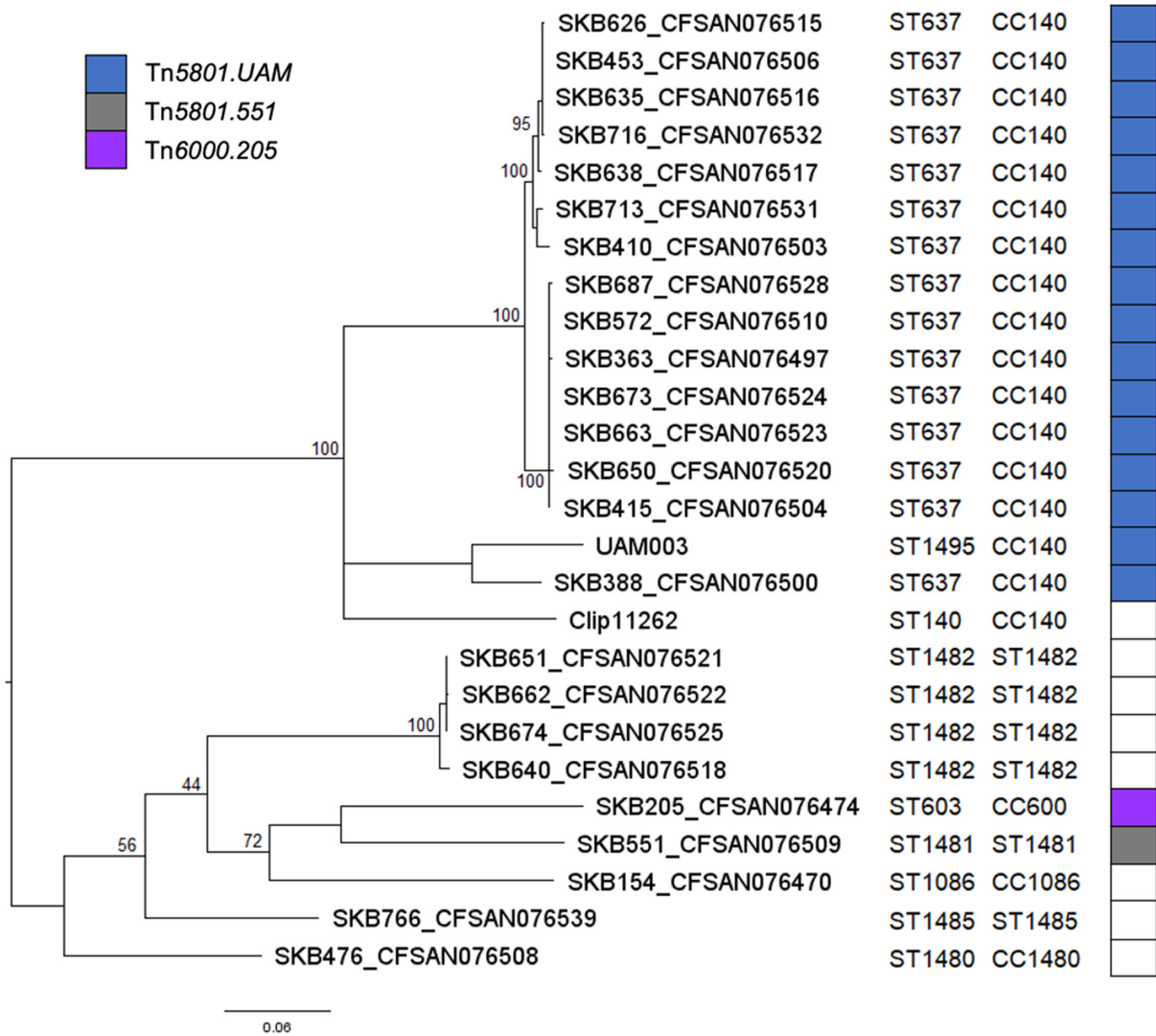
FIG 4 Phylogenetic relationships among the sequenced bear-derived *Listeria innocua* strains investigated in this study. The reference strain *L. innocua* CLIP11262 is included. Strain designations are in the format "strain name_CFSAN number." SKB115 (ST1481) is not shown. Maximum likelihood tree was constructed using MEGA, with bootstrapping (*n* = 500); the scale bar indicates distance as assessed by the Tamura-Nei method. Blue, gray, and purple boxes denote the presence of Tn*5801.UAM*, Tn*5801.551*, and Tn*6000.205*, respectively; white boxes denote the absence of tetracycline resistance elements.

needed to determine whether *L. innocua* ST637 harboring Tn*5801.UAM* continues to be repeatedly isolated from black bears in this region and to also elucidate the distribution of Tn*5801.UAM* in *L. innocua* from other sources and regions.

Tet(M)-harboring element Tn5801.551

A different chromosomal *tet*(M)-harboring element was identified in the bear-derived strains *L. innocua* SKB115 and SKB551 of the novel ST1481, phylogenetically highly distinct from ST637/ST1495 (Table 2; Fig. 2B). This approx. 21-kb element (designated Tn*5801.551*) was earlier detected in *L. innocua* SKB551 in the course of the analysis of LIPI-3 distribution and genomic content in the *guaA* hotspot in *L. innocua* from bears and surface water (58). Tn*5801.551* is integrated in the same *guaA* site as Tn*5801.UAM*, and is a component of a larger genomic island of approx. 63-kb with GC content of 31.3% (Fig. 3). Interestingly, a partial LIPI-3 consisting of *llsAGHX* was also identified in this island, adjacent to Tn*5801.551* (58). BLAST of the entire 63-kb island against the NCBI database suggested that it was unique to SKB115 and SKB551, as there were no matches with high

identity and over 65% coverage. SKB115 and SKB551 were highly related, harboring only 76 core-genome MLST differences and 160 allele differences based on whole-genome MLST analysis but were isolated from two different bears almost 2 years apart (Table 2). These findings suggest that *L. innocua* with the novel ST1481 harboring the 63-kb island with Tn*5801.551* may be established in the wild bears in this region, even though they are less commonly encountered than strains of ST637.

Tn*5801.551* had high similarity with Tn*5801.UAM*, but two ORFs (annotated as a hypothetical protein and a transposase) were only present in SKB115 and SKB551, while two others (annotated as a DNA helicase and an endonuclease) were lacking (Fig. 3). Interestingly, Tn*5801.551* was found via BLAST analysis to be identical (100% nucleotide sequence identity and coverage) to strains of *L. monocytogenes* ST5 (serotype 1/2b), including strain L2624 and other ST5 strains implicated in a major multistate outbreak of human listeriosis in 2011 in the United States, involving whole cantaloupe (59, 60). In the ST5 *L. monocytogenes* strain L2624 from this outbreak, Tn*5801.551* was integrated in this same *guaA* location that we found to harbor Tn*5801.UAM* and Tn*5801.551* in *L. innocua* (Fig. 3).

Similarly to Tn*5801.UAM*, Tn*5801.551* was widely disseminated among Gram-positive bacteria, with highly conserved counterparts (100% coverage and >99.9% identity) in *Lactobacillus amylophilus* and the pathogen *Streptococcus agalactiae*. These findings, together with the detection of this element in outbreak-associated strains of *L. monocytogenes*, suggest potential roles of non-pathogenic species such as *L. innocua* in the acquisition and circulation of AMR among pathogenic bacteria. However, Tn*5801.551* remains relatively uncommon in *Listeria*, and additional work is required to determine if this is due to recent acquisition, limitations on transferability, or potential fitness costs.

Interestingly, a segment of the novel genomic island that harbors Tn*5801.551* in the two ST1481 strains was also identified in the *guaA* hotspot in *L. innocua* SKB662, a member of the novel ST1472, and all other ST1482 strains, and further BLAST analysis indicated that this shared segment (shown in turquoise in Fig. 3) corresponded to the partial LIPI-3 (*llsAGHX*) (Fig. 3 and data not shown). The ST1482 strains were isolated from three different bears in the summer of 2016 (Table 2) and were included in the analysis because three of the four exhibited the same level of resistance to cadmium, while the fourth was susceptible to cadmium but from the same animal that yielded a cadmium-resistant strain (Table 2; Fig. 2B). They are closely related to each other (Fig. 4) and phylogenetically quite distinct from ST1481, with none of the alleles in the seven-locus MLST scheme shared between STs 1481 and 1482 (Fig. 2A). The organization of this region in *L. innocua* SKB115, SKB551, SKB662, and the other three ST1482 strains supports the hypothesis that this chromosomal hotspot may have progressively accumulated novel genomic content, including Tn*5801.551*.

Novel *tet*(S)-harboring element in *Listeria*, Tn*6000.205*

One of the 19 tetracycline-resistant *L. innocua* strains (strain SKB205, ST603) was found to harbor *tet*(S) on an element of approx. 30-kb with a GC content of 34.6%. The *tet*(S)-harboring element was located in the intergenic region between *lin2691* and *lin2692* and was designated Tn*6000.205* (Fig. 5). The Tn*6000.205*-harboring strain SKB205 was not closely related to any other strains in our panel (Fig. 2B; Fig. 4). BLAST analysis with Tn*6000.205* indicated strong matches (100% coverage, >99.9% nucleotide identity) with certain *L. monocytogenes* strains of ST/CC 288 (serotype 1/2b) and CC2 (serotype 4b) from food and clinical sources, as well as exact matches with certain strains of *L. innocua*. Strong matches (100% coverage, >99.9% nucleotide identity) with Tn*6000.205* were noted with several other Gram-positive bacteria including *Enterococcus faecalis*, *Enterococcus gallinarum*, *Enterococcus casseliflavus*, *E. faecium*, *Enterococcus raffinosus*, and *Streptococcus canis*.

Tetracycline resistance is the most common antimicrobial resistance trait encountered in *Listeria*, and it is most commonly conferred by *tet*(M), while *tet*(S) and other determinants such as *tet*(L) and *tet*(A) are encountered infrequently (19, 31, 61). While
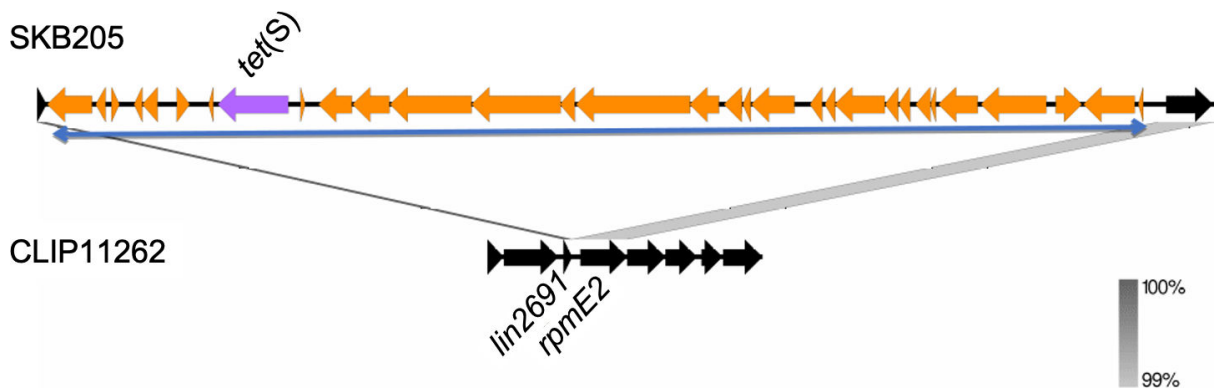
FIG 5 Genomic location and genetic organization of the Tn*7056*-harboring region in bear-derived *Listeria innocua* SKB205. The Tn*6000*-like element Tn*6000.205* is indicated with the blue double-pointed arrow. ORFs conserved in the reference strain *L. innocua* CLIP11262 are in black. The tetracycline resistance determinant *tet*(S) is in purple. The gray scale indicates nucleotide sequence identity (%) in the flanking sequences. Comparative genomic organizations were determined and visualized as in the legend of Fig. 1.

there have been numerous surveys of AMR among clinical and food-related isolates of *L. monocytogenes* and other *Listeria* spp. (60, 62), AMR among strains from wildlife remains poorly understood. Acquired AMR in *Listeria* appears to be generally uncommon, regardless of the source (24). This is supported by our findings, which indicated low prevalence of acquired AMR among *L. monocytogenes* from black bears. Interestingly, we detected a considerably higher prevalence of tetracycline resistance among non-pathogenic *Listeria* spp. from the black bears than among *L. monocytogenes* from the same animals. The extent to which this finding may also pertain to *Listeria* from environmental sources such as soil or water remains to be determined. Another intriguing finding was that, in contrast to the bear-derived tetracycline-resistant *L. monocytogenes* strains which harbored a single tetracycline resistance element, three different elements were identified among tetracycline-resistant *L. innocua* from the black bears.

Of special interest was the Tn*5801.UAM* element harbored at the same chromosomal location in genetically related strains of *L. innocua* CC140 isolated thousands of kilometers apart, from multiple individuals and diverse sample types, suggesting a tetracycline-resistant *L. innocua* clone highly efficient in colonizing this wildlife host in the United States. Another noteworthy finding was the discovery of *L. innocua* that harbored Tn*5801.551* identical to a tetracycline resistance element in *L. monocytogenes* of ST5 implicated in a major produce-associated outbreak in the United States in 2011 (59, 60). This is of particular interest, since this appears to be the first major outbreak of listeriosis involving strains with acquired AMR. This finding, together with the noticeably higher prevalence of tetracycline resistance and diversity of the corresponding genetic elements among non-pathogenic *Listeria* spp. than *L. monocytogenes*, support the hypothesis that non-pathogenic species of *Listeria* may contribute to the dissemination of AMR elements to *L. monocytogenes* strains, which may subsequently become implicated in human listeriosis. The trajectories via which strains harboring the AMR elements may eventually contaminate the human food supply currently remain poorly understood. Potential dissemination of AMR by non-pathogenic *Listeria* species may be also suggested by the fact that tetracycline resistance elements harbored by the bear-derived *L. innocua* strains were identical or highly similar to those detected among several other Gram-positive pathogens. Additionally, the average GC content of each of the three tetracycline resistance elements from *L. innocua* was below the average GC content of *L. innocua* or *L. monocytogenes* (approx. 38%) (52, 63), supporting acquisition via horizontal gene transfer from other organisms. While non-pathogenic *Listeria* species are not a direct threat to human health, they may be able to serve as reservoirs and vehicles for the retention, amplification, and dissemination of resistance elements in natural ecosystems, with serious implications for human and animal health. Further

investigations are needed to elucidate the roles of wildlife and non-pathogenic *Listeria* species in the circulation of these AMR determinants in the natural environment (e.g., water and soil) and at the human-wildlife interface.

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acquisition, Investigation, Project administration, Visualization, Writing – original draft, Writing – review and editing

DATA AVAILABILITY

Fastq reads for strains of *Listeria monocytogenes* SKB111, SKB121, SKB297, SKB461, SKB537, and SKB542 harboring Tn*916.1039* have been deposited in the National Center for Biotechnology Information (NCBI) short-read archive under accession numbers SRR13643220, SRR13642654, SRR6395255, SRR6395704, SRR6397048, and SRR6395509, respectively. *Listeria innocua* strains SKB363, SKB410, SKB415, SKB453, SKB572, SKB626, SKB635, SKB638, SKB650, SKB663, SKB673, SKB687, SKB713, SKB716, and UAM003-1A harboring Tn*5801.UAM* have been deposited under accession numbers SRR1364323, SRR13643206, SRR13643216, SRR13643183, SRR13643006, SRR13642913, SRR13642681, SRR13642689, SRR13642916, SRR13642642, SRR13642647, SRR13642667, SRR13642991, SRR13642980, and SRR9827491, respectively. *L. innocua* strains SKB 115 (Tn*5801.551*), SKB551(Tn*5801.551*), and SKB205 (Tn*6000.205*) have been deposited under accession numbers SRR22299583, SRR13643008, and SRR13642649, respectively. FASTA sequences of the transposon regions for Tn*916.1039*, Tn*5801.UAM*, Tn*5801.551*, and Tn*6000.205* were deposited in the NCBI database under accession numbers OP846513, OP846514, OP846515, and OP846516, respectively.

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