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Ecology of Antibiotic Resistance Genes: Characterization of Enterococci from Houseflies Collected in Food Settings†

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In this project, enterococci from the digestive tracts of 260 houseflies (*Musca domestica* L.) collected from five restaurants were characterized. Houseflies frequently (97% of the flies were positive) carried enterococci (mean, 3.1×10^3 CFU/fly). Using multiplex PCR, 205 of 355 randomly selected enterococcal isolates were identified and characterized. The majority of these isolates were *Enterococcus faecalis* (88.2%); in addition, 6.8% were *E. faecium*, and 4.9% were *E. casseliflavus*. *E. faecalis* isolates were phenotypically resistant to tetracycline (66.3%), erythromycin (23.8%), streptomycin (11.6%), ciprofloxacin (9.9%), and kanamycin (8.3%). Tetracycline resistance in *E. faecalis* was encoded by *tet*(M) (65.8%), *tet*(O) (1.7%), and *tet*(W) (0.8%). The majority (78.3%) of the erythromycin-resistant *E. faecalis* isolates carried *erm*(B). The conjugative transposon Tn*916* and members of the Tn*916*/Tn*1545* family were detected in 30.2% and 34.6% of the identified isolates, respectively. *E. faecalis* carried virulence genes, including a gelatinase gene (*gelE*; 70.7%), an aggregation substance gene (*asa1*; 33.2%), an enterococcus surface protein gene (*esp*; 8.8%), and a cytolysin gene (*cylA*; 8.8%). Phenotypic assays showed that 91.4% of the isolates with the *gelE* gene were gelatinolytic and that 46.7% of the isolates with the *asa1* gene aggregated. All isolates with the *cylA* gene were hemolytic on human blood. This study showed that houseflies in food-handling and -serving facilities carry antibiotic-resistant and potentially virulent enterococci that have the capacity for horizontal transfer of antibiotic resistance genes to other bacteria.

Multidrug resistance in clinical isolates has become a serious problem due to a progressive decline in the number of antibiotics that are effective for treatment of human infections (38, 66). It has been suggested that there is a connection between the antibiotic resistance of food animal origin, the antibiotic resistance of clinical isolates, and community health (19, 50, 58); however, this remains a controversial issue (43–45, 61) because the ecology of antibiotic resistance and virulence genes in the environment is not well understood.

Insects, such as houseflies (HF) (*Musca domestica* L.), that develop in decaying organic material may transmit antibiotic-resistant bacteria from the manure of animals and other decaying organic substrates to residential settings. The habitats in which it develops (e.g., manure), its dependence on a live microbial community, its feeding mechanism (regurgitation), its attraction to human food, and its ability to fly long distances make this insect a very good candidate for dissemination of fecal bacteria, including human and animal pathogens (4, 26, 69) and possibly antibiotic-resistant strains.

The ubiquity of enterococci in animal and human digestive tracts, their medical importance, their frequent multiple-antibiotic resistance, and their seemingly limitless capacity for horizontal gene transfer via numerous mobile genetic elements (24) make this bacterial group an ideal group for investigating the ecology of antibiotic resistance genes. While some enterococci are used as probiotics (21), other species are important opportunistic and nosocomial pathogens of humans (34). At present, the genus *Enterococcus* comprises 26 species, and two species, *E. faecalis* and *E. faecium*, are responsible for the majority of human infections (35). The recent discovery of transfer of the transposon Tn*1546* conferring vancomycin resistance from *E. faecalis* to a clinical isolate of *Staphylococcus aureus* (64) highlights the importance of horizontal gene transfer among bacteria from the clinical perspective as well as the ecological perspective.

Many studies have addressed the importance of enterococci as a reservoir of antibiotic resistance genes in the environment (24); however, less information is available about enterococci from the food safety perspective, particularly for ready-to-eat food. Enterococci have previously been isolated from milk, cheese, and meat (21, 30, 36), as well as from raw produce (21, 32). To our knowledge, no study has examined the significance of insects, including HF, in the ecology of antibiotic resistance and virulence genes associated with enterococci.

In this study, enterococci from HF collected from five fast-food restaurants in Kansas were isolated, quantified, identified, and screened for antibiotic resistance and virulence genes by phenotypic and genotypic approaches.

MATERIALS AND METHODS

Sample collection and isolation of enterococci. HF from five fast-food restaurants (restaurants A, B, C, D, and E) located 0.5 to 3 km apart in a town in northeastern Kansas were collected from June to September using sticky traps and/or sweep nets and were processed for enterococcus isolation on the same day. Individual HF ($n = 260$) were surface sterilized with sodium hypochlorite and ethanol (69), homogenized in 1 ml of potassium buffer saline (pH 7.2; ICN Biomedicals, Ohio), serially diluted in potassium buffer saline, drop plated on mEnterococcus agar (Becton Dickinson, Massachusetts), and incubated at 37°C for 48 h. Up to four presumptive enterococcal colonies with different colony

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TABLE 1. Primers and PCR conditions used in this study

Primer type	Positive control	Direction[a]	Sequence (5′–3′)	Primer concn (pmol)	Annealing temp (°C)	Product size (bp)	Reference
Species identification (multiplex PCR)							
E. gallinarum (*vanC1*)	ATCC 49579	F	GGTATCAAGGAAACCTC	2.5	54	822	33
		R	CTTCCGCCATCATAGCT				
E. casseliflavus (*vanC2*/*C3*)	ATCC 25788	F	CGGGGAAGATGGCAGTAT	2.5	54	484	33
		R	CGCAGGGACGGTGATTTT				
E. faecalis	ATCC 19433	F	TCAAGTACAGTTAGTCTTTATTAG	5.0	54	941	18
		R	ACGATTCAAAGCTAACTGAATCAGT				
E. faecium	ATCC 19434	F	TTGAGGCAGACCAGATTGACG	1.25	54	658	18
		R	TATGACAGCGACTCCGATTCC				
16S rRNA gene		F	GGATTAGATACCCTGGTAGTCC	2.5	54	320	33
		R	TCGTTGCGGGACTTAACCCAAC				
Virulence gene (multiplex PCR)							
asa1	*E. faecalis* MMH 594	F	GCACGCTATTACGAACTATGA	0.1	56	375	62
		R	TAAGAAAGAACATCACCACGA				
gelE	*E. faecalis* MMH 594	F	TATGACAATGCTTTTTGGGAT	0.1	56	213	62
		R	AGATGCACCCGAAATAATATA				
cylA	*E. faecalis* MMH 594	F	ACTCGGGGATTGATAGGC	0.2	56	688	62
		R	GCTGCTAAAGCTGCGCTT				
esp	*E. faecalis* MMH 594	F	AGATTTCATCTTTGATTCTTGG	0.2	56	510	62
		R	AATTGATTCTTTAGCATCTGG				
Antibiotic resistance gene Multiplex PCR							
tet(A) group I	*E. coli* HB101(RP1)	F	GCTACATCCTGCTTGCCTTC	1.0	55	210	41
		R	CATAGATCGCCGTGAAGAGG				
tet(C) group I	*E. coli* DO7(pBR322)	F	CTTGAGAGCCTTCAACCCAG	0.25	55	418	41
		R	ATGGTCGTCATCTACCTGCC				
tet(K) group II	*Bacillus subtilis* BD99(pT181)	F	TCGATAGGAACAGCAGTA	1.25	55	169	41
		R	CAGCAGATCCTACTCCTT				
tet(O) group II	Cloned plasmid (O) pGEM-tet	F	AACTTAGGCATTCTGGCTCAC	1.25	55	515	41
		R	TCCCACTGTTCCATATCGTCA				
tet(S) group II	Cloned plasmid pAT451	F	CATAGACAAGCCGTTGACC	0.5	55	667	41
		R	ATGTTTTTGGAACGCCAGAG				
tet(Q) group I	Cloned plasmid pBT-1	F	TTATACTTCCTCCGGCATCG	1.25	55	904	41
		R	ATCGGTTCGAGAATGTCCAC				
Single PCR							
tet(W)	Cloned plasmid pGEM	F	GAGAGCCTGCTATATGCCAGC	25	64	168	6
		R	GGGCGTATCCACAATGTTAAC				
erm(B)	*E. faecalis* MMH594	F	GAAAAGGTACTCAACCAAATA	25	55	639	60
		R	AGTAACGGTACTTAAATTGTTTAC				
tet(M)	Cloned plasmid pFD310	F	AGTTTTAGCTCATGTTGATG	25	55	1,862	15
		R	TCCGACTATTTGGACGACGG				
int (Tn*916*/Tn*1545*)	*E. faecalis* OG1RF(pCF10)	F	GCGTGATTGTATCTCACT	25	50	1,046	15
		R	GACGCTCCTGTTGCTTCT				
ORF13 (T*n916*)	*E. faecalis* OG1RF(pCF10)	F	GGCTGTCGCTGTAGGATAGAG	25	50	589	7
		R	GGGTACTTTTAGGGCTTAGT				

[a] F, forward; R, reverse.

TABLE 2. Prevalence and identification of enterococci isolated from HF collected from five fast-food restaurants

Restaurant	No. of HF analyzed/ no. positive	CFU/HF (10^3, mean ± SEM)	Total no. of isolates analyzed	No. of isolates identified (%)	No. (%) of isolates		
					E. faecalis	*E. faecium*	*E. casseliflavus*
A	53/53	2.1 ± 0.26	74	39 (52.7)	38 (97.4)	0	1 (2.6)
B	52/52	2.0 ± 0.15	73	43 (58.9)	41 (95.4)	1 (2.3)	1 (2.3)
C	50/50	4.6 ± 0.65	67	28 (41.8)	25 (89.3)	2 (7.1)	1 (3.6)
D	63/55	4.0 ± 0.67	60	27 (45.0)	20 (74.1)	7 (25.9)	0
E	50/50	3.0 ± 0.39	81	68 (84.0)	57 (83.8)	4 (5.9)	7 (10.3)
Total or mean	268/260	3.1 ± 0.43	355	205 (57.7)	181 (88.2)	14 (6.8)	10 (4.9)

morphologies from each sample were streaked on Trypticase soy agar (Becton Dickinson, Massachusetts), incubated at 37°C for 24 h, and stored at 4°C until further analysis.

Identification and phenotypic screening for antibiotic resistance. The presumptive identities of enterococcal colonies were confirmed at the genus level by the esculin hydrolysis test using Enterococcossel broth (Becton Dickinson, Massachusetts) and by growth at 44.5°C in Trypticase soy broth (Becton Dickinson, Massachusetts) with 5% sodium chloride. Multiplex PCR was used to identify four common species, *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum* (33). The strains used as positive controls and the primer sequences are listed in Table 1. *E. mundtii* ATCC 43186 was used as a negative control. All isolates that were identified were characterized further by screening for antibiotic resistance and virulence determinants by PCR, as well as by phenotypic tests.

Identified isolates were screened for antibiotic sensitivity by the disk diffusion method on Mueller-Hinton agar (Becton Dickinson, Massachusetts) using six antibiotics, tetracycline (30 μg/ml), chloramphenicol (30 μg/ml), ciprofloxacin (5 μg/ml), erythromycin (15 μg/ml), vancomycin (30 μg/ml), and ampicillin (10 μg/ml). High-level resistance to aminoglycosides was assessed by the agar dilution technique using 2,000 μg/ml of streptomycin and 2,000 μg/ml kanamycin in brain heart infusion agar (Becton Dickinson, Massachusetts). *E. faecalis* ATCC 19433 was used as a quality control strain. The protocols used followed the guidelines of the Clinical and Laboratory Standards Institute (12).

Screening for antibiotic resistance, virulence, and integrase genes. Multiplex or single PCRs were used to screen all identified isolates for tetracycline and erythromycin resistance genes. The group I multiplex reaction included the *tet*(A), *tet*(C), and *tet*(Q) genes, while the group II multiplex reaction included the *tet*(M), *tet*(S), *tet*(K), and *tet*(O) genes (41, 63) (Table 1). Each reaction mixture consisted of 25 μl Master mix (Promega, Madison, WI), 4 mM $MgCl_2$ (group I) or 3 mM $MgCl_2$ (group II), and 3 μl of supernatant from freshly boiled cells. The PCR conditions have been described previously (41, 63). Single PCRs were used to screen *tet*(W) (6) and *erm*(B) (60) (Table 1). The PCR program for *erm*(B) consisted of 94°C for 3 min (initial denaturing step), 94°C for 3 min, 55°C for 3 min, 72°C for 30 min, and 72°C for 4 min (final extension) (59).

All identified isolates were screened for four putative virulence determinants, *gelA* (gelatinase), *asa1* (aggregation substance), *cylA* (cytolysin), and *esp* (enterococcus surface protein), using multiplex PCR (62). The integrase gene (*int*) was used for detection of the Tn*916*/Tn*1545* conjugative transposon family (15, 22). Primers targeting ORF13 were used for detection of Tn*916* specifically (7) (Table 1). To confirm the identities of the determinants, one randomly selected PCR product for each resistance, virulence, or transposon determinant was purified, sequenced using either the PCR primers or M13 primers after cloning of the PCR product, and compared to the sequences in the GenBank database using BLAST (Basic Local Alignment Search Tool) (5).

Screening for virulence genes by phenotype. Trypticase soy agar with 3% skim milk was used for detection of gelatinase activity. All identified isolates were streaked and after 24 h of incubation at 37°C were examined for a clearance zone surrounding the colonies (23).

For phenotypic expression of the *asa1* gene, *E. faecalis* JH2-2 was grown for 6 h at 37°C in Todd-Hewitt broth (Becton Dickinson, Massachusetts). The broth was then centrifuged at 6,000 rpm for 10 min, and the pheromone-containing supernatant that induced pheromone-responsive plasmids was removed and autoclaved for 15 min. Test isolates were grown in Todd-Hewitt broth (5 ml) for 6 h at 37°C. After incubation, 1 ml of the supernatant from *E. faecalis* JH2-2 was added to each tube and incubated at 37°C overnight in a shaker. Isolates that showed clumping (as determined by the naked eye and with a compound microscope) were considered positive for aggregation substance expression (17). *E. faecalis* OG1RF(pCF10) was used a positive control.

Phenotypic assays for cytolysin were conducted using Columbia blood agar base (Becton Dickinson, Massachusetts) with 5% cattle and human blood. Isolates were streaked and incubated at 37°C for 48 to 72 h. Isolates that had a complete clearance zone around the colonies (beta-hemolysis) were considered positive for cytolysin expression (23). *E. faecalis* MMH594 was used as a positive control.

Restriction fragment length polymorphism of *tet*(M). *tet*(M) PCR amplicons (1,862 bp) from 54 *E. faecalis* isolates and four *E. faecium* isolates were separately digested with four restriction enzymes, RsaI, DdeI, MseI, and AluI (15), which provided a restriction footprint of 7.5%. Restriction digests were visualized on an 8% polyacrylamide gel stained with ethidium bromide.

Statistical analysis. The differences among the three enterococcal species in terms of the prevalence of antibiotic resistance and virulence factors (genotype and phenotype) were analyzed using chi-square analysis of contingency tables and Fisher's exact test ($P = 0.05$) (52). Species with zero prevalence of antibiotic resistance and virulence factors (genotype and phenotype) were not included in the analysis.

RESULTS

Identification, quantification, and phenotypic screening of enterococci for antibiotic resistance. Enterococci were detected in the digestive tracts of 260 (97%) HF collected in five fast-food restaurants. Individual HF contained high concentrations of enterococci [1×10^2 to 2.4×10^4 CFU per fly; mean, $(3.1 \pm 0.43) \times 10^3$ CFU per fly] (Table 2). A total of 355 randomly selected enterococcal colonies were screened by multiplex PCR for species identification, which resulted in identification of 205 (57.7%) isolates. These isolates were *E. faecalis* (88.2%), *E. faecium* (6.8%), and *E. casseliflavus* (4.9%) isolates (Table 2). The highest number of *E. faecium* isolates was detected in flies from restaurant D, and the highest number of *E. casseliflavus* isolates was found in flies from restaurant E (Table 2); no other apparent differences in species diversity were observed among the sites sampled.

Many identified isolates were phenotypically resistant to tetracycline (Tet^r) and erythromycin (Em^r); *E. faecalis* isolates were most frequently Tet^r and Em^r (66.3% and 23.8%, respectively), followed by *E. faecium* isolates (35.7% and 14.3%, respectively) (Fig. 1). *E. casseliflavus* strains were sensitive to tetracycline and erythromycin but frequently exhibited resistance to ciprofloxacin (40.0%) (Fig. 1). High-level resistance to the aminoglycosides streptomycin and kanamycin was detected in *E. faecalis* (11.6% and 8.3%, respectively) and *E. faecium* (7.1% and 7.1%, respectively). None of the identified isolates was resistant to vancomycin, and very few isolates (*E. faecalis*) were resistant to ampicillin (0.6%) and chloramphenicol (2.2%) (Fig. 1).

Of the 205 isolates identified, 67.3% were resistant to at least one antibiotic. The majority of *E. faecalis* isolates (70.7%) were resistant to at least one antibiotic. For multidrug resis-

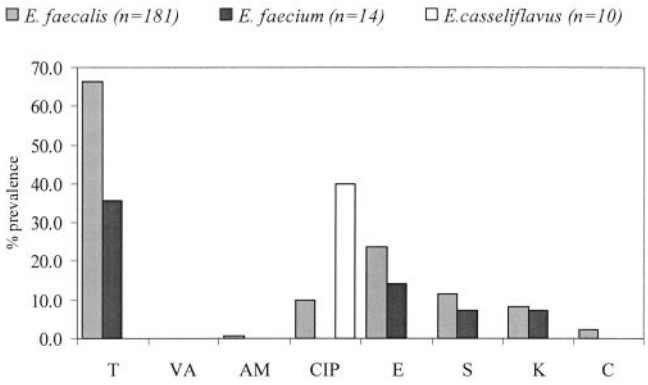

FIG. 1. Antibiotic resistance screening of identified isolates by phenotype. T, tetracycline; VA, vancomycin; AM, ampicillin; CIP, ciprofloxacin; K, kanamycin; E, erythromycin; S, streptomycin; C, chloramphenicol.

tance (resistance to two or more antibiotics), the combination of Tet^r and Em^r in *E. faecalis* was the most common (13.3%), followed by the combination of Tet^r, Em^r, Str^r, and Kan^r (5.5%). Several *E. faecium* isolates (14.3%) were also resistant to multiple drugs, specifically tetracycline and erythromycin.

Prevalence of Tet^r, Em^r, Tn*916*, and Tn*916*/Tn*1545* determinants. Several different Tet^r genes were detected in phenotypically resistant *E. faecalis* isolates; these genes included *tet*(M) (65.8%), *tet*(O) (1.7%), and *tet*(W) (0.8%) (Fig. 2). No tetracycline resistance determinants were found in 30.8% of the phenotypically Tet^r isolates.

The ribosomal protection protein mechanism encoded by *tet*(M) was detected most frequently and also was mostly frequently expressed in both *E. faecalis* and *E. faecium* (Table 3). *tet*(O) was carried by very few *E. faecalis* and *E. casseliflavus* isolates and was expressed only in *E. faecalis* (Table 3).

The *erm*(B) determinant was found in 78.3% of Em^r *E. faecalis* isolates and 50% of Em^r *E. faecium* isolates (Fig. 2). No silent erythromycin resistance genes were detected (Table 3).

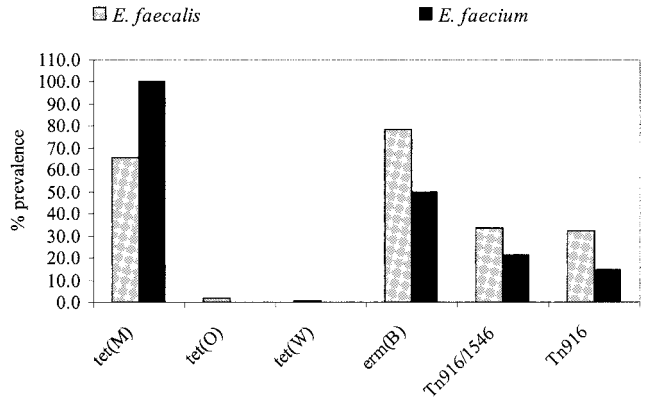

FIG. 2. Distribution of *tet*(M), *tet*(O), *tet*(W), and *erm*(B) genes in *E. faecalis* and *E. faecium* isolates phenotypically resistant to tetracycline (n = 120 and n = 4, respectively) and erythromycin (n = 46 and n = 2, respectively) and distribution of Tn*916* and Tn*916*/Tn*1545* family transposons in all *E. faecalis* (n = 181) and *E. faecium* (n = 14) isolates.

TABLE 3. Distribution of *tet*(M), *tet*(O), *tet*(W), *erm*(B), Tn*916*, and Tn*916*/Tn*1545* family determinants among *E. faecalis* (n = 181), *E. faecium* (n = 14), and *E. casseliflavus* (n = 10) isolates

Combination of determinants	Total no.	% of isolates	Correlation with phenotype (Tet^r/Em^r)
E. faecalis			
tet(M) plus (n = 79):			
Tn*916*	15	8.3	100
Tn*916*/Tn*1545*	1	0.6	0
Tn*916*, *erm*(B)	29	16.0	100/100
erm(B)	6	3.3	100/100
tet(M) alone	28	15.5	78.5
Other (n = 25)			
tet(O)	2	1.1	100
erm(B)	1	0.6	100/100
Tn*916*, *tet*(W)	1	0.6	100
Tn*916*/Tn*1545* family	6	3.3	NA[a]
Tn*916*	15	8.3	NA
Isolates with no detected determinants	77	42.5	35.1/5.2
E. faecium (n = 14)			
tet(M), Tn*916*	1	7.1	100
tet(M), Tn*916*/Tn*1545* family	1	7.1	100
tet(M), *erm*(B), Tn*916*	1	7.1	100/100
tet(M) alone	1	7.1	100
Tn*916*/Tn*1545* family alone	1	7.1	NA
Isolates with no detected determinants	9	64.3	NA
E. casseliflavus (n = 10)			
tet(O) alone	3	30.0	0
Isolates with no detected determinants	7	70.0	NA

[a] NA, not applicable.

Many (45.9%) of the *E. faecalis* isolates carried at least one resistance determinant; the most common determinant was *tet*(M) (43.7%) alone or in combination with other determinants, followed by *erm*(B) (19.8%) (Table 3). Several *E. faecalis* isolates carried multiple resistance determinants, specifically *tet*(M) and *erm*(B) (Table 3).

The Tn*916*/Tn*1545* conjugative transposon family, likely carrying *tet*(M) (11), was found in 71 (34.6%) identified isolates, and 62 (87.3%) of these isolates carried specifically Tn*916*. The Tn*916*/Tn*1545* family determinant was commonly detected in *E. faecalis* (37.0%), and the majority of the isolates (89.6%) contained Tn*916*. *E. faecium* isolates (28.6%) also carried Tn*916*/Tn*1545*, and one-half of these isolates specifically carried Tn*916* (Fig. 2).

The most common *E. faecalis* genotypes based on a combination of antibiotic resistance determinants and transposons were as follows: *tet*(M) plus *erm*(B) plus Tn*916* (16.0%), *tet*(M) alone (15.5%), and *tet*(M) plus Tn*916* (7.7%) (Table 3). Several *E. faecalis* isolates were positive for Tn*916* (8.3%) and for Tn*916*/Tn*1545* (3.3%) with no resistance determinant detected (Table 3).

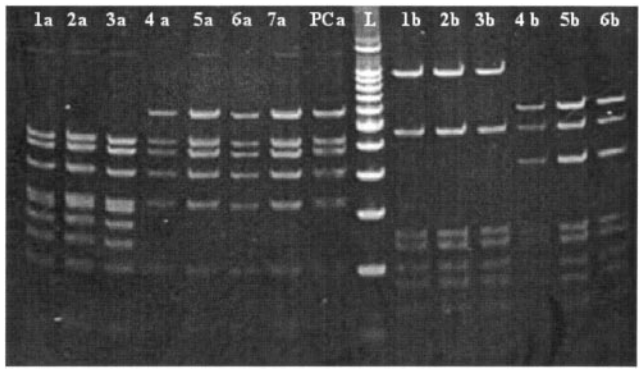

FIG. 3. Restriction fragment length polymorphism of *tet*(M), determined using RsaI and MspI. Lane L, 100-bp molecular marker; lane PCa, *tet*(M) from *E. faecalis* OG1RF(pCF10); lanes 1a to 7a, *E. faecalis* isolates digested with RsaI; lanes 1b to 6b, *E. faecalis* isolates digested with MspI.

Polymorphism of *tet*(M) gene from *E. faecalis*. Based on restriction enzyme profiles, two different genotypes of the *tet*(M) gene were identified (Fig. 3). The majority (66.7%) of the isolates carried type A, similar to *tet*(M) from *E. faecalis* OG1RF(pCF10), and 33.3% of the isolates were positive for type B.

Virulence determinants. Virulence determinants were found mostly in *E. faecalis*; 70.7% of the isolates contained *gelE*, 33.1% contained *asa1*, 8.8% contained *esp*, and 8.8% contained *cylA* (Fig. 4A). Multiple virulence determinants were detected in several *E. faecalis* isolates; four factors were found in 6.1% of the isolates, three factors were found in 4.4% of the isolates, and two factors were found in 21.0% of the isolates. One *E. faecium* isolate carried *gelE* (7.1%), one *E. casseliflavus* isolate carried *gelE*, and one *E. casseliflavus* isolate carried *asa1*.

In order to assess how many isolates expressed the virulence genes, phenotypic tests for gelatinase, aggregation substance, and hemolysis (cytolysin) were conducted for all identified isolates (Fig. 4B). Phenotypic tests for gelatinase revealed that the majority (64.6%) of *E. faecalis* isolates expressed *gelE*; 6.0% of *E. faecalis* isolates carried the silent gene, and 8.8% carried an unknown gelatinase gene(s). Many *E. faecium* isolates (78.6%) exhibited gelatinase activity, but only 7.1% of these isolates were positive for *gelE* (Fig. 4). The majority (80%) of *E. casseliflavus* isolates were positive for gelatinase activity, but *gelE* was detected in only 10% of the isolates (Fig. 4).

The clumping/aggregation assay revealed that 56.7% of *E. faecalis* isolates expressed the aggregation substance (Fig. 4B). None of the *E. faecium* isolates carried *asa1* or aggregated in the phenotypic assay (Fig. 4).

The test for hemolysis (cytolysin expression) with *E. faecalis* showed that there was a 100% correlation between *cylA* and beta-hemolysis on human blood. In contrast, none of the isolates that were positive for *cylA* were hemolytic on cattle blood agar. In addition, 2.2% and 0.6% of the *E. faecalis* isolates that were positive for beta-hemolysis on human and cattle blood, respectively, carried an unknown determinant(s). Two *E. faecium* isolates were beta-hemolytic but negative for *cylA*. None

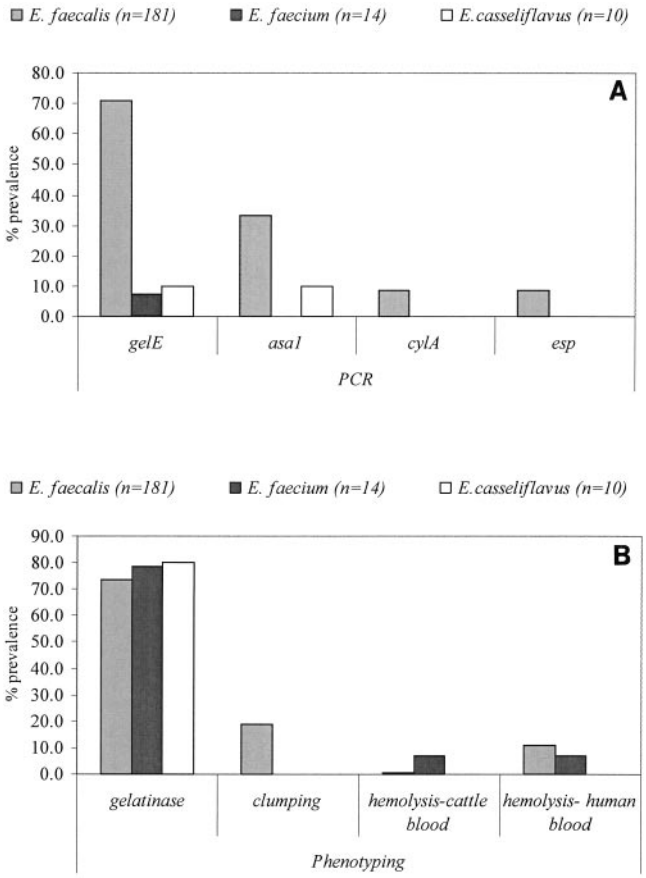

FIG. 4. Prevalence of virulence genes *gelE*, *asa1*, *cylA*, and *esp* in identified isolates. (A) Data from multiplex PCRs. (B) Data from phenotypic tests (except *esp* data).

of the *E. casseliflavus* isolates carried *cylA* or were beta-hemolytic.

E. faecalis isolates were significantly different from *E. faecium* and/or *E. casseliflavus* in terms of the prevalence of the following factors: phenotypic resistance to tetracycline ($P = 0.005$) and ciprofloxacin ($P = 0.004$), hemolysis on cattle blood agar ($P = 0.018$), and *gelE* ($P < 0.001$). Little variation was observed for antibiotic resistance and virulence factors (phenotype and genotype) within individual houseflies (data not shown).

DISCUSSION

Frequent use of antibiotics in medicine and in food animal production has resulted in an increase in the prevalence of bacterial strains resistant to these antimicrobial agents (37–39, 50, 67). The decreased effectiveness of antibiotics results in infections that are more difficult to treat and have higher economic cost (29). In the medical community, the need for prudent use of antibiotics is accepted worldwide. Furthermore, the European Union has banned the use of several antibiotics as growth promoters (avoparcin, bacitracin, spiramycin, tylosin, and virginiamycin) in the animal industry, and there are proposals to withdraw more antibiotics by 2006 (3). In contrast, in the United States, antimicrobial agents are used widely as food

additives to improve growth and feed conversion in many types of animal operations, including poultry, swine, and cattle operations. As a result, antibiotic resistance in the bacterial communities in the intestinal tracts of domestic animals has become common (1, 2, 28, 42). The horizontal transfer of antibiotic resistance genes from the gastrointestinal tracts of domestic animals to the gastrointestinal tract of humans remains a controversial subject (43–45, 58, 61) because the ecology and horizontal transfer of antibiotic resistance and virulence determinants in the environment are poorly understood.

Insects, such as HF, that develop in animal manure and other decaying organic materials can play an important role in the ecology and dissemination of bacteria in agricultural and urban environments. Larvae of muscoid flies develop in animal manure and other decaying organic substrates, where they require a microbial community for growth and survival (69). Consequently, due to the habitat in which they develop, their feeding mechanism (regurgitation), their unrestricted movement, and their attraction to residential areas, HF can play an important role in the ecology and transmission of bacteria, including enterococci, and associated antibiotic resistance and virulence genes. It has been shown previously that HF can carry potential pathogens, such as *Yersinia pseudotuberculosis* (68), *Helicobacter pylori* (27), *Campylobacter jejuni* (56), *Escherichia coli* O157:H7 (4, 53), and *Salmonella* spp. (40). Several studies showed that there was a positive correlation between the incidence of food-borne diarrhea and the density of fly populations. For example, suppression of flies in military camps in the Persian Gulf resulted in an 85% decrease in shigellosis and a 42% reduction in the incidence of other diarrheal diseases (13). Esrey reported a 40% reduction in the incidence of diarrheal infections in children after suppression of a fly population (20).

Our data show that houseflies in urban fast-food restaurants carry a large population of enterococci with resistance to several antibiotics. The most frequently identified species was *E. faecalis*, followed by *E. faecium*; these taxa are the two most important enterococcal species from the clinical perspective (24, 35). It is important to point out that 42% of the isolates were not identified by the multiplex PCR protocol. These isolates represent other enterococcal species that were not included in the additional analysis in this study. The majority (67%) of identified enterococci were phenotypically resistant to at least one antibiotic, and many isolates were multidrug resistant. The most frequent resistance was resistance to tetracycline and erythromycin, although resistance to high levels of aminoglycosides (streptomycin and kanamycin) and ciprofloxacin was also detected. The origin of the HF used in this study is unknown; however, our data suggest that the HF captured in restaurants developed in or were in contact with manure and feces of domestic animals, although other sources for contamination of the flies with antibiotic-resistant enterococci, including the feces of dogs and cats (9, 49), are also possible. Contamination of houseflies with enterococci from human feces, including feces from hospitals, is highly unlikely due to strict regulations for insect infestations in hospitals and sewer systems in the United States. Houseflies can develop in any decaying organic material, including trash and compost piles in residential areas; however, the high prevalence of *E. faecalis* and *E. faecium* in houseflies in this study indicates that these insects developed in or were in contact with fecal material.

Further analysis focusing on the diversity of tetracycline and erythromycin resistance revealed that *tet*(M) (two different genotypes) was the most prevalent tetracycline resistance gene, although 35.1% of Tet^r isolates (based on phenotype) were negative for the resistance genes that we selected in this study. The majority (78.3%) of the erythromycin-resistant *E. faecalis* isolates carried *erm*(B). These findings are in agreement with the findings of other studies which showed that *tet*(M) and *erm*(B) are widespread in antibiotic-resistant enterococci (1, 24). In addition, screening of the isolates for the conjugative transposon Tn*916* and the Tn*916*/Tn*1545* conjugative transposon family indicated that many isolates carried the frequently detected resistance determinants, including *tet*(M) and *erm*(B), on this class of transposons (11, 46, 51). This is important because Tn*916* and Tn*1545* have very broad host ranges and could be horizontally transferred to a variety of gram-positive and gram-negative bacteria in a human gastrointestinal microbial community (8, 11, 47, 48, 55) and possibly to human pathogens. For example, Tn*916* has been transferred from *E. faecalis* to *Butyrivibrio fibrisolvens* and *Escherichia coli* (8, 31), and Tn*1545* has been transferred from *E. faecalis* to *Listeria monocytogenes* (16). In our study, several *E. faecalis* isolates without Tn*916* and Tn*916*/Tn*1545*were positive for *tet*(M) and *erm*(B), indicating that these genes were carried on other transposons or plasmids or on the chromosome. Clearly, horizontal transfer of genes on conjugative transposons and plasmids has played a major role in the dramatic spread and increase in the number of multidrug-resistant bacteria in the environment over the past 40 years (10, 48, 51, 54, 57).

Our study also shows that virulence determinants are relatively common in environmental isolates and that these virulence determinants are comparable to the virulence determinants of some clinical strains (23). Many of the genes were expressed, as shown in our phenotypic assays. The majority of *E. faecium* and *E. casseliflavus* isolates were positive for gelatinase activity, but only a few of them were positive for *gelE*, indicating that other unknown gelatinase determinants were present. Some of the genes encoding virulence determinants, including cytolysin and aggregation substance, can be carried on plasmids, such as pAD1 (23), which indicates that there is potential for horizontal transfer to other *E. faecalis* isolates in the human gastrointestinal tract.

In most studies investigating the prevalence of antibiotic-resistant enterococci in food, the workers have focused on food products before they were prepared and cooked (21, 25, 30, 36, 65). HF are very common from spring to fall and commonly enter buildings, including restaurants, in the urban environment in search of food. Qualitative and quantitative assessments of the contamination of ready-to-eat food by HF in restaurants, as well as assessments of the potential for horizontal transfer of resistance and virulence genes of enterococci from food to the human gastrointestinal community, should be performed. Although contamination of ready-to-eat food by enterococci from HF has not been assessed, other studies have demonstrated that this insect has great potential to contaminate human food by regurgitation and defecation (14, 53).

In summary, our study showed that HF in urban fast-food restaurants commonly carried a large and genetically diverse

population of enterococci with antibiotic resistance and virulence genes that are frequently expressed and likely carried on mobile genetic elements, such as the conjugative transposon Tn*916*. This suggests that HF play a role in the ecology of antibiotic-resistant bacteria in the environment, including food-handling and -serving facilities.

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