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

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Abstract

Aims: To investigate the transfer of antibiotic resistance from a donor *S.* Typhimurium DT104 strain to a recipient *E. coli* K12 strain .

Methods and Results: Mating experiments were conducted in broth, milk and ground meat (beef) at incubation temperatures of 4, 15, 25 and 37°C for 18 and 36 h. Ampicillin resistance transfer was observed at similar frequencies in all transfer media at 25 and 37°C (10^{-4} to 10^{-5} $\log_{10}$ cfu/ml/g, transconjugants per recipient) for 18 h. At 15°C, transfer was observed in ground meat in the recipient strain (10^{-6}, $\log_{10}$ cfu/g, transconjugants per recipient), but not in broth or milk. At 4°C, transfer did not occur in any of the examined mediums. Further analysis of the *E. coli* K12 nal^R transconjugant strain revealed the presence of a newly acquired β-lactamase gene bla_{TEM}. Transconjugants isolated on the basis of resistance to ampicillin did not acquire any other resistant markers.

Conclusion: This study demonstrates the transfer of antibiotic resistance in food matrices at mid-range temperatures.

Significance and Impact of the Study: It highlights the involvement of food matrices in the dissemination of antibiotic resistant genes and the evolution of antibiotic resistant bacteria.

Introduction

Salmonella Typhimurium DT104 is a major cause of enteric infections world-wide (Beaudin *et al.* 2002). This serovar typically expresses resistance to ampicillin, chloramphenicol/florfenicol, streptomycin/spectinomycin, sulfonamides and tetracycline (ACSSuT) (Golding *et al.* 2007). Such multi-drug resistant (MDR) *S.* Typhimurium DT104 are of concern especially in immuno-comprised patients, as it restricts clinical options in the treatment of salmonellosis. In more general terms, it has potential consequences in relation to transfer and dissemination of antibiotic resistant material to other bacteria including pathogens.

Class A β-lactamases are the most widespread enzymes in Gram-negative bacteria (Chochani *et al.* 2006, Bonnet, 2004). These enzymes are widely distributed in *Salmonella* spp. and are frequently associated with the above (ACSSuT) penta resistance profile (Guerri *et al.* 2004). In *Salmonella,* β-lactamase resistance is often conferred by the presence of the bla_{TEM} or bla_{PSE} gene (Randall *et al.* 2004). Of these, the $bla_{\text{TEM-1}}$ gene which is generally plasmid-mediated (Larson and Ramphal, 2002) is considered the most frequently expressed β-lactamase in *Salmonella* in Europe (Guerri *et al.* 2004; Tzouvelekis *et al.* 2003).

A number of studies have investigated the transfer of antibiotic resistance in food-borne bacterial pathogens such as *Salmonella,* but few studies have examined gene transfer between bacteria in-*situ* in food. Reports of gene transfer (by transformation) of kanamycin in *Bacillus subtilis* in milk (Kharazmi *et al.,* 2002) and the transfer of vancomycin resistant genes among enterococcal strains by conjugation during cheese

and sausage fermentation (Cocconelli *et al.,* 2003) have been cited. However, the majority of studies on the transfer of antibiotic resistance have been carried out in liquid systems (McMahon *et al.* 2007; Wilcks *et al.,* 2005; Chen *et al.,* 2004; Allen and Poppe, 2002) or on filters (Hummel *et. al.,* 2007; Gevers *et al.,* 2003; Pourshaban *et al.,* 2002) and thus do not reflect the in-*situ* dynamics of food matrices. This balance of activity is unfortunate, as liquid or filter based systems have been reported to underestimate the rates of genes transfer compared to more complex matrices (Netherwood *et al.,* 1999). Moreover, concern has been raised about food being an important and under-estimated avenue for antibiotic resistance dissemination and evolution (Wang *et al.* 2005).

The objective of this study was to ascertain if antibiotic resistance gene transfer can occur between a donor *S.* Typhimurium DT104 and *E. coli* K12, in broth, milk and ground meat under different temperature and time conditions encountered in food processing.

Material and Methods

Bacterial strains

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

Preparation of nalidixic acid resistant mutants and antibiogram profiling

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

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

Preparation of inoculum

Protect beads coated with *S.* Typhimurium DT104 and *E. coli* K12 nal^R were incubated in 30 ml volumes of LB Broth (Miller, Germany) at 37^{o}C for 18 h, to form stationary phase cultures containing approximately 10^9 cfu/ml. A 1.0 ml aliquot from each stationary phase culture was serially diluted in 9 ml volumes of Maximum Recovery Diluent (MRD, Oxoid) to form inocula containing approximately 10^6 cfu/ ml^{-1} culture.

Control experiments

The donor and recipient strains were grown independently, inoculated and recovered from each food matrix during each experiment, by plating on to TSA-ampicillin (50 μg/ml) (to recover the no. of donor strains) and TSA-nalidixic acid (50 μg/ml) (to recover the no. of transconjugant strains). The number of colonies recovered from these plates was then used to calculate the frequency of transfer of antibiotic resistance.

Antibiotic resistance transfer experiment in broth or milk

Nine ml volumes of LB Broth (Miller) or retail pasteurised milk were inoculated with 1 ml volumes of the above inocula to form suspensions containing approx. 10^5 cfu/ml of donor (*S.* Typhimurium DT104) or recipient (*E. coli* K12 nal^R) suspensions. Donor and recipient cell suspensions were mixed in 1:1 ratios, incubated for 18 h at 4, 15, 25 or 37^{o}C and then plated directly on TSA containing 50 μg/ml of ampicillin and 50 μg/ml of nalidixic acid and incubated at 37^{o}C for 24 h, to recover potential 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

individual sterile bags, incubated at temperatures 4, 15, 25 and 37^{o}C for 18 h (and also 36 h for ground meat). At these time intervals, bags for each different temperature were retrieved and microbiologically examined. Following incubation the contents of each bag were stomached for 2 min with 225 ml Maximum Recovery Diluent (MRD, Oxoid) in a stomacher bag fitted with an integral filter (Seward Ltd., London). The resultant filtrate was serially-diluted in 9 ml aliquots of MRD, plated onto TSA containing ampicillin (50 μg/ml) and nalidixic acid (50 μg/ml) and incubated at 37^{o}C for 24 h. Plates from samples co-inoculated or singly inoculated with *E. coli* K12 nal^{R} (as potential recipient), were overlayed with Mac Conkey No. 3 (Oxoid). All plates were then incubated at 37^{o}C for 24 h and the numbers of colonies per plate was counted.

The antibiotic resistance profiles of all recovered recipient, donor and presumptive transconjugant strains of *S.* Typhimurium DT104 and *E. coli* K12 nal^{R} were confirmed using the Bauer-Kirby disc diffusion method as described above.

This experiment was replicated on three different occasions using separate batches of broth, milk and ground meat, fresh inocula and all of the antibiotic resistance profiles were rechecked (by disc diffusion) on each occasions. The average of these three replicate experiments was used to calculate the frequency of antibiotic resistance transfer.

Stability of transconjugants

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

Molecular detection of ß-lactamase genes

DNA isolation

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

Amplification of β-lactamase-encoding *bla*$_{TEM}$ gene by PCR

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

Calculation of frequency of antibiotic resistance transfer

The frequency of antibiotic resistance transfer was calculated as follows;

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

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

Results

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

The frequencies of transfer of ampicillin resistance from the donor strain (*S.* Typhimurium DT104) into the recipient strain (*E. coli* K12 nal^R) in each of the matrices (broth, milk or ground meat), at a range of incubation temperatures (4, 15, 25, or 37^{o}C) and times (18 or 36 h) are presented in Table 2. When transfer was found to occur under defined conditions, it was reproducible for each of three independent replicate experiments. Newly acquired antibiotic resistance profiles in the transconjugant strains (as detected by disc diffusion analysis), were found to be consistent for each independent replicate experiment.

At 25 and 37^{o}C, ampicillin resistance transfer was observed in all matrices at a rate of between 10^{-2} to 10^{-4} cfu/ml/g transconjugants per recipient at 37^{o}C and 10^{-4} to 10^{-5} cfu/ml/g transconjugants per recipient at 25^{o}C (Table 2). The highest frequency of transfer observed in this study was observed at 37^{o}C, from *S.* Typhimurium DT104 to *E. coli* K12 nal^R (10^{-2} cfu/ g^{-1}transconjugants per recipient) in ground meat stored for 36 h.

At 15^{o}C, ampicillin resistance transfer was observed in ground meat, but was not observed in milk or broth mating experiments. At 4^{o}C, ampicillin resistance transfer was not observed in any the examined matrices.

Antibiotic Resistance transfer and stability of transconjugants

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

Molecular detection of ß-lactamase genes.

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

Discussion

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

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

again suggesting that the nature of the transfer environment may not significantly impact on the frequency of antibiotic resistance transfer at non-stress temperatures.

Our study observed lower frequencies of ampicillin resistance transfer (10^6 $\log_{10}$ cfu/g tranconjugants per recipient) at stressed temperatures such as 15^{o}C in ground meat and no transfer in broth or milk. Lower rates of antibiotic resistant transfer at non-optimal temperatures have been previously reported (McMahon *et al.* 2007). At 15^{o}C, the nature of the transfer environment was found to influence the frequency of ampicillin resistance from the donor to the recipient strains. However, while reduced rates of transfer were expected, it is interesting that ampicillin resistance transfer occurred at 15^{o}C in ground meat, but not in broth or milk. Cocconcelli *et al.* (2003) reported the transfer of antibiotic resistance by conjugation at 10^{o}C in sausage and cheese. However, unlike the current study, Cocconcelli *et al.* (2003) did not try to transfer antibiotic resistance within a liquid system at this temperature (10^{o}C) making it difficult to compare these data. A knowledge gap exists in area of transfer of antibiotic resistance in food making comparison with other studies difficult.

No ampicillin resistance transfer was found to occur in any matrices examined at 4^{o}C. This appears to be due to the overall reduction in the metabolic rates of the (mesophilic) donor and recipient strains used. However, McMahon *et al*. (2007) reported antibiotic resistance transfer in broth between *E. coli* at 5^{o}C, suggesting that the occurrence or absence of such transfer may be related to the characteristics of the donor and/or recipient organisms (and possible mating criteria), rather than simple thermodynamic (Q_{10}) factors. This is underpinned by Frischer *et al* (1993) who reported transfer at temperatures between 4 and 33°C while working with a marine

Vibro spp., but significantly reduced rates of gene transfer at 37°C. It would therefore be unwise to assume that effective maintenance of correct chill-chain conditions during food production will prevent gene transfer among all bacterial species in food and the food chain.

PCR analysis confirmed the transfer of the β-lactamase gene bla_{TEM} (conferring ampicillin resistance) from *S.* Typhimurium DT104 to *E. coli* K12 nal^R. It also confirmed that the acquired resistance persisted in the transconjugants for some considerable time. This observation significantly increases the potential importance of such transfers, in terms of the general persistence and dissemination of antibiotic resistance in food and food environments and in more specific terms, confirming the exchange of clinically significant antibiotic resistance within the food chain. Such processes mean that antibiotic resistance genes entering the human food chain may be spread among other bacteria, allowing the persistence and dissemination of antibiotic resistance to other more directly significant pathogens in food production and the processing chain.

Examination of the wider antibiotic resistant profile of the *E. coli* K12 nal^R transconjugants revealed that the recipient bacteria had acquired stable resistance to ampicillin *via* (the generally plasmid mediated) bla_{TEM} gene. Transconjugants isolated on the basis of resistance to ampicillin did not acquire any other resistant markers (chloramphenicol, streptomycin, sulphonamides and tetracycline) of the donor strain, suggesting no movement of the *Salmonella* Genomic Island 1 (SGI-1) from the donor *S.* Typhimurium DT104 strain. Similarly a study by Guerri *et al.* (2004), demonstrated the transfer of ampicillin resistance (only) *via* the bla_{TEM} gene from *S.*

Typhimurium DT104 (SGI-1 containing) to *E. coli* during conjugation. However, Doublet *et al.* (2005) and Mulvey *et al.* (2006) report that the SGI-1 can be mobilized from *S.* Typhimurium DT104 to non-SGI-1 containing *Salmonella* and *E. coli via* a helper IncC plasmid R55, highlighting the possibility of such transfer in food.

In conclusion, this study has established that antibiotic resistance genes can be transferred between bacteria in common food systems, suggesting that food matrices can play a role in gene transfer, dissemination and persistence. Antibiotic resistance transfer was not observed at 4^{o}C, suggesting that effective chill chain conditions would reduce the rate and significance of gene transfer in refrigerated foods for the bacteria examined in this study. However, gene transfer can occur at relatively low temperatures i.e. 15^{o}C in some food matrices (ground meat) and more rapidly in a wider range of food matrices at/or above room temperature. The transfer of antibiotic resistance among Gram-negative bacteria including commensals and clinically significant pathogens is a matter of public health concern. Such transfers, the factors governing their rates, stability and the linkages between virulence and antibiotic resistance, deserve greater research attention in terms of reducing clinical risks and as an emerging element in the wider dissemination and persistence of antibiotic resistant genes in the human environment.

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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^{o}C-2 min	54^{o}C-1 min	72^{o}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 ^{o}C	0.00	0.00	4 ^{o}C	0.00	0.00
15 ^{o}C	0.00	0.00	15 ^{o}C	0.22×10^{0}	2.0×10^{-6}
25 ^{o}C	4.13×10^{0}	2.9×10^{-4}	25 ^{o}C	1.93×10^{0}	4.7×10^{-5}
37 ^{o}C	4.45×10^{0}	5.8×10^{-4}	37 ^{o}C	3.49×10^{0}	4.7×10^{-4}
Milk (18 h)			**Meat (36 h)**		
4 ^{o}C	0.00	0.00	4 ^{o}C	0.00	0.00
15 ^{o}C	0.00	0.00	15 ^{o}C	0.52×10^{0}	8.1×10^{-6}
25 ^{o}C	3.17×10^{0}	2.6×10^{-4}	25 ^{o}C	3.08×10^{0}	5.8×10^{-4}
37 ^{o}C	4.07×10^{0}	2.6×10^{-4}	37 ^{o}C	4.23×10^{0}	3.3×10^{-2}

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