Food commensal microbes as a potentially important avenue in transmitting antibiotic resistance genes

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Abstract

The rapid emergence of antibiotic-resistant (ART) pathogens is a major threat to public health. While the surfacing of ART food-borne pathogens is alarming, the magnitude of the antibiotic resistance (AR) gene pool in food-borne commensal microbes is yet to be revealed. Incidence of ART commensals in selected retail food products was examined in this study. The presence of 10^2–10^7 CFU of ART bacteria per gram of foods in many samples, particularly in ready-to-eat, 'healthy' food items, indicates that the ART bacteria are abundant in the food chain. AR-encoding genes were detected in ART isolates, and *Streptococcus thermophilus* was found to be a major host for AR genes in cheese microbiota. *Lactococcus lactis* and *Leuconostoc* sp. isolates were also found carrying AR genes. The data indicate that food could be an important avenue for ART bacterial evolution and dissemination. AR-encoding plasmids from several food-borne commensals were transmitted to *Streptococcus mutans* via natural gene transformation under laboratory conditions, suggesting the possible transfer of AR genes from food commensals to human residential bacteria via horizontal gene transfer.

Introduction

Resistant pathogens to various antibiotics are emerging rapidly. Surfacing of these resistant pathogens, untreatable by antibiotics, constitutes a real threat to public health. To effectively combat this problem, a comprehensive understanding of the major pathways in antibiotic resistance (AR) gene dissemination as well as the key mechanisms in the evolution of antibiotic-resistant (ART) bacteria is essential.

Horizontal gene transfer among pathogens in the hospital environment has been recognized as an important avenue for the rapid spread of AR genes among pathogens. It is also believed that horizontal transmissions of AR genes between commensal and pathogenic microorganisms in ecosystems are much more likely events than direct AR gene dissemination from one pathogen to another (Andremont, 2003). The presence of AR gene reservoirs in commensal microbes in various environmental and host ecosystems (Gilliver *et al.*, 1999; Österblad *et al.*, 2001; Lancaster *et al.*, 2003; Ready *et al.*, 2003; Nandi *et al.*, 2004; Salyers *et al.*, 2004; Smith *et al.*, 2004), the illustration of commensals as facilitators for AR gene dissemination (Luo *et al.*, 2005b), and the correlation of antibiotics usage in animals with increased AR in human microbiota (Levy *et al.*, 1976; Smith *et al.*, 2002) suggest the importance of commensals in mediating the dissemination of AR genes. The isolation of AR genes in food-borne pathogens from retail products exemplified the potential contribution of the food chain in transmitting ART pathogens to humans (Charpentier & Courvalin, 1999; Zhao *et al.*, 2001; Luo *et al.*, 2005a). The studies on commensal bacteria, however, are limited and primarily focused on the opportunistic pathogen enterococci (Cocconcelli *et al.*, 2003; Johnston & Jaykus, 2004). A standard laboratory enrichment procedure (http://www.fda.gov/cvm/Documents/AppendicesA-6.pdf) is often used to detect the presence of the ART bacteria, masking the real magnitude of the AR problem associated with the food chain.

To examine the AR risks associated with the food chain, this study aimed at revealing the distribution spectrum and magnitude of ART commensal bacteria and AR gene pool in retail foods, by targeting total food microbiota instead of a particular group of microorganisms or pathogens. Therefore, food samples were analyzed without any laboratory enrichment procedures. Microbial resistance to tetracycline

(Tet) and erythromycin (Em), which are still used in animal production and human therapy (Chopra & Roberts, 2001; Roberts, 2004), was investigated. The presence of several AR markers including *erm*B, *erm*C, *tet*S/M and *tet*A, encoding ribosomal modification and Tet efflux mechanisms, in selected food isolates was examined and main AR gene hosts were identified.

Materials and methods

Food sample preparation and enumeration of total and ART populations

Food samples (Fig. 1) were purchased from local grocery stores and analyzed within the products' sell-by dates. Fresh raw milk was obtained from the dairy pilot plant at OSU and analyzed the same day as shipped. Five grams of each sample were aseptically removed from the product packaging and placed in disposable plastic bags containing 10 mL of sterile 0.1% peptone water. Bagged samples were hand massaged for 10 min. Homogenized samples or rinsing liquids were serially diluted and plated on nonselective plate count agar (PCA, Becton Dickinson and Company, Sparks, MD) for nonselective total microbial counting, and on PCA plates containing 16 μg mL^{-1} of Tet or 8 μg mL^{-1} of Em (Fisher Biotech, Fair Lawn, NJ). Plates were incubated at conditions as indicated (Fig. 1) for up to 48 h for assessing Tet- and Em-resistant population. The levels of antibiotics used in selective agar plates were based on that used to screen for ART enterococci (http://www.fda.gov/cvm/Documents/AppendicesA-6.pdf). Serially diluted samples were also plated on Difco Lactobacilli MRS Agar (MRS, Becton Dickinson and Company) and *Pseudomonas* isolation agar (PIA, EMD Chemicals Inc., Gibbstown, NJ) plates with proper antibiotics to recover ART lactic acid bacteria and *Pseudomonas* species, respectively. The cell numbers reported (CFU g^{-1} of food, Figs 1a and b) were the mean values from duplicates.

Antibiotic resistance gene detection and host isolates identification

Conventional PCR was conducted to detect AR genes in the ART isolates. Bacterial cells from single colonies were resuspended in 300 μL sterile dH_2O containing 100 μg of 1 : 1 mixture of 0.5 μm diameter and 0.1 μm diameter glass beads (Biospec Products Inc., Bartlesville, OK). The sample mixtures were homogenized using the Mini-Bead-Beater-8 (Biospec Products Inc.) for 2 min at maximum speed. The resulting cell extracts were placed in a boiling water bath for

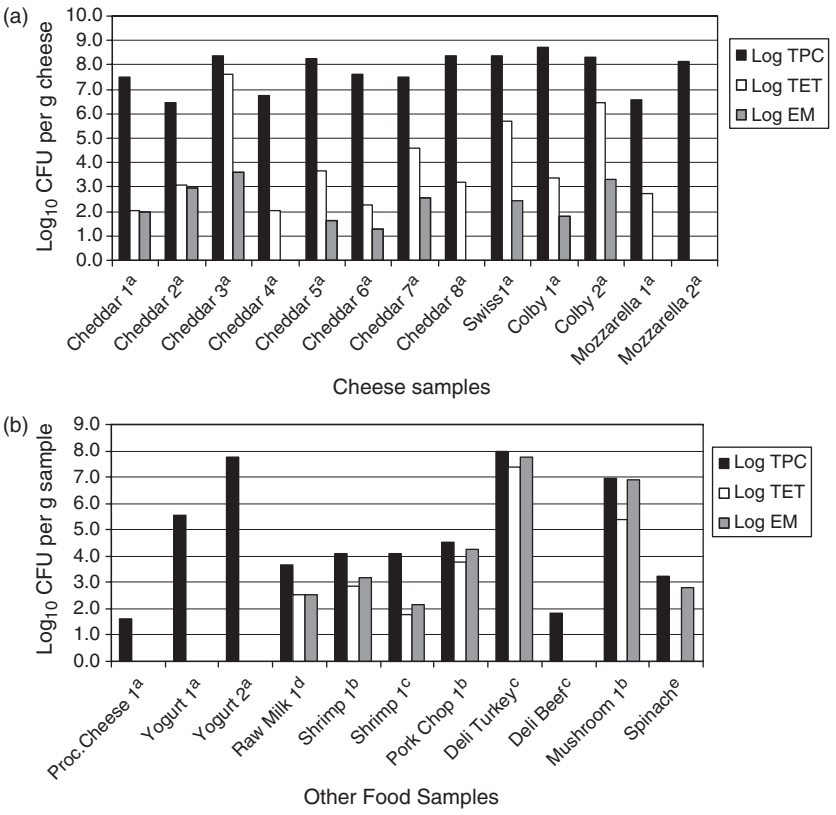

Fig. 1. Prevalence of antibiotic-resistant microbes in retail foods. (a) Representative cheese samples. (b) Other representative foods. TPC, total plate count; Tet, Tet^r population screened on agar plates containing 16 μg mL^{-1} tetracycline; Em, Em^r population screened on agar plates containing 8 μg mL^{-1} (a–d) and 50 μg mL^{-1} (e) erythromycin. Data were means of duplicated results, and the standard deviations were less than 10% of the mean values. [a]Microorganisms were recovered by plating on MRS agar plates and incubated anaerobically at 30 °C. [b]Microorganisms were recovered by plating on plate count agar (PCA) agar plates and incubated aerobically at 20 °C. [c]Microorganisms were recovered by plating on PCA agar plates and incubated aerobically at 37 °C. [d]Microorganisms were recovered by plating on PCA agar plates and incubated aerobically at 20 °C. [e]Microorganisms were recovered from spinach samples by plating on PCA agar plate and incubated anaerobically at 30 °C.

10–15 min and 5 μL of the supernatant were used as PCR templates. The PCR primers *tet*A-FP 5′-GCTACAT CCTGCTTGCCTTC-3′ and *tet*A-RP 5′-CATAGATC GCC GTGAAGAGG-3′ were used to amplify the 220 bp *tet*A fragment (Ng *et al.*, 2001), *tet*S-FP 5′-CATAGACAAGC CGTTGACC-3′ and *tet*S-RP 5′-ATGTTTTTGGAACGCCA-GAG-3′ for the 667 bp *tet*S/M fragment (Ng *et al.*, 2001), *erm*B-FP 5′-GGAACAGGTAAAGGGC-3′ and *erm*B-RP 5′-GGTTTAGGATGAAAGC-3′ for the 389 bp *erm*B fragment (this study), and *erm*C FP 5′-GCTAATATTGTTTAAATC GTCAAT-3′ and *erm*C RP 5′-TCAAAACATAATATAGA-TAAA-3′ for the 640 bp *erm*C fragment (Chung *et al.*, 1999). PCR was conducted using reagents and conditions as described previously (Luo *et al.*, 2004). PCR products with expected sizes were purified using the QIAquick® kit (Qiagen, Valencia, CA) following manufacturer's instruction. DNA sequences of the 16S rRNA gene, *erm*C, *tet*A gene fragments and 50% of the *erm*B and *tet*S/M gene fragments were determined using a DNA analyzer (ABI PRISM® 3700, Applied Biosystems, Foster City, CA) at the Plant Genome Sequence Facility, The Ohio State University. The DNA sequences were compared with published Tet or Em resistance gene sequences deposited in the NCBI database. ART isolates containing the resistance genes were identified by PCR amplification of the 16S rRNA gene fragment and sequence analysis following procedures as described previously (Connor *et al.*, 2005).

Minimum inhibition concentration (MIC) profiles of ART isolates

The MIC profiles of selected ART isolates were determined using a commercial kit (Sensititre® 18–24 h MIC and Breakpoint Susceptibility Plates; TREK Diagnostic Systems, Cleveland, OH) following the manufacturer's instructions, with modifications. MRS or brain heart infusion (BHI) broth instead of the standard Mueller–Hinton broth was used to culture fastidious organisms. The MIC panels were incubated at either 30 or 37 °C for 24–48 h. Additional 96-well microtiter plates with wells containing up to 256 μg mL^{-1} of Tet and Em were used to determine the MIC of Em^r or Tet^r isolates which exhibited positive growth in wells containing 16 μg mL^{-1} of Tet or 8 μg mL^{-1} of Em on the Sensititre® plates. The MICs were reported as the minimum concentration of the antibiotic that inhibited visible growth, as indicated by increased turbidity or by deposition of cells at the bottom of the wells. Control strains used in the study include *Staphylococcus aureus* ATCC 29213 (American Type Culture Collection (ATCC), Manassas, VA), *Pseudomonas aeruginosa* ATCC 27853 (ATCC), *Lactococcus lactis* ML3 (Kuhl *et al.*, 1979), and *Streptococcus thermophilus* LMD-9 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=13773).

Plasmid isolation and natural gene transformation

Lactococcus sp. CZ-T4 (Tet^r) and CZ-T8 (Tet^r) were isolated from commercial cheddar cheese, while strain RMK-T14 (Tet^r) was obtained from raw milk (this study). The multi-drug-resistant *L. lactis* K214 was isolated from soft cheese made from raw milk (Perreten *et al.*, 1997). The strains were grown in MRS broth or M17 broth with 0.5% glucose, supplemented with 5 μg mL^{-1} Tet, and the mixture were incubated at 30 °C for 24 h. Plasmids were isolated from these strains following the method of Anderson & McKay (1984) and were used in the natural transformation experiments, following procedures as described by Li *et al.* (2001). For the selection of Tet^r transformants, BHI plates were supplemented with 5 μg mL^{-1} Tet. Plates were incubated in a 5% CO_2 incubator at 37 °C for 48 h. Transformation efficiency was calculated based on the ratio of Tet^r transformants to the total number of viable cells.

Results

Prevalence of ART bacteria in food samples

Antibiotic-resistant microbes were detected in majority of the retail foods examined, from raw food materials such as meat and shrimp to ready-to-eat items such as cheeses and salad. No detectable ART microbes were found in processed cheese (heat treated during manufacture) and yogurt samples, with representative data illustrated in Fig. 1. Twenty out of the 23 cheese samples analyzed contained Tet^r and/or Em^r microbes ranging from 10^2 to 10^7 CFU g^{-1} of food, which are equivalent to 10^3–10^8 CFU ART microbes per slice of cheese (about 20 g). In general, the number of Tet^r microbes was greater in cheeses than that of Em^r bacteria. It is worth noting that the study was conducted using limited incubation conditions, and the antibiotic concentrations used to screen for resistant organisms might not be optimal for all bacteria.

Detection of AR genes and ART isolates identification

To confirm that most of the ART organisms detected by growth on the selective agar plates were resistant bacteria because of the possession of AR determinants, conventional PCR was conducted to detect the presence of selected AR genes in these organisms and the results were summarized in Table 1. Among the Tet^r isolates recovered from cheese, about 10% contained the *tet*S/M gene. Seven out of 11 *tet*S/M$^+$ cheese isolates identified were *Staphylococcus thermophilus*; two *tet*S/M$^+$ isolates were found to be *Lactococcus lactis*. Two additional cheese isolates CZ-T4 and CZ-T8 had 97% 16S rRNA gene sequence identity to unidentified

Lactococcus sp., and particularly had 93–94% identity to *Lactococcus garvieae* and *L. lactis*, similar with that of the raw milk ART isolate RMK-T14, suggesting this might be a common organism from milk. Therefore, it is possible that the *Lactococcus* sp. *tet*S/M$^+$ isolates from cheese originated from milk (pasteurized but not sterile) or dairy processing environment during cheese fermentation. Another *tet*S/M$^+$ isolate from raw milk was identified as *Leuconostoc* sp. In addition, the *tet*A gene was found in two cheese isolates CZ-T3, CZ-T7 and several isolates from raw pork meat. These isolates were all identified as *Pseudomonas* sp.

Among the Em^r isolates from cheese, more than 50% contained the *erm*B gene, and the carrier organisms identified so far include *Staphylococcus* sp. (five out of 28) and *S. thermophilus* (23 out of 28). Both *tet*S/M and *erm*C genes were found in the isolate CX-I EM from packaged sliced chicken lunchmeat, suggesting a multidrug resistance phenotype of the strain. CX-I EM was identified as *Pseudomonas* sp. ART bacteria were isolated sporadically in lunchmeat (data not shown), which is probably because of occasional contamination during the processing of the meat.

Minimum inhibition concentration analysis

Minimum inhibition concentration tests of selected cheese isolates showed that *Lactococcus* sp. CZ-T4 and CZ-T8 (*tet*S/M$^+$) were resistant to at least 128 μg mL^{-1} Tet, and *S. thermophilus* E4 (*erm*B$^+$) was resistant to Em (> 256 μg mL^{-1}), clarithromycin (> 8 μg mL^{-1}), and clindamycin (> 4 μg mL^{-1}). *S. thermophilus* BOR-COCZ-T19 (*tet*S/M$^+$) was resistant to Tet (> 128 μg mL^{-1}). *Staphylococcus* sp. C202 was resistant to both Em (> 256 μg mL^{-1}) and Tet (> 32 μg mL^{-1}), suggesting the possible possession of both resistance determinants in this isolate. The control strains *Lactococcus lactis* ML3, *Staphylococcus thermophilus* LMD-9, two other commercial *S. thermophilus* starters and *Staphylococcus aureus* ATCC 29213 were sensitive (< 2 μg mL^{-1}) to the above antibiotics.

Lactococcus sp. RMK-T14 (*tet*S/M$^+$) from raw milk was resistant to Tet (> 128 μg mL^{-1}), Em (> 64 μg mL^{-1}), clarithromycin (> 8 μg mL^{-1}), and clindamycin (> 4 μg mL^{-1}). Therefore this isolate likely carried multidrug-resistant determinants or multidrug-resistant mechanism(s). The raw milk isolate *Streptococcus uberis* RMK-T22W exhibited resistance to Tet (> 128 μg mL^{-1}).

All of the *Pseudomonas tet*A$^+$ isolates recovered from pork and cheese exhibited resistance to Tet (> 128 μg mL^{-1}) and vancomycin (> 32 μg mL^{-1}); The *Pseudomonas* sp. CX-I EM (*erm*C$^+$*tet*S/M$^+$) from packaged sliced chicken lunchmeat was resistant to Tet (> 64 μg mL^{-1}) and its tolerance to Em (> 256 μg mL^{-1}) was much higher than the control strain *P. aeruginosa* ATCC 27853 (Tet < 16 μg mL^{-1}, Em < 32 μg mL^{-1}).

Horizontal transfer of the AR gene from food isolates to oral residential bacterium

The *tet*S/M-containing lactococcal isolates CZ-T4 and CZ-T8, recovered from cheese, and RMK-T14, isolated from raw milk, contained a plasmid with an approximate size of 20–25 kb. To assess the potential risk of the food-borne ART bacteria in disseminating AR genes to human microbiota, plasmids isolated from the above strains were used for natural transformation of the oral cariogenic pathogen *Streptococcus mutans* under laboratory conditions. The *tet*S/M gene was successfully transferred to *S. mutans* UA159 at frequencies ranging from 1.9×10^{-7} to 2.8×10^{-5}, 4.7×10^{-7} to 2.3×10^{-6}, and 3.8×10^{-7} to 2.1×10^{-6} transformants per recipient cell using CZ-T4, CZ-T8 and RMK-T14 plasmid extracts, respectively. In addition, the multidrug-resistant plasmid pK214 from the cheese isolate *L. lactis* K214 was also successfully transformed into *S. mutans* UA159 at frequencies of 1.1×10^{-6}–1.2×10^{-5} transformants per recipient cell. PCR amplification confirmed the presence of the *tet*S/M gene in the streptococcal transformants. MIC test showed that the transformants had significantly increased resistance to Tet (> 128 μg mL^{-1}) compared with the parental strain UA159 (< 4 μg mL^{-1}). These results illustrated that the *tet*S/M gene from food isolates can lead to resistance in residential host bacteria or pathogens, if acquired by horizontal gene transfer.

Table 1. Screening of antibiotic resistance genes from dairy samples and identification of host strains

Food	Antibiotic-resistant trait	Resistance gene (no. of carriers/no. of isolates screened)	16S rRNA gene identity (no. of organisms/no. of identified)
Cheese	Tet	*tet*S/M (11/113)	*Lactococcus* sp. (2/11) *Streptococcus thermophilus* (7/11) *Lactococcus lactis* (2/11) (including *Lactococcus* sp. CZ-T8, CZ-T4, *S. thermophilus* BOR-COCZ-T19)
		*tet*A (2/33)	*Pseudomonas* sp. (2/2) (including *Pseudomonas* sp. CZ-T3, CZ-T7)
	Em	*erm*B (32/56)	*Staphylococcus* sp. (5/28) *Streptococcus thermophilus*. (23/28) (including *S. thermophilus* E4, *Staphylococcus* sp. C202)
Raw milk	Tet	*tet*S/M (9/108)	*Lactococcus* sp. (3/9) *Streptococcus* sp. (2/9) *Leuconostoc* sp. (1/9) (including *Lactococcus* sp. RMK-T14, *Streptococcus uberis* RMK-T22W)

Discussion and conclusion

Despite the fact that this current study only screened for a limited number of resistance markers, it illustrated the prevalence of ART commensals and AR genes in retail foods. Many ART bacteria-containing ready-to-eat products are consumed without further cooking or processing. Consequently, human are routinely inoculated with ART bacteria through daily food intake, including opportunistic pathogens and commensals such as *Pseudomonas* sp., *Streptococcus* sp. and *Staphylococcus* sp. The detection of high numbers (i.e. up to 10^8 CFU per serving of food) in several products is alarming, suggesting that food can be a potentially important avenue transmitting ART bacteria. This finding is in agreement with a previous report showing that consuming sterile foods can significantly decrease the presence of ART bacteria in the gastrointestinal system (Levy, 1998). While further research is needed to establish the direct correlation between the ART microbes from foods and the ART population in the host ecosystems, it is evident that a constant supply of ART bacteria, partnered with occasional colonization and horizontal gene transfer, are at least partially responsible for the increased AR profiles seen in humans. Such an intrinsic AR gene pool could have significant impact on pathogen resistance in susceptible population, particularly those receiving antibiotic treatment.

Oral cavity could be an important area where many initial interactions between food microbes and human microbiota, including horizontal gene transfer events such as conjugation and transformation, might take place during the retention of food residues in the oral cavity. Our data are consistent with results from recent studies showing that the microbiota in children and adults is becoming increasingly resistant to antibiotics, even in the absence of antibiotic treatment (Lancaster *et al.*, 2003; Ready *et al.*, 2003; Villedieu *et al.*, 2004). In fact, the *tet*S/M and *erm*B genes were found to be abundant in bacteria isolated from foods, which is in agreement with the prevalence of these Tet- and Em-resistance genes in human oral microflora (Roberts, 1998). Successful transmission of the resistance genes from the food isolates to the oral residential bacterium *S. mutans*, by natural gene transformation, further confirmed the functionality of the mobile resistance-encoding elements from food isolates, if acquired by horizontal gene transfer.

Identification of the key pathways in AR gene transfer is critical, but developing a strategy to combat this problem is even more important. The identification of ART bacteria in cheeses often associated with raw milk, such as *Lactococcus* sp., *Streptococcus* sp. and *Staphylococcus* sp., suggests that cheese fermentation is a susceptible process during which ART bacteria could evolve and proliferate. Improving sanitation and milk heat treatment are thereby essential steps in reducing ART bacteria. While it is a major challenge to track the direct and indirect gene transfer events among microbes in complicated ecosystems (Andremont, 2003), identifying key AR gene host organisms in foods, and likely in other ecosystems, not only reveals the ultimate consequence of these events in the food chain and the organisms involved in horizontal gene transfer, but opens the door for further characterization of conditions in these ecosystems that might facilitate horizontal gene transfer and features of the organisms that might grant their fitness in such ecological niches (Luo *et al.*, 2005a). Such understanding would be critical for effective counteractive strategies to interfere with the detrimental gene swapping in both natural and host ecosystems. An industrially important lactic acid bacterium, *S. thermophilus*, was found to be a dominant host for both Tet and Em genes. ART *L. lactis* was also isolated from cheese. Genetic screening and MIC tests of three commercial *S. thermophilus* starter cultures as well as the control *L. lactis* strain showed that they are free of the above AR genes, suggesting the susceptibility of these starter cultures to horizontal gene transfer, at least during certain cheese fermentation processes. The potential health impact of these organisms thus needs to be carefully evaluated. Although it would be a tedious and likely long-term effort to clean up the AR gene pool in the environment, interrupting the transmission of ART bacteria into human by focusing our efforts on the food chain could be an effective strategy to combat the AR challenge in humans.

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