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Functional Cloning and Characterization of Antibiotic Resistance Genes from the Chicken Gut Microbiome

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Culture-independent sampling in conjunction with a functional cloning approach identified diverse antibiotic resistance genes for different classes of antibiotics in gut microbiomes from both conventionally raised and free-range chickens. Many of the genes are phylogenetically distant from known resistance genes. Two unique genes that conferred ampicillin and spectinomycin resistance were also functional in *Campylobacter*, a distant relative of the *Escherichia coli* host used to generate the genomic libraries.

Zoonotic bacterial pathogens cause diseases in animals and/or humans, consequently threatening animal production, food safety, and public health (22, 27, 29). Increasing evidence indicates that antimicrobial usage in animals promotes the emergence of resistant zoonotic pathogens, greatly compromising the effectiveness of antibiotic treatments (7, 24). It has been proposed that normal intestinal microbiotas play a critical role in antibiotic resistance (AR) development and transmission via horizontal gene transfer (HGT) (13, 26). However, the majority of intestinal microbiotas cannot be cultivated using traditional culturing methods, greatly impeding our understanding of the AR reservoir in gut microbiotas. Recently, Sommer et al. (21) functionally characterized the AR reservoir in the human gut microbiota; many AR genes identified using a culture-independent approach are evolutionarily distant from known resistance genes, first demonstrating the immense diversity of AR genes in the human microbiome. Based on this recent human study, the gut microbiota in food animals may also contain diverse and novel AR genes that could be accessible to clinically relevant pathogens. However, the reservoirs of mobile AR genes in the gut microbiomes from food animals are still largely unknown. In addition, it is still not clear if the novel AR genes identified in the gut microbiome can function normally in a new host or a distant relative when HGT successfully occurs, a key issue for the emergence of AR in bacterial pathogens.

In this study, we examined AR genes in gut microbiomes of chickens from different production systems and determined the functional compatibility of the identified novel AR genes. The major bacterial strains and plasmids used in this study are listed in Table 1. We first isolated total genomic DNA directly from cecal contents of two free-range chickens that have never been treated with antimicrobials (kindly provided by a free-range farm in Dandridge, TN) and two conventionally raised chickens that have received antibiotic-containing feed (from Koch Foods, Inc., Morristown, TN). The sheared DNA fragments were cloned into expression vector pZE21-MCS (12) and transformed into *Escherichia coli* One Shot TOP10 electrocompetent cells (Epicentre) for library construction as described by Sommer et al. (21). The total sizes of the 4 expression libraries ranged from 0.4×10^8 bp to 2.4×10^8 bp. Using the same screening protocol as Sommer et al. (21), we selected AR clones from each library by plating culture on Luria broth agar plates containing the antibiotic of interest at the appropriate concentration (50 μg/ml of ampicillin, 10 μg/ml of tetracycline, 25 μg/ml of chloramphenicol, 75 μg/ml of spectinomycin, 12.5 μg/ml of norfloxacin, or 10 μg/ml of ciprofloxacin). Fifty inserts conferring resistance were sequenced by using primers pZE-F and pZE-R (Table 2); the sequences were annotated and analyzed as described by Sommer et al. (21).

As shown in Table 3, 3 AR genes that conferred resistance to ampicillin or spectinomycin were identified in the gut microbiomes of two free-range chickens, while a total of 11 AR genes were identified from conventionally raised chickens (Table 3). The novel AR genes that shared low sequence similarity (58% to 76% at the amino acid level) with the known resistance genes constitute a substantial proportion of the AR genes identified in this study (6 of 14) (Table 3). Notably, among the 6 novel genes, 3 were unique and even shared low sequence similarities (<68% at the amino acid level) with the novel AR genes recently identified from the human gut microbiome using the same functional cloning approach (21); these 3 genes are *FRAmp1.1*, *FRSpe1.1*, and *CRChl2.2* (Table 3). Discovery of the 3 AR genes from free-range chickens in this study strongly supports a recent theory that environmental bacteria harbor the readily available and diverse pool of AR genes regardless of antibiotic exposure (2). This finding is also consistent with a recent work directly showing that antibiotic resistance is a natural phenomenon that predates the modern selective pressure of clinical antibiotic use (4). It is not surprising that more AR genes, in terms of number and diversity, were identified in the conventionally raised chickens than in the free-range chickens. The extensive antimicrobial usage in conventionally raised chickens may enrich AR genes in the gut microbiome, consequently increasing likelihood and ease of identification of AR genes using the functional cloning approach. However, given the limitation of the small sample size of this study (2 birds for each production system), this finding needs to be confirmed in future large-scale experiments using animals from diverse sources.

To better understand the uniqueness of the identified novel AR genes, we further analyzed the phylogenetic relationship of FRAmp1.1, a novel β-lactamase identified in the gut microbiome

Received 19 September 2011 **Accepted** 20 January 2012
Published ahead of print 27 January 2012
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doi:10.1128/AEM.06920-11

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source
Strains		
E. coli strains		
DH5α	F$^-$ *ϕ80lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_{K^-} m_{K^-}) *supE44 thi-1 gyrA relA1*	Invitrogen
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *ϕ80lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
JL519	DH5α containing pZE21-MCS	This study
JL48	Conjugation helper strain; DH5α containing pRK2013	32
JL860	TOP10 containing pZE21-MCS-*FRAmp1.1*	This study
JL861	TOP10 containing pZE21-MCS-*FRSpe1.1*	This study
JL850	DH5α containing pZW	This study
JL851	DH5α containing pZW1	This study
JL852	DH5α containing pZW2	This study
JL853	DH5α containing pZW3	This study
C. jejuni strains		
JL241	NCTC 11168; human isolate	16
JL242	*C. jejuni* 81-176; human isolate	3
JL854	JL242 containing pZW	This study
JL855	JL242 containing pZW1	This study
JL856	JL242 containing pZW2	This study
JL857	JL242 containing pZW3	This study
Plasmids		
pZE21-MCS	Cloning and expression vector; Kan^r	12
pRY111	*E. coli-C. jejuni* shuttle vector; Chl^r	30
pRK2013	Helper plasmid for triparental conjugation	14
pZE21-MCS-*FRAmp1.1*	pZE21-MCS with cloned fragment containing ampicillin resistance gene *FRAmp1.1*	This study
pZE21-MCS-*FRSpe1.1*	pZE21-MCS with cloned fragment containing spectinomycin resistance gene *FRSpe1.1*	This study
pZW	pRY111 containing *flaA* promoter	This study
pZW1	pZW containing *FRAmp1.1*	This study
pZW2	pZW containing *FRSpe1.1*	This study
pZW3	pZW containing kanamycin resistance gene *KAN-2*	This study

[a] Kan^r, kanamycin resistant; Chl^r, chloramphenicol resistant.

of a free-range chicken (Table 3), to 25 presently identified β-lactamases that represent 21 different families. As shown in Fig. 1, FRAmp1.1 and the β-lactamases recently identified in the human gut microbiome (21) were phylogenetically distant from most known β-lactamases that were characterized in cultivated organisms. In particular, FRAmp1.1 was very distantly related to the β-lactamases (Cj0229, Cam1, CJS_0285, and CJE0344) (Fig. 1) that have been identified in *Campylobacter*, an important foodborne bacterial pathogen with chicken as a major animal host (10, 11) and a distant relative of the *E. coli* host used here to generate genomic libraries. This finding prompted us to explore another significant issue of antibiotic resistance by taking advantage of the identified novel AR genes: can novel AR genes function normally in different bacterial species if the HGT barrier is successfully overcome? To address this issue, we first constructed an *E. coli-Campylobacter* shuttle vector (pZW) (Table 1) carrying a *Campylobacter* promoter according to the procedure described by Larsen et al. (8). The two selected novel AR gene products (FRAmp1.1, conferring ampicillin resistance, and FRSpe1.1, conferring spectinomycin resistance) shared low sequence similarity to the major determinants of ampicillin resistance (Cj0299) and spectinomycin resistance (AadA) in *Campylobacter* (6, 17). *FRAmp1.1* and *FRSpe1.1* were PCR amplified using proper primers pairs (Table 2), and the PCR fragments were cloned into pZW, creating pZW1 and pZW2, respectively. The promoterless kanamycin resistance gene *KAN-2* from a *Campylobacter jejuni* strain (11) was also cloned into pZW to generate pZW3 (Table 1). All plasmids described above were transferred into *C. jejuni* 81-176 (JL242) (Table 1) using triparental conjugation as described previously (32). All the *C. jejuni* constructs were subjected to MIC tests for differ-

TABLE 2 PCR primers used in this study

Name	Sequence[a]
pZE-F	5′-GAA TTC ATT AAA GAG GAG AAA GGT-3′
pZE-R	5′-TTT CGT TTT ATT TGA TGC CTC TAG-3′
Sig28-F	5′-GCT CTA GAG CGT AAA ATT GAA GAT GAA AGA GAG-3′ (XbaI)
Sig28-R	5′-CGG GAT CCC GTT TTA AAT CCT TTT AAA TAA TTT C-3′ (BamHI)
CAm-BamHIF	5′-CGG GAT CCC ATC GCA AGT GAA ATG ACA TCA GTA C-3′ (BamHI)
CAm-EcoRIR	5′-CGG AAT TCC TCC TTA ACT CCT AAA ATT TAA CTT C-3′ (EcoRI)
CSp-BamHIF	5′-CGG GAT CCC AAT GAA ATG ATA ATA TG-3′ (BamHI)
CSp-EcoRIR	5′-CGG AAT TCC ATT TTA AGC AAA ACT TTA CAG CC-3′ (EcoRI)
CKa-F	5′-CGG GAT CCG TAA TAC AAG GGG TGT TAT G-3′ (BamHI)
CKa-R	5′-CGG AAT TCA TTA GAA AAA CTC ATC GAG C-3′ (EcoRI)

[a] Restriction sites are underlined in the primer sequences, and the corresponding names are in parentheses.

TABLE 3 Antibiotic resistance genes identified in the cecal contents of free-range and conventionally raised chickens[a]

Antibiotic[b]	Gene[c]	Length (bp)	Annotation	% amino acid similarity to NCBI[d]	% amino acid similarity to Sommer et al.[e]
AMP	*FRAmp1.1*	902	β-Lactamase	59	61
	FRAmp2.1	861	β-Lactamase	100	59
	CRAmp1.1	861	TEM β-lactamase	99	59
	CRAmp1.3	525	TEM β-lactamase	91	56
	CRAmp2.1	504	CblA β-lactamase	58	100
	CRAmp2.2	642	HGF-1 β-lactamase	63	99
	CRAmp2.3	510	CblA β-lactamase	67	98
	CRAmp1.4	756	TEM β-lactamase	100	62
TET	*CRTet2.1*	936	TetW tetracycline resistance protein	99	100
	CRTet2.2	660	TetW tetracycline resistance protein	99	100
CHL	*CRChl2.1*	804	Chloramphenicol acetyltransferase	92	77
	CRChl2.2	639	Chloramphenicol acetyltransferase	76	68
SPE	*FRSpe1.1*	786	Adenyl transferase	70	NH
	CRSpe2.1	774	Adenyl transferase	99	NH
NOR	–	–	–	–	–
CIP	–	–	–	–	–

[a] –, no genes identified or parameter not available.
[b] Abbreviations: AMP, ampicillin; TET, tetracycline; CHL, chloramphenicol; SPE, spectinomycin; NOR, norfloxacin; CIP, ciprofloxacin.
[c] Each gene identified in this study was named with a two-letter code corresponding to the source (FR, free-range chicken; CR, conventionally raised chicken) followed by a three-letter code referring to the three initial letters of the antibiotic used for screening, the library number corresponding to a specific chicken, and a number used to distinguish each unique gene against the specific antibiotic.
[d] The highest percent similarity when compared with the AR genes deposited in GenBank (excluding those identified by Sommer et al. [21]).
[e] The highest percent similarity when compared with the AR genes discovered by Sommer et al. (21) using the same culture-independent sampling and functional cloning approach. NH, no homologs found based on alignment.

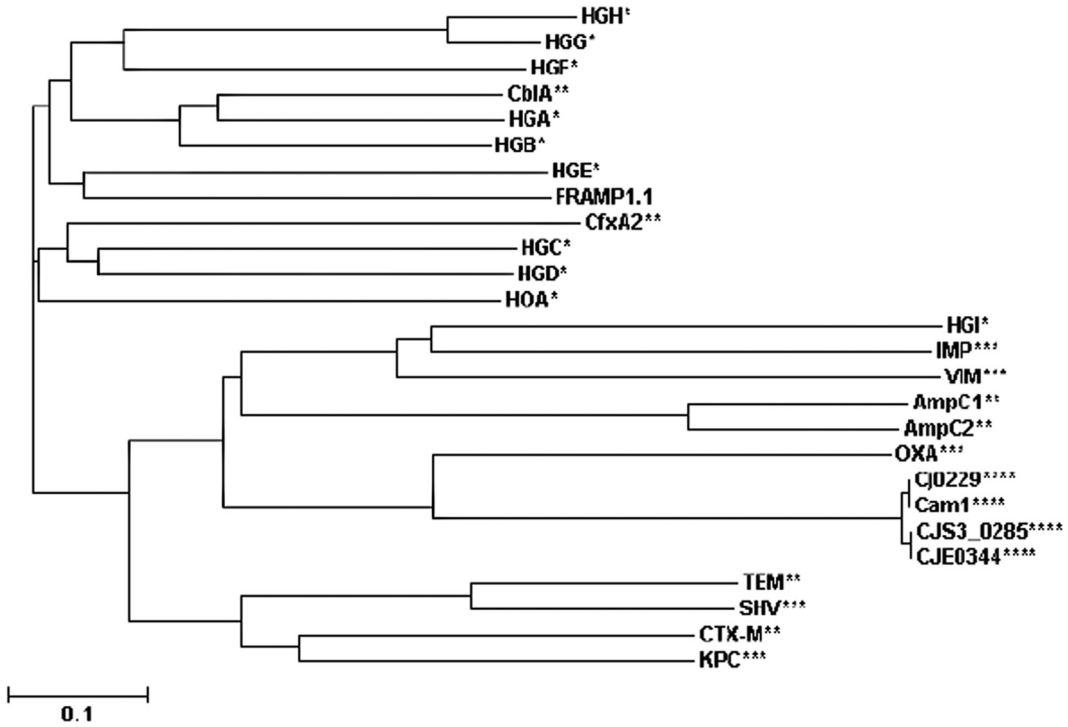

FIG 1 Phylogenetic relationship of β-lactamases from different sources. Unrooted phylogenetic trees were generated from ClustalW2 alignments using the neighbor-joining algorithm. FRAmp1.1, discovered in this study, was analyzed together with 25 identified β-lactamases. *, the novel β-lactamases belonging to different families that were discovered from the human gut microbiome by Sommer et al. (21); **, the β-lactamases that were discovered from the human microbiome by Sommer et al. (21) that share high similarity to the previously characterized β-lactamase families; ***, the β-lactamases representing previously characterized common β-lactamase families that were not analyzed by Sommer et al. (21) (SHV [25], OXA-10 [accession number AY841859], IMP [15], VIM [9], and KPC [31]); ****, the β-lactamases identified in different *C. jejuni* strains, including Cj0299 (accession number YP_002343737), CJE0344 (accession number AAW34933), CJS3_0285 (accession number ADT72049), and Cam1 (accession number AAT01092).

TABLE 4 MICs of different β-lactam antibiotics for the *C. jejuni* JL865 strain that carries resistance gene *FRAmp1.1* and for the control strain JL854

β-Lactam agent	MIC (μg/ml)	
	JL855	JL854
Ampicillin	8	1
Amoxicillin	128	16
Penicillin G	64	4
Ticarcillin	32	8
Carbenicillin	32	8
Cloxacillin	>256	256
Cefotaxime	>256	128

ent antibiotics as determined by a standard microtiter broth dilution method (10). The JL857 strain (*C. jejuni* 81-176 containing pZW3) displayed a dramatically enhanced resistance to kanamycin (MIC > 128 μg/ml) compared to that of the control strain JL854 (MIC = 8 μg/ml), indicating that pZW was an effective expression vector for *Campylobacter*. The constructs harboring the novel AR genes (JL855 and JL856) (Table 1) also showed significantly higher levels of resistance to the corresponding antibiotics. Specifically, the JL856 strain containing the *FRSpe1.1* gene displayed dramatically enhanced resistance to spectinomycin (MIC > 256 μg/ml) compared to that of the control strain JL854 (MIC = 8 μg/ml). Expression of *FRAmp1.1* in JL855 also led to enhanced ampicillin resistance compared to that of the control JL854 strain (8-fold increase) (Table 4). The JL855 strain also displayed significantly increased resistance to a variety of β-lactams in *C. jejuni*, including those (e.g., cefotaxime) to which Cj0299 fails to confer high resistance (6) (Table 4). Together, these findings further support a previous hypothesis that if a barrier to gene transfer exists between the constituents of gut microbiomes and pathogens, it must be attributed to processes other than functional compatibility (21).

Characterization of novel AR genes from animal gut microbiomes is important in several ways. First, the genes that are divergent in sequence can help to assemble a more complete image of the evolutionary history of AR genes (2). Second, further functional and structural study of novel AR genes will improve our understanding of the relationship between sequence diversity and the resistance spectrum/level (28). In particular, identification of novel AR genes using the functional cloning approach would greatly complement modern high-throughput pyrosequencing of the metagenome by improving annotation power. Third, since the novel AR genes may function effectively in zoonotic pathogens, thorough examination of novel AR gene pools in food animals and even other agriculture ecosystems (e.g., manure) will greatly facilitate development of more-powerful molecular diagnostic tools to monitor AR in zoonotic pathogens and examine the development and transmission of AR (20). Finally, considering the fact that genes for antibiotic biosynthesis were sometimes clustered with AR genes, identification of novel AR genes may lead to the discovery of potentially novel antibiotics (19).

Although the novel gut AR genes have been successfully identified in both conventionally raised and free-range chickens in this study, the following limitations and issues should be addressed for future large-scale investigation of the AR reservoir. First, the sizes of expression libraries are a limiting factor of the likelihood of identifying more novel AR genes. The sizes of the libraries used in both a recent human study (~10^9 bp per library) (21) and this study (~10^8 bp per library) cover only a proportion of the gut microbiome (18). Thus, increasing library size, for instance by cloning larger insertions into the vector, will provide a greater chance to identify more novel AR genes. Second, we used chickens as a model here to analyze the gut AR reservoir in food animals. The functional cloning method can be extended into the investigation of the AR gene reservoir in other habitats, including the guts of other food animals as well as specific niches of agricultural ecosystems (e.g., manure, soil, water, lagoon). Recent studies for the identification of specific AR genes in soils also showed the feasibility of the similar methodology in revealing a natural AR gene reservoir, which exhibited a higher level of diversity than previously expected (1, 5, 19). Finally, the complex mechanisms of HGT should be elucidated in parallel to the effort for identification of novel AR gene reservoirs. The novel AR genes need to overcome multiple HGT barriers (23) before being able to confer resistance to the pathogens. AR gene reservoirs and HGT are equally important issues to be addressed in the future to improve our understanding of the development, transmission, and evolution of AR genes.

Nucleotide sequence accession numbers. The full coding sequences of the 14 AR genes identified in this study were deposited in the GenBank database under accession numbers JN625754, JN625755, JN625756, JN625757, JN625758, JN625759, JN625760, JN625761, JN625762, JN625763, JN625764, JN625765, JN625766, and JN625767.

ACKNOWLEDGMENTS

We thank Andree A. Hunkapiller for technical support.

This study was supported by Microbiology across Campuses Educational and Research Venture (M-CERV) and AgResearch at The University of Tennessee.

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