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Tn6198, a novel transposon containing the trimethoprim resistance gene *dfrG* embedded into a Tn916 element in *Listeria monocytogenes*

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Objectives: To characterize Tn6198, a novel conjugative transposon from the clinical *Listeria monocytogenes* strain TTH-2007, which contains the tetracycline and trimethoprim resistance genes *tet(M)* and *dfrG*, respectively, and to assess its transferability *in vitro* and *in situ*.

Methods: The complete sequence of Tn6198 was determined using a primer walking strategy. Horizontal gene transfer studies were performed by filter matings, as well as on the surface of smear-ripened cheese and smoked salmon. The presence of Tn916-like circular intermediates was determined by PCR. Antibiotic resistance was determined by the broth microdilution method and microarray hybridization.

Results: Sequencing of Tn6198 revealed that a 3.3 kb fragment containing *dfrG* was integrated between open reading frames 23 and 24 of Tn916. Furthermore, an additional copy of Tn916 was present in *L. monocytogenes* TTH-2007. Both elements were transferred simultaneously and separately *in vitro* to recipients *L. monocytogenes* 10403S and *Enterococcus faecalis* JH2-2 by conjugation, resulting in either tetracycline- and trimethoprim-resistant or solely tetracycline-resistant transconjugants. On the surface of cheese and salmon, only *L. monocytogenes* 10403S transconjugants were detected.

Conclusions: This study reports the first Tn916-like element associated with a trimethoprim resistance gene, as well as the first fully characterized transposon conferring multidrug resistance in *L. monocytogenes*. This is of concern, as trimethoprim is administered to listeriosis patients with β -lactam allergy and as Tn6198 has a large potential for dissemination, indicated by both intra-species and inter-genus transfer.

Keywords: *tet(M)*, *L. monocytogenes*, conjugative transposon, multidrug resistance, horizontal gene transfer

Introduction

The Tn916 family of conjugative transposons is a growing group of mobile genetic elements and is widespread mainly among Firmicutes.¹ Tn916, originally discovered in an *Enterococcus faecalis* strain, was transferable in the absence of plasmids.² Most members harbour the tetracycline resistance gene *tet(M)* which encodes a ribosomal protection protein. However, Tn6000 from *Enterococcus casseliflavus*³ contains the *tet(S)* gene and is widespread among enterococci,⁴ whereas the previously assumed *tet(S)* gene in Tn916S from *Streptococcus intermedius*⁵ was recently shown to be a mosaic *tet(S/M)* gene.⁶ Interestingly, next to the many more Tn916-like elements with additional resistance genes that have been reported,¹ no resistance genes could be identified in Tn5386 from *Enterococcus faecium*.⁷

Tetracycline resistance, whenever detected in *Listeria monocytogenes* isolates, is mostly associated with the *tet(M)* gene

located on Tn916^{8,9} and less frequently with *tet(S)* carried by plasmids.¹⁰ Transferability of the *tet(M)* gene from *L. monocytogenes* was first shown for foodborne isolates in Italy.¹¹ Generally, the prevalence of *L. monocytogenes* with acquired resistance is relatively low.¹² However, these strains can hamper the difficult treatment of human listeriosis, a disease that seems to be increasing, particularly in elderly people.¹³ It was estimated that in 99% of the cases people had been infected with *L. monocytogenes* after ingestion of contaminated foodstuffs.¹⁴ Especially when these bacteria are present on ready-to-eat foods like smoked salmon or cheese, the lack of a heating step before consumption constitutes a potential health risk. Therefore, next to the human gut, where resistance gene transfer to *Listeria* spp. most probably can occur, as demonstrated in a gut model,¹⁵ food matrices also have to be taken into account as platforms for horizontal gene transfer due to high bacterial concentrations. Conjugative transfer of erythromycin and tetracycline resistance

genes from *E. faecalis* to enterococci, pediococci and coagulase-negative staphylococci during sausage fermentation was recently demonstrated.¹⁶

L. monocytogenes is naturally susceptible to trimethoprim;¹⁷ therefore, this bacteriostatic antibiotic is often administered together with sulfamethoxazole as co-trimoxazole to patients with allergies to penicillins.¹⁸ Resistance of *L. monocytogenes* against trimethoprim was first detected in France in an environmental isolate containing the *dfrD* gene encoded on the 3.7 kb plasmid pIP823.¹⁹ Recently, two further isolates from France were reported to contain this resistance gene, namely a human clinical isolate²⁰ and a foodborne isolate.¹² The foodborne isolate additionally harboured the *tet(M)* gene on a Tn916-like transposon. So far, there are no reports of resistance in *Listeria* spp. caused by *dfrG*, a gene first detected in *Staphylococcus aureus*²¹ and highly prevalent in *Staphylococcus pseudointermedius*.²²

The aim of this study was to characterize the molecular mechanism of the trimethoprim and tetracycline resistance in a clinical isolate of *L. monocytogenes* and to determine whether both resistances were transferable by conjugation.

Materials and methods

Bacterial strains

Strain TTH-2007 was isolated by haemoculture in 2007 in Switzerland and provided by Professor Jacques Bille from the Centre Hospitalier Universitaire Vaudois (CHUV) in Lausanne. Strains used in this study were cultured on brain heart infusion (BHI) agar medium (Biolife Italiana S.r.l., Milan, Italy) at 37°C and stored in BHI medium containing 33% glycerol at –80°C.

DNA extraction and PCR assays

Genomic bacterial DNA was isolated as described before.²³ The GeneJet™ Plasmid Miniprep Kit (Fermentas) with lysozyme (10 g/L as final concentration) treatment at 37°C for 30 min was used to isolate circular intermediates of transposons from 2 mL of overnight cultures grown in BHI with 26 mg/L tetracycline, as well as to investigate the presence of plasmids.

Primers were purchased from Microsynth AG (Balgach, Switzerland) and Sanger sequencing analyses were performed with the cycle sequencing method by Microsynth AG and GATC Biotech AG (Konstanz, Germany) using capillary electrophoresis. DNA was previously purified with the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). PCR assays with primers used in this study are listed in Table S1 (available as Supplementary data at JAC Online). PCR Master Mix (2×) with *Taq* DNA polymerase (Fermentas) was applied for templates up to 3 kb; for longer targets GoTaq® Long PCR Master Mix (Promega) or Phusion™ High-Fidelity DNA Polymerase (New England Biolabs) was used.

Phenotypic and genotypic characterization of antibiotic resistance

Antibiotic resistance phenotypes were investigated by broth microdilution susceptibility testing in cation-adjusted Mueller–Hinton broth (Becton, Dickinson and Company) with 2.5% (volume/volume) laked horse blood (Oxoid) at 37°C for 48 h, based on the CLSI guidelines M45-A2 for *L. monocytogenes*.²⁴ The following antibiotics were tested: minocycline, rifampicin, streptomycin, tetracycline and trimethoprim. *L. monocytogenes* DSM 20600^T and *Streptococcus pneumoniae* ATCC 46919 were

used as reference/quality control strains. Antibiotic resistance genotypes were determined with biotin-labelled DNA hybridizing on a custom-made microarray (AMR+ve-2 ArrayTubes™, Alere Technologies GmbH, Jena, Germany) targeting > 100 antibiotic resistance genes of Gram-positive bacteria, including *dfrG*. This microarray was based on a previous publication.²⁵ PCR assays (Table S1, available as Supplementary data at JAC Online) were applied to confirm the presence of antibiotic resistance genes, as well as of the transposon integrase gene *int*.

Antibiotic resistance gene transfer by filter mating

In vitro conjugation experiments on nitrocellulose membrane filters (0.45 µm; Millipore AG) were performed as described before²⁶ using streptomycin-resistant *L. monocytogenes* 10403S²⁷ and rifampicin-resistant *E. faecalis* JH2-2²⁸ as recipients. Briefly, 1% of overnight cultures of donor and recipient were incubated in fresh BHI medium for 6 h before conjugation experiments, a mixture (1:3) was passed through a sterile filter followed by a washing step with 2 mL of diluent [0.85% NaCl, 0.1% peptone from casein (VWR), pH 8.0] and incubation overnight at 37°C. Cells were removed from the filter with 2 mL of diluent in a 50 mL reaction tube under shaking on a vortex-mixer. Isolation of transconjugant and donor colonies was obtained by spreading appropriate dilutions onto selective agar medium, supplemented with corresponding antibiotics (13 mg/L rifampicin, 26 mg/L streptomycin, 26 mg/L tetracycline and 13 mg/L trimethoprim). *L. monocytogenes* transconjugants were selected on PALCAM agar (Oxoid) at 37°C and *E. faecalis* transconjugants were selected on Difco™ KF Streptococcus Agar (Becton, Dickinson and Company) at 43°C, respectively. The transfer rate was calculated as the number of transconjugants per donor.

Antibiotic resistance gene transfer on salmon and cheese surfaces

In situ conjugation experiments were performed on the surface of smoked salmon and of smear-ripened cheese on the basis of the filter mating experiments. Swiss semi-hard cheese with a fully developed smear after a medium ripening time (90 days) was used. Cells of donor and recipients were grown for 6 h in BHI medium, 2 mL were centrifuged and the pellets were resuspended in the same volume of diluent. Thereafter, 0.1 mL of donor and 0.3 mL of recipient suspensions (~10⁶–10⁷ cells each) were spread onto the salmon or cheese surface (about 70 cm²), respectively, and the food samples were stored for 5 days in closed boxes containing distilled water in a side compartment to retain a humid environment. Salmon samples were stored both at 9°C and 22°C, respectively, and cheese samples at 22°C. The concentration of inocula was determined by plating onto BHI agar medium and calculated as cfu/cm². After the incubation period, the cheese smear was scraped off with a sterile knife and stomached in 10 mL of diluent, whereas the complete salmon sample (15–20 g) was stomached in 20 mL of diluent. Appropriate dilutions were plated onto selective agar medium.

Identification of transconjugants

After every mating experiment, three donor colonies and up to 10 transconjugant colonies were isolated from corresponding selective agar media. PCR assays (Table S1, available as Supplementary data at JAC Online) were applied to determine species affiliation, as well as presence of antibiotic resistance genes. Phenotypic resistance was tested by the broth microdilution method as described above. As both TTH-2007 and 10403S are *L. monocytogenes* strains with serovar 1/2a, transconjugants were differentiated from donor and recipient strains by the presence of antibiotic resistance genes and rep-PCR fingerprinting, amplifying repetitive extragenic palindromic elements, which allows discrimination between bacterial strains.²⁹ Furthermore, the highly polymorphic

virulence gene *actA* was partially sequenced, as it allows effective differentiation of *L. monocytogenes* strains.³⁰ Presence of circular forms of Tn916-like transposons was tested by PCR (Table S1, available as Supplementary data at JAC Online).

Results

Characterization and sequencing of Tn6198

L. monocytogenes TTH-2007 exhibited phenotypic resistances to tetracycline (64 mg/L), minocycline (32 mg/L) and trimethoprim (>320 mg/L), which were attributed to *tet(M)* and *dfrG*, respectively. Presence of the transposon integrase gene *int* and absence of plasmids suggested association with the Tn1545-Tn916 family of conjugative transposons.³¹ By long PCR with combinations of primers derived from *tet(M)*, *dfrG*, *int* and Tn916 (GenBank accession no. U09422) together with a primer walking strategy, most of the sequence of the novel transposon was obtained. Ring closure was achieved with DNA of its circular intermediate. Tn6198 had a length of 21322 bp (GenBank accession no. JX120102) and comparison with Tn916 (Figure 1a) revealed the integration of a 3285 bp DNA fragment including the *dfrG* gene between open reading frames (ORFs) 23 and 24 of Tn916. This fragment (named F23-24) was identical to a nucleotide sequence (GenBank accession no. AB205645) of *S. aureus* strain CM.S2, in which the trimethoprim resistance dihydrofolate reductase gene *dfrG* was first described.²¹ Therefore, the ORFs next to *dfrG* were named *orfU1* as designated in the above-mentioned publication and *orfU2*, respectively (Figure 1a). Apart from *dfrG* fragment F23-24, the sequences of Tn6198 and Tn916 share a similarity of >99.9%.

Direct repeat sequences of 11 bp, which mark the point of insertion of *dfrG* fragment F23-24, as well as inverted repeats of 27 bp were located (Figure 1b). The circular form of this fragment was detected by PCR with primers A and B (Figure 1a and Figure S1, available as Supplementary data at JAC Online).

Furthermore, by using an established PCR assay including primers L1 and R1³² we demonstrated the presence of the circular form of a Tn916-like antibiotic resistance carrier in TTH-2007 (Figure S1). However, the location of primer R1 to the left of the inserted *dfrG* fragment F23-24 (Figure 1a) did not enable differentiation between the circular intermediate of Tn916 and Tn6198. Therefore, a PCR assay with primers C and D was applied leading to both a DNA fragment of 770 bp lacking the *dfrG* insertion (Figure 1c and Figure S1, available as Supplementary data at JAC Online) and a fragment of about 4 kb containing the insertion (Figure 1d and Figure S1). This demonstrated the presence of an additional copy of Tn916 next to Tn6198 in strain TTH-2007. The detection of two different joint sequences, which are known to be formed by the flanking regions of a transposon before its excision,³³ confirmed the presence of both transposons. The 6 bp sequence between the termini of Tn916 comprised the bases AAGTAA and for Tn6198 it comprised the bases ATTATA (nucleotides 1–6 in JX120102).

In vitro and in situ gene transfer studies

MICs of antibiotics obtained by broth microdilution testing were a prerequisite for correct experimental selection of donor and recipient cells. Strain TTH-2007 showed high MIC values for tetracycline and trimethoprim (see above), but low levels for rifampicin (0.016 mg/L) and streptomycin (0.25 mg/L). Recipient *L. monocytogenes* 10403S had a high MIC value of streptomycin (>256 mg/L) and *E. faecalis* JH2-2 of rifampicin (32 mg/L), respectively; however, both were susceptible to tetracycline and trimethoprim with values ≤ 0.5 mg/L.

Horizontal gene transfer was observed between *L. monocytogenes* strains TTH-2007 and 10403S after mating on filters, where transfer rates between 1.3×10^{-8} and 3.0×10^{-6} transconjugants per donor were detected. We were able to assign transconjugant colonies to strain 10403S because of an additional

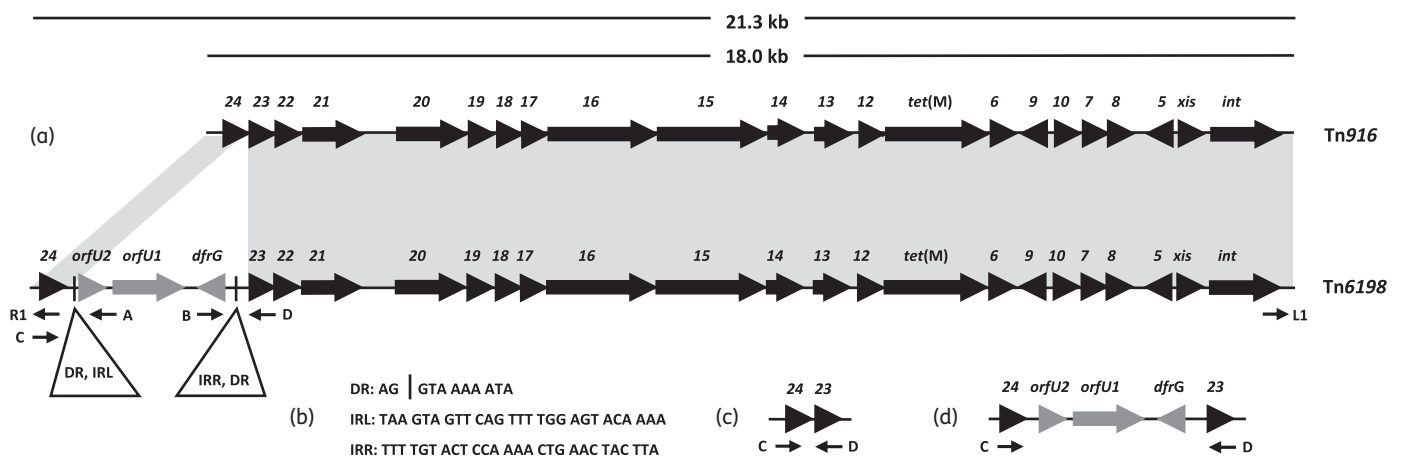


Figure 1. (a) Comparative schematic genetic organization of *E. faecalis* transposon Tn916 (GenBank accession no. U09422) and the novel *L. monocytogenes* transposon Tn6198 (GenBank accession no. JX120102). Grey areas indicate regions with >99.9% sequence identity. Black arrows indicate ORFs in Tn916 nomenclature. Grey arrows show ORFs of the integrated *dfrG* fragment F23-24 and black vertical lines indicate its insertion site, flanked by direct repeats (DR) and inverted repeats (IRL and IRR). Lengths of transposons are illustrated in kb and primers (A, B, C, D, L1 and R1) by small black arrows. (b) Sequences of direct and inverted repeats (DR, IRL and IRR); the black vertical line indicates the integration site. (c) PCR product with primers C and D for Tn916, without *dfrG* fragment F23-24. (d) PCR product with primers C and D for Tn6198, with *dfrG* fragment F23-24.

band at about 350 bp in the fingerprint obtained by rep-PCR compared with strain TTH-2007. Furthermore, 2% difference in their *actA* sequences enabled distinction between both strains (data not shown). In the *in situ* experiments on salmon and cheese surfaces, the concentration of the donor and recipient strains was stable during the incubation time (10^5 – 10^6 cfu/cm²) and transconjugants were obtained with transfer rates of 10^{-7} – 10^{-6} transconjugants per donor, similar to the filter mating experiments. There was no significant difference between storage of the salmon at 9°C or 22°C. Fewer transconjugants were obtained when *E. faecalis* JH2-2 was used as recipient strain in the filter matings, resulting in a transfer rate of 10^{-9} . No *E. faecalis* transconjugants were found on the salmon or cheese surface and enterococcal counts decreased up to 2 logs during storage.

Both Tn6198 and Tn916 were present in the donor, so we tested whether the transposons were transferable together or separately *in vitro* and *in situ*. Therefore, selection of transconjugants was performed by application of 26 mg/L tetracycline and 13 mg/L trimethoprim, as well as 26 mg/L tetracycline or 13 mg/L trimethoprim alone, respectively. The different types of transconjugants are listed in Table 1. Interestingly, the two transposons were detected together in several transconjugants selected with tetracycline and trimethoprim, although Tn6198 alone already confers resistance to both antibiotics. In contrast, after selection with only 13 mg/L trimethoprim, all transconjugants harboured Tn6198 but no additional copy of Tn916. Selection with 26 mg/L tetracycline alone led to three different types of transconjugants, including transfer of solely Tn916, providing only resistance to tetracycline.

The sequence of Tn6198 obtained from the donor strain TTH-2007 (Figure 1a) was identical to the corresponding sequence in an *L. monocytogenes* transconjugant to which Tn916 was not transferred. This control demonstrated the correctness of the sequence of Tn6198.

Discussion

Tn6198 is the first Tn916-like element harbouring a trimethoprim resistance gene (*dfpG*) next to the *tet(M)* gene. The presence of this transposon in *L. monocytogenes* is notable because such

transferable elements are rare in this species. Integration of accessory resistance genes into Tn916 was reported previously for, among others, *Streptococcus pneumoniae*,³⁴ where *erm(B)* confers resistance to erythromycin (Tn6002, originally found in *Streptococcus cristatus* and Tn3872) and *aphA-3* in addition to kanamycin (Tn1545³⁵ and Tn6003). A combination of the resistance genes *tet(M)*, *tet(L)* and *erm(T)* was recently detected on Tn6079 in the gut of an infant.³⁶ Apart from antibiotic resistance, Tn6009 harbouring the mercury resistance gene *mer* next to *tet(M)* was described in *S. aureus*³⁷ and Tn6087 conferring resistance to both tetracycline and the antiseptic cetrimide bromide seems to be widespread among *Streptococcus oralis* strains.³⁸ Generally, the presence of more than one antibiotic resistance gene seems to be an exception in *L. monocytogenes*, as only a few cases have been reported, from France, Greece and Switzerland. In all these incidents, resistance was plasmid mediated.²⁰ Concerning the recent report of a tetracycline- and trimethoprim-resistant foodborne *L. monocytogenes* isolate from France, the authors did not elaborate whether *dfpD* is encoded on plasmid pIP823 or whether there is a connection to *tet(M)*, which seems to be present on a Tn916-like transposon.¹² Until now, *dfpG* was mainly detected in enterococci³⁹ and staphylococci,⁴⁰ where it was characterized and reported in 2005.²¹ As the fragment with *dfpG* and *orfU1* detected in *S. aureus* CM.S2 was flanked by inverted and direct repeats, the authors suggested involvement of an insertion sequence (IS). Due to a high similarity of the fragment to plasmid pMG1 from *E. faecium*, they assumed that it might originate from this species. However, its equivalent in pMG1 additionally contains two ORFs in the central region that correspond to *orfA* and *orfB* of IS1485, encoding for transposases.⁴¹ They seem to have been lost through a deletion that turned the element into a mobile insertion cassette where passenger genes with unknown functions are present instead of transposases.⁴² This process might be an explanation of how fragment F23-24 ended up in Tn916, turning it into Tn6198. Recently, the same fragment was also detected in the genome of clinical *Streptococcus pyogenes* isolates in India.⁴³

We demonstrated that both Tn6198 and Tn916 are present in *L. monocytogenes* TTH-2007. The fact that nearly identical Tn916-like elements can coexist in bacterial strains was observed

Table 1. Presence or absence of different types of transconjugants after matings on filters, cheese and salmon from donor *L. monocytogenes* TTH-2007 to recipients *L. monocytogenes* 10403S and *E. faecalis* JH2-2

Recipient	Selection	Filter mating, <i>L. m.</i> TTH-2007 as donor of:			Cheese mating, <i>L. m.</i> TTH-2007 as donor of:			Salmon mating, <i>L. m.</i> TTH-2007 as donor of:		
		Tn6198	Tn916	Tn6198 and Tn916	Tn6198	Tn916	Tn6198 and Tn916	Tn6198	Tn916	Tn6198 and Tn916
<i>L. m.</i> 10403S	TMP ₁₃	+	–	–	–	–	–	+	–	–
<i>L. m.</i> 10403S	TET ₂₆	+	+	+	ND	ND	ND	+	+	+
<i>L. m.</i> 10403S	TET ₂₆ +TMP ₁₃	+	–	+	+	–	+	+	–	+
<i>E. f.</i> JH2-2	TMP ₁₃	–	–	–	–	–	–	–	–	–
<i>E. f.</i> JH2-2	TET ₂₆	–	+	+	–	–	–	–	–	–
<i>E. f.</i> JH2-2	TET ₂₆ +TMP ₁₃	+	–	+	–	–	–	–	–	–

L. m., *L. monocytogenes*; *E. f.*, *E. faecalis*; TMP₁₃, 13 mg/L trimethoprim; TET₂₆, 26 mg/L tetracycline; +, presence of transconjugants; –, absence of transconjugants; ND, not determined.

before for clinical *E. faecium* isolates.⁴⁴ Both transposons were transferable together or separately by conjugation *in vitro*. In contrast, although we detected *dfrG* fragment F23-24 as a circular form (Figure S1, available as Supplementary data at JAC Online), it seems not to be transferable on its own. This is in accordance with the previous finding that intra-species conjugal transfer of *dfrG* from donor strain *S. aureus* CM.S2 was not successful.²¹ In our study, *dfrG* was only transferred together with the Tn916 part of Tn6198, which provided the required elements for conjugation.

In addition to horizontal gene transfer by mating on filters, transmission of Tn6198 and Tn916 was demonstrated within the smear of semi-mature cheese stored at 22°C and on the surface of smoked salmon at both 9°C and 22°C. This is the first description of such an event on cheese and salmon surfaces. However, further tests with different storage times and temperatures should be performed to detect the physical parameters influencing the transferability of Tn6198. The transfer of the conjugative transposon Tn6198 on cheese surfaces is an especially important finding, as *Listeria* spp. can be in close contact with high concentrations of enterococci and other bacteria during cheese ripening and storage. Despite the fact that total bacterial counts on salmon are generally lower than on cheese, both food matrices provide favourable conditions for horizontal gene transfer where *Listeria* can act both as donor and recipient. Even though it is not known where the clinical strain TTH-2007 received Tn6198, as almost all *L. monocytogenes* infections in humans are caused by the consumption of food, it is important to determine the transferability of mobile genetic elements in the foodstuffs where *Listeria* spp. occasionally cause problems.

In conclusion, we detected a clinical multidrug-resistant *L. monocytogenes* isolate associated with a novel conjugative Tn916-like transposon Tn6198. In addition to providing its complete sequence (GenBank accession no. JX120102), we demonstrated its transferability both *in vitro* and *in situ* on the surface of salmon and cheese, respectively, indicating further opportunities for spread.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figure S1 are available as Supplementary data at JAC Online (<http://jac.oxfordjournals.org>).

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