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2008

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Recommended Citation

Walsh, C. et al. (2008) Transfer of Ampicillin Resistance from S. Typhimurium DT104 to E. coli K12 in food. *Letters in applied microbiology, 46, 210-215, 2008.* DOI: 10.1111/j.1472-765X.2007.02288.x

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1 **Transfer of Ampicillin resistance from *S.* Typhimurium DT104 to *E. coli* K12 in**

2 **food**

3

4

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20

21 **Running title:** β-lactamase Transfer in Food

22 **Key Words**: Ampicillin Resistance, *S.* Typhimurium DT104, *E. coli* K12, β-

23 lactamase, bla_{TEM}

24

25

26 **Abstract**

27 **Aims:** To investigate the transfer of antibiotic resistance from a donor *S.*

28 Typhimurium DT104 strain to a recipient *E. coli* K12 strain .

29 **Methods and Results:** Mating experiments were conducted in broth, milk and ground

30 meat (beef) at incubation temperatures of 4, 15, 25 and 37°C for 18 and 36 h.

31 Ampicillin resistance transfer was observed at similar frequencies in all transfer

32 media at 25 and 37°C (10^{-4} to 10^{-5} $\log_{10}$ cfu/ml/g, transconjugants per recipient) for 18

33 h. At 15°C, transfer was observed in ground meat in the recipient strain (10^{-6}, $\log_{10}$

34 cfu/g, transconjugants per recipient), but not in broth or milk. At 4°C, transfer did not

35 occur in any of the examined mediums. Further analysis of the *E. coli* K12 nal^R

36 transconjugant strain revealed the presence of a newly acquired β-lactamase gene

37 bla_{TEM}. Transconjugants isolated on the basis of resistance to ampicillin did not

38 acquire any other resistant markers.

39 **Conclusion:** This study demonstrates the transfer of antibiotic resistance in food

40 matrices at mid-range temperatures.

41 **Significance and Impact of the Study:** It highlights the involvement of food

42 matrices in the dissemination of antibiotic resistant genes and the evolution of

43 antibiotic resistant bacteria.

44

45

46 **Introduction**

47 *Salmonella* Typhimurium DT104 is a major cause of enteric infections world-wide

48 (Beaudin *et al.* 2002). This serovar typically expresses resistance to ampicillin,

49 chloramphenicol/florfenicol, streptomycin/spectinomycin, sulfonamides and

50 tetracycline (ACSSuT) (Golding *et al.* 2007). Such multi-drug resistant (MDR) *S.*

51 Typhimurium DT104 are of concern especially in immuno-comprised patients, as it

52 restricts clinical options in the treatment of salmonellosis. In more general terms, it

53 has potential consequences in relation to transfer and dissemination of antibiotic

54 resistant material to other bacteria including pathogens.

55

56 Class A β-lactamases are the most widespread enzymes in Gram-negative bacteria

57 (Chochani *et al.* 2006, Bonnet, 2004). These enzymes are widely distributed in

58 *Salmonella* spp. and are frequently associated with the above (ACSSuT) penta

59 resistance profile (Guerri *et al.* 2004). In *Salmonella*, β-lactamase resistance is often

60 conferred by the presence of the bla_{TEM} or bla_{PSE} gene (Randall *et al.* 2004). Of these,

61 the $bla_{\mathrm{TEM-1}}$ gene which is generally plasmid-mediated (Larson and Ramphal, 2002) is

62 considered the most frequently expressed β-lactamase in *Salmonella* in Europe

63 (Guerri *et al.* 2004; Tzouvelekis *et al.* 2003).

64

65 A number of studies have investigated the transfer of antibiotic resistance in food-

66 borne bacterial pathogens such as *Salmonella,* but few studies have examined gene

67 transfer between bacteria in-*situ* in food. Reports of gene transfer (by transformation)

68 of kanamycin in *Bacillus subtilis* in milk (Kharazmi *et al.,* 2002) and the transfer of

69 vancomycin resistant genes among enterococcal strains by conjugation during cheese

70 and sausage fermentation (Cocconelli *et al.,* 2003) have been cited. However, the
71 majority of studies on the transfer of antibiotic resistance have been carried out in
72 liquid systems (McMahon *et al.* 2007; Wilcks *et al.,* 2005; Chen *et al.,* 2004; Allen
73 and Poppe, 2002) or on filters (Hummel *et. al.,* 2007; Gevers *et al.,* 2003; Pourshaban
74 *et al.,* 2002) and thus do not reflect the in-*situ* dynamics of food matrices. This
75 balance of activity is unfortunate, as liquid or filter based systems have been reported
76 to underestimate the rates of genes transfer compared to more complex matrices
77 (Netherwood *et al.,* 1999). Moreover, concern has been raised about food being an
78 important and under-estimated avenue for antibiotic resistance dissemination and
79 evolution (Wang *et al.* 2005).
80
81 The objective of this study was to ascertain if antibiotic resistance gene transfer can
82 occur between a donor *S.* Typhimurium DT104 and *E. coli* K12, in broth, milk and
83 ground meat under different temperature and time conditions encountered in food
84 processing.
85

86

87 **Material and Methods**

88 **Bacterial strains**

89 A previously characterised *S.* Typhimurium DT104 strain resistant to ampicillin,
90 chloramphenicol, streptomycin, sulfonamides and tetracycline (R-type ACSSuT) was
91 obtained from the culture collection at Ashtown Food Research Centre, Dublin. An
92 antibiotic susceptible strain of *E. coli* K12 (NC10538:06) was obtained from the
93 Health Protection Agency (HPA), London. All strains were stored on cryoprotect
94 beads (Technical Consultant Services Ltd., Heywood, Lancashire, UK) at –20°C.

95

96 **Preparation of nalidixic acid resistant mutants and antibiogram profiling**

97 *E. coli* K12 were rendered chromosomally resistant to 50 µg/ml of nalidixic acid, by
98 the method of Blackburn and Davies (1994). In brief, this involved growing each
99 strain separately in nutrient broth (Oxoid) containing nalidixic acid 50 µg/ml and
100 plating directly onto nutrient agar plates containing the same drug concentration.
101 Resultant colonies were serially subcultured to confirm mutant stability. The above
102 procedures produced nalidixic acid resistant *E. coli* K12 recipient isolates, which
103 could be easily differentiated and recovered during subsequent studies. This strain is
104 referred to as *E. coli* nal^R in our study.

105

106 The antibiogram profiles of all donor and recipient strains were established by the
107 Bauer-Kirby Disc Diffusion method, following the Clinical and Laboratory Standards
108 Institute recommended method (Anon., 2004) as described by Walsh *et al.* (2001).
109 Donor and recipient strains were maintained on cryoprotect beads, as described
110 above.

111

112 **Preparation of inoculum**

113 Protect beads coated with *S.* Typhimurium DT104 and *E. coli* K12 nal^R were

114 incubated in 30 ml volumes of LB Broth (Miller, Germany) at 37°C for 18 h, to form

115 stationary phase cultures containing approximately 10^9 cfu/ml. A 1.0 ml aliquot from

116 each stationary phase culture was serially diluted in 9 ml volumes of Maximum

117 Recovery Diluent (MRD, Oxoid) to form inocula containing approximately 10^6 cfu/

118 ml^{-1} culture.

119

120 **Control experiments**

121 The donor and recipient strains were grown independently, inoculated and recovered

122 from each food matrix during each experiment, by plating on to TSA-ampicillin (50

123 µg/ml) (to recover the no. of donor strains) and TSA-nalidixic acid (50 µg/ml) (to

124 recover the no. of transconjugant strains). The number of colonies recovered from

125 these plates was then used to calculate the frequency of transfer of antibiotic

126 resistance.

127

128 **Antibiotic resistance transfer experiment in broth or milk**

129 Nine ml volumes of LB Broth (Miller) or retail pasteurised milk were inoculated with

130 1 ml volumes of the above inocula to form suspensions containing approx. 10^5 cfu/ml

131 of donor (*S.* Typhimurium DT104) or recipient (*E. coli* K12 nal^R) suspensions. Donor

132 and recipient cell suspensions were mixed in 1:1 ratios, incubated for 18 h at 4, 15, 25

133 or 37°C and then plated directly on TSA containing 50 µg/ml of ampicillin and 50

134 µg/ml of nalidixic acid and incubated at 37°C for 24 h, to recover potential

135 transconjugants. Single strain suspensions of each strain were incubated at the test

136 temperatures for 18 h in broth and milk (10^5 cfu/ml) and were used as controls in this

137 experiment.

138

139 **Antibiotic resistance transfer experiment in ground meat**

140 Beef trimmings (70% w/w visible lean), obtained from a beef abattoir in the Dublin

141 area were minced (Crypto Ltd., London) divided into 30 g portions, blast frozen at –

142 30°C for 2 h (Woods $M3C_3$, Avon Refrig. Co. Ltd. U.K.) and stored at –20°C.

143 Samples from each batch of ground meat were confirmed as *Salmonella* free (ISO

144 method 6579) and *E. coli* free (ISO method 6649-2). Prior to use, ground meat

145 samples were defrosted overnight at 4°C. Duplicate 25 ml volumes of the donor

146 strain inoculum (10^7 cfu/ml) (*S.* Typhimurium DT104) and 25 ml of the respective

147 recipient strain inocula (10^7 cfu/ml) (*E. coli* K12 nal^R), were added to 450 ml volumes

148 of MRD (1:10 dilution), to form combined inoculating suspensions containing 10^6

149 cfu/ml. Single strain inoculating suspensions were prepared by adding 50 ml of donor

150 or recipient inocula to 450 ml volumes of MRD to give a final suspension of 10^6

151 cfu/ml of donor or recipient cells. The single strain suspensions of the donor and

152 recipient cultures were also incubated at 37°C for 18 or 36 h and were used as controls

153 in this experiment.

154

155 Thirty gram ground meat samples (retained within a sterile sieve) were immersed in

156 each of the above single or combined inoculating suspensions for 1 min and recovered

157 by removal of the sieve from the inoculation suspensions, allowed to drain and then

158 reminced (in a sterilise mincer). Preliminary studies established that this process

159 resulted in ground meat samples with an inoculum of approximately $\log_{10} 10^5$ cfu/g

160 (data not shown). Samples (25 g) of inoculated ground meat were placed in

161 individual sterile bags, incubated at temperatures 4, 15, 25 and 37°C for 18 h (and also
162 36 h for ground meat). At these time intervals, bags for each different temperature
163 were retrieved and microbiologically examined. Following incubation the contents of
164 each bag were stomached for 2 min with 225 ml Maximum Recovery Diluent (MRD,
165 Oxoid) in a stomacher bag fitted with an integral filter (Seward Ltd., London). The
166 resultant filtrate was serially-diluted in 9 ml aliquots of MRD, plated onto TSA
167 containing ampicillin (50 μg/ml) and nalidixic acid (50 μg/ml) and incubated at 37°C
168 for 24 h. Plates from samples co-inoculated or singly inoculated with *E. coli* K12
169 nal^{R} (as potential recipient), were overlayed with Mac Conkey No. 3 (Oxoid). All
170 plates were then incubated at 37°C for 24 h and the numbers of colonies per plate was
171 counted.
172
173 The antibiotic resistance profiles of all recovered recipient, donor and presumptive
174 transconjugant strains of *S.* Typhimurium DT104 and *E. coli* K12 nal^{R} were
175 confirmed using the Bauer-Kirby disc diffusion method as described above.
176
177 This experiment was replicated on three different occasions using separate batches of
178 broth, milk and ground meat, fresh inocula and all of the antibiotic resistance profiles
179 were rechecked (by disc diffusion) on each occasions. The average of these three
180 replicate experiments was used to calculate the frequency of antibiotic resistance
181 transfer.
182

183 **Stability of transconjugants**

184 The stability of the *E. coli* K12 nal^R transconjugant was assessed by daily sequential
185 subculture on TSA containing ampicillin (50 µg/ml) and nalidixic acid (50 µg/ml) for
186 14 days.

187

188 **Molecular detection of ß-lactamase genes**

189 **DNA isolation**

190 DNA was purified from donor, recipient and transconjugant strains using the Wizard
191 Genomic DNA purification kit (Promega, Madison, WI), according to the
192 manufacturers recommendations. In each case, the amount of recovered (template)
193 DNA was spectrophotometrically determined (O'Mahony *et al.,* 2005). The integrity
194 of each DNA sample was assessed by conventional agarose gel [1.5%, (w/v)]
195 electrophoresis in 1 X tris-EDTA-acetic acid (TEA) buffer containing 0.5 µg/ ml
196 ethidium bromide (EtBr). DNA preparations were stored at 4˚C.

197

198 **Amplification of β-lactamase-encoding bla_{TEM} gene by PCR**

199 DNA samples were examined for the presence of the bla_{TEM} encoding β-lactamase
200 genes, using the PCR primers and cycle conditions, previously reported by Arlet and
201 Philippon (1991) as shown in Table 1.

202

203

204 **Calculation of frequency of antibiotic resistance transfer**

205 The frequency of antibiotic resistance transfer was calculated as follows;

$$\frac{\text{No. of transconjugants } \log_{10} \text{cfu per ml/ g}}{\text{No. of recipient cells } \log_{10} \text{cfu per ml/g}}$$

206

207 (McMahon *et al.* 2007; Ohlsen *et al.* 2003; Netherwood *et al.* 1999).

208

209 **Results**

210 **Ampicillin resistance transfer experiment in broth, milk and ground meat.**

211 The frequencies of transfer of ampicillin resistance from the donor strain (*S.*

212 Typhimurium DT104) into the recipient strain (*E. coli* K12 nal^R) in each of the

213 matrices (broth, milk or ground meat), at a range of incubation temperatures (4, 15,

214 25, or 37°C) and times (18 or 36 h) are presented in Table 2. When transfer was

215 found to occur under defined conditions, it was reproducible for each of three

216 independent replicate experiments. Newly acquired antibiotic resistance profiles in

217 the transconjugant strains (as detected by disc diffusion analysis), were found to be

218 consistent for each independent replicate experiment.

219

220 At 25 and 37°C, ampicillin resistance transfer was observed in all matrices at a rate of

221 between 10^{-2} to 10^{-4} cfu/ml/g transconjugants per recipient at 37°C and 10^{-4} to 10^{-5}

222 cfu/ml/g transconjugants per recipient at 25°C (Table 2). The highest frequency of

223 transfer observed in this study was observed at 37°C, from *S.* Typhimurium DT104 to

224 *E. coli* K12 nal^R (10^{-2} cfu/ g^{-1}transconjugants per recipient) in ground meat stored for

225 36 h.

226 At 15°C, ampicillin resistance transfer was observed in ground meat, but was not
227 observed in milk or broth mating experiments. At 4°C, ampicillin resistance transfer
228 was not observed in any the examined matrices.
229

230 **Antibiotic Resistance transfer and stability of transconjugants**

231 Disc diffusion analysis confimed that the transconjugant isolates of *E. coli* K12 nal^{R}
232 possessed (newly acquired) resistance to ampicillin. Transconjugants isolated on the
233 basis of resistance to ampicillin did not acquire any other resistance markers
234 (resistance to chloramphenicol, streptomycin, sulphonamides and tetracycline) present
235 in the donor strain. The *E. coli* K12 nal^{R} transconjugant isolates continued to express
236 ampicillin resistance (as determined by continuous culture in the presence of
237 ampicillin and nalidixic acid) for 14 consecutive days.
238

239 **Molecular detection of ß-lactamase genes.**

240 PCR analysis demonstrated the presence of the β-lactamase gene *bla*$_{TEM}$ in the donor
241 strain/*S.* Typhimurium DT104, but not in original recipient strain of *E. coli* K12 nal^{R}.
242 After mating, PCR analysis revealed the newly acquired *bla*$_{TEM}$ gene in the *E. coli*
243 K12 nal^{R} transconjugant.
244

245 **Discussion**

246 This study found that ampicillin resistance could be transferred among bacterial
247 species in meat systems, at temperatures which occur within the food processing and
248 the distribution chain. The current study detected ampicillin resistance gene transfer
249 at temperatures as low as 15°C, suggesting that conditions/components of the meat
250 matrix are favourable for gene transfer at low temperatures. It is not yet clear, which
251 aspects of the meat matrix are important to facilitate gene transfer. However, ground
252 meat provides a large (non-motile) area for bacterial attachment in contrast to mating
253 experiments in liquids. The advantages of solid matrix for mating experiments have
254 been previously reported (Lagido *et al.* 2003; Molin and Tolker-Nielsen, 2003). Hirt
255 *et al.* (2002), also reported that the presence of plasma increased tetracycline
256 resistance transfer in *Enterococcus faecalis* in a rabbit endocarditis model, suggesting
257 that the composition of the meat matrix may present a favourable environment for
258 gene transfer. All of these factors may have contributed to ground meat being the
259 most suitable matrix for ampicillin resistance gene transfer in this study.

260

261 Resistance to ampicillin was found to be transferable from *S.* Typhimurium DT104 to
262 *E. coli* K12 nal^{R} in broth, milk and ground meat, at 25 and 37°C. At these
263 temperatures, similar rates of transfer (10^{-4} to 10^{-5} cfu/ml/g transconjugants per
264 recipient) were observed in all three matrices after 18 h. These results suggest that at
265 non-stress (optimal 37°C and sub-optimal 25°C) temperatures, the transfer frequency
266 of the organisms studied were not significantly affected by the nature of the transfer
267 environment (broth, milk or ground meat). This is agreement with a study by
268 Cocconelli *et al.* (2003), who found similar frequencies of transfer during sausages
269 and cheese fermentation (10^{-7} to 10^{-8} cfu/ml/g, transconjugants per recipient) at 30°C,

270 again suggesting that the nature of the transfer environment may not significantly
271 impact on the frequency of antibiotic resistance transfer at non-stress temperatures.
272
273 Our study observed lower frequencies of ampicillin resistance transfer (10^6 $\log_{10}$ cfu/g
274 tranconjugants per recipient) at stressed temperatures such as 15°C in ground meat
275 and no transfer in broth or milk. Lower rates of antibiotic resistant transfer at non-
276 optimal temperatures have been previously reported (McMahon *et al.* 2007). At
277 15°C, the nature of the transfer environment was found to influence the frequency of
278 ampicillin resistance from the donor to the recipient strains. However, while reduced
279 rates of transfer were expected, it is interesting that ampicillin resistance transfer
280 occurred at 15°C in ground meat, but not in broth or milk. Cocconcelli *et al.* (2003)
281 reported the transfer of antibiotic resistance by conjugation at 10°C in sausage and
282 cheese. However, unlike the current study, Cocconcelli *et al.* (2003) did not try to
283 transfer antibiotic resistance within a liquid system at this temperature (10°C) making
284 it difficult to compare these data. A knowledge gap exists in area of transfer of
285 antibiotic resistance in food making comparison with other studies difficult.
286
287 No ampicillin resistance transfer was found to occur in any matrices examined at 4°C.
288 This appears to be due to the overall reduction in the metabolic rates of the
289 (mesophilic) donor and recipient strains used. However, McMahon *et al*. (2007)
290 reported antibiotic resistance transfer in broth between *E. coli* at 5°C, suggesting that
291 the occurrence or absence of such transfer may be related to the characteristics of the
292 donor and/or recipient organisms (and possible mating criteria), rather than simple
293 thermodynamic (Q_{10}) factors. This is underpinned by Frischer *et al* (1993) who
294 reported transfer at temperatures between 4 and 33°C while working with a marine

295 *Vibro* spp., but significantly reduced rates of gene transfer at 37°C. It would therefore
296 be unwise to assume that effective maintenance of correct chill-chain conditions
297 during food production will prevent gene transfer among all bacterial species in food
298 and the food chain.
299
300 PCR analysis confirmed the transfer of the β-lactamase gene bla_{TEM} (conferring
301 ampicillin resistance) from *S.* Typhimurium DT104 to *E. coli* K12 nal^R. It also
302 confirmed that the acquired resistance persisted in the transconjugants for some
303 considerable time. This observation significantly increases the potential importance
304 of such transfers, in terms of the general persistence and dissemination of antibiotic
305 resistance in food and food environments and in more specific terms, confirming the
306 exchange of clinically significant antibiotic resistance within the food chain. Such
307 processes mean that antibiotic resistance genes entering the human food chain may be
308 spread among other bacteria, allowing the persistence and dissemination of antibiotic
309 resistance to other more directly significant pathogens in food production and the
310 processing chain.
311
312 Examination of the wider antibiotic resistant profile of the *E. coli* K12 nal^R
313 transconjugants revealed that the recipient bacteria had acquired stable resistance to
314 ampicillin *via* (the generally plasmid mediated) bla_{TEM} gene. Transconjugants isolated
315 on the basis of resistance to ampicillin did not acquire any other resistant markers
316 (chloramphenicol, streptomycin, sulphonamides and tetracycline) of the donor strain,
317 suggesting no movement of the *Salmonella* Genomic Island 1 (SGI-1) from the donor
318 *S.* Typhimurium DT104 strain. Similarly a study by Guerri *et al.* (2004),
319 demonstrated the transfer of ampicillin resistance (only) *via* the bla_{TEM} gene from *S.*

320 Typhimurium DT104 (SGI-1 containing) to *E. coli* during conjugation. However,

321 Doublet *et al.* (2005) and Mulvey *et al.* (2006) report that the SGI-1 can be mobilized

322 from *S.* Typhimurium DT104 to non-SGI-1 containing *Salmonella* and *E. coli via* a

323 helper IncC plasmid R55, highlighting the possibility of such transfer in food.

324

325 In conclusion, this study has established that antibiotic resistance genes can be

326 transferred between bacteria in common food systems, suggesting that food matrices

327 can play a role in gene transfer, dissemination and persistence. Antibiotic resistance

328 transfer was not observed at 4°C, suggesting that effective chill chain conditions

329 would reduce the rate and significance of gene transfer in refrigerated foods for the

330 bacteria examined in this study. However, gene transfer can occur at relatively low

331 temperatures i.e. 15°C in some food matrices (ground meat) and more rapidly in a

332 wider range of food matrices at/or above room temperature. The transfer of antibiotic

333 resistance among Gram-negative bacteria including commensals and clinically

334 significant pathogens is a matter of public health concern. Such transfers, the factors

335 governing their rates, stability and the linkages between virulence and antibiotic

336 resistance, deserve greater research attention in terms of reducing clinical risks and as

337 an emerging element in the wider dissemination and persistence of antibiotic resistant

338 genes in the human environment.

339

340

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Table 1 **Thermocycler amplification conditions for the β-lactamase gene**: bla_{TEM} **(Arlet and Philippon, 1991)**

Gene	Denaturation	Annealing	Extensions	No. of Cycles	Primers	
bla_{TEM}	94°C-2 min	54°C-1 min	72°C-30 sec	30	Fwd	5`-TTG GGT GCA CGA GTG GGT TA-3`
					Rev	5`-TAA TTG TTG CCG GGA AGC TA-3`

Table 2 **Frequency of transfer from *S.* Typhimurium DT104 to *E. coli* K12 nal^R in broth (log_{10} cfu/ml), milk (log_{10} cfu/ml) for 18 h, and ground meat (log_{10} cfu/g) for 18 and 36 h.**

	Trans	Trans/Recpt		Trans	Trans/Recpt
Broth (18 h)			**Meat (18 h)**		
4 °C	0.00	0.00	4 °C	0.00	0.00
15 °C	0.00	0.00	15 °C	0.22×10^0	2.0×10^{-6}
25 °C	4.13×10^0	2.9×10^{-4}	25 °C	1.93×10^0	4.7×10^{-5}
37 °C	4.45×10^0	5.8×10^{-4}	37 °C	3.49×10^0	4.7×10^{-4}
Milk (18 h)			**Meat (36 h)**		
4 °C	0.00	0.00	4 °C	0.00	0.00
15 °C	0.00	0.00	15 °C	0.52×10^0	8.1×10^{-6}
25 °C	3.17×10^0	2.6×10^{-4}	25 °C	3.08×10^0	5.8×10^{-4}
37 °C	4.07×10^0	2.6×10^{-4}	37 °C	4.23×10^0	3.3×10^{-2}

*Trans: Transconjugants, Trans/Recpt: Transconjugants per recipient cells