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Atypical *Listeria innocua* strains possess an intact LIPI-3

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**Abstract**

**Background:** *Listeria monocytogenes* is a food-borne pathogen which is the causative agent of listeriosis and can be divided into three evolutionary lineages I, II and III. While all strains possess the well established virulence factors associated with the *Listeria* pathogenicity island I (LIPI-1), lineage I strains also possess an additional pathogenicity island designated LIPI-3 which encodes listeriolysin S (LLS), a post-translationally modified cytolytic peptide. Up until now, this pathogenicity island has been identified exclusively in a subset of lineage I isolates of the pathogen *Listeria monocytogenes*.

**Results:** In total 64 *L. innocua* strains were screened for the presence of LIPI-3. Here we report the identification of an intact LIPI-3 in 11 isolates of *L. innocua* and the remnants of the cluster in several others. Significantly, we can reveal that placing the *L. innocua* lls genes under the control of a constitutive promoter results in a haemolytic phenotype, confirming that the cluster is capable of encoding a functional haemolysin.

**Conclusions:** Although the presence of the LIPI-3 gene cluster is confined to lineage I isolates of *L. monocytogenes*, a corresponding gene cluster or its remnants have been identified in many *L. innocua* strains.

**Background**

*Listeria monocytogenes* is a food-borne pathogen which is the causative agent of listeriosis [1-5]. It has long been known that the characteristic haemolytic phenotype of *L. monocytogenes* is attributable to the activity of listeriolysin O (LLO), encoded by the *hly* gene located within *Listeria* Pathogenicity Island I (LIPI-1) [5]. However, more recently, it has also been revealed that several strains of lineage I *L. monocytogenes* (of four evolutionary lineages, serotype 4b strains within lineage I have been most commonly associated with outbreaks [6]) also possess an additional pathogenicity island (designated LIPI-3) which encodes a second haemolysin, designated listeriolysin S (LLS) [7-9]. Listeriolysin S (LLS) is not normally expressed in *vitro*, and *hly* mutants give a non-haemolytic phenotype on blood agar. LLS is one of a growing number of post-translationally modified cytolysins (post-translationally modified haemolytic peptides) that include the *Streptococcus pyogenes*-associated Streptolysin S (SLS) and the *Clostridium botulinum/Clostridium sporogenes*-associated Clostriadiolysin S and is a member of the broader family of thiazole/oxazole modified microcins (TOMMs) [9]. It has been established that LLS plays a role in the survival of *L. monocytogenes* in PMNs and also contributes to virulence in the murine model [8]. LIPI-3 consists of 8 genes arranged in the following order: llsAGHXBYDP. LlsA is the structural peptide; LlsB, Y and D are enzymes proposed to perform the post-translational modifications; LlsGH is an ABC transporter; LlsP is a protease; while LlsX is of unknown function [7,8]. The associated promoter, P_{llsA}, which is situated upstream of *llsA*, is not transcribed in standard laboratory media but is induced by oxidative stress. It has been suggested that expression of the LIPI-3 genes may be induced in the phagosome of macrophages [8]. When P_{llsA} is replaced by a constitutive promoter (P_{HELP}), a strongly haemolytic/cytolytic phenotype is revealed under laboratory conditions [8]. The inducible nature of LLS and its absence in many *L. monocytogenes* strains is probably responsible for the fact that this virulence factor has gone undetected until recently.

*Listeria innocua* is an avirulent species within the Genus *Listeria*. It has been proposed that *L. innocua* and *L. monocytogenes* have evolved from a common ancestor.
and differ predominantly due to the loss of virulence genes by *L. innocua* [10,11]. This is supported by the existence of atypical *L. innocua* isolates which retain LIPI-1 and other virulence factors [12,13]. In a previous investigation we demonstrated that none of 11 *L. innocua* isolates examined (one of which was initially classified as an *L. grayi* isolate) possessed the equivalent of the LIPI-3 [7,8]. In this study we extended our analysis to a larger collection of strains, which has revealed that several strains possess the remnants of a LIPI-3. In fact, 11 strains possess fully intact LIPI-3 which gives rise to a haemolytic phenotype when the genes are constitutively expressed.

**Methods**

**Strains and growth conditions**

Tables 1, 2, and 3 list the panel of *Listeria* strains used in this study. Strains were obtained from the Food Microbiology Microbial Collection (University College Cork) and the Special *Listeria* Culture Collection (SLCC). All strains were cultured at 37°C for 16 h in Brain Heart Infusion (BHI) broth or agar (Oxoid, Hampshire, UK) unless otherwise stated. Where necessary, the characterisation of strains as *L. innocua* was confirmed biochemically by means of the API listeria kit (BioMérieux, Lyon, France) and 16S ribosomal DNA (rDNA) with CO1 and CO2 primer pairs previously described by Simpson et al. [14]. *Escherichia coli* EC101 was used as an intermediate vector host. Antibiotics were incorporated as follows [8]: Erythromycin (Ery) 150 μg/ml *E. coli*, 5 μg/ml *L. innocua*. Chloroamphenicol (Cm) 10 μg/ml *E. coli* and *L. innocua*. Ampicillin (Amp) 100 μg/ml *E. coli*. 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) was incorporated at a concentration of 40 μg/ml.

**Sequence analysis**

A PCR-based strategy, employing the primer pair *llsA*For-*llsA*Rev, was employed to screen for the presence of the LLS structural gene, *llsA*. These and other primers corresponding to regions both within (1113for, 1114rev, 1115 rev, 1118rev, 1120rev) and surrounding (*araC* rev) the LIPI-3 of *L. monocytogenes* F2365 were employed to amplify flanking DNA sequences which were subsequently sequenced (MWG Biotech) (Table 4). Primer LinIn1080_F1, which was designed to amplify from the conserved gene, corresponding to *lin1080* in strain CLIP11262, was used to determine the position of LIPI-3 in *L. innocua* strains relative to this locus. Overlapping sequences were assembled and a consensus sequence was determined using

| Table 1 | LIPI-3 positive SLCC *L. monocytogenes* strains |
|----------|-----------------------------------------------|
| UCC strain ID | SLCC strain ID | Lineage* | Logged date | Source | Country of isolation | City of isolation |
| 63 | SLCC4352 | I | 28/04/1975 | Human | Spinal fluid | France | Nantes |
| 74 | SLCC4563 | I | 26/11/1975 | Human | Unknown | France | Rouen |
| 75 | SLCC4330 | I | 17/03/1975 | Human | Spinal fluid | France | Nantes |
| 79 | SLCC4309 | I | 14/02/1975 | Human | Liquor | Germany | Munich |
| 86 | SLCC3829 | I | 15/01/1973 | Animal | Goat | unknown | Unknown |
| 87 | SLCC3734 | I | 10/11/1972 | Food/animal | Milk | Denmark | Copenhagen |
| 89 | SLCC4580 | I | 15/12/1975 | Human | Unknown | France | Rouen |
| 94 | SLCC3659 | I | 26/05/1972 | Animal | Brain, Sheep | Germany | Frankfurt |
| 101 | SLCC6254 | I | 05/06/1985 | Feed | Silage (grass) | Norway | Unknown |
| 102 | SLCC6104 | I | 13/10/1984 | Environmental | Sewage | Germany | Unknown |
| 105 | SLCC3733 | I | 10/11/1972 | Food/animal | Milk | Denmark | Copenhagen |
| 106 | SLCC3606* | I | 06/03/1972 | Human | Unknown | Belgium | Bruxelles |
| 110 | SLCC2503 | I | 1966 | Human | CFS | Germany | Stuttgart |
| 113 | SLCC6088 | I | 13/10/1984 | Environmental | Sewage | Germany | Unknown |
| 118 | SLCC3834 | I | 15/01/1973 | Animal | Sheep, brain | Germany | Frankfurt |
| 121 | SLCC3760 | I | 24/11/1972 | Human | New born, liver | Peru | Lima |
| 133 | SLCC6606 | I | 02/06/1986 | Feed | Silage | Switzerland | Unknown |
| 143 | SLCC6092 | I | 13/10/1984 | Environmental | Sewage | Germany | Unknown |
| 148 | SLCC3732 | I | 10/11/1972 | Food/animal | Milk | Denmark | Copenhagen |
| 154 | SLCC3106 | I | 09/02/1970 | Human | Liquor | Germany | Idar-Oberstein |
| 156 | SLCC4157 | I | 09/05/1974 | Animal | Cow, Brain | Germany | Freiburg |

*Lineages revealed by allele specific oligonucleotide (ASO)-PCR [15].
## Table 2  *IlsA* negative *L. monocytogenes* strains

| UCC strain ID | SLCC ID | Lineage* | Logged date  | Source          | Country of isolation | City of isolation |
|---------------|---------|----------|--------------|------------------|----------------------|-------------------|
| 64            | SLCC3996 | I        | 31/08/1973   | Human            | Spinal fluid         | France            | Nantes            |
| 65            | SLCC4410 | II       | 15/07/1975   | Human            | Blood                | France            | Nantes            |
| 66            | SLCC4068 | II       | 08/01/1973   | Animal           | Red deer, faeces     | Germany           | Freiburg          |
| 67            | SLCC6303 | II       | 05/06/1985   | Feed             | Silage (grass)       | Norway            | Unknown           |
| 68            | SLCC6374 | II       | 05/06/1985   | Feed             | Silage (grass)       | Norway            | Unknown           |
| 69            | SLCC6342 | II       | 05/06/1985   | Feed             | Silage               | Norway            | Unknown           |
| 70            | SLCC4274 | I        | 26/11/1974   | Human            | Unknown              | Germany           | Freiburg          |
| 71            | SLCC4280 | II       | 16/12/1974   | Unknown          | Unknown              | Slovak Republic   | Bratislava        |
| 73            | SLCC4063 | II       | 08/01/1974   | Animal           | Cattle, faeces       | Germany           | Freiburg          |
| 76            | SLCC4349 | II       | 28/04/1975   | Human            | Blood                | France            | Nantes            |
| 77            | SLCC4290 | II       | 16/12/1974   | Unknown          | Unknown              | Slovak Republic   | Bratislava        |
| 78            | SLCC4400 | II       | 05/03/1974   | Animal           | Sheep, brain         | Germany           | Stuttgart         |
| 80            | SLCC4481 | II       | 27/10/1975   | Unknown          | Unknown              | Spain             | Madrid            |
| 81            | SLCC4077 | I        | 15/02/1974   | Human            | Blood                | France            | Nantes            |
| 82            | SLCC3852 | II       | 09/04/1973   | Animal           | Lamb, brain          | Germany           | Stuttgart         |
| 83            | SLCC4235 | II       | 16/09/1974   | Animal           | Hare, caecum         | Denmark           | Copenhagen        |
| 84            | SLCC4209 | II       | 12/08/1974   | Human            | Intestine            | Germany           | Heidelberg        |
| 85            | SLCC4230 | II       | 16/09/1974   | Animal           | Hare, caecum         | Denmark           | Copenhagen        |
| 88            | SLCC4592 | I        | 15/12/1975   | Human            | Unknown              | France            | Rouen             |
| 93            | SLCC3738 | II       | 10/11/1972   | Animal           | Horse                | Denmark           | Copenhagen        |
| 95            | SLCC4455 | II       | 10/09/1975   | Unknown          | Unknown              | Hungary           | Szolnok           |
| 96            | SLCC4439 | II       | 10/09/1975   | Unknown          | Unknown              | Hungary           | Szolnok           |
| 97            | SLCC4315 | I        | 14/02/1975   | Human            | Liquor               | Australia         | North Adelaide    |
| 98            | SLCC4234 | II       | 16/09/1974   | Animal           | Hare, caecum         | Denmark           | Copenhagen        |
| 99            | SLCC6108 | I        | 13/10/1984   | Environmental    | Sewage               | Germany           | Unknown           |
| 100           | SLCC643  | II       | 01/01/1958   | Human            | csf                   | USA               | Georgia           |
| 103           | SLCC6340 | II       | 05/06/1985   | Feed             | Silage               | Norway            | Unknown           |
| 104           | SLCC293  | III      | 01/01/1955   | Unknown          | Unknown              | USA               | Maryland          |
| 107           | SLCC3631 | I        | 12/04/1972   | Animal           | Sheep, brain         | Germany           | Frankfurt         |
| 108           | SLCC2671 | III      | 01/01/1967   | Unknown          | Unknown              | USA               | California        |
| 109           | SLCC2634 | III      | 1934         | Animal           | Ruminant             | USA               | Unknown           |
| 111           | SLCC6255 | II       | 05/06/1985   | Feed             | Silage (grass)       | Norway            | Unknown           |
| 112           | SLCC6202 | II       | 05/06/1985   | Feed             | Silage (grass)       | Norway            | Unknown           |
| 114           | SLCC6605 | II       | 02/06/1986   | Feed             | Silage (maize)       | Switzerland       | Unknown           |
| 115           | SLCC4138 | II       | 23/04/1974   | Animal           | Lymph node           | Togo              | Lome              |
| 116           | SLCC4617 | II       | 28/12/1975   | Unknown          | Unknown              | Switzerland       | Basel             |
| 117           | SLCC4618 | II       | 28/12/1975   | Unknown          | Unknown              | Switzerland       | Basel             |
| 119           | SLCC4101 | II       | 05/03/1974   | Animal           | Sheep, brain         | Germany           | Stuttgart         |
| 120           | SLCC4070 | II       | 08/01/1974   | Animal           | Cattle, faeces       | Germany           | Freiburg          |
| 123           | SLCC3939 | II       | 09/07/1973   | Human            | Blood                | Belgium           | Bruxelles         |
| 125           | SLCC3847 | II       | 09/04/1973   | Animal           | Fox, brain           | Slovenia           | Ljubljana         |
| 125           | SLCC3864 | II       | 09/04/1973   | Animal           | Calf, organs         | Germany           | Freiburg          |
| 126           | SLCC4079 | II       | 15/02/1974   | Human            | Meconium             | France            | Nantes            |
| 127           | SLCC4294 | II       | 16/12/1974   | Unknown          | Unknown              | Slovak Republic   | Bratislava        |
the Seqmanager programme (Lasergene 6) and deposited in Genbank (accession numbers KJ394487, KJ394488, KJ394489 and KJ394490). Putative open reading frames (ORFs) were identified and pair-wise alignment of protein sequences was carried out using Needleman-Wunsch global alignment algorithms accessed via the European Bioinformatics Institute (EBI) web server. Shading of multiple-aligned sequences was carried out using the Boxshade programme (version 3.2) accessed via the European Molecular Biology web server (EMBnet).

Constitutive expression of the LIPI-3 cluster of *L. innocua* strain FH2051

The *L. innocua* FH2051 *lls* genes were placed under the control of the strong constitutive synthetic promoter PHELP using the pORI-based repA-negative plasmid system as previously described by Cotter et al., with some modification [8]. Briefly, PHELP DNA was amplified with the primer pair PhelpFsoe(LI)/PhelpRsoe from the plasmid pPL2luxPHelp [16] and fused between two DNA fragments amplified from the regions flanking PllsA by splicing by overlap extension (SOE) PCR [17]. The upstream region was amplified with the primer pair PllsAchgA(LI) and PllsAchgB(LI) and the downstream region was amplified with primers PllsAchgC and PllsAchgD. All PCRs were performed using Vent DNA polymerase (NEB, New England Biolabs, MA, USA). The SOE PCR product was cloned into the multiple cloning site (MCS) of pORI280 following *PstI* and *EcoRI* (NEB) digestion and ligation with the Ligafast rapid DNA ligation system (Promega, Madison, USA). The sequence of the cloned product was verified with MCS primers pORI280For/Rev by MWG Biotech, Germany [18]. Pellet-paint (Novagen) precipitated plasmid was subsequently transformed into the intermediate repA-positive host *E. coli* EC101. The plasmid was co-transformed into *L. innocua* FH2051 with the highly temperature-sensitive plasmid pVE6007 which supplies RepA in trans. Transformed cells appeared as blue colonies following plating on BHI-Ery-Xgal at 30°C. The integration of pORI280 by single crossover homologous recombination was stimulated by picking a single blue colony from the transformation plate and incubating it on BHI-Ery-Xgal at 30°C for 24 h and subcultured twice on BHI-Ery-Xgal at 42°C. A second crossover event, resulting in the introduction of PHELP in place of PllsA and the eventual loss of the pORI280 vector, was screened for following multiple subcultures in the absence of antibiotic selection. The introduction of PHELP upstream of *llsA* in Ery resistant Cm sensitive colonies was confirmed by PCR. A haemolytic phenotype was determined by spotting 10 μL of an overnight culture of this strain onto Columbia blood agar (Oxoid) containing 5% defibrinated horse blood (TCS Biosciences, Buckingham, UK) and 1 mU/ml sphingomyelinase (Sigma) and examining after 24 h.

### Table 2 *llsA* negative *L. monocytogenes* strains (Continued)

| No. | Strain Code | Lineage | Date | Source | Species | Country | City |
|-----|-------------|---------|------|--------|---------|---------|------|
| 128 | SLCC4442    | II      | 10/09/1975 | Unknown | Unknown | Hungary | Szolnok |
| 129 | SLCC4444    | II      | 10/09/1975 | Unknown | Unknown | Hungary | Szolnok |
| 130 | SLCC3278    | I       | 03/09/1970 | Animal | Duck, liver | Denmark | Copenhagen |
| 131 | SLCC3270    | II      | 03/09/1970 | Animal | Hare, pus | Denmark | Copenhagen |
| 132 | SLCC3258    | II      | 02/09/1970 | Unknown | Unknown | Belgium | Bruxelles |
| 135 | SLCC5203    | II      | 17/11/1977 | Feed | Silage | Netherlands | Unknown |
| 136 | SLCC3683    | II      | 22/06/1972 | Environmental | Fir needle | Germany | Unknown |
| 137 | SLCC6611    | II      | 02/06/1986 | Environmental | Soil | Switzerland | Unknown |
| 138 | SLCC4153    | I       | 09/05/1974 | Animal | Faeces | Germany | Freiburg |
| 139 | SLCC3269    | II      | 03/09/1970 | Animal | Hare, spleen | Denmark | Copenhagen |
| 141 | SLCC3214    | II      | 18/06/1970 | Human | Spinal fluid | France | Lyon |
| 144 | SLCC6343    | II      | 05/06/1985 | Feed | Silage | Unknown | Unknown |
| 146 | SLCC3629    | I       | 04/04/1972 | Human | New born; intestine, liver | Peru | Lima |
| 147 | SLCC3569    | II      | 08/02/1972 | Animal | Hen | France | Alfort |
| 149 | SLCC3458    | I       | 08/07/1971 | Human | Unknown | France | Rouen |
| 150 | SLCC3457    | II      | 08/07/1971 | Human | Unknown | France | Rouen |
| 152 | SLCC3366    | I       | 11/03/1971 | Animal | Pig, brain | Germany | Freiburg |
| 153 | SLCC3277    | II      | 03/09/1970 | Animal | Bird, liver | Denmark | Copenhagen |

*Lineages revealed by allele specific oligonucleotide (ASO)-PCR [15].

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis was carried out following the CDC standardized PulseNet protocol for
Table 3  *Listeria innocua* strains used in this study

| UCC strain ID | SLCC strain ID | Serotype | Logged date | Source       | Country of isolation | City of isolation | IIsA PCR | LIPI-3 PCR |
|---------------|----------------|----------|-------------|--------------|----------------------|-------------------|----------|------------|
| 1             | SLCC7157*      | 6a       | 08/12/1986  | Animal       | Switzerland          | Bern              | ✓        | X          |
| 2             | SLCC7199       | 6b       | 18/12/1986  | Food         | Germany              | Munich            | ✓        | ✓          |
| 3             | SLCC6483       | 6b       | 05/03/1986  | Food         | Switzerland          | St.Gallen         | X        | ✓          |
| 4             | SLCC6109       | 6a       | 13/10/1984  | Sewage       | Germany              | Braunschweig      | X        | X          |
| 5             | SLCC6814       | 4c       | 07/05/1986  | Human        | UK                   | London            | ✓        | ✓          |
| 6             | SLCC6270       | 6b       | 05/06/1985  | Animal       | Norway               | Minde             | ✓        | X          |
| 7             | SLCC6276       | 6b       | 05/06/1985  | Animal       | Norway               | Minde             | ✓        | ✓          |
| 8             | SLCC6362       | 6b       | 05/06/1985  | Animal       | Norway               | Minde             | ✓        | X          |
| 9             | SLCC6370*      | 6b       | 05/06/1985  | Animal       | Norway               | Minde             | ✓        | X          |
| 10            | SLCC6382       | 6b       | 05/06/1985  | Animal       | Norway               | Minde             | ✓        | X          |
| 11            | SLCC6285*      | 6b       | 05/06/1985  | Feed         | Norway               | Minde             | ✓        | X          |
| 12            | SLCC6373       | 6b       | 05/06/1985  | Feed         | Norway               | Minde             | ✓        | X          |
| 13            | SLCC6098       | 6a       | 13/10/1984  | Sewage       | Germany              | Braunschweig      | X        | X          |
| 14            | SLCC6007       | 6a       | 10/08/1984  | Brasil       | Rio de Janeiro       |                   |          |            |
| 15            | SLCC6099       | 6a       | 13/10/1984  | Sewage       | Germany              | Braunschweig      | X        | X          |
| 16            | SLCC6364       | 6b       | 05/06/1985  | Animal       | Norway               | Minde             | ✓        | X          |
| 17            | SLCC6317*      | 6b       | 05/06/1985  | Animal       | Norway               | Minde             | ✓        | X          |
| 18            | SLCC7030       | 6a       | 14/11/1986  | Food         | Germany              | Munich            | ✓        | X          |
| 19            | SLCC6297*      | 6b       | 05/06/1985  | Feed         | Norway               | Minde             | ✓        | X          |
| 20            | SLCC6356       | 6b       | 05/06/1985  | Food/animal  | Norway               | Minde             | ✓        | X          |
| 21            | SLCC6235       | 6b       | 05/06/1985  | Silage       | Norway               | Minde             | ✓        | X          |
| 22            | SLCC6298       | 6b       | 05/06/1985  | Feed         | Norway               | Minde             | ✓        | ✓          |
| 23            | SLCC6203       | 6b       | 05/06/1985  | Silage       | Norway               | Minde             | ✓        | ✓          |
| 24            | SLCC7116       | 6a       | 17/11/1986  | Food         | Austria              | Innsbruck         | ✓        | X          |
| 25            | SLCC6353       | 6b       | 05/06/1985  | Food/animal  | Norway               | Minde             | ✓        | X          |
| 26            | SLCC6409       | 6b       | 05/06/1985  | Feed         | Norway               | Minde             | ✓        | X          |
| 28            | SLCC6541       | 6a       | 23/04/1986  | Food         | Germany              | Munich            | ✓        | X          |
| 29            | SLCC6927       | 6b       | 22/09/1986  | Food         | Germany              | Vienna            | ✓        | X          |
| 31            | SLCC6228       | 6b       | 05/06/1985  | Silage       | Norway               | Minde             | ✓        | X          |
| 30            | SLCC6749       | 6b       | 31/07/1986  | Food         | Germany              | Munich            | ✓        | ✓          |
| 32            | SLCC6322       | 6a       | 05/06/1985  | Feed         | Norway               | Minde             | ✓        | X          |
| 33            | SLCC5916       | 6a       | 16/03/1984  | Switzerland  | Lausanne            |                   | ✓        | X          |
| 34            | SLCC5226       | 6a       | 09/03/1979  | USA          | Richmond, Virginia   |                   | ✓        | X          |
| 35            | SLCC6283       | 6b       | 05/06/1985  | Feed         | Norway               | Minde             | ✓        | X          |
| 36            | SLCC6246       | 6b       | 05/06/1985  | Feed         | Norway               | Minde             | ✓        | X          |
| 37            | SLCC3533       | 4b       | 06/12/2010  | Environment  | Germany              | Freiburg          | X        | X          |
| 38            | SLCC6466       | 6b       | 30/01/1986  | Food         | Switzerland          | St.Gallen         | ✓        | ✓          |
| 39            | SLCC6359       | 6b       | 05/06/1985  | Animal       | Norway               | Minde             | ✓        | X          |
| 40            | SLCC6286       | 6b       | 05/06/1985  | Feed         | Norway               | Minde             | ✓        | X          |
| 41            | SLCC6294       | 6b       | 05/06/1985  | Animal       | Norway               | Minde             | ✓        | X          |
| 42            | SLCC6371       | 6b       | 05/06/1985  | Animal       | Norway               | Minde             | ✓        | X          |
| 43            | SLCC6119       | 6a       | 10/12/1984  | Human        | Germany              | Goettingen        | X        | X          |
| 44            | SLCC3947       | 4f       | 27/07/1973  | Human        | Germany              | Cologne           | X        | X          |
*L. monocytogenes* with *Aci* and *ApaI* as the restriction endonucleases. The PFGE patterns were analyzed using BioNumerics software [19].

**Results and discussion**

**Screening *L. monocytogenes* and *L. innocua* for homologues of *llsA***

To date LIPI-3 has been identified in ~60% (27 of 46) of lineage I *L. monocytogenes* but was absent from all lineage II (n = 23) and lineage III (n = 5) isolates tested [8]. As a consequence of gaining access to the Seeliger collection of *Listeria* isolates [20], we were provided with the opportunity to screen for the presence of LIPI-3 among an additional 83 *L. monocytogenes* isolates including 30 lineage I, 50 lineage II and 3 lineage III strains. The *llsA* gene was not identified in any lineage II or lineage III strain, consistent with our previous observations (Table 1). However, the *llsA* gene was identified in 70% of lineage I *L. monocytogenes* screened (21 of 30) and, on the basis of PCR amplification, in all cases the full complement of LIPI-3 genes was present. All LIPI-3 positive isolates belonged to Lineage I as verified by an allele specific oligonucleotide PCR multiplex (actA1-f, actA1-r, plcB2-f, plcB2-r, actA3-f, plcB3-r) based on the *prfA* virulence gene cluster [15], thus verifying previous observations with respect to the distribution of LIPI-3 among different evolutionary lineages of *L. monocytogenes* [7,8].

Access to the Seeliger collection and other strains also facilitated a further investigation of the LIPI-3 status of *L. innocua*. As stated, a previous analysis of 11 strains of *L. innocua* indicated that all lacked genes associated with LIPI-3 [7,8]. However, screening a larger collection of 64 *L. innocua* strains using *llsA* specific primers revealed that 45 strains (70.3%) were *llsA*-positive (Table 3). Further PCR-based analysis of these isolates, employing a variety of primers designed to amplify across and within the LIPI-3 (*llsA*For, *llsA*Rev, 1113for, 1114rev, 1115rev, 1118rev, 1120 rev, *araC*rev) revealed that 11 of these strains possess a cluster which is comparable in size, gene content and gene organisation to that of the LIPI-3 cluster found in a subset of lineage I *L. monocytogenes* strains. These 11 isolates originated from a number of European countries between 1984 and 2000, and were isolated from varied sources including processed chicken [1], cheese [7], sheep [7], silage [7] and human [1] (Table 3). Further analysis revealed that 25 *L. innocua* isolates possess a truncated LIPI-3 with no PCR product generated for *llsBYDP*. Sequencing the region confirmed that these genes are absent.

**Table 3 Listeria innocua strains used in this study (Continued)**

| No. | Code   | Host | Source                  | Country | City            | prfA Result | prfA Result |
|-----|--------|------|-------------------------|---------|-----------------|-------------|-------------|
| 45  | SLCC6519 | 6a   | Food                    | Cheese  | Germany Munich  | ✘           | ✘           |
| 46  | SLCC6408* | 6b   | Feed                    | Silage (grass) | Norway Minde | ✓           | ✘           |
| 47  | SLCC6296 | 6b   | Feed                    | Silage (grass) | Norway Minde | ✓           | ✓           |
| 48  | SLCC5328 | 6b   | 09/03/1979 Norway Minde | USA Richmond, Virginia | ✘           | ✘           |
| 49  | SLCC6279 | 6b   | 05/06/1985 Animal Sheep | Norway Minde | Minde         | ✓           | ✓           |
| 50  | SLCC6318 | 6b   | 05/06/1985 Animal Sheep | Norway Minde | Minde         | ✘           | ✘           |
| 51  | SLCC6542 | 6a   | 23/04/1986 Food Cheese  | Germany Munich | Minde         | ✓           | ✓           |
| 52  | SLCC6272 | 6b   | 05/06/1985 Animal Goat  | Norway Minde | Minde         | ✓           | ✓           |
| 53  | SLCC3835* | 6b   | 08/02/1973 Human        | Germany Cologne | Minde         | ✓           | ✓           |
| 54  | SLCC5998 | 6a   | 16/07/1984 Animal Cattle | Belgium Bruxelles | Minde         | ✘           | ✘           |
| 55  | SLCC6670 | 6a   | 02/06/1986 Food Milk    | Switzerland Bern | Minde         | ✘           | ✘           |
| 56  | SLCC6667 | 6a   | 02/06/1986 Food Milk    | Switzerland Bern | Minde         | ✘           | ✘           |
| 57  | SLCC5753* | 6b   | 16/11/1982 Slovak Republic | Bratislava | Minde         | ✓           | ✘           |
| 58  | SLCC7113 | 6b   | 17/11/1986 Food Cheese  | Austria Vienna | Minde         | ✘           | ✘           |
| 59  | SLCC6103 | 6b   | 13/10/1984 Sewage Sewage | Germany Braunschweig | Minde         | ✓           | ✘           |
| 60  | SLCC6543 | 6a   | 23/04/1986 Food Cheese  | Germany Munich | Minde         | ✓           | ✓           |
| 61  | SLCC6977* | 6c   | 13/10/1986 Food Cheese  | Germany Munich | Minde         | ✓           | ✓           |
| 62  | SLCC6921 | 6a   | 22/09/1986 Food Milk    | Switzerland Bern | Minde         | ✘           | ✘           |
| FH2034 | N/A | Unknown | 2000 Food Raw mince | Ireland Cork | Minde         | ✓           | ✓           |
| FH1836 | N/A | Unknown | 2000 Food Spinach canneloni | Ireland Cork | Minde         | ✓           | ✓           |
| FH2051 | N/A | Unknown | 2000 Food Chicken nuggets | Ireland Cork | Minde         | ✓           | ✓           |

*Possess *llsA* but not other LIPI-3 associated genes.*
in at least two isolates (SLCC6270 and SLCC6382). With the exception of \textit{llsP}, these genes have previously been found to be essential for LLS production in \textit{L. monocytogenes} [7]. Of the remaining 28 strains, 9 were found to contain \textit{llsA} but attempts to amplify across or within other LIPI-3 associated genes were unsuccessful and another 19 isolates lacked all LIPI-3 genes.

Two \textit{L. innocua} isolates, SLCC6382 and SLCC6270, containing a truncated LIPI-3, were selected for further analysis. Both SLCC6382 and SLCC6270 shared 98\% homology with respect to the structural peptide LlsA. The putative LlsG, LlsH and LlsX proteins from both strains shared 96\%, 99\% and 95\% identity with their \textit{L. monocytogenes} counterparts. \textit{llsB}, \textit{llsY}, \textit{llsD} and \textit{llsP} are absent from both isolates, while the AraC-like regulatory protein determinant was present with 98\% identity to the \textit{L. monocytogenes} cluster. As in \textit{L. monocytogenes}, the \textit{L. innocua} cluster is located downstream of a putative glutamine hydrolyzing GMP synthase protein (GuaA). However, the island in SLCC6382 and SLCC6270 commences 600 bases immediately downstream of \textit{guaA} and thus is not flanked by glyoxylase encoding genes, thereby contrasting with LIPI-3 in \textit{L. monocytogenes}.

Three strains (SLCC6466, SLCC6294, FH2051) possessing an entire LIPI-3 cluster were also selected for a more extensive investigation. Eight complete ORFs were identified, each corresponding to their homologue in the \textit{L. monocytogenes} LIPI-3 cluster (\textit{llsAGHXBYDP}). Sequence alignments confirmed considerable homology at the protein level (Figure 1). The structural peptide LlsA shared

| Table 4 Primers used in this study |
|-----------------------------------|
| **Primer name** | **Sequence (5’ to 3’)*** |
| PllsAchgA(LI) | GCCGCAGATCTGCCCCGTTCCTG |
| PllsAchgB(LI) | GAGGTTTCTGCTTGTCGGT |
| PhelpFsoe(LI) | GATGATGCTCTCTGTCAAG |
| PhelpRsoe | GCCTGATTTCCATCTGTC |
| PllsAchgC | ATGAATATTAAATACATCATC |
| PllsAchgD | TGGAATTCGCCGCTCCATCTGTC |
| pOR280For | CTTGTTCCATTAACCCCT |
| pOR280Rev | CGCTTCTTCCCCCAT |
| Lin1080_F1 | CGGTACCGAGTTGTTAGTTAGT |
| llsAFor | CGATTTCACAATGTGATAGGATG |
| llsARev | GCCGCTGCACACTCATAC |
| 1113for | GTTATGAGGTGAGTGC |
| 1114rev | GTCTGCGGATATGATCC |
| 1115rev | CACTAGCAGATTTGTTAGG |
| 1118rev | CAGACAGCAGCTGCTGTAAAG |
| 1120rev | CGTCCCCGCTTCTTGGACAG |
| araCrev | CTCTCCTTATTGCCTG |
| actA1-f | AATACAAACAGTGAACAAAGC |
| actA1-r | TATCACGTACCACCTTAC |
| plcB2-f | TGTTGATGAACTTACAAAC |
| plcB2-r | TTTGTACGATGTCTTCC |
| actA3-f | CGGGCAACCTACACAT |
| actA3-r | TTGGAATTTGCTGTCG |

*Restriction site in bold and SOE overhang italicised.
98% homology in the case of the three strains mentioned above to the \textit{L. monocytogenes} equivalent. These \textit{L. innocua} clusters also encode homologs of the putative two component ABC transport system LlsG and LlsH, with LlsG sharing 95.3% (FH2051) and 95% (SLCC6466, SLCC6294) identity, and 98.8% (FH2051) and 99% (SLCC6466, SLCC6294) with respect to LlsH. The putative LlsX homolog, which is of unknown function, is 97% identical to its \textit{L. monocytogenes} counterpart for all three isolates. This gene is believed to be specific to LIPI-3 since no homologue exists among other \textit{sag}-like gene clusters [7]. A corresponding cluster of putative Lls homologs, all of which are predicted to encode biosynthetic enzymes, were also identified [8]: LlsB (99% in the case of all three strains), LlsY (95.4% FH2051, 95% SLCC6466 and SLCC6294) and LlsD (98.4% FH2051, 98% SLCC6466 and SLCC6294). Finally, the \textit{L. innocua} cluster also carries putative LlsP and LmoF2365_1120 homologs, annotated as a CAAX amino-terminal putative metalloprotease and AraC-like regulatory protein which share 93.8% FH2051, 91% SLCC6466 and SLCC6294 and 91.3% FH2051, 94% SLCC6466 and SLCC6294 identity to the \textit{L. monocytogenes} cluster, respectively. PFGE was carried out to assess the relatedness of the 11 \textit{L. innocua} LIPI-3+ isolates. On the basis of this analysis, all LIPI-3’ isolates share a high degree of similarity, with the majority of strains (SLCC6466, SLCC6814, SLCC6749, SLCC6276, SLCC6279, SLCC6294, FH2051, SLCC6296 and SLCC6298) displaying 80% similarity and strains SLCC6203 and SLCC7199 sharing 76% identity (Figure 2).

The LIPI-3’ \textit{L. innocua} FH2051 is non-haemolytic when grown on Columbia blood agar (Figure 1). This is not surprising given that \textit{L. innocua} strains do not produce LLO and the fact that it has previously been established that LLS is not produced by wild type \textit{L. monocytogenes} in laboratory media. It has been established that the latter is due to the fact that \textit{P}_{llsA} is not transcribed under standard laboratory conditions [8]. It has been noted previously that \textit{P}_{llsA} is induced under oxidative stress but, unfortunately, the requirement for an oxidizing agent prevents an assessment of associated haemolytic activity on blood agar [7]. Thus, to investigate the functionality of the LIPI-3 cluster in \textit{L. innocua}, here we constitutively expressed LIPI-3 through the introduction of the constitutive Highly Expressed \textit{Listeria} Promoter [\textit{P}_{HELP}, \textit{LLS}C] upstream of \textit{llsA} in \textit{L. innocua} FH2051, to create FH2051LLSC. Examination of the resultant strain revealed that the \textit{L. innocua} LIPI-3 is indeed functional as evidenced by a clear haemolytic phenotype on Columbia blood agar (Figure 3).

**Conclusion**

In conclusion, we have established that although the presence of the LIPI-3 gene cluster is confined to lineage I isolates of \textit{L. monocytogenes}, a corresponding gene cluster or its remnants can be identified in many \textit{L. innocua}. It is now generally accepted that \textit{L. innocua} and \textit{L. monocytogenes} evolved from a common ancestor, with \textit{L. innocua} having lost virulence genes since this division. Although rare, \textit{L. innocua} isolates exist which possess the LIPI-1 gene cluster and another \textit{L. monocytogenes} associated virulence gene, \textit{inlA} [12,13]. Nonetheless, the retention of the LIPI-3 cluster by a large proportion of strains is unexpected. The LIPI-3 clusters in the various \textit{L. innocua} strains seem to be at various stages of reductive evolution with a number of stains possessing an intact island, others showing clear evidence of disintegration and yet another group in which the island is completely absent. It is not clear, however, whether the gradual loss of LIPI-3 from \textit{L. innocua} strains is a slow process that has been underway since the existence of the last common ancestor of...
L. monocytogenes and L. innocua or if it was initiated following a more recent acquisition of LIPI-3 by L. innocua from L. monocytogenes.

Competing interests
The authors have declared that no competing interests exist.

Authors’ contributions
EC contributed to study design, laboratory investigations, data analysis and manuscript preparation, KD contributed to laboratory investigations, data analysis and manuscript preparation, CG contributed to data analysis, PDC, CH and RPR conceived the study, contributed to study design, data analysis and manuscript preparation. All authors have read and approved the final manuscript.

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