Extreme genetic diversity in the type VII secretion system of *Listeria monocytogenes* suggests a role in bacterial antagonism

Kieran Bowran and Tracy Palmer*

Abstract

The type VII protein secretion system (T7SS) has been characterized in members of the phyla Actinobacteria and Firmicutes. In mycobacteria the T7SS is intimately linked with pathogenesis and intracellular survival, while in Firmicutes there is mounting evidence that the system plays a key role in interbacterial competition. A conserved membrane-bound ATPase protein, termed EssC in *Staphylococcus aureus*, is a critical component of the T7SS and is the primary receptor for substrate proteins. Genetic diversity in the *essC* gene of *S. aureus* has previously been reported, resulting in four protein variants that are linked to specific subsets of substrates. Here we have analysed the genetic diversity of the T7SS-encoding genes and substrate proteins across *Listeria monocytogenes* genome sequences. We find that there are seven EssC variants across the species that differ in their C-terminal region; each variant is correlated with a distinct subset of genes for likely substrate and accessory proteins. EssC1 is most common and is exclusively linked with polymorphic toxins harbouring a YeeF domain, whereas EssC5, EssC6 and EssC7 variants all code for an LXG domain protein adjacent to *essC*. Some *essC1* variant strains encode an additional, truncated *essC* at their T7 gene cluster. The truncated EssC, comprising only the C-terminal half of the protein, matches the sequence of either EssC2, EssC3 or EssC4. In each case the truncated gene directly precedes a cluster of substrate/accessory protein genes acquired from the corresponding strain. Across *L. monocytogenes* strains we identified 40 LXG domain proteins, most of which are encoded at conserved genomic loci. These loci also harbour genes encoding immunity proteins and sometimes additional toxin fragments. Collectively our findings strongly suggest that the T7SS plays an important role in bacterial antagonism in this species.

INTRODUCTION

Protein secretion systems are found in almost all bacteria and are critical for processes such as nutrient capture and niche adaptation. To date, 10 distinct protein secretion systems (named types I–X) have been identified [1–3]. While most of these are exclusive to Gram-negative bacteria, the type VII secretion system (T7SS) is encoded by many Gram-positive Actinobacteria and Firmicutes [4, 5]. The T7SS has been heavily studied in pathogenic mycobacteria, where it is essential for virulence [6–8]. It is also linked to virulence in *Staphylococcus aureus*, supporting abscess formation during persistent infection [9, 10].

The T7SS comprises two subtypes – the T7a found in Actinobacteria and T7b in Firmicutes, that although related, have distinct differences [11, 12]. A hexameric membrane-bound ATPase, termed EccC in Actinobacteria and EssC

in Firmicutes, is common to both systems and is a critical component of the T7SS [13, 14]. It lies at the centre of a multi-subunit membrane complex that mediates protein transport across the cytoplasmic membrane [15–17]. The protein has four ATP-binding domains in its cytoplasmic C-terminus, each of which is essential for secretion activity [13, 14, 16–19], and the most C-terminal of these domains also plays a role in substrate recognition [13, 20, 21]. Substrates are recognized through a short C-terminal signal sequence, and secretion appears to be post-translational because at least some substrates are exported in a folded form [22–24].

In Firmicutes three additional membrane proteins, EsaA, EssA and EssB, and the cytoplasmic protein EsaB function alongside EssC to mediate T7 secretion [9, 25–29] (Fig. 1). Characterized substrates of the T7b system include proteins from the WXG and LXG families [9, 24, 30, 31]. In all T7b

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Author affiliations: *Microbes in Health and Disease Theme, Newcastle University Biosciences Institute, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK.

***Correspondence:** Tracy Palmer, tracy.palmer@newcastle.ac.uk

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Abbreviations: CC, clonal complex; ST, sequence type; Tm, transmembrane helix; T7SS, Type VII secretion system.

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Fig. 1. The Firmicutes T7b secretion system. (a) Loci encoding the functional components of the T7b secretion system in *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus subtilis*. The gene numbers for *L. monocytogenes* relate to the reference strain EGDe. (b) Subcellular location and topology of the T7b components. The two forkhead-associated domains (FHA1/FHA2) and the four ATPase domains (D0, D1, D2 and D3) of EssC are indicated. (c) The *ess* (T7) gene cluster of *S. aureus* strain NCTC8325. Genes encoding functional components are shaded in blue, secreted substrates in yellow, a chaperone protein in white and EsaG family immunity proteins in turquoise. (d) Schematic representation of *L. monocytogenes* EssC. Amino acid positions of the predicted domains are shown and the region that is variable across strains is indicated. Amino acid 771, shown boxed in red, is the position at which the truncated EssC proteins encoded in some *L. monocytogenes* *essC1* strains start to align with the full-length protein.

systems a conserved WXG protein, EsxA, is present. In *S. aureus* EsxA is essential for T7 secretion activity and may act as a structural/functional component of the secretion system [9, 25]. The genes encoding these essential T7 components are arranged contiguously (Fig. 1), and are often found in gene clusters encoding secreted substrates and accessory proteins [9, 29, 32].

The *S. aureus* T7SS is encoded at the *ess* locus. Although part of the core genome, it was noted that there is significant genetic diversity at the *ess* region across *S. aureus* strains [33]. Sequence divergence occurs part way through *essC*, giving rise to four *EssC* variants that differ in their C-terminal domains. Each *essC* variant is associated with a specific set of genes encoding T7 substrate proteins, and the variable C-terminal domain of *EssC* has been implicated in the recognition of strain-specific substrates [21, 33].

The most commonly studied *S. aureus* strains encode the *EssC1* variant (Fig. 1c). These strains produce a T7-secreted nuclease, *EsaD*, a member of the YeeF protein family, and are protected from self-intoxication by an immunity protein, *EsaG* [34, 35]. A chaperone protein, *EsaE*, interacts with both *EsaD* and *EssC* and likely targets the nuclease to the secretion machinery [34, 36]. Strains harbouring *essC1* account for approximately 50% of sequenced *S. aureus* [33]. Analysis of *EsaD* sequences across these strains shows that the nuclease domain is polymorphic, suggestive of an anti-bacterial toxin [34]. All *S. aureus* strains, including *essC2*, *essC3* and *essC4* strains that do not encode *EsaD*, have multiple genes coding for *EsaG* homologues at their *ess* loci, suggesting that *EsaD* targets staphylococci (Fig. 1c) [33, 34]. An anti-staphylococcal role for *EsaD* has been confirmed in competition experiments, where strains were sensitized to killing by the nuclease when *esaG* genes were deleted [34, 37].

Since the identification of *EsaD*, a growing number of T7-secreted antibacterial toxins have been described. *TspA*, a second polymorphic toxin found in *S. aureus*, has membrane-depolarizing activity that is neutralized by the *TsaI* immunity protein [37]. *Streptococcus intermedius* produces at least three antibacterial toxins, of which *TelB* is an NADase and *TelC* a lipid II phosphatase [30]. Moreover, recent reports indicate that the T7b systems of *Bacillus subtilis* and *Enterococcus faecalis* also have antibacterial activity [38, 39], indicating that bacterial antagonism is a common feature of several Firmicutes T7SSs.

Listeria monocytogenes is a Gram-positive firmicute and human foodborne pathogen that is able to invade and replicate intracellularly [40]. A T7SS is encoded in the genome and has been partially characterized in strains EGDe and 10403s, where it was shown to be dispensable for virulence and host cell invasion [32, 41]. Here we have analysed the genetic diversity at the T7 gene clusters of sequenced *L. monocytogenes* strains. We show that seven variants of *EssC*, which differ in their C-terminal region, are encoded across *L. monocytogenes*. Each variant is associated with specific genes encoding likely substrate proteins. We identify polymorphic toxins that fall into the YeeF (*EsaD*) and LXG

families and clusters of immunity proteins for self and non-self toxins. Our observations point to a major role for the T7SS in interbacterial competition.

METHODS

L. monocytogenes EGDe serovar 1/2a (NC_003210.1) was used as the reference strain in this analysis. *EssC*, encoded by *lmo0061*, was used as the query for Basic Local Alignment Search Tool Protein (BLASTP) analysis using the National Center for Biotechnology Information's (NCBI's) Reference Sequence (RefSeq) database of non-redundant protein sequences [42]. All identified *L. monocytogenes* *EssC* homologues were assigned a percentage identity to *lmo0061* using the BLOSUM62 scoring matrix and an expect threshold of 10. *EssC* sequence variants were aligned using the CLUSTALW algorithm of the European Bioinformatics Institute (EBI) via the Clustal OMEGA software (v.1.2.4) [43], and protein sequence alignments were viewed in Jalview [v.2.11.1.0] [44]. *EssC* sequences were further analysed through the construction of maximum-likelihood phylogenetic trees using the MEGAX software package (v.10.1.8) [45, 46]. The Pasteur Institute's Listeriomics tool [47] was used to acquire sequences of *EssCs* across 60 annotated *L. monocytogenes* genomes with information on lineage, clonal clusters and serotypes. The presence of sigma 70 promoters and terminator regions within the T7b locus was evaluated using BPROM and FindTerm, respectively [48]. Candidate Rho-independent terminator regions were analysed using ARNold [49].

Protein accession numbers were subjected to flanking genes (FlaGs) analysis [50] to establish the conservation of T7b genes across multiple *L. monocytogenes* strains. Gene products were analysed using BLASTP analysis, and the presence of transmembrane regions was predicted using TMHMM [51]. Homology modelling was achieved using the Protein Homology/analogy Recognition Engine (PHYRE [52]).

RESULTS

The T7SS gene cluster of *L. monocytogenes*

The functional components of the *L. monocytogenes* T7SS are encoded contiguously from *lmo0056* (*esxA*) through to *lmo0061* (*essC*; Fig. 1a; all gene numbers relate to type strain EGDe), with the gene order mirroring that of the *S. aureus* *ess* locus. In *S. aureus*, a peptidoglycan hydrolase, *EssH*, is encoded divergently to *esxA* and is essential for T7 substrate secretion across the cell wall [53]. Analysis of the genomic region immediately upstream of *esxA* in *L. monocytogenes* did not identify a homologue of *essH* at this locus, or elsewhere on the chromosome. A stem-loop structure is present in the *S. aureus* *esxA-esaA* intergenic region [25]. A similar stem-loop with an estimated Gibbs free energy of $-13.6 \text{ kcal mol}^{-1}$ and sharing 62% identity with that of *S. aureus*, is conserved across the sequenced *L. monocytogenes* genomes. In *S. aureus* the stem-loop plays a role in regulating the expression level of the genes immediately downstream of *esxA*, which are transcribed 10–100 fold lower than *esxA* [25, 54]. It likely

Table 1. Seven EssC variants identified encoded across *L. monocytogenes* genome sequences present in RefSeq

Variant	Sequence length (aa)	C-terminal amino acid sequence	No. of sequences	Percentage frequency
EssC1	1498	IAPNQ	71	26%
EssC2	1498	YEEEV	56	23%
EssC3	1496	KIPKG	56	16%
EssC4	1501	RMPKH	36	14%
EssC5	1483	IKLPN	33	13%
EssC6	1500	VKEEE	7	3%
EssC7	1490	VLSKS	12	5%

has a similar role in *L. monocytogenes*, as it has been reported that *essC* and *essB* are much more weakly expressed than *esxA* in strain EGDe [41].

Seven distinct EssC variants are encoded across *L. monocytogenes* strains

Analysis of the amino acid sequence of the T7 core components indicates that five of them (*EsxA*, *EsaA*, *EsaB*, *EssA* and *EssB*) have high sequence conservation (approximately 97% identity across strains present in RefSeq). By contrast, *EssC* sequences are much more variable, with less than 80% identity between strains. To further analyse the sequence variability of *EssC*, all *L. monocytogenes* full-length *EssC* sequences (274 in total) were extracted from NCBI RefSeq and aligned. It was observed that the proteins fall into seven different groups, here named *EssC1*–*7*. The sequences of *EssC1* and *EssC3* diverge from the other *EssC* groups at around the start of domain D2 (Figs 1a and S1, available in the online version of this article). The five other *EssC* variants are almost invariant in their proximal D2 sequences, starting to diverge from one another approximately 180 amino acids into this domain. As shown in Table 1, *EssC1* is the most common variant, being encoded in 26% of sequenced *L. monocytogenes* strains, and *EssC6* the least common, with only 3% occurrence.

To assess the distribution of *essC* variants across clonal complexes and evolutionary lineages [55, 56], the 65 complete *L. monocytogenes* *EssC* sequences were extracted from the Pasteur Institute's publicly available annotated collection and used to construct a maximum-likelihood phylogenetic tree (Fig. 2). While *essC6* strains are notably absent from the phylogenetic tree, the six other *EssC* variants are represented. The tree divides into two main branches, with the first comprising *EssC2*, *EssC4*, *EssC5* and *EssC7*, and the second solely comprising *EssC1*. As with the NCBI RefSeq database, the majority of the Pasteur Institute sequences encode *EssC1* or *EssC2*, which both show distribution across evolutionary lineages I and II. The remaining *essC* variants are confined to a single lineage, with the exception of *L. monocytogenes* FSL J1-208, which is the only *essC7* strain to belong to lineage IV out of those analysed. The four *essC3* strains belonging to CC69 of lineage III cluster outside of the main two branches away from the other variants.

Two variable genomic regions are found at the T7 gene cluster of *L. monocytogenes*

It was noted for *S. aureus* that genetic diversity at the *ess* locus starts within the *essC* gene and continues downstream [33]. Inspection of the genomic region 3' of *L. monocytogenes* *essC* reveals that it is exceptionally diverse across different strains, in agreement with prior studies that identified this region as a hypervariable hotspot [57, 58]. We identified two closely spaced regions of high variability (Figs 3a and S2). The first of these variable regions is bounded by *essC* at the 5' side and at the 3' side by a cluster of housekeeping genes (*lmo0075-lmot01*; encoding a predicted phosphoenolpyruvate mutase, 6-O-methylguanine DNA methyltransferase, a protein of the YjbI superfamily, a predicted D-isomer-specific 2-hydroxyacid dehydrogenase and a tRNA, respectively). The second variable region lies between *lmot1* at the 5' end and *lmo0082* (encoding a hypothetical membrane protein) at the 3' end (Fig. 3a).

YeeF family toxins are encoded in variable region 1 of *L. monocytogenes* *essC1* strains

Although variable region 1 is highly diverse across *L. monocytogenes*, strains with the same *essC* variant show some common features. For example, a shared set of genes are encoded immediately downstream of *essC* in all *essC1* strains (Figs 3b and S2). The first three of these genes, *lmo0062*, *lmo0063* and *lmo0064*, are syntenous with the three genes that are adjacent to *essC1* in *S. aureus* strains (Fig. 1c), and encode homologues of the substrate proteins *EsxC* (*Lmo0062*) and *EsxB* (*Lmo0063*), and the chaperone protein *EsaE* (*Lmo0064*). *Lmo0065* is also common to all *essC1* strains and encodes a small helical protein with some limited similarity to *S. aureus* *EsxD*.

The *essC1* sequences start to diverge at *lmo0066*. In each *essC1* strain this gene codes for a protein of the YeeF superfamily, to which the *S. aureus* toxin *EsaD* also belongs. However, we note that the encoded proteins have a highly variable C-terminal toxin domain across variant 1 strains. In total, 10 different toxin domains could be identified (Fig. S3, Table 2). In each case, the N-terminal 400 amino acids (the 'YeeF domain') are highly conserved and this region is predicted to be almost completely α -helical. In *S. aureus*

Fig. 2. Phylogenetic distribution of *essC* variants across strains of *L. monocytogenes*. Maximum-likelihood phylogenetic tree showing the presence of multiple *essC* variants across the four evolutionary lineages (I–IV) of *L. monocytogenes*. CC, clonal complex; ST, sequence type. The scale bar represents the number of substitutions per site.

Table 2. Ten distinct YeeF family toxin variants are encoded in variable region 1 of *essC1* strains

Toxin	Sequence length (aa)	Predicted function	Probable immunity
Toxin 1	604	VIP2 superfamily: ADP ribosyltransferase	Im1: ADP ribosyl glycohydrolase
Toxin 2	557	Unknown	Im2: unknown
Toxin 3	577	Predicted ribonuclease	Im3: SUKH-1 family immunity protein
Toxin 4	565	Predicted ribonuclease	Im4: SUKH-1 family immunity protein
Toxin 5	564	Structurally related to Tne2: NADase?	Im5: bipartite? haemagglutinin-related protein/Ssp translocon-related protein
Toxin 6	598	Ntox15 superfamily: predicted ribonuclease	Im6: DUF1851 domain-containing protein: structurally related to type VI immunity protein Atu4351 from <i>Agrobacterium tumefaciens</i>
Toxin 7	563	Structurally related to Tne2: NADase?	Im7: unknown
Toxin 8	609	Structurally related to <i>Streptococcus pyogenes</i> Spn: NAD ⁺ glycohydrolase?	Im8: unknown
Toxin 9	556	Unknown	Im9: unknown
Toxin 10	578	Unknown	Im 10: unknown

variant 1 strains, the EsaE chaperone recognizes the YeeF domain of EsaD, and it is likely that this highly conserved domain in *L. monocytogenes* *essC1* toxins is also recognized by EsaE. The toxin domains are predicted to have functions such as ADP ribosyltransferase, ribonuclease or NADase activity (Table 2), and each toxin is always paired with the same candidate immunity protein encoded directly downstream. Some *essC1* strains encode two YeeF domain proteins within this region, and some also encode a protein with a tuberculosis necrotizing toxin (TNT) domain, although this lacks a detectable YeeF domain (Figs 3b and S2). Other genes found within this region of *essC1* strains encode toxin fragments and orphan immunity proteins.

A conserved set of genes are found downstream of *essC2*

All *essC2* strains have a common set of genes immediately downstream of *essC* (Fig. 3c; numbered 21–28 for variant 2 genes 1–8). Most of these genes encode small proteins (>150 amino acids) of unknown function. The largest protein encoded in this cluster, represented by B0647_RS15290 (gene 26 in Fig. 3c) is approximately 320 amino acids in size and is strongly predicted to be a D-isomer-specific 2-hydroxyacid dehydrogenase family protein. Curiously, this is the same predicted function as that for the gene encoded by *lmo0078* in the invariant housekeeping cluster, although the two proteins have no detectable sequence identity. Two of the genes in the *essC2* variable region 1 cluster encode related proteins (24 in Fig. 3c). This duplication is seen in all strains, and there is always an approximate 500 bp intergenic region between the last two genes in the cluster.

Genes 24–28 in this cluster seem to form a module of six genes (always in the order 24–25–26–27–28–24; with an extended intergenic region between genes 28 and 24). This

module is often but not always found in variable region 1 of other *essC* variants, and sometimes additional copies of gene 24 also flank this cluster (Fig. S2). Within this module genes 24 and 25 may form a pair, as they are occasionally co-occur away from the other genes of the module. Many orphan copies of 24 are also found in the T7 gene clusters of most *essC* variants, suggesting that it may encode an immunity protein.

Downstream of these nine conserved genes the *essC2* strains are much more variable, but many strains encode multiple predicted antitoxins, including to the YeeF and LXG domain toxins present in other *L. monocytogenes* strain variants.

essC3 strains may encode a novel toxin

Many *essC3* strains analysed have a very short variable region 1, comprising two genes of unknown function (Fig. 4a). The second gene of the pair encodes a protein of approximately 660 amino acids, which is a similar size to other T7b toxins, although it is unrelated to either the LXG or YeeF domain proteins. It therefore may represent a novel toxin. A few *essC3* strains have larger gene clusters in variable region 1, with a few carrying the six-gene module [24, 25] that is present in all *essC2* strains. Orphan immunity proteins to other T7b toxins are also found in this region in some strains.

Seven conserved genes are found downstream of *essC4*

The first seven genes downstream of *essC4* are conserved in all *essC4* strains (41–47 in Fig. 4b). Most of the encoded proteins are <200 amino acids; the largest protein encoded by the cluster, at 433 amino acids, is the product of gene 44. Most of the proteins lack any identifiable domains, with the exception of 41, which is a member of the SMC_prok_A

Fig. 5. An LXG toxin is encoded in variable region 1 of *essC5*, *essC6* and *essC7* strains. (a, c) Two genetic arrangements of variable region 1 from (a) *essC5* and (c) *essC7*. (b) A single genetic arrangement is seen for *essC6* strains. Genes encoding candidate toxins are shaded yellow with the cognate immunity gene in green. Probable orphan immunity proteins are shown in peach (with likely immunity indicated where identified). Genes numbered 61–64 are common to *essC6* strains. Genes numbered 24–28 are common to *essC2* strains and those numbered 44–47 are common to *essC4* strains. Gene sizes and intergenic regions not to scale.

region. Several *essC4* strains also encode a copy of the *essC2* six-gene module at this locus (Fig. 4b).

***essC5*, *essC6* and *essC7* strains encode an LXG toxin in variable region 1**

Variable region 1 of *essC5*, *essC6* and *essC7* strains shares some superficial similarity (Fig. 5). An LXG family toxin is always

encoded by the third gene downstream of *essC*, followed by a probable immunity protein. The three toxins (toxin A in *essC5* strains, toxin B in *essC6* strains and toxin C in *essC7* strains) share some limited sequence identity (approximately 15–20%; Table 3, Fig. S4) in the first ~270 amino acids, but are less similar in the C-terminal toxin domains. Nonetheless, all three proteins can be structurally modelled with

Table 3. LXG toxins encoded in *L. monocytogenes* strains

LXG toxin	Sequence length (aa)	Predicted function	Probable immunity: function	Occurrence/location
Toxin A	458	Structurally related to colicin Ia: membrane depolarizing?	ImA: polytopic membrane protein	<i>essC5</i> strains variable region 1
Toxin B	423	Structurally related to colicin Ia: membrane depolarizing?	ImB: DUF5082 domain protein	<i>essC6</i> strains variable region 1
Toxin C	537	Structurally related to colicin Ia: membrane depolarizing?	ImC: unknown	<i>essC7</i> strains variable region 1
Toxin D	492	Unknown	ImD: unknown	<i>essC1</i> , <i>essC2</i> , <i>essC3</i> , <i>essC4</i> , <i>essC5</i> , <i>essC6</i> , <i>essC7</i> strains variable region 2
Toxin E	493	Unknown	ImE: unknown	<i>essC1</i> , <i>essC2</i> , <i>essC4</i> , <i>essC5</i> strains variable region 2
Toxin F	507	Structurally related to Thz2: NADase?	ImF: unknown	<i>essC1</i> , <i>essC2</i> , <i>essC3</i> , <i>essC7</i> strains variable region 2
Toxin G	479	Structurally related to colicin D/E5 nuclease domain	ImG: unknown	<i>essC1</i> , <i>essC2</i> , <i>essC3</i> , <i>essC5</i> , <i>essC7</i> strains variable region 2
Toxin H	462	Unknown	ImH: DUF1871 family protein	Cluster of ABC transporter-encoding genes
Toxin I	466	Unknown	ImI: unknown	Cluster of ABC transporter-encoding genes
Toxin J	435	Structurally related to colicin D/E5 nuclease domain	ImJ: unknown	Cluster of ABC transporter-encoding genes
Toxin K	436	Unknown	ImK: unknown	Cluster of ABC transporter-encoding genes
Toxin L	463	Structurally related to colicin D/E5 nuclease domain	ImL: unknown	Cluster of ABC transporter-encoding genes
Toxin M	468	Structurally related to NAD ⁺ glycohydrolase	ImM: immunity protein 74; pfam15603	Cluster of ABC transporter-encoding genes
Toxin N	478	EndoU-nuclease	ImN: unknown	Cluster of ABC transporter-encoding genes
Toxin O	465	Unknown	ImO: unknown	Cluster of ABC transporter-encoding genes
Toxin P	477	EndoU-nuclease	ImP: unknown	Cluster of ABC transporter-encoding genes
Toxin Q	475	EndoU-nuclease	ImQ: unknown	Cluster of ABC transporter-encoding genes
Toxin R	490	EndoU-nuclease	ImR: unknown	Cluster of ABC transporter-encoding genes
Toxin S	491	Unknown	ImS: unknown	Insertion between DNA-directed RNA polymerase and transketolase-encoding genes
Toxin T	517	Unknown	ImT: unknown	Insertion between DNA-directed RNA polymerase and transketolase-encoding genes
Toxin U	509	Unknown	ImU: unknown	Insertion between DNA-directed RNA polymerase and transketolase-encoding genes
Toxin V	482	Unknown	ImV: unknown	Insertion between DNA-directed RNA polymerase and transketolase-encoding genes
Toxin W	469	Structurally related to colicin D/E5 nuclease domain	ImW: cysteine-rich CPCC superfamily protein	Insertion between DNA-directed RNA polymerase and transketolase-encoding genes

Continued

Table 3. Continued

LXG toxin	Sequence length (aa)	Predicted function	Probable immunity: function	Occurrence/location
Toxin X	478	Unknown	ImX: unknown	Insertion between DNA-directed RNA polymerase and transketolase-encoding genes
Toxin Y	551	Structurally related to <i>Legionella</i> effector SdeA	ImY: immunity 70 family protein	Insertion between DNA-directed RNA polymerase and transketolase-encoding genes
Toxin Z	458	Unknown	ImZ: unknown	Insertion between leucine-rich repeat surface protein family BspA and oxetanocin A resistance protein OxrB
Toxin α	470	Unknown	Imα: unknown	Insertion between leucine-rich repeat surface protein family BspA and oxetanocin A resistance protein OxrB
Toxin β	491	Structurally related to the of the toxic C-terminal Tip2 of CdiA from <i>Pseudomonas aeruginosa</i> – haemagglutinin	Imβ: member of the prominin superfamily of membrane proteins	Insertion between nitronate monooxygenase and cation-translocating P-type ATPase-encoding genes
Toxin γ	433	Unknown	Imγ: unknown	Insertion between haem oxygenase IsdG and NUDIX hydrolase-encoding genes
Toxin δ	457	Unknown	Imδ: unknown	Insertion between haem oxygenase IsdG and NUDIX hydrolase-encoding genes
Toxin ε	492	EndoU-nuclease	Imε: unknown	Insertion between haem oxygenase IsdG and NUDIX hydrolase-encoding genes
Toxin ζ	502	Structurally related to TelC – probable lipid II phosphatase	Imζ: unknown	Insertion between effector binding domain-containing protein and DeoR/GlpR transcription factor encoding genes
Toxin η	512	Unknown	Imη: unknown	Insertion between effector binding domain-containing protein and DeoR/GlpR transcription factor encoding genes
Toxin θ	540	Unknown	Imθ: polytopic membrane protein	Insertion between effector binding domain-containing protein and DeoR/GlpR transcription factor encoding genes
Toxin λ	466	Unknown	Imλ: unknown	Insertion between glutamine-hydrolysing GMP synthase and α/β hydrolase-encoding genes*
Toxin μ	481	Unknown	Imμ: unknown	Insertion between glutamine-hydrolysing GMP synthase and α/β hydrolase-encoding genes*
Toxin π	462	Unknown	Imπ: unknown	Insertion between glutamine-hydrolysing GMP synthase and α/β hydrolase-encoding genes*
Toxin ρ	514	Structurally related to <i>Legionella</i> effector SdeA	Imρ: unknown	Insertion between chitinase ChIB and ABC transporter ATP-binding protein-encoding genes
Toxin σ	491	Fic superfamily	Imσ: unknown	Insertion between lytic polysaccharide monooxygenase and TetR/AcrR family regulator-encoding genes
Toxin φ	485	Unknown	Imφ: unknown	Insertion between histidine phosphatase family protein and ATP-dependent RNA helicase DbpA-encoding genes

*Two LXG toxins are often encoded at this locus in a head-to-head arrangement.

99% confidence using colicin Ia as a template (PHYRE [52]). Recently it has been shown that TspA, a T7-secreted protein from *S. aureus* that can also be modelled on the colicin Ia, is a membrane-depolarizing toxin [37], suggesting that these three *L. monocytogenes* toxins may also have a similar mode of action. The candidate immunity proteins for these toxins fall into three unrelated protein families (Figs 5 and S2, Table 3).

The *essC5* strains always encode a pair of small proteins of unknown function in-between *essC5* and the toxin A gene. Downstream of the candidate immunity gene the *essC5* strains start to diverge (Fig. 5a), with some strains encoding the six-gene module found in *essC2* strains, while others have the five-gene module found in *essC4*.

All *essC6* strains sequenced to date have an identical set of genes in variable region 1 (Fig. 5b). The first gene in this region encodes a 367 amino acid product that appears to be structurally related to a metallopeptidase (match to the crystal structure of the m16b metallopeptidase subunit from *Sphingomonas* sp. a1 with 96% confidence; HYRE [52]). The second gene encodes a 93 amino acid peptide that is a member of the DUF3130 family. Following the toxin and immunity gene are a cluster of five genes encoding proteins of between 136 and 151 amino acids. The final four genes in this cluster form a module (61–64) that is also found in variable region 1 of some *essC5* and *essC7* strains (Figs 5c and S2). The first two genes of the module are also found in some *essC3* strains (Fig. 4).

The majority of *essC7* strains have a relatively short variable region 1, comprising genes encoding two small proteins (115 and 113 amino acids, respectively) alongside the toxin- and immunity-encoding genes (Fig. 5c). A few strains also have the *essC6* four gene module at this region (Fig. 5c).

Some *essC* variant 1 strains encode truncated *EssC2*, *EssC3* and *EssC4* variants

During our analysis we observed that some *essC1* strains encode the conserved sets of genes found in variable region 1 of other *essC* variant strains. For example, strain N11-1255 encodes the complete eight-gene module [21–28] found in *essC2* strains, strain FSL L7-0763 encodes the candidate toxin found in *essC3* strains and strain CFSAN026581 encodes the full seven-gene module from *essC4* strains (Fig. 6a). In each of these cases, a truncated *EssC* protein comprising the C-terminal ~728 amino acids is also encoded immediately preceding these ‘foreign’ genes. The truncated *EssCs* (which are annotated as pseudogenes on the FlaGs output in Fig. S2) are much shorter than the full-length *EssC1* protein encoded by these strains; each one aligns to the canonical *EssC* sequence at residue 771 (Fig. 1d) and has a potential initiator methionine a few codons away. Strikingly, these truncated *EssCs* do not have the *EssC1* variable region, instead the variable region is from the same *essC* variant as the foreign genes they harbour (i.e. *essC2* for N11-1255, *essC3* for FSL L7-0763 and *essC4* for CFSAN026581).

We also noted that some *essC1* strains only encode part of the *essC2* and *essC4* conserved modules (Fig. 6b). For

example, strain PNUSAL000361 harbours genes 24–28 of the *essC2* module but lacks genes 21–23, and strain 25B09 encodes the final four genes [44–47] of the *essC4* conserved cluster, including the shorter version of gene 44 (Fig. S2). In these instances, no truncated *EssC* is encoded at the gene cluster. These findings suggest that the first three genes at these conserved clusters may be involved in mediating interaction with the variable region of the cognate *EssC*, potentially to target substrates or to promote functional assembly of the secretion system.

An LXG toxin is encoded at variable region 2 of many strains

The second variable region lies between the tRNA gene *lmoT01* and *lmo0082*, which encodes a small membrane protein of unknown function (Fig. 3a). We noted that while approximately 25% of strains have no ‘insertion’ in this region, many other strains have a small locus of two or three genes relating to the T7SS (Figs 7 and S2). Commonly the locus comprises a gene encoding an LXG toxin, along with one or two genes encoding probable immunity proteins (Fig. 7). Four different toxins were identified in variable region 2 across strains (Table 3; toxins D–G; Fig. S5). All four toxins have a highly conserved N-terminal region – the first 330 or so amino acids are almost invariant, but the sequences diverge substantially in the toxin domain (Fig. S5).

A small number of strains (approximately 4% of those we analysed) have two genes at this locus encoding a protein of 262 amino acids with three predicted transmembrane domains at its N-terminus and a one of 207 amino acids with an N-terminal Tir domain (Fig. S2). We also observed that approximately 5% of strains had undergone a recombination event next to the tRNA gene to introduce a recombinase gene at this locus (Fig. S2).

LXG proteins are encoded at other loci on the *L. monocytogenes* chromosome

To determine whether there may be LXG proteins encoded outside of the *L. monocytogenes* T7 gene cluster, we selected 14 strains (2 of each *essC* variant) from those listed in Fig. S2 and searched the genome annotations for the term ‘LXG’. This yielded 44 protein sequences containing this term, 17 of which we had not previously identified. We next entered the multiple protein accessions collectively into a single round of analysis using the FlaGs tool to see whether any of the encoding genes shared a common genomic location. The output (shown in Fig. S6 and summarized in Table 3) indicates that there are common genomic regions where toxins are likely to be encoded.

To ensure we had covered the diversity of LXG proteins in *L. monocytogenes*, we next searched RefSeq under the term ‘*Listeria monocytogenes* LXG protein’ which returned 569 protein accession numbers. Following manual inspection to see whether they differed from previously identified toxins, we found a further 10 unique protein sequences.

Fig. 6. An additional truncated EssC protein is encoded in some *essC1* strains. (a) Genetic arrangement of some example *essC1* strains encoding truncated EssCs derived from *essC2*, *essC3* and *essC4* strains as indicated. (b) Genetic arrangement of some example *essC1* strains that harbour conserved T7 genes from *essC2* and *essC4* strains but no truncated EssC. (b) Genes encoding toxins are shaded yellow with the cognate immunity gene in green. Probable orphan immunity proteins are shown in peach (with likely immunity indicated where identified) and genes encoding toxin fragments in hatched grey and yellow shading. Genes numbered 21–28 are common across all *essC2* strains; genes numbered 41–47 are common across all *essC4* strains. Gene sizes and intergenic regions not to scale.

To identify the chromosomal loci where these additional LXG proteins were encoded, we used two accessions for each toxin that we identified that is encoded outside of the T7 locus and collectively submitted them for flanking gene analysis (shown in Fig. S7 and summarized in Table 3).

It is clear that there are preferred chromosomal locations where LXG proteins are encoded, shown on a map in Fig. 8. We noted that a cluster of ABC transporter genes (*lmo0135/lmo0152*) is a hotspot for LXG protein-encoding genes, and across *L. monocytogenes* strains we detected

Fig. 7. LXG toxins are often encoded in variable region 2. Three genetic arrangements of variable region 2. Genes encoding candidate toxins are shaded yellow with the cognate immunity gene in green. Probable orphan immunity proteins are shown in peach (with likely immunity indicated where identified). Gene sizes and intergenic regions not to scale.

11 different candidate toxins (toxins H–R in Table 3) encoded at that region. Seven unique LXG protein-encoding sequences were also identified at an insertion site between DNA-directed RNA polymerase- and transketolase (*lmo0334/lmo0342*)-encoding genes (toxins S–Y in Table 3). As far as we could determine, each individual LXG protein is encoded at the same chromosomal locus across the strains. Other hotspots were also identified, although these generally encode fewer unique sequences.

The loci share a number of common features; insertions generally encode a single LXG protein, with the exception of the insertion between the glutamine-hydrolysing GMP synthase and α/β hydrolase-encoding genes, where two LXG protein-encoding genes are arranged in a head-to-head organization (Fig. S7). Also encoded at the LXG loci are the likely cognate immunity proteins and often additional orphan immunities and toxin fragments. Interestingly, in almost all instances the LXG-encoding gene is preceded by a pair of genes encoding the same two small proteins (numbered 4 and 3 in Figs S6 and 1 and 2 in Fig. S7). Although neither of these is classified as being in

the WXG100 superfamily, structural prediction suggests that they share the same WXG100 protein fold, and gene number 4 in Fig. S6/2 in Fig. S7 encodes a protein of the DUF3130/SACOL2603 family that has been linked to the T7SS by phylogenetic profiling.

It is striking to note that all of the LXG proteins encoded next to this gene pair share a similar LXG domain sequence (Fig. S8), although proteins encoded at the same loci are clearly more closely related. Whitney *et al.* [30] have previously shown that WXG proteins bind to their cognate LXG proteins to promote export by the T7SS. It is very likely that one or both of these small proteins plays a similar role, presumably interacting with the highly conserved N-terminal region. We also observed that three LXG proteins, toxins θ , μ and ϕ , are quite divergent in their N-terminal sequence (Fig. S8). In the case of toxins θ and ϕ , they are preceded by a different pair of small genes (346/349 and 319/324, respectively, in Fig. S7) that may encode cognate export partners. No candidate small genes immediately precede toxins σ and μ , and they therefore may rely on partner proteins encoded elsewhere in the genome.

Fig. 8. Chromosomal positions of LXG protein-encoding genes in *L. monocytogenes*. The positions of the loci at which LXG proteins may be encoded are shown on a genomic map of strain EGDe. LXG protein encoding in EGDe is shown as blue arrows, with the toxin assignment indicated. ** indicates the truncated variant of toxin α . The position of the T7 locus is indicated in green.

Finally, when we analysed the *bspA/oxrB* (*lmo0460/lmo0476*) locus in the genome of EGDe where we had observed LXG protein-encoding genes in other strains, we observed that this strain encodes an unusual truncated LXG domain protein at this locus (Fig. S7). The protein, Lmo0473, lacks the first 130 amino acids of a canonical LXG domain protein and is preceded by a gene encoding a protein with a bacteriophage abortive infection AbiH domain. A second AbiH domain protein is also encoded nearby, and it appears that EGDe and a few other strains may have undergone a genomic rearrangement or insertion event at the locus. A manual search using the sequence of Lmo0473 revealed that several *L. monocytogenes* strains encode an otherwise sequence identical protein at this locus, but it is longer and is a bona fide LXG protein (toxin α on the sequence alignment shown in Fig. S8). Curiously, these longer proteins are not found in the RefSeq database, which is used as input for the FlaGs program (and are therefore missing from Fig. S7), although there are several entries in the NCBI's non-redundant protein sequence database. It is not clear whether the truncated variant encoded by EGDe and other strains would be a substrate for the T7SS or if it may use an alternative secretion pathway.

DISCUSSION

In this study we have undertaken a genomic survey of the T7SS of *L. monocytogenes*. Our results show that there is exceptional diversity across strains in both the identity of the core component EssC and the likely secreted substrates.

We identified seven variants of EssC based on the sequence of the C-terminal 500–600 amino acids. The most common variant is EssC1, which is encoded in just over a quarter of sequenced *L. monocytogenes* strains, including the reference strain EGDe. The genetic organization of *essC1* to some extent mirrors that of *S. aureus* *essC1* strains, with genes encoding the secreted substrates EssxC and EssxB and the chaperone protein EsaE common between the two, and sharing conserved positioning. A toxin with an N-terminal YeeF domain is found in all *L. monocytogenes* *essC1* strains. In *S. aureus* EsaE interacts with the YeeF domain of EsaD and with EssC1 and appears to target the toxin to the secretion machinery [34, 36]. It is likely that the YeeF domain toxins are similarly targeted in *L. monocytogenes*. Since we failed to identify an EsaE-like protein encoded in any of the other *essC* variants, we speculate that other substrate proteins, such as the LXG domain proteins, do not require an EsaE-like protein for targeting to the T7SS.

Strains encoding the EssC5, EssC6 and EssC7 variants are the least common. These strains share some similarity at the *essC* locus; all three strains encode an LXG domain protein and likely immunity protein downstream of *essC*. In each case the LXG protein-encoding gene is separated from *essC* by two intervening genes. Based on the positioning of these genes and the fact that one or both encode small proteins, we speculate that they may be LXG partners required to mediate export.

For the other three *essC* variants the identity of substrate protein(s) encoded immediately downstream of *essC* is not obvious. Strains harbouring *essC3* variants always encode the same two proteins downstream, the larger of which may represent a novel secretion substrate. The *essC2* and *essC4* variants have clusters of genes in the vicinity of *essC* that mainly encode proteins of unknown function. Some or all of these genes are frequently found at the *essC* locus of other variant strains. Often a smaller module of five genes (*essC2*) or four genes (*essC4*) is found across other strain variants, including some *essC1* strains. Curiously, however, some other *essC1* strains contain the full complement of 'foreign' genes – in this case the strains always adjacently encode an 'orphan' truncated *EssC* covering the C-terminal 728 amino acids, which shares the same sequence as the *essC* variant from where the genes were acquired. It is not clear whether these shorter *EssC* proteins (which lack transmembrane domains) are functional and can integrate into the T7SS. Alternatively, they may serve as a site of recombination with the full-length *essC* gene, which would splice out the intervening genes and convert an *essC1* variant into an *essC2/essC4* strain.

We identified a second variable locus at the T7 gene cluster that is present in most, but not all, strains. One of four different proteins, each sharing a highly conserved LXG domain, was found to be encoded at this region. Outside of the T7 cluster we also identified additional loci where LXG proteins are encoded. In total, across all strains we identified 40 different LXG proteins. Although none of these LXG proteins have yet been characterized, some of them could be structurally modelled on nuclease, NAD⁺ glycohydrolase and membrane-depolarising toxins. Taken together, our findings imply an important role for the T7SS secretion system in bacterial antagonism and kin discrimination in *L. monocytogenes*. We note that prior studies failed to demonstrate a function for the T7SS in virulence and host cell invasion, if anything finding that it had a negative impact on *L. monocytogenes* infection. Intriguingly, genes encoding some of the T7SS components and the YeeF domain toxin are upregulated when strain EGDe is used to infect germ-free mice that have been colonized with *Lactobacillus* [59], which would be consistent with a role in interspecies competition. Our findings provide a framework for the functional analysis of the T7SS across the species.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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