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Characterisation of *Listeria monocytogenes* food-associated isolates to assess environmental fitness and virulence potential.

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Abstract

The ability of *Listeria monocytogenes* isolates to survive within the food production environment (FPE), as well as virulence, varies greatly between strains. There are specific genetic determinants that have been identified which can strongly influence a strains ability to survive in the FPE and/or within human hosts. In this study, we assessed the FPE fitness and virulence potential, including efficacy of selected hygiene or treatment intervention, against 52 *L. monocytogenes* strains isolated from various food and food environment sources. Phenotypic tests were performed to determine the minimum inhibitory concentration of cadmium chloride and benzalkonium chloride and the sensitivities to five clinically relevant antibiotics. A genomic analysis was also performed to identify resistance genes correlating to the observed phenotypic resistance profiles, along with genetic determinants of interest which may elude to the FPE fitness and virulence potential. A transposon element containing a novel cadmium resistance gene, *cadA7*, a Tn*916* variant

28 insert in the hypervariable *Listeria* genomic island 1 region and an LGI2 variant were
29 identified. Resistance to cadmium and disinfectants was prevalent among isolates in this
30 study, although no resistance to clinically important antimicrobials was observed. Potential
31 hypervirulent strains containing full length *inlA*, LIPI-1 and LIPI-3 were also identified in this
32 study. Cumulatively, the results of this study show a vast array of FPE survival and
33 pathogenicity potential among food production-associated isolates, which may be of concern
34 for food processing operators and clinicians regarding *L. monocytogenes* strains colonising
35 and persisting within the FPE, and subsequently contaminating food products then causing
36 disease in at risk population groups.
37
38 Keywords: Disinfectant, cadmium, pathogenicity, antimicrobial, plasmid, transposon.
39
40 **1. Introduction**
41 *Listeria monocytogenes* is a saprophyte and a human pathogen. *L. monocytogenes* can
42 cause the severe disease, listeriosis, in at risk populations that includes the elderly, immuno-
43 compromised, pregnant women and neonates with an associated mortality rate of almost 30
44 % (Havelaar et al., 2015; Todd and Notermans, 2011). *L. monocytogenes* can enter the food
45 production environment (FPE) through multiple vectors, such as the introduction of
46 contaminated raw ingredients, or the ingress of staff or pests carrying the bacterium, which
47 can then progress to the colonisation of the production environment and subsequent cross
48 contamination of food products, particularly ready to eat (RTE) items (Camargo et al., 2017).
49 Although not all strains which enter the FPE will go on to cause listeriosis, there are a variety
50 of genetic and phenotypic traits/mechanisms which can indicate a strains ability to survive or
51 cause disease. An understanding of the various factors influencing colonisation, survival and
52 pathogenicity is thus important.
53 Within the FPE there are intrinsic (food-related) and extrinsic (intentionally applied to reduce
54 microbial contamination and spoilage) stress factors utilised to control *L. monocytogenes*
55 strains including high osmolarity, temperature and pH, disinfectants, sanitisers and episodes

56 of desiccation (Jordan et al., 2018). *L. monocytogenes*’ ability to survive various
57 processing/hurdle technologies influences its ability to colonise and persist in the FPE,
58 making it an important foodborne pathogen. Survival throughout the FPE can result in cross
59 contamination of products like RTE foods, which are considered high risk products due to
60 the lack of further cooking prior to consumption, and common vehicles for listeriosis
61 outbreaks (Camargo et al., 2017; Jordan et al., 2018). Importantly, it has been suggested
62 conditions present within the FPE may promote the development of survival strategies like
63 cross protection and interconnectedness between tolerance or resistance to multiple
64 stressors (Bucur et al., 2018). At refrigeration temperatures, or lower ambient temperatures
65 used in many FPEs, an increase in tolerance of associated *L. monocytogenes* strains to cold
66 temperatures, osmotic and oxidative stressors has been noted (Manso et al., 2020; Schmid
67 et al., 2009). Recently identified genomic islands, stress survival islet (SSI)-1, and SSI-2, are
68 responsible for tolerance to acid, salt, bile, gastric, alkaline and/or oxidative stress, further
69 highlighting the diverse genomic arsenal which supports niche adaptation, survival and
70 persistence of *L. monocytogenes* in the FPE (Harter et al., 2017; Ryan et al., 2010). In
71 addition, the presence of plasmids and prophage elements also increases an isolates fitness
72 within the FPE (Schmitz-Esser et al., 2015; Verghese et al., 2011).
73 An understanding of the pathogenic potential of *L. monocytogenes* strains isolated from the
74 FPE, as well as their resistance to antibiotics, is also important. The listerial infection cycle is
75 the result of several essential virulence factors, predominately a six gene virulence cluster
76 also known as *Listeria* pathogenic island 1 (LIPI-1) (Hadjilouka et al., 2016; Karthikeyan et
77 al., 2015; Luo et al., 2013; Osman et al., 2020; Poimenidou et al., 2018). In addition, there
78 are other important genes involved in virulence, including *inlA* and *inlB*, which are required
79 for initial invasion (Autret et al., 2001). However, not all isolates in the FPE contain functional
80 virulence genes, with mutations in key virulence genes like *prfA* or *inlA* resulting in a reduced
81 pathogenic potential (Miner et al., 2008; Nightingale et al., 2008). Determining the presence
82 and the degree of diversity can provide an overview of the pathogenicity potential of
83 transient or persistent isolates within the FPE (Poimenidou et al., 2018). Differences in

84 virulence is an important public health concern as highly virulent strains can be associated
85 with outbreaks and severity of illness, and therefore the sensitivity to clinically relevant
86 antibiotics is also required. Importantly, resistance in *L. monocytogenes* isolates have been
87 increasingly reported, along with the presence of genetic determinants being identified for
88 various classes of antibiotics, some of clinical relevance (Grayo et al., 2008; Rakic-Martinez
89 et al., 2011; Wilson et al., 2018); therefore continued surveillance of antibiotic sensitivity is
90 required. Typically, the pathogenic potential of clinical isolates is looked at, with less studies
91 considering food-isolated *L. monocytogenes* ability to both cause disease, in addition to the
92 carriage of genes that may support their survival within the FPE. The aim of this study was to
93 characterise *L. monocytogenes* isolates from food and food-environment sources using
94 phenotypic and genomic methods to determine their ability to survive within the food
95 production environment, their potential to cause infection and their susceptibility to frequently
96 used antibiotics in the treatment of listeriosis.
97
98 **2. Materials and methods**
99 *2.1 Bacterial strains, isolation and molecular characterisation*
100 A total of 52 *L. monocytogenes* isolates from 12 sequence types (ST, up to five isolates per
101 ST) representative of multi-locus sequence types commonly associated with the food chain
102 were chosen from a variety of sources (dairy, meat, vegetable, mixed food and environment;
103 Supplementary Table 1) across a span of 18 years (1998 to 2016). Isolates selected each
104 possessed unique pulsed field gel electrophoresis pulsotypes to increase strain variance
105 (data not shown). Isolates were stored in a -80 °C freezer, and resuscitated on Brain Heart
106 Infusion (BHI, Oxoid, Australia) agar at 37 °C for 24 hours, prior to experimental
107 manipulations.
108
109 2.2 Phenotypic characterisation
110 *2.2.1 Antimicrobial sensitivity*

111 The sensitivity of *L. monocytogenes* strains to five antibiotics used for the treatment of
112 listeriosis was determined on Muller Hinton Agar (Oxoid, Australia) supplemented with 5%
113 (v/v) defibrinated sheep blood (MHSBA; Thermo Fisher Scientific, Australia). Ciprofloxacin,
114 trimethoprim/sulfamethoxazole and penicillin G (0.002-32 µg/mL) along with gentamicin and
115 amoxicillin (0.016-256 µg/mL), were assessed. Bacterial suspensions adjusted to 0.5
116 McFarland in maximum recovery diluent (MRD) were inoculated onto MHSBA using a sterile
117 swab in three directions and incubated at 37 °C for 24-48 hr. The Minimum Inhibitory
118 Concentration (MIC) for each antibiotic was assessed using Etest strips (Biomerieux,
119 Australia) and MIC values were interpreted according to breakpoints provided by the Clinical
120 Laboratory Standards Institute (CLSI) (CLSI, 2014, 2016) or European Committee on
121 Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2019) or the literature (Noll et al.,
122 2018) for *L. monocytogenes* where available; or staphylococcal species MICs were used in
123 the absence of breakpoints from the aforementioned sources. The MIC value for
124 susceptibility was defined as ≤ 1 µg/mL for amoxicillin, ≤ 1 µg/mL for gentamicin and
125 ciprofloxacin, ≤ 2 µg/mL for penicillin and ≤ 0.064 µg/mL for trimethoprim/sulfamethoxazole.
126 The control strains used were *S. aureus* ATCC 29213 and *Streptococcus pneumoniae*
127 ATCC 49619.
128

129 *2.2.2 Disinfectant and heavy metal sensitivity*

130 Benzalkonium chloride (BC) (Sigma Aldrich, Australia) was used to determine sensitivity of
131 *L. monocytogenes* strains to an important industrial quaternary ammonium compound
132 disinfectant, using a broth microdilution method with the following modifications: briefly,
133 *L. monocytogenes* strains were grown overnight in Mueller Hinton broth (MHB) and diluted to
134 ~10^3 CFU/mL, then 190 µL was inoculated into 96 well microtitre plate. For each BC stock
135 concentration, 10 µL was added to the microtitre plates to achieve final concentrations of 50,
136 40, 30, 20, 10, 5, 2.5, 2, 1.5, 1 and 0.5 µg/mL. All plates were incubated at 37 °C for 24 hr.
137 Growth was monitored immediately following inoculation (T0) and again at 24 hr (T24) at
138 OD_{600} using EnSpire™ multilabel plate reader 2300 (PerkinElmer, Singapore). The T24

139 reading was subtracted from T0 to determine if isolates were capable of growth. The
140 bacteriostatic or bactericidal effect of BC was tested for any isolate with growth under OD_{600}
141 0.1 with 10 µL spotted onto Brain Heart Infusion agar (BHIA) and incubated at 37 °C. After
142 24 hr plates were checked for growth or no growth. A minimum of two biological replicates
143 were performed.
144 Cadmium chloride ($CdCl_2$) was used to determine *L. monocytogenes* sensitivity to the heavy
145 metal cadmium, using a previously optimized method (Ratani et al., 2012). Mueller Hinton
146 agar (Oxoid, Australia) was supplemented with $CdCl_2$ (Sigma Aldrich, Australia) at the
147 following final concentrations: 10, 35, 40, 70, 140 and 150 µg/mL. Isolates were grown
148 overnight on BHIA and a 1 µL loopful was inoculated into 2 mL phosphate-buffered saline
149 (PBS) and vortexed until completely suspended. The PBS and isolate solution were streaked
150 with a cotton swab onto the $CdCl_2$ plates in three directions and incubated at 37 °C. Plates
151 were visually assessed for growth or no growth after 48 hr. Two biological replicates were
152 performed.
153
154 *2.3 Genomic characterisation*
155 Genomic characterisation was predominately performed in Geneious (2020). Genes of
156 interest were downloaded from NCBI with searches performed in Geneious using Megablast
157 or tblastn, with positive results for hits displaying >85% query coverage and pairwise identity.
158 A phylogenetic tree was created based upon raw reads using Snippy and Snippy-core
159 (Seemann, 2015) in Galaxy Australia (Jalili et al., 2020) utilising the genbank file of isolate
160 7943 as the reference genome and reconstructed with RAxML (v8.2.4) (Stamatakis, 2014),
161 utilising substitution model 'GTRCAT' and the remainder with default parameters.
162
163 *2.3.1 Genetic determinants of virulence potential and FPE stress survival*
164 The LIPI-1 virulence cluster and a selection of genetic determinants identified in the literature
165 were chosen to assess the potential of the isolates to survive various stress conditions
166 encountered within the FPE, and the potential to cause disease should a contaminated food

167 product be consumed (Table 1). The protein or gene was downloaded from NCBI and a
168 BLAST search of the genetic determinants occurred in Geneious utilising the above criteria.
169 EasyFig 2.2.5 (Sullivan et al., 2011) was used to visualise gene comparisons.
170
171 *2.3.2 Antimicrobial and virulence gene databases*
172 Mass screening of acquired antimicrobial and virulence genes were performed using
173 Abricate in Galaxy (v1.0.1) (Seemann, 2016) against the associated databases NCBI
174 AMRFinder Plus (Feldgarden et al., 2019), CARD (Alcock et al., 2020; Jia et al., 2017),
175 ARG-ANNOT (Gupta et al., 2014), Resfinder (Zankari et al., 2012) and VFDB (Chen et al.,
176 2016).
177
178 *2.3.3 Mobile Genetic Elements characterisation*
179 Plasmids were identified using PlasmidFinder 2.1 against the Gram-Positive database
180 (Carattoli et al., 2014). Prophage elements were identified using the online platform
181 PHASTER (Arndt et al., 2016; Zhou et al., 2011). Draft nucleotide sequences were utilised
182 for both analyses. Confirmation of plasmid and prophage results were performed in
183 Geneious (2020), through contig interrogation and read-mapping. Comparison of closed
184 plasmids was visualised using BRIG (Alikhan et al., 2011), with the following combinations:
185 ST8 plasmids with pLM1686 as the reference plus an additional section from p7922 from this
186 study, ST121 and ST321 utilising pLM6179 for reference and ST3, ST9, ST155 and ST204
187 were compared to pN1-011A and pR479a plasmids.
188
189 *2.3.4 Data Availability*
190 Draft genome sequences for strains from this project have been deposited in the NCBI
191 genome database or sequence read archive under BioProjects: PRJNA725037,
192 PRJNA320339, PRJNA377767, PRJNA295145, PRJNA295464, PRJNA692370 and
193 PRJNA422580; accession numbers are displayed in Supplementary Table 1.
194

195 **3. Results**

196 *3.1 Genomic composition of L. monocytogenes isolates*

197 An overview of the genomic composition of the 52 *L. monocytogenes* isolates included in
198 this study is shown in Supplementary Table 2. The draft genome sizes ranged between 2.61
199 and 3.08 Mb, with the GC percentage between 37.7 and 38.1%. The number of coding DNA
200 sequences ranged from 2,668 to 3,165.

201

202 *3.2 Cadmium Chloride and Benzalkonium Chloride Phenotypes*

203 The sensitivity of the 52 *L. monocytogenes* strains to various concentrations of BC and
204 $CdCl_2$ is shown in Figure 1. When assessed against BC only two isolates (7544 and 7546)
205 were unable to grow at the lowest concentration (0.5 µg/mL), however when subsequently
206 spotted onto BHI agar they were able to produce colonies indicating BC at 0.5 µg/mL had a
207 bacteriostatic effect on these two isolates. Assessment against varying concentrations of
208 $CdCl_2$ resulted in 10 isolates unable to grow at the lowest concentration with the remainder
209 of the isolates growing at various concentrations between 10 and 140 µg/mL, however not at
210 the highest concentration (150 µg/mL). Isolate 7920 contained a $CdCl_2$ resistant gene,
211 however it was only able to grow to 10 µg/mL. There were also nine isolates which had no
212 *cadA* genes but were able to grow at 10 µg/mL.

213

214 *3.3 Antimicrobial susceptibility*

215 Five antibiotics used for the treatment of listeriosis were tested against the
216 *L. monocytogenes* isolates (Figure 1). All the *L. monocytogenes* isolates displayed sensitivity
217 to the antibiotics tested in this study (amoxicillin, gentamicin, penicillin, ciprofloxacin and
218 trimethoprim/sulfamethoxazole).

219

220 *3.4 Food production stress determinants*

221 All isolates were assessed for the presence of genetic determinants relating to various stress
222 conditions experienced within the food production environment, used as a mitigation strategy

223 to reduce growth and/or survival of *L. monocytogenes*. These stress conditions included
224 thermal treatment, low temperatures, acidification, oxidation, osmotic stress, the use of
225 bacteriocins or nisin, and high hydrostatic pressure (HHP). A screening database of genetic
226 determinants associated with each condition identified within the literature was selected to
227 determine the potential of strains to survive within the FPE. The genetic determinants
228 selected for heat, acid, cold, osmotic, bacteriocin/nisin and HHP stresses were present in all
229 isolates (Supplementary Table 3). The stress survival islets (SSI) were also assessed, with
230 SSI-1 present in 34 of the 52 isolates (65.4%) from ST3, 7, 8, 9, 12, 155, 204 and 321
231 (Figure 1). The five isolates from ST121 were positive for SSI-2 (9.6%), and an SSI
232 genotype harbouring an *LMOf2365_0481* gene homolog was present in 13 of the 52 isolates
233 (25.0%) from ST1, 2 and 101.
234
235 *3.5 Cadmium and disinfectant genes*
236 The *L. monocytogenes* isolates were analysed for the presence of cadmium genes *cadA1C-*
237 *A6C* (Figure 1). There were 19 isolates which had no *cadA* genes present; the *cadA1* gene
238 was present in 20 isolates, *cadA2* was represented in five isolates, four isolates had *cadA4*
239 and five isolates had *cadA5* present. No isolates had the *cadA3* or *cadA6* gene. Isolate 7929
240 had two *cadA* genes present, *cadA2* and *cadA4*. Two *cadA* genes were also present in
241 isolate 7533, *cadA2* and interestingly, it also contained a transposon with a novel *L.*
242 *monocytogenes* cadmium resistance gene, referred to here as *cadA7*. A nucleotide BLAST
243 search of NCBI nucleotide database identified four other *L. monocytogenes* strains also
244 contain this transposon and the novel *cadA7* gene homologue. This transposon was also
245 identified in Enterobacteriaceae strains suggesting direct or indirect horizontal gene transfer
246 occurring between *Enterococcus* and *Listeria* (Figure 2). No other isolates within this study
247 contained the *cadA7* gene. Amino acid sequence identity of the published cadmium genes
248 and the novel *cadA7* gene was determined, with *cadA7* sharing the highest amino acid
249 percentage identity with *cadA2*, 75.74% (Supplementary Figure 1); however, when

250 compared to *E. faecalis* ATCC 29212 genome the novel *cadA7* displayed 100% coverage
251 and 99.95% nucleotide identity.
252
253 A variety of genes and mechanisms providing resistance to disinfectants were assessed
254 against the isolates in this study (Figure 1). All isolates were found to contain the *mdrL* and
255 *lde* efflux pumps. The *bcrABC* cassette was present in 13 isolates covering ST1, 3, 9, 155,
256 204 and 321. Six isolates in total were positive for *ermB* and *qacH* from ST8, 9 and 121. The
257 *emrC* gene was present in six isolates, one isolate from ST7 and all the isolates from ST101.
258 The ST101 isolates were the only whole ST group in which all contained the same
259 disinfectant resistance gene.
260
261 *3.6 Plasmids, Prophages and Transposons*
262 Plasmid replicons were identified in 26 of the 52 isolates with PlasmidFinder. The identified
263 regions were further interrogated in the draft genomes. A total of 13 closed plasmids and 12
264 draft open plasmids were identified (Table 1). Plasmids were present in ST3, ST8, ST9,
265 ST121, ST155, ST204 and ST321 isolates (Figure 3). ST121 was the only group in which
266 plasmids were found in all five isolates and displayed a 95.9% pairwise identity with
267 pLM6179, however only three of these plasmids were closed following sequence analysis.
268 Genes shared across the plasmids, and not restricted to a single ST, included heavy metal
269 and disinfectant resistance genes including the *bcrABC* operon *cadAC* operon, and genes
270 for copper, zinc and arsenic resistance; stress response genes including UV damage repair
271 protein, oxidative and heat stress response genes; invasion related genes; toxin/anti-toxin
272 genes; genes involved in DNA replication, translation, recombination and conjugation;
273 transposon genes; however most genes were hypothetical proteins.
274
275 Phaster identified 52 intact phage regions across 43 isolates. Nine isolates had no phage
276 regions, most isolates had a single phage region, two regions were identified in 15 isolates
277 and three and four regions identified in four and one isolate respectively. An additional

278 analysis of the *comK* phage insertion site identified 21 isolates with a full length *comK* gene
279 and 31 isolates with a *comK* prophage disruption (Figure 1). Transposon elements were
280 identified in 27 of the 52 isolates. Transposon Tn*6188* was present in five isolates, Tn*5422*
281 was identified in 20 isolates, Tn*ILP* was present in two isolates and Tn*yfbR* was identified in
282 six strains. Seven isolates contained two transposons.
283
284 *3.7 Virulence determinants and genomic islands*
285 The *Listeria* pathogenicity and genomic islands were assessed against the panel of isolates
286 (Figure 1). The LIPI-1 virulence cluster was present in all isolates, along with the *inlB* gene.
287 The LIPI-3 element was found in three isolates (7523, 7550 and 8122) from ST1 and two
288 isolates (7514 and 7583) from ST3. No isolates harboured LIPI-4; this island has only been
289 identified in CC4 isolates, which were not included in this study. The LGI2 was present in
290 eight isolates, in either one of two insertion locations; within the *LMOSA2140* (homolog of
291 *LMOf2365_2257*) gene originally identified in the strain ScottA (Lee et al., 2013), or within
292 the *yfbR* gene (Fox et al., 2016). The three isolates with the *LMOSA2140* LGI2 insertion
293 region were from ST2 and three ST204 strains contained the *yfbR* LGI2 insert. Interestingly,
294 an LGI2 variant was also identified from two ST1 isolates within a transmembrane protein
295 that displays distant homology to *ydbT* gene. This LGI2 variant harbours an additional gene,
296 a *metC* homolog, within the LGI2 region (Figure 4). The recently reported *Listeria* genomic
297 island 3 was partially identified in the five ST101 isolates only; however, they were missing
298 the 6,248 bp region containing the *cadAC* homolog, recombinase and Tn*3* family
299 transposase.
300
301 *3.8 Internalin A (inlA) Analysis*
302 The *inlA* gene was assessed for the presence of mutations resulting in premature stop
303 codons (PMSCs), truncation or a full length *inlA* gene (Figure 1 and Supplementary Figure
304 2). Thirty-eight of the isolates contained a full length *inlA* gene. Thirteen isolates contained
305 PMSCs. All ST121 isolates (7425, 7475, 7495, 7987 and 8113) contained mutation type 6 at

306 AA 492, as previously described (Van Stelten et al., 2010). Isolates 8123 and 7535
307 contained a PMSC at AA 685, resulting in mutation type 11. Both these isolates are ST9,
308 from which this mutation type has been previously associated with (Van Stelten et al., 2010).
309 Mutation type 12, the result of a PMSC at AA 576 was identified in three isolates from ST9
310 (8117, 8118 and 8119) and mutation type 3, the result of a PMSC at AA 700, was identified
311 in isolates 8126 and 7943 from ST321. A novel PMSC was identified in isolate 7452, the
312 result of a frame shift mutation from an AG insertion at nucleotide position 183 producing an
313 *inlA* gene of 67 AA in length. Isolate 7456 contained an in-frame 70 AA deletion within the B-
314 repeat region (B- repeat region 2 and 3).
315
316 *3.9 Listeria genomic island 1 analysis*
317 None of the isolates harboured the *Listeria* genomic island 1 (Figure 1). To confirm this, we
318 manually inspected the hypervariable region between *lmo1703-lmo1702* locus, an RNA
319 methyltransferase gene and the fosfomycin resistance gene *fosX* respectively, for inserts in
320 all isolates. A phage (ϕRNA-MT) insert was present in six of the isolates (7514, 7535, 7553,
321 7583, 7945 and 8123) and a novel insert was harboured in isolate 8115. This novel insert
322 was not present in any of the other isolates. A BLAST search resulted in a 64% query
323 coverage and 90.73 % nucleotide identity with Tn*916* from *Bacillus subtilis* (which has over
324 98% identity with Tn*6198* of *L. monocytogenes* TTH-2007) and a 64% query coverage and
325 91% identity with an integrated chromosomal element ICESpnIC1 identified in *St.*
326 *pneumoniae* isolate 9611+04103 (Figure 5). This Tn*916* variant insert between *lmo1703* and
327 *lmo1702* in isolate 8115 is approximately 23,275 bp in length, has a GC content of 37.5 %
328 and contains a Clp protease ATP-binding subunit *clpA*, as well as coding sequences for
329 FtsK/SpoIIIE family protein, a Tn*916* transcriptional regulator, an anti-restriction protein,
330 lipoprotein, a *XRE* family transcriptional regulator, an efflux *ABC* transporter, and a site-
331 specific recombinase with the remaining genes being hypothetical proteins.
332
333 *3.10 Antimicrobial resistance determinants*

334 The *L. monocytogenes* isolates were analysed for the presence or absence of a variety of
335 antimicrobial genes associated with conferring resistance to frequently used antibiotics in the
336 treatment of listeriosis or other diseases (Supplementary Table 4). Resistance genes from
337 the following selected antibiotics classes: trimethoprim, tetracycline except for the *tetA-like*
338 resistance protein, vancomycin, erythomycin, amoxicillin and aminoglyosides along with the
339 penicillin *mecC* protein were not detected in any isolates. The AMR genes selected relating
340 to fluroquinolones (second generation), penicillin (except *mecC*), sulfonamide, fosfomycin,
341 lincomycin, fusidine, quinolone and cephalosporins were present within all isolates. There
342 were no AMR genes or class specific to an isolate, lineage or ST. Potential resistance
343 mechanism beyond known AMR genes were not examined.
344

345 **4. Discussion**

346 This study utilised phenotypic and genotypic analyses of whole genome sequences to
347 assess the potential of *L. monocytogenes* to survive within the FPE, cause disease and
348 provide insights into antimicrobial resistance relevant to control in the FPE, or to treatment of
349 human infection.
350

351 *4.1 Virulence potential*

352 Clonal complex 1 (CC1) and 2 (CC2) are well established as being associated with clinical
353 infections (Maury et al., 2016; Yin et al., 2019), and therefore the presence of genomic
354 regions relating to hypervirulence in food isolates is of interest. In this study ST1 and ST2
355 isolates did not contain the SSI-1 or SSI-2 islands, however, the SSI-alternative,
356 *LMOf2365_0481* homologue, was present in all isolates. A study by Harter and colleagues
357 (Harter et al., 2017) reported the presence of this SSI-alternative to be common among
358 clinical strains, however its function at this stage is undetermined. While CC1 and CC2 did
359 not contain SSI-1 or SSI-2, the isolates did contain all but one of the stress resistance-
360 associated genes screened, with a role in heat, cold, acid, osmotic, oxidative or nisin stress
361 response, suggesting that they are capable of surviving within the FPE, or in food. Horlbog

362 et al (Horlbog et al., 2018) found CC1 strains were able to recover more quickly after salt
363 stress, suggesting these strains could be able to proliferate faster within food environments.
364 All isolates of CC1 and CC2 contained full length *inlA* genes; three of the CC1 isolates
365 contained LIPI-3, suggestive of increased virulence potential.
366 Within Australia, CC3 and CC204 are widely distributed (Jennison et al., 2017), with these
367 strains analysed in this study all containing LIPI-1, full length *inlA*, SSI-1, and all the various
368 processing related stress genes. Neither CC3 nor CC204 contained the LGI1, however,
369 three of the CC3 strains and one of the CC204 strains contained a phage insert in the LGI1
370 hypervariable position between *lmo1703* and *lmo1702*. Interestingly, two of the CC3 isolates
371 contained the phage insert instead of LGI1. Increased bacterial colonisation and
372 hypervirulence has been reported in isolates which encode a full length *inlA*, LIPI-1 and LIPI-
373 3 (Maury et al., 2016; Yin et al., 2019), suggesting the isolates in this study which contain
374 these have increased virulence potential, and with the addition of SSI-1 may also have
375 increased FPE fitness. The CC204 strains which were negative for LGI1 and the ϕRNA-MT
376 phage insert, contained LGI2. *Listeria* genomic island 2 contains cadmium and arsenic
377 resistance genes, providing increased environmental survival potential; interestingly, also in
378 both this study and Lee et al (Lee et al., 2017), LGI2 was common in CC2 strains, which are
379 also prevalent in human cases. Although LGI2 requires further characterisation, it is
380 prevalent in clonal complexes linked to hypervirulence and has been suggested to contribute
381 to virulence (Lee et al., 2017).
382 Premature stop codons and mutations in the *inlA* gene resulting in secretion of *inlA* instead
383 of being attached to the bacterial cell wall have been associated with reduced invasion and
384 virulence ability (Ferreira da Silva et al., 2017; Gelbíčová et al., 2015; Van Stelten et al.,
385 2016). In this study, 13 isolates were identified to carry one of five types of mutations,
386 suggesting these isolates may have a reduced virulence potential. We identified a novel
387 PMSC at 67 AA resulting from an AG insertion at position 183 bp producing a frameshift,
388 referred to as mutation type 22. This mutation occurs within the signal cap region of the *inlA*
389 protein. Mutation types 4 and 15 occurring at AA positions 9 and 77 respectively have been

390 shown to affect invasion ability (Van Stelten et al., 2010), indicating mutation type 22 has the
391 potential to also have reduced invasiveness, however, in vitro cell invasion assays and/or
392 mouse model virulence assays will be required to confirm this theory. In addition, a 70 AA
393 deletion within the B-repeat region was identified in isolate 7456. Deletion of the B-repeat
394 region between AA 517 and 707 by Lecuit et al (Lecuit et al., 1997) resulted in similar
395 invasiveness level to those with the WT *EGD-e inlA* protein, suggesting this deletion within
396 the B-repeat region does not contribute to a strains ability to invade cells. All ST121 isolates
397 contained the type 6 mutation and ST321 isolates contained mutation type 3. In addition, all
398 but two *inlA* mutant isolates had cadmium resistance, contained either SSI-1 or SSI-2, and
399 harboured plasmids. All of these isolates harboured LIPI-1. The majority of *inlA* mutants are
400 commonly associated with the FPE and food isolates (Nightingale et al., 2005; Van Stelten
401 et al., 2016), therefore the presence of these genes in the *inlA* mutant isolates are
402 suggestive of increased survival within the FPE.
403
404 *4.2 FPE survival potential*
405 Agricultural practices and industrial pollution have resulted in increased levels of various
406 heavy metals in the environment, and as such bacteria require resistance determinants in
407 order to tolerate these substances, particularly heavy metals which are not required for
408 cellular processes. Cadmium resistant determinants are widely distributed and are
409 commonly associated with *L. monocytogenes* strains repeatedly isolated from food sources
410 (Parsons et al., 2017). In this study, 32 isolates were capable of growing at levels above 40
411 µg/ml $CdCl_2$, which was associated with the presence of at least one cadmium resistant
412 determinant, with similar results observed in previous studies (Haubert et al., 2019; Lee et
413 al., 2013; Mullapudi et al., 2010; Ratani et al., 2012; Xu et al., 2019). Interestingly, in this
414 study we did not identify the *cadA3* resistant determinant which is present as an integrating
415 chromosomal element in a variable genomic region, that in other strains may contain diverse
416 cassettes like LIPI-3 (Parsons et al., 2019).

417 The novel *cadA7*, identified in this study, contained all three key motifs, DKTGT, CPC and
418 CTNCA, characteristic of the *cadA* protein family (Bal et al., 2003; Parsons et al., 2017).
419 Parsons et al (Parsons et al., 2017) identified an amino substitution in the CTNCA →
420 CANCA motif in *cadA4* suggesting this substitution most likely, in conjunction with other
421 elements, influencing *cadA4*'s reduced tolerance of 35 μg/mL to cadmium. In this study, the
422 three key motifs of the novel *cadA7* matched the *cadA1-cadA3* sequences, which are
423 purportedly associated with cadmium resistance of 140 μg/mL or higher, potentially
424 suggesting *cadA7* may confer similar levels of resistance (Parsons et al., 2017). The single
425 isolate harbouring *cadA7* in this study also contained *cadA2*; as such, further research is
426 required to establish the resistance level conferred by *cadA7*, and to determine if it has a
427 potential role in virulence.
428 The presence of disinfectant resistant genes in this study was associated with resistance to
429 BC (≥ 5 μg/mL) in all but two isolates, with 50% of the isolates displaying a MIC of 5 μg/mL
430 or higher. The capability of isolates to grow at higher levels of disinfectants like BC is being
431 increasingly reported (Mullapudi et al., 2008; Møretrø et al., 2017). In addition, the
432 *L. monocytogenes* strains' tolerance to disinfectants has been correlated with cadmium
433 resistance and increased survival within the FPE being associated with subinhibitory levels
434 of disinfectants (Martinez-Suarez et al., 2016; Mullapudi et al., 2008; Ortiz et al., 2014).
435 Therefore, an evaluation of the level of resistance of cadmium and BC is important to
436 understand the survival potential *L. monocytogenes* may have in the FPE.
437 Genomic islands have the potential to contain genes to improve the fitness of an isolate,
438 while also being implicated in potential horizontal gene transfer (Palma et al., 2020);
439 therefore, the presence of these islands might lead to increased FPE survival or pathogenic
440 potential. Of the genomic islands identified in *L. monocytogenes*, LGI1 and LGI3 have been
441 associated with survival and persistence in the FPE (Kovacevic et al., 2016; Palma et al.,
442 2020), with LGI2 potentially providing increase survival and persistence within the FPE as
443 well as virulence potential (Lee et al., 2013; Lee et al., 2017).

444 In this study, LGI2 was the only full-length island present in six isolates, inserted within one
445 of two genes, *LMOSA2140* or *yfbR*. This has the potential to provide increased virulence and
446 environmental fitness. In addition, an LGI2 variant was identified in two ST1 isolates within a
447 transmembrane protein that displays distant homology to the *ydbT* gene from *Bacillus*. The
448 *ydbT* in *Bacillus subtilis* strains has been reported to provide resistance to bacteriocins
449 produced by *B. amyloliquefaciens*, an important function particularly in natural environmental
450 reservoirs like soil (Butcher and Helmann, 2006). The LGI2 variant shows high homology
451 with LGI2 and maintains the arsenic and cadmium resistant determinants along with various
452 metabolism, transport, stress resistance, transposon and regulatory genes. However, the
453 LGI2 variant contains an additional cystathionine β-lyase (*metC*) gene. A previous study
454 demonstrated that disruption of *metC* in *Salmonella*, reduced strain virulence in a mouse
455 model (Ejim et al., 2004). This suggests the LGI2 variant may play a role in virulence as well
456 as survival within food and the food environment; however, this needs to be further
457 confirmed experimentally.
458
459 In this study, the LGI3 variant which lacks the *cadA1C* cassette was identified in all ST (CC)
460 101 isolates. The LGI3 element was first identified in CC101 isolates by Palma et al (Palma
461 et al., 2020) and found to harbour a *cadA1C* cassette; however, a search of the NCBI
462 genome database identified a smaller LGI3 variant lacking the *cadA1C* cassette in the *L.*
463 *monocytogenes* strain ATCC 51775 (ST222). In comparison, the CC101 isolates from this
464 study also contained the LGI3 variant, suggesting CC101 strains may display either LGI3
465 genotype.
466
467 Instead of LGI1, six isolates contained a phage insert and one isolate contained a Tn*916*
468 variant insert, in the associated insertion locus. This Tn*916* variant shares similarity with
469 Tn*916* and Tn*6198*; however, it lacks the tetracycline (*tetM*) and the trimethoprim (*dfrG*)
470 resistant genes. Interestingly, an efflux ABC transporter is present on the Tn*916* variant,
471 which shares homology to efflux systems. Further experimental work is required to

472 determine its function in this transposon, and the ability of this transposon to transfer to other
473 *Listeria* strains, or other bacterial species.
474
475 *4.3 Therapeutic treatment potential*
476 Traditionally, listeriosis is treated with a β-lactam (penicillin, ampicillin or amoxicillin) either
477 alone or in combination with an aminoglycoside, typically gentamicin (Grayo et al., 2008;
478 Knudsen et al., 2013; Olaimat et al., 2018; Temple and Nahata, 2000) or trimethoprim and
479 sulfamethoxazole combination for patients with a β-lactam sensitivity(Bertrand et al., 2016;
480 Wilson et al., 2018). While in this study all the isolates were sensitive to the five clinically
481 relevant antibiotics tested, there has been reports in the literature of resistance to
482 gentamicin, penicillin, ciprofloxacin and trimethoprim/sulfamethoxazole along with a variety
483 of other antibiotics and importantly reports of resistance to multiple antibiotic classes (Arslan
484 and Özdemir, 2020; Kuan et al., 2017; Obaidat et al., 2015; Obaidat and Stringer, 2019;
485 Welekidan et al., 2019). Our study provides a timely contribution to the current state of AMR
486 in *L. monocytogenes* and does not highlight any resistance concern among food isolates in
487 this study.
488
489 In this study, we identified a novel cadmium gene, *cadA7* as part of a transposon insert, a
490 variant of LGI2, as well as a novel insert in the hypervariable region LGI1, in the latter
491 sharing similarity to a Tn*916* transposon. The identification of these novel genes and inserts
492 contributes to our understanding of the *L. monocytogenes* pangenome, in particular to
493 elements relating to survival ability and pathogenic potential. The isolates analysed in this
494 study showed potential to survive and persist within the FPE, with all isolates containing one
495 of the SSIs, various genes relating to stressors present in the FPE to reduce bacteria, in
496 addition to a high portion of strains containing cadmium or disinfectant resistance genes.
497 Hypervirulent strains of *L. monocytogenes* have been previously reported, with some
498 isolates from CC1 and CC3 in this study harbouring genes associated with this virulence
499 status, suggesting a concern to public health.

500

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504

505 **Author Contributions**

506 JG, PSC and EF conceived and designed the study. JG performed the experiments. Data

507 was analysed by JG, EF, PSC, JPB, MK and CK. JG and EF drafted the manuscript. All

508 authors corrected and approved the manuscript.

509

510 **Declarations of Interests**

511 None.

512

513 **Appendix A. Supplementary Information**

514 Supplementary Table 1. Isolate information.

515 Supplementary Table 2. Genome assembly and genetic subtype details.

516 Supplementary Table 3. Presence of genetic determinants related to food processing

517 environment stressor tolerance.

518 Supplementary Table 4. Antibiotic resistant determinants among isolates in this study.

519 Supplementary Figure 1. *CadA1-A7* amino acid comparison.

520 Supplementary Figure 2. Protein alignment of isolates with *inlA* mutation.

521

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786

787 **Table 1**. Plasmids identified among isolates in this study.

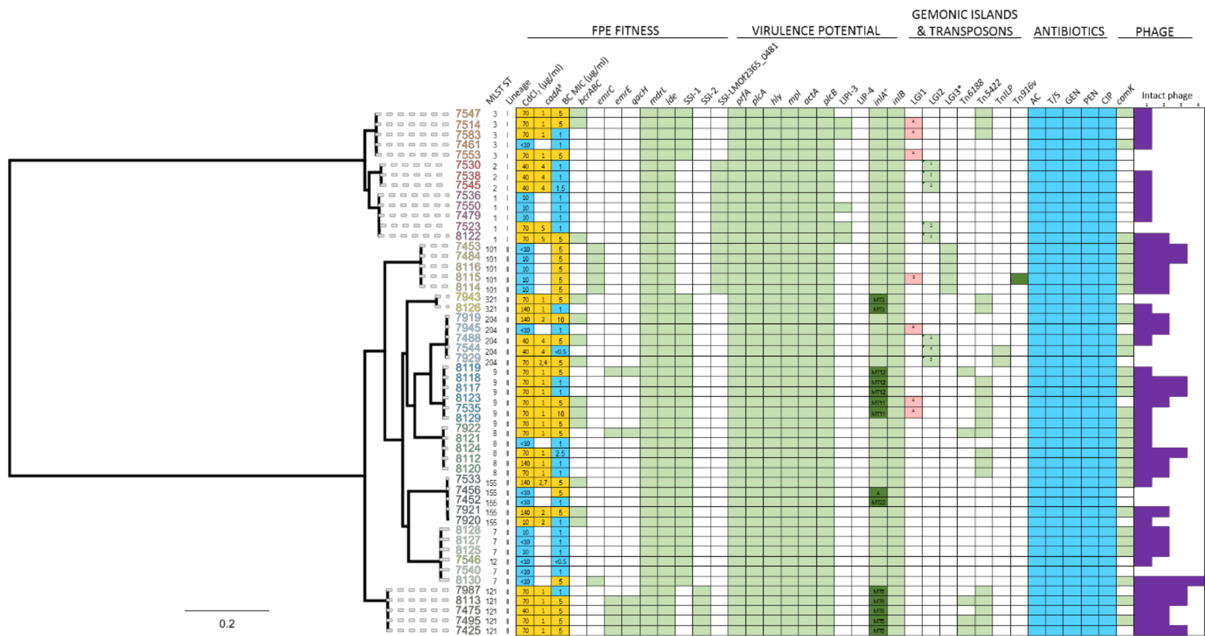
		Plasmid		
Isolate	MLST	Closed/ Open	Size (bp)	GC (%)
7514	3	Open	59,826	35.2
7547	3	Open	32,307	36.1
7553	3	Closed	4,176	34.1
7583	3	Open	56,146	36
7922	8	Closed	88,290	36.5
8112	8	Closed	79,144	36.7
8120	8	Open	85,394	36.9
8124	8	Closed	79,180	36.7
8117	9	Closed	25,550	36.5
8118	9	Closed	25,550	36.5
8119	9	Closed	25,550	36.5
8123	9	Open	49,141	36.3
8129	9	Open	49,281	35.8
7425	121	Closed	62,207	36.5
7475	121	Open	60,666	36.7
7495	121	Open	62,191	36.5
7987	121	Closed	60,923	36.6
8113	121	Closed	62,207	36.5
7533	155	Open	64,751	38.1
7920	155	Closed	77,756	37.5
7921	155	Closed	80,184	37.4
7488	204	Open	48,687	37.4
7919	204	Closed	38,191	37.3
7929	204	Open	91,345	37.7
7943	321	Open	66,904	36.5
8126	321	Open	60,124	36.7

788

789

790 **Figure 1. Phylogeny and genetic determinants of 52 *L. monocytogenes* isolates**
791 **relating to survival within the FPE, virulent potential and therapeutic treatment**
792 **potential**. Character designations are as follows: #, numbers designated *cadA* gene type; ^,
793 pLi0048 – elements of the pLi100 are present however we were unable to close or identify
794 full plasmid. This plasmid is known to carry cadmium resistant genes; +, *inlA* PMSC type; Δ,
795 70 AA deletion; A, phage insert in the *lmo1703-lmo1702* region; B, transposon insert in the
796 *lmo1703-lmo1702* region (*Tn916* variant); 1, LGI2 insert within the *EGD-e LMO2257* gene;
797 2, LGI2 variant; 3, LGI2 insert in the *yfbR* gene; *, LGI3 lacking the *cadA1C* cassette; yellow,
798 resistant phenotype; light green, gene is present; dark green, gene is present – does not
799 match wildtype; light red, LGI1 is absent however there is alternative genes present within
800 the *lmo1703-lmo1702* region; blue, sensitive phenotype; orange, *comK* phage is present;
801 purple, number of intact phage regions present.

802



803 **Figure 2. The novel *cadA7* gene.** Transposon identified in isolate 7533 inserted between
804 30S ribosomal protein S9 and *lmo2595*, compared to *E. faecalis* ATCC 29212. Integrase
805 genes are in light blue, replication genes are in red, heavy metal resistance genes are in
806 purple. Hypothetical genes or those with an unknown function are shaded grey. Nucleotide
807 sequence identity of transposon where shared, ranged from 80 to 100 % as depicted by the
808 percentage homology bar.

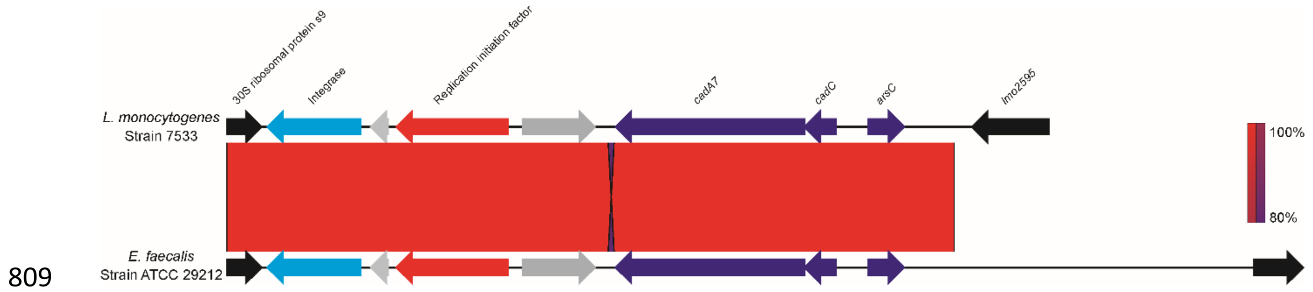
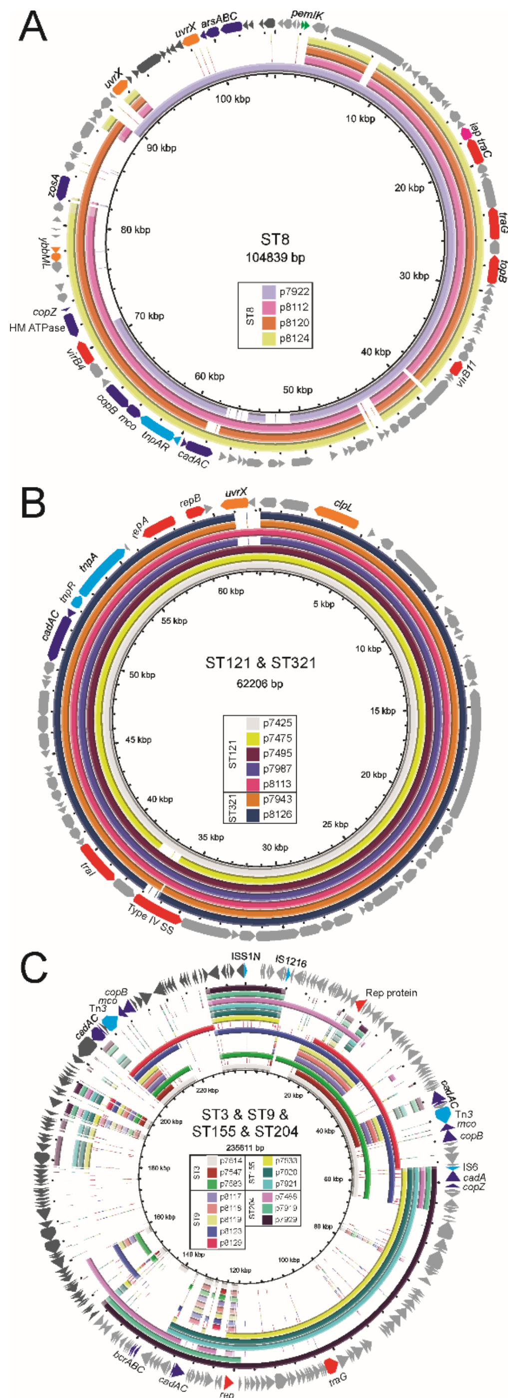
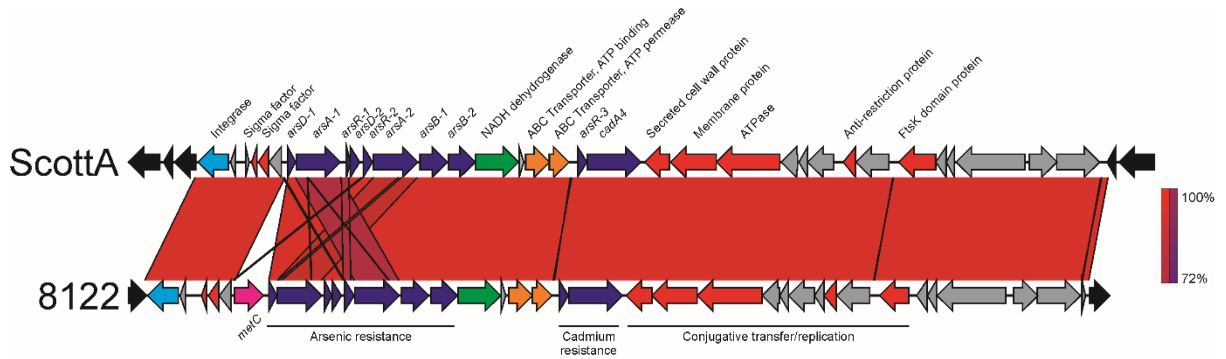
809 

Figure 3. BRIG comparison of plasmids identified within 26 isolates within this study. Each ring represents the plasmids of the individual isolates. The plasmid pangenome reference contains all the unique genetic features of the closest related plasmid identified in NCBI and segments of plasmids from the respective pST groups from this study which are not represented in NCBI, combined into a single contig reference (outside ring). **(A)** pST8 utilises the genome of pLM1686 (MK134858) (light grey) and p7922 (dark grey) as reference pangenome. **(B)** pST121 and pST321 utilises the p6179 as reference genome. **(C)** pST3, pST9, pST155 and pST204 utilises the genome of pN1-011A (light grey) and pR479a (dark grey) as reference pangenomes. Annotated genes are colour coded to represent genetic markers as follows: red – replication, light Blue – transposases, dark Blue – heavy metals, orange – stress response, pink – invasion associated, green – toxin/antitoxin and grey – hypothetical proteins.



823 **Figure 4. LGI2 variant identified in isolate 8122**. A variant of LGI2 inserted in a
824 transmembrane protein within isolate 8122 and 7523. Annotated genes are colour coded to
825 represent genetic markers as follows: black – flanking genes, light blue – integrase, dark
826 blue – heavy metal and antimicrobial resistance, orange – metabolism and transport, red -
827 transposon system and regulatory genes, pink - virulence , green – stress resistance , grey –
828 hypothetical proteins. Sequence identity where shared, ranged from 72 to 100 % as
829 determined by the percentage homology bar.

830



831 **Figure 5. Novel insert identified in isolate 8115.** The insert was identified in the
832 hypervariable region of isolate 8115 between genes *lmo1703*-*lmo1702*, compared to Tn*916*,
833 Tn*6198* and *S. pneumoniae* 9611+04103 ICESpnIC1. Sequence identity where shared,
834 ranged from 78 to 100 % as determined by the percentage homology bar. EGD-e flanking
835 genes, *lmo1703* and *lmo1702* are shown in black; red genes are transposon systems and
836 regulatory genes, light blue – integrase, dark blue – heavy metal/antimicrobial resistance,
837 green – stress response and grey – hypothetical proteins.

838