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

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ABSTRACT

A recent study using human fecal samples in conjunction with a culture-independent approach revealed immense diversity of antibiotic resistance (AR) genes in the human gut microflora. We hypothesize that food animal gut microflora also contain diverse and novel AR genes which could contribute to the emergence and transmission of AR in pathogens important in animal and human health. To test this, we examined AR reservoir in chicken gut microflora using a metagenomic, functional cloning method. Total genomic DNA was extracted from individual cecal contents of two free range chickens and two conventionally raised chickens. The DNAs were physically sheered into 1 to 3 kb fragments, cloned into expression vector pZE21-MCS, and transformed into *E. coli* TOP10 host strain, resulting in four metagenomic libraries of a total size of 10^8 base pairs per library. The AR transformants from the libraries were selected on plates containing the specific antibiotic of interest; six antibiotics including ampicillin, tetracycline, chloramphenicol, spectinomycin, ciprofloxacin and norfloxacin were used for screening. Plasmids from selected transformants were extracted and subjected to sequence analysis of inserted fragments. Identified AR genes were annotated and aligned with homologs that have been deposited in GenBank. A total of 12 AR genes and 3 AR genes were identified from the microbiome in conventionally raised chickens and free-range chickens, respectively. Of the identified 15 AR genes, 8 genes that confer resistance to ampicillin, spectinomycin or chloramphenicol shared low sequence similarity (58% - 76% at amino acid level) with the corresponding AR genes previously identified using culture-dependent approaches. Notably, among the 8 novel AR genes identified in this study, 4 genes also shared low sequence similarities (59%-76% at amino acid level) with recently identified AR genes in

human gut. An *E. coli-Campylobacter* shuttle vector bearing the *flaA* sigma 28 promoter was constructed. Two novel genes conferring resistance to ampicillin (*FRAmp1.1*) and spectinomycin (*FRSpe1.1*) were cloned into this new expression vector, respectively. The derived vectors have conferred increased AR in *C. jejuni*, a leading zoonotic bacterial pathogen causing human gastroenteritidis in many industrialized countries. Together, findings from this study showed the effectiveness of the metagenomic approach for examination of AR reservoir in food animals, revealed novel AR resistance genes in chicken gut microflora, and demonstrated the functionality of such AR genes in foodborne human pathogens.

Key words: gut microflora, antibiotic resistance, metagenomics, functional cloning

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REVIEW OF LITERATURE

Zoonotic pathogens and infectious diseases in food animals

Zoonotic pathogens, including viruses, bacteria, and parasites, are a major cause of animal infectious diseases. For domesticated animals, the consequences of infectious disease include the loss in productivity, loss of incomes from activities using animal resources, cost of disease control as well as impacts on food safety and quality. Viral infections are responsible for some highly contagious animal diseases. For instance, avian infectious bronchitis virus causes avian infectious bronchitis (Kahn 2006), a highly contagious respiratory disease in poultry, seriously affecting growth and performance of broilers as well as egg production and quality in layers and breeders. Aphthovirus, a severe plague for animal farming, is highly contagious and can be spread through contact with various sources of contamination and even aerosols. This virus causes Foot-and-Mouth disease in cloven-hoofed animals (Martinez-Salas 2008), named after its typical symptom of oral and feet blisters. A serious outbreak of foot-and-mouth disease in the United Kingdom in 2001 caused an economic loss of $16 billion.

Bacterial pathogens also cause many infectious diseases in food animals. For instance, *Clostridium perfringens* causes necrotic enteritis in poultry, which is one of the most common and financially devastating diseases in broiler flocks (Van Immerseel, De Buck *et al*. 2004). *C. perfringens* causes growth depression, and in acute forms, even death without clinical signs in birds. The average cost for this disease can be as high as 5 US cents per bird (Anonymous 2000). *Salmonella* species can induce enteric lesions in chickens (Porter 1998). *Salmonella pullorum* causes life-long infections and induces pullorum disease that is most lethal in young

birds and can be transmitted through various mechanisms. *Salmonella gallinarum* causes fowl typhoid that has similar clinical signs with pullorum disease. Paratyphoid group of *Salmonella* can also cause systematic infection in young chickens with high mortality (Porter 1998).

Zoonotic pathogens, food safety and public health

Besides the infection of animals, zoonotic pathogens also have the potential to infect humans, thus resulting in public health problems. Throughout human history, zoonotic pathogens have played vital roles in large infectious events. For example, Black death is believed to be caused by *Yersinia pestis* carried by rodent. The 1918 flu pandemic is thought to be caused by an Encephalitis virus carried by various animal vectors. The HIV virus is hypothesized to evolve from closely related Simian immunodeficiency virus carried by non-human primates (Lederberg 1997). More than half of the recognized human pathogens are zoonotic, and almost all of the most important human pathogens have animal origins (Taylor, Latham *et al*. 2001; Woolhouse and Gowtage-Sequeria 2005; Wolfe, Dunavan *et al*. 2007). Transmission of zoonotic pathogens to humans is facilitated by the close interaction between human and animals, especially after a large set of animals became domesticated. Besides physical, airborne and fecal-oral contacts, which are mainly involved in disease transmission in food production, food contamination becomes a major route of transmission of zoonotic pathogens to humans. More than 250 different foodborne diseases have been described, most of which are infections caused by a variety of bacteria, viruses and parasites (CDC 2010). CDC estimates that 76 million Americans get sick, more than 300,000 are hospitalized, and 5,000

people die from foodborne illnesses each year (CDC 2010). *Campylobacter* infection is the most commonly reported bacterial cause of human enteritidis in the United States. Chickens and turkeys are major reservoirs of *Campylobacter* and therefore the major source for human campylobacteriosis (Nachamkin 2008). There are 2.1- 2.4 million estimated cases of campylobacteriosis in the United States each year, and the annual medical and productivity costs resulting from *Campylobacter* infection are estimated at 1.5- 8.0 billion dollars (Altekruse, Stern *et al*. 1999; Mead, Slutsker *et al*. 1999). *Salmonella* infection, the second most common cause of foodborne enteric infections, is caused mainly by the paratyphoid group of *Salmonella* including *Salmonella enteritidis*, which are found generally in chicken carcasses, eggs, and egg products. In August 2010, 380 million eggs coming from Wright County Egg of Galt, Iowa were recalled in several states because of suspected *Salmonella* contamination. *Salmonella* infections in humans account for 1.4 million salmonellosis, 415 deaths, and $2.65 billion cost each year (Mead, Slutsker *et al*. 1999) in the United States. *E.coli* O157:H7, commonly found in intestines of healthy goats and cattle, can be spread through fecal-oral routes. *E. coli* O157:H7 causes no disease in cattle and has been found in all age groups (USDA 1997). Contaminated beef, water and vegetables are major sources of human *E.coli* O157:H7 infections. *E.coli* O157:H7 causes 73,000 cases, 61 deaths, and a $478.4 million cost annually in the United States (Mead, Slutsker *et al*. 1999).

Antibiotic usage and development of antibiotic resistance

Originally produced by or derived from fungi, bacteria or other microorganisms,

antibiotics have been successfully used for treatment of bacterial infections in human and animals. Through therapeutic application of antibiotics, mortality rates associated with bacterial infections decreased significantly, compared with before the 1930s when antibiotics had not been introduced (Overbye and Barrett 2005). In food animals, antibiotics are more widely used comparing with the therapeutic use in humans (van den Bogaard and Stobberingh 1999). Application of antibiotics in domestic animals includes both therapeutic and non-therapeutic uses. The therapeutic uses of antibiotics in domestic animals are under strict regulations. These antibiotics include amoxicillin, sulfonamides, tylosin, fluoroquinolones, lincosamides, aminoglycosides, tetracyclines, colistin and pleuromutilins (Gyles 2008). In most western countries, these antimicrobials can only be applied to a particular group of animals, for a defined period of time, and only upon prescription from a veterinarian to cure or prevent some specific bacterial infections (van den Bogaard and Stobberingh 1999). However, usage of antibiotics as non-therapeutic antimicrobial growth promoters (AGP) is much less restricted. These antibiotics include zinc bacitracin, procaine penicillin, tetracyclines, tylosin, virginamycin and monensin (Gyles 2008). According to the European Federation of Animal Health Industries, of the World Animal Health Product Market 44% were therapeutic pharmaceutical products and 41% AGP feed additives (Miller 1993). According to an estimate by Levy (1998), more than 80% of all tetracycline and benzylpenicillin were used as AGP in the US. Given that epidemiological studies have linked AGP application to the emergence of antibiotic resistant bacteria (Wegener 2003), Denmark banned all AGPs in 1998 and European Union member nations banned all AGPs in 2006 (Wegener 2003; Dibner and Richards 2005). Although there has been little regulatory activity regarding AGP use in the United States, consumer pressure, market limitations, and export restrictions are causing commerce to

withdraw AGPs from market (Dibner and Richards 2005)

Antibiotic resistance (AR) is a phenomenon where microorganisms become insensitive to exposure to antibiotics. It is initially developed in antibiotic producing organisms in order to help them to resist the antimicrobial effect of their own products. For instance, *Streptomycetes* produce beta-lactamases to resist beta-lactam produced by themselves (Forsman, Haggstrom *et al*. 1990). It has been estimated that there exist over 20,000 potential AR genes in bacteria with 400 different classes (Liu and Pop 2009).

AR is an especially serious issue in pathogenic bacteria. When pathogens develop AR, the infections are harder to cure, and alternative and effective drugs are often not readily available. Nowadays, development of AR is accelerating (Alene and Bennett 1996), nonetheless the speed of the discovery and introduction of new antimicrobial drugs are slowing down (Lapara 2006). An estimated 38 deaths happen per day caused by hospital acquired antibiotic resistant pathogens alone among Americans (WHO 2000). The economic impact is also severe, for instance, the costs to bring a new drug onto the market are estimated at a minimum of $ 1.3-1.7 billion (Collier 2009), while the overall annual cost considering issues like therapeutic treatment, development of new drugs, maintenance of the life of old drugs and reduction of forces leading to resistance was estimated to be at least $ 4 to 5 billion (McGowan 2001).

It is believed that antibiotic usage in both human and animals may select for AR bacteria, either in commensal or pathogenic bacteria. For human, imprudent use of antimicrobial drugs is deemed as a cause for the rapid development of drug resistant pathogens.

Community usage of antibiotics is expanding AR gene reservoirs (van den Bogaard and Stobberingh 1999). Intensive care units (ICUs) are still considered to be the major breeding grounds of resistant pathogens because of the effect of surgery, manipulation as well as prolonged hospital stay on the host, and most importantly the patients' exposure to multiple antimicrobial drugs which selects for AR pathogens. Nosocomial infections caused by AR micro-organisms can date back to the discovery of penicillin resistant *Staphylococcus aureus* in the 1950s (Wilson and Cockcroft 1952). In the 1960s, multidrug resistant Gram-negative bacilli became responsible for many nosocomial infections (Swartz 1994; Itokazu, Quinn *et al*. 1996). The extensive usage of methicillin brought about the methicillin resistant Coagulase-negative staphylococci *S. aureus*, as important nosocomial pathogens in the 1980s (Wilkinson, Maxwell *et al*. 1980). Use of vancomycin as a cure for these infections then aroused vancomycin resistant enterococci (Dixson, Brumfitt *et al*. 1985). Antibiotic usage in animals has significant influences on selection of AR bacterial pathogens. For example, use of enrofloxacin rapidly selected high-level fluoroquinolone resistant *Campylobacter* in poultry (Luo, Sahin *et al*. 2003). In animals, antibiotics used as AGPs lead to a long-term selective pressure at sub-inhibitory concentrations. There are several examples of the direct impact of AGP usage in antimicrobial resistance development. For example, poultry and pigs fed tylosin and bacitracin shed enterococci that were resistant to the antibiotics (Rosen 1995). An increase in resistance against quinupristin dalfopristin in fecal *E. faecium* was observed in turkeys given virginiamycin as AGP. Fecal *E.coli* tends to display enhanced resistance against olaquindox when fed to pigs (Rosen 1995). Feeding chicken with tylosin at a growth promoting dose resulted in the emergence of erythomycin resistant *Campylobacter* (Lin, Yan *et al*. 2007).

Mechanism of AR in bacteria.

Although the exact AR mechanisms and genes underlying mechanisms are widely diversified, there are three general mechanisms of AR resistance in bacteria. First, mutations on the drug target may decrease binding affinity of drugs to the target, hindering the proper function of the drug. Similarly, protection proteins may be produced to cover the drug target from being bound by drugs. Second, bacteria may destroy the correct structure of drug molecules by synthesizing enzymes that catalyzes the covalent modification or degradation of drug molecules. Third, bacteria may decrease drug uptake by altering membrane permeability, or increase active efflux of the drug through the function of drug efflux pumps. Although some of the drug efflux systems are substrate-specific, several else can confer multi-drug resistance, and even resistance against natural molecules other than antibiotics (Piddock 2006). The following are mechanisms of bacterial resistance to specific group of antibiotics.

Beta-lactam resistance

Beta-lactam is one of the most prevalent groups of antibiotics. It is a general term regarding all antibiotic agents which have a lactam with a heteroatomic ring structure (beta-lactam ring). The general mode of action of beta-lactams is inhibition of peptidoglycan synthesis of bacterial cell wall by binding to and blocking transpeptidase in the periplasm. Three general mechanisms may result in beta-lactam resistance in bacteria: enzyme degradation of the compound, mutations in the transpeptidase, and drug efflux as well as changes in membrane permeability. Except for the alternations of membrane permeability that

is mainly observed only in Gram-negative bacteria, other mechanisms are adopted by both Gram-positive and Gram-negative bacteria.

Among the three mechanisms, enzyme degradation is the most commonly observed mechanism. Generally, inactivation of beta-lactams is accomplished through hydrolyzing the beta-lactam rings in the compounds by beta-lactamases. The beta-lactamase is a large group of hydrolase. According to different criteria, it can be classified into four functional groups (functional group 1-4) or four molecular classes (classes A-D) (Bush, Jacoby *et al*. 1995). The evolution of beta-lactamases expands their resistance spectrum. Penicillinases are the earliest to be identified; they confer resistance to penicillins, but often sensitive to other beta-lactams like methicillin. Plasmid encoded penicillinases like TEM-1, TEM-2 and SHV-1 are initially responsible for resistance only to penicillins. Therefore, new generation beta-lactam called ‘cephems' are often used as an alternative treatment (Sougakoff 1988). However, point mutations in these enzymes soon extended their resistance spectrum. Arising then are the Extended Spectrum Beta-Lactamases (ESBLs) that hydrolyzes not only penicillins but also extended spectrum cephems (mainly 3rd and 4th generation cephalosporins). For instance, TEM-3 beta-lactamase, which is derived from TEM-2 with only two amino acid substitution in the active site, can confer resistance to the cephems (Sougakoff 1988; Bradford 2001). Other types of ESBLs include CTM-X beta-lactamases which show higher activity against cefotaxime compared with other cephems (Seputiene, Linkevicius *et al*. 2010) and beginning to dominate the ESBLs (Seputiene, Linkevicius *et al*. 2010), and OXA beta-lactamases which show high activity against oxacillin and cloxacillin (Paterson and Bonomo 2005).

In an attempt to enhance antimicrobial effectiveness of beta-lactam antibiotics to

bacteria producing beta-lactamases, beta-lactamase inhibitors are combined with beta-lactam to overcome resistance. Beta-lactamase inhibitors are typically identical to beta-lactams in chemical structure. These inhibitors like clavulanic acid and sulbactams can bind irreversibly to beta-lactamases as suicide inhibitors (Bush 1988; Totir, Helfand *et al*. 2007). However, the beta-lactamases that were not susceptible to inhibitors were detected in clinical isolates of various bacteria in Europe (Bradford 2001). In addition, AmpC beta-lactamases, another distinctive family of beta-lactamse, can hydrolyze third-generation cephems (Sanders 1996) and are also resistant to inhibitors (Bradford 2001). Peptide-based inhibitors are being developed to overcome these resistance (Huang, Beharry *et al*. 2003). Still, commonly encountered beta-lactamases are hardly capable of hydrolyzing Carbapenems. Nonetheless, carbapenemases, a diverse group of beta-lactamases, have been discovered and found to be plasmid-mediated (Bradford 2001). Carbapenemases are found in *Klebsiella pneumoniae* from different geographical regions (Koh, Sng *et al*. 2001; Wong-Beringer 2001; Giakkoupi, Xanthaki *et al*. 2003; Bradford, Bratu *et al*. 2004; Woodford, Tierno *et al*. 2004).

Mutations in transpeptidase, alteration of membrane permeability and possession of drug efflux systems contribute more to intrinsic resistance and tend to be comparatively moderate in resistance level. They often coordinate with other mechanisms to confer resist the inhibiton by beta-lactams. For instance, membrane barrier often works synergistically with inducible beta-lactamases to enhance resistance in *Pseudomonas aeruginosa* (Li, Ma *et al*. 1994). Also, active drug-efflux by itself does not confer a high level of resistance to beta-lactamases, and is often coupled with the production of beta-lactamases (Li, Ma *et al*. 1994; Cavallo, Plesiat *et al*. 2002).

Chloramphenicol resistance

Chloramphenicol exerts its bacteriostatic effect through binding to the 50S ribosomal subunit and inhibiting protein synthesis. Chloramphenicol has a wide antibacterial spectrum against a number of Gram-positive and Gram-negative bacteria.

Resistance to chloramphenicol comes in mainly four forms: mutation of the chloramphenicol binding site on the ribosome, reduced membrane permeability, enzymatic drug modification that confers high level of resistance, and expression of drug efflux pumps (Vecchione, Alexander *et al*. 2009). Chloramphenicol modification is mainly achieved by acetyl-CoA based acetylation on the compound's hydroxyl group catalyzed by chloramphenicol acetyltransferase (CAT) (Shaw, Packman *et al*. 1979; Engel and Prockop 1991). CAT holoenzyme is a trimer of subunits at 25kDa. Three chloramphenicol molecules are bound to one holoenzyme, with each of the molecule located in a binding pocket at the interface of two subunit. The histidine residue (His 195) located in the subunit's C-terminal is vital for the catalysis (Leslie 1990).

Tetracycline resistance

Tetracycline group of antibiotics, including oxytetracycline, doxycycline, methacycline, etc., are polyketide antibiotics that inhibit protein synthesis. Tetracyclines can bind to the 30S ribosomal subunit thus blocking the localization of aminoacyl-tRNA to the A site of the ribosome.

The usefulness of tetracycline is compromised greatly by the revealing of tetracycline resistance in both Gram-negative and Gram-positive bacteria. Tetracycline resistance comes in

three general modes of action: drug efflux, ribosomal protection, and drug modification. Drug-efflux genes constitute the largest proportion of tetracycline resistance genes in both Gram-negative and Gram-positive bacteria, and more than 25 genes have been discovered already (http://faculty.washington.edu/marilynr/tetweb4.pdf). Activities of the tetracycline efflux pumps are inducible by presence of tetracycline. In Gram-positive bacteria, the induction is achieved by the release of the repressor protein (due to the binding to tetracycline) from the operator region of efflux genes(Paulsen, Brown *et al*. 1996). In Gram-negative bacteria, the induction is achieved through the regulation of mRNA structure changes by the presence of tetracycline molecule (Paulsen, Brown *et al*. 1996).

Ribosome protection is also widely adopted. More than 10 ribosome protection proteins have been found (Brown, Mitchell *et al*. 2008). Ribosome binding proteins show considerable sequence identity to bacterial elongation factor G especially at the GTP binding site (Sanchez-Pescador, Brown *et al*. 1988; Burdett 1991). TetM has been determined to have ribosome-dependent GTPase activity, which suggests an evolutionary relationship between the ribosome protection protein and the elongation factor G (Burdett 1991).

TetX exemplifies a tetracycline modification enzyme, putatively an oxidoreductase (Speer, Bedzyk *et al*. 1991; Speer, Shoemaker *et al*. 1992). Its activity requires oxygen and NADPH, and functions in *E. coli* but not in its natural host bacteroides. The clinical significance of tetracycline modification is not as high as the other two mechanisms (Speer, Bedzyk *et al*. 1991; Speer, Shoemaker *et al*. 1992).

Aminoglycoside resistance

Aminoglycosides are antibiotics with amino-modified sugar structure. This group includes kanamycin, spectinomycin, neomycin, streptomycin, gentamycin, etc. The antimicrobial activity of aminoglycosides has several potential mechanisms including increasing the rate of nonsense suppression in genes by interfering with the DNA proofreading process, inhibiting protein synthesis by binding to the 30s, or sometimes 50s ribosome(Lorian 1996; Champney 2001), and interfering with the integrity of bacterial cell membrane (Shakil, Khan *et al*. 2008).

Aminoglycoside resistance has four major mechanisms: alternation in membrane permeability, efflux of drugs, mutations in ribosome binding site, and enzyme-based modification of the drugs. Reduced drug uptake (eg. transport defect), alternation in membrane permeability and drug efflux mechanisms all contribute to intrinsic resistance (Mingeot-Leclercq, Glupczynski *et al*. 1999). The resistance level is moderate, but the spectrum of resistance is wide, and these mechanisms usually need to couple with a secondary mechanism to function effectively (Nikaido 1996; Mingeot-Leclercq, Glupczynski *et al*. 1999). For instance, membrane impermeability of *P. aeruginosa* was proposed to be responsible for aminoglycoside resistance, but later it was elucidated that it needs to work together with the MexX-MexY-OpmG efflux pump to function (Nikaido 1996; Jo, Brinkman *et al*. 2003).

Enzymatic modification is the most common resistance mechanism for aminoglycoside resistance. More than 50 enzymes have been discovered to introduce covalent modifications on the amino (N-modification) or hydroxyl (O-modification) groups of aminoglycosides. The N-modifications are catalyzed by acetyltransferases, which introduces

CoA-dependent acetylation at site 3, 2' or 6'. O-modifications are catalyzed by adenyltransferase or phosphotransferase, which introduces ATP- dependent adenylation or phosphorylation at site 6, 4', 2" or 3" for adenylation and 6, 3', 2" or 3" for phosphorylation. Modifications introduced by different enzymes at different sites will result in distinctive resistance phenotypes regarding the spectrum of aminoglycoside resistance. Certain genes have different transferases fused together to achieve bifunctional modifications. For instance, N6'-acetyl-O2"- phosphotransferase has been discovered and shown to confer resistance to all of the aminoglycosides with the exception of streptomycin (Chow 2000).

Quinolone resistance

Quinolones are a group of synthetic antibiotics that inhibit the unwinding and duplicating of DNAs through binding to the gyrase in Gram-negative bacteria, typeII topoisomerases and topoisomerase IV in Gram-positive bacteria (Gellert, Mizuuchi *et al*. 1977).

Mechanisms of quinolone resistance were once deemed to be mainly mutations in gyrase and topoisomerase (target alternations), gyrase protection, and permeability barrier in the cell membrane (Ruiz 2003). For instance, plasmid-mediated Qnr proteins can bind to and protect gyrase from being targeted by quinolones (Nordmann and Poirel 2005). Later, drug-efflux mechanisms arouse attention. For example, the *norA* gene in *Staphylococcus aureus* encodes a multidrug efflux pump that excludes quinolone from the cell. Inhibitors of the NorA pump can improve the activity of quinolone drugs like norfloxacin and ciprofloxacin (Ng, Trucksis *et al*. 1994).

The synthetic nature of quinolones made it believed that enzymatic modification of

the compound is not likely to be developed. However, quinolone acetylation mechanism was reported to be responsible for enhanced resistance against ciprofloxacin. Resistance was conferred by the product of gene aac(6')-Ib, which was adapted from aminoglycoside acetyltransferases (Robicsek, Strahilevitz *et al*. 2006). This acetyltransferase characterizes an enzyme that, by introducing modifications onto the drugs, confers resistance to two distinctive classes of antibiotics. The report of the enzyme also refreshes the understanding of the speed of the development of antibiotic resistance in bacteria, even against a synthetic one.

Role of horizontal gene transfer in AR development and transmission

Horizontal gene transfer (HGT) (Davies 1994) appears a highly efficient mechanism in development and transmission of AR in bacteria. HGT is a process in which an organism incorporates genetic material from another non-parental organism. HGT can be facilitated by a number of mechanisms, including transformation (direct uptake and expression of foreign genetic material), transduction (phage facilitated transfer), and conjugation (transfer through cell-cell physical contact). Interestingly, the first report of HGT is an observation of the transfer of AR genes between different bacterial strains and species (Ochiai 1959). In HGT-based development of AR, donors of AR genes are readily available all around. For instance, microorganisms in the environment, like soil or water, constitute an environmental antibiotic resistome (Baysarowich, Koteva *et al*. 2008). These enormous resistomes are potentially accessible to pathogens, as it has already been determined that many resistance mechanisms in pathogens can also be found in the resistome (Wright 2007; Baysarowich, Koteva *et al*. 2008). Another group of important donors for AR genes are animal commensals. Regarding efficiency of HGT, a major advantage of commensal bacteria over environmental

bacteria is that transformation and conjugation appear to occur more frequently in densely packed cells with physical association, which are typified by commensal bacterial communities like on the tooth surface and intestinal tracts (Marshall 2009).

Diversity of gut microflora

Because of its large surface area and nutrient-rich nature, the mammalian gastrointestinal tract (GI tract) turns to be a favorable habitat for microorganisms. The animal GI tract is most condensely colonized by microorganisms comparing with any other parts of the body surface. The gut microflora participates in different physiological functions including metabolic activities, trophic effects on intestinal epithelia and immune system, and barricading the invasion of alien microbes including pathogens (Guarner and Malagelada 2003).

The gut microflora contains an enormous quantity of microorganisms. In humans, the gut microflora is estimated to contain about 10^{14} cells, which is 10 times the number of cells consisting the human body (Bjorksten, Sepp *et al*. 2001; Guarner and Malagelada 2003), and 100 times as many genes as there are in the human genome (Qin, Li *et al*. 2010). Even compared with the microbiota on other parts of the mammalian body, the gut microflora is still dominant in quantity. It has been estimated that the colon alone holds 70% of all microbes carried by an individual (Whitman, Coleman *et al*. 1998; Ley, Peterson *et al*. 2006).

Microorganisms in the gut microflora are composed of bacteria, archaea, viruses and unicellular eukaryotes. Dominated by bacteria, the gut microflora contains more than 50 bacteria phyla, and more than 500 bacterial species (Xu and Gordon 2003). The vast majority

are strict anaerobes, followed by facultative anaerobes and aerobes. In humans, most of the organisms from the gut microflora are members of the Firmicutes and Bacteroidetes phyla (Hayashi, Sakamoto *et al*. 2002; Eckburg, Bik *et al*. 2005). Most Firmicutes falls in the Clostridia class (Eckburg, Bik *et al*. 2005). Substantial proportions of Firmicutes are butyrate-producting bacteria and involved in maintenance of normal colonic epithelium (Pryde, Duncan *et al*. 2002; Eckburg, Bik *et al*. 2005). The Bacteroidetes are the second largest population (Eckburg, Bik *et al*. 2005), and are involved actively in functions like nutrient absorption and epithelial cell maintenance (Hooper, Wong *et al*. 2001; Eckburg, Bik *et al*. 2005). Other phyla like Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, and Cyanobacteria constitute a comparatively smaller proportion of the microflora (Eckburg, Bik *et al*. 2005).

The composition of microorganisms in the gut is not stable over time and not homogeneous among species, individuals, and different parts of the intestine. Development of the gut microflora is dynamic, and is influenced by parents, environment and age. In poultry, Torok *et al* (2007) showed that the gut microbial community composition changes greatly within the first 2-3 weeks of age before stabilizing until 5-6 weeks of age, when a final change in community composition was observed. The gut microflora composition also show significant differences between chickens raised under conventional poultry conditions and low pathogen load isolator, suggesting the effect of environment on the microflora difference (Torok 2007). In mammals, parental inoculation plays an important role in the development of the gut microflora, thus posing differences between individuals with different parents (Mandar and Mikelsaar 1996). The intestine shows longitudinal and latitudinal heterogeneity.

Firmicutes and *Actinobacteria* are more prevalent in the small intestine, while *Bacteriodetes* and *Lachnospiraceae* are more prevalent in the colon (Frank, St Amand *et al*. 2007). In humans, bacterial species in the intestinal lumen differ from those on the mucus layer; *Clostridium, Lactobacillus* and *Enterococcus* were found at both sites while *Enterobacteriacea* and *Ruminococcus* are only present in the lumen (Swidsinski, Loening-Baucke *et al*. 2005).

Our understanding of the gut microflora is largely incomplete. One of the most important reasons is that in the gut microflora, a large proportion of the bacterial species are unculturable in the laboratory. This theory was first proposed by Langendijk, who found that only 15% DAPI (4'-6-diamidino-2-phenylindole) counts belonged to culturable bacteria by fluorescence in situ hybridization (FISH) with bifidobacterium probes (Langendijk, Schut *et al*. 1995). This suggests that the proportion of unculturable bacteria in the gut is substantial, and very likely constitutes the majority of the microflora. Thus, conventional culture-dependent methodologies inevitably introduce bias against unculturable bacteria when used to study gut microflora. In the past decade, culture-independent molecular techniques, particularly 16S ribosomal RNA gene (rDNA)-based approaches for prokaryotes have been increasingly used to examine diversity of gut microbota and significant progress has been made to understand the structure and diversity of microbial communities in the gut (Eckburg, Bik *et al*. 2005). Several 16S rDNA-based molecular approaches have been used widely to study intestinal microbiota and these approaches include sequencing of cloned 16S rDNA amplicons from 16S rDNA library, quantitative real-time PCR, denaturing gradient gel electrophoresis (DGGE), restriction fragment length polymorphism (RFLP), terminal restriction fragment length polymorphism (T-RFLP), and FISH (Zoetendal, Vaughan *et al*. 2006).

Although 16S rDNA-based approaches have been applied widely in microbial ecology, these methods do not provide any clue of the functional potential in an ecosystem, particularly with respect to the physiology of uncultivated microbes. A metagenomic approach based on genome sequences from an entire community provides a wealth of sequence information and has been successfully used to study the phylogenetic, physical and functional properties of the microbiome (Zoetendal, Vaughan *et al*. 2006). Metagenomic approaches have been used to examine gut microbiomes in humans (Vacharaksa and Finlay 2010) and animals (Walter, Mangold *et al*. 2005). These recent studies demonstrate that metagenomic approaches are promising for unraveling the significance and functionality of gut microbiota and information from metagenomic analysis will facilitate our development of novel strategies to improve gut health. Notably, Sommer *et al*. (2009) characterized the AR gene reservoir in human gut microflora using metagenomic-based functional cloning method. They identified a large number of novel AR genes failed to be discovered in the culture-dependent studies in the past. Their results renewed our understanding of the gut microflora; it is an underexplored microbiota with even larger genetic diversity than we might have estimated.

INTRODUCTION

Zoonotic bacterial pathogens are not only a major cause of diseases in animals but may be transmitted to humans, threatening food safety and public health (Taylor, Latham *et al*. 2001; Woolhouse and Gowtage-Sequeria 2005; Wolfe, Dunavan *et al*. 2007). For example, it has been widely accepted that many human bacterial pathogens, such as *E. coli* O157:H7, *Salmonella*, *Campylobacter*, and *Yersinia* are transmitted from various food animals such as chicken, swine, and cattle (CDC 2010). Antibiotics have been used widely in food animals for the purpose of disease prevention and control as well as for growth promotion (van den Bogaard and Stobberingh 1999; Gyles 2008). Increasing evidence indicates that antimicrobial use in animals selects for resistant bacteria including those that could be transmitted to humans, greatly compromising the effectiveness of antibiotic treatments of these pathogens. Therefore, examination of mechanisms of antibiotic resistance (AR) development and transmission in zoonotic bacteria has a significant impact on animal health and human health.

Genes involved in AR can be transferred horizontally between diverse bacteria, such as from gut normal microflora to pathogens (Witte 2000). Thus, intestinal microflora, which contains more than 1,000 different species with total population up to 100 trillion microorganisms in human, play a significant role in AR development and transmission. There is increasing interest in elucidating the reservoir of gut AR genes that may be horizontally transferred to clinically relevant pathogens. In particular, bacteria in the gut are generally densely packed, which enhances the efficiency of transformation and conjugation for horizontal gene transfer (Marshall 2009). However, the majority of intestinal microflora cannot

be cultivated using traditional culturing methods, greatly impeding our understanding of the AR gene reservoir in gut microflora. Recently, Sommer *et al*. (2009) used a culture-independent method in conjunction with a functional cloning approach to examine the AR gene reservoir in human gut microflora. Strikingly, many AR genes identified by the functional cloning shared very low similarity to the known AR genes, indicating these genes are novel and the immense diversity of AR genes in the human microflora may contribute to future emergence of antibiotic resistance in human pathogens.

Based on this recent finding in the human gut microflora, we hypothesize that the gut microflora in food animals also contains diverse and novel AR gene reservoir, which plays an important role in the emergence of AR pathogens. Notably, given that many prevalent human foodborne pathogens, such as *E. coli* O157:H7, *Salmonella*, and *Campylobacter*, are from the intestinal tract of food animals, the gut microflora in food animals should also serve as a significant AR gene pools for AR human pathogens. Despite its significant impact on animal health and public health, the AR gene reservoir in gut microflora of food animals is still largely unknown.

Using total genomic DNA extracted from chicken cecal contents and an efficient *E. coli* expression vector pZE21-MCS (Lutz and Bujard 1997), in this study, we constructed four metagenomic libraries for functional screening of AR genes against six antibiotics that represent five major classes of antibiotics. We identified novel AR genes from gut microflora in both free range and conventionally raised chickens. In addition, an *E. coli-Campylobacter* shuttle vector pZW bearing a *flaA* promoter (sigma 28) was constructed. The identified novel AR genes (*FRAmp1.1* and *FRSpe1.1*) were cloned successfully into this shuttle vector and

demonstrated to confer increased resistance to corresponding antibiotics in *C. jejuni*, an important foodborne bacterial pathogen with chicken as a major animal host.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

The major bacterial strains and plasmids used in this study are listed in Table 1. *Campylobacter* strains were grown routinely in Mueller-Hinton (MH) broth (Difco, Sparks, MD) or on agar at 42^{o}C under microaerobic conditions, which were generated using a CampyGen Plus (Oxoid, Hampshire, England) gas pack in an enclosed jar. *Escherichia coli* was grown in Luria-Bertani (LB) broth with shaking (250 rpm) or on agar at 37 ^{o}C overnight.

Extraction of total genomic DNA from chicken cecal content

Total genomic DNAs from cecal contents of two free-range chickens (kindly provided by Mr. Wade Wisner, Dandridge, TN) and two conventionally raised chickens (from KOCH Foods, Inc., Morristown, TN) were extracted separately using the PowerMax Soil DNA Isolation Kit (Mobio Laboratories Inc.) with minor modifications. Briefly, 5 g of cecal content from each individual chicken were used for extraction. Cell lysis mix was treated by incubation in 65^{o}C water bath for 5 min, followed by 5 min vortexing; this procedure was repeated one time. A total 5 ml of the elution buffer (Solution C6, TE buffer, pH 8.0) was used to elute pure DNA bound to the provided column. As a control for comparison of DNA yield and quality, the total DNA was also extracted from ileal contents of free-range chickens. The DNA concentration and ratio of absorbance at 260 nm and 280 nm were measured using NanoDrop Spectrophotometer (Thermo Scientific).

Construction of metagenomic libraries

Genomic DNAs obtained from four individual chickens were concentrated to about 30 ng/μl by Vacufuge concentrator (Eppendorf) and sheared physically to fragment size about 3,000 base pairs (The shearing service was provided by Covaris Inc.). The fragment size was determined by running on 0.8% EB stained agarose gel together with 1kb DNA ladder for 40 min at 80 volts in 1x TAE buffer. Gel slices corresponding to fragments in a size ranging from 1,000 to 4,000 bp were excised and the DNAs were extracted from the gel slice using QIAquick® Gel extraction kit (Qiagen). Extracted fragments were then blunt-ended by using the End-it® end repair kit (Epicentre). End-repaired fragments were subsequently purified from the reaction mix by using QIAquick PCR purification kit (Qiagen) for ligation described below.

The expression plasmid vector pZE21-MCS (Lutz and Bujard 1997) was digested by HincII (Promega). Linearized vector DNA was visualized by 0.8% agarose gel. The HincII-digested, linearized vector was extracted from the gel slice using QIAquick® Gel extraction kit (Qiagen).The linearized vector DNA was then dephosphorylated using Promega® Alkaline Phosphatase Calf Intestinal (HC), which was further purified using QIAquick PCR purification kit (Qiagen).

Both the above end-repaired DNA fragments and linearized vector DNA were concentrated to a final concentration of 250 ng/μ l. The end-repaired DNA fragment from each individual chicken was ligated to HincII-digested pZE21-MCS using Fast-Link® ligation kit

(Epicentre) in a reaction mix containing 4μl of vector DNA, 12μl of sheared DNA, 2 μl of Fast-Link buffer, 1 μl of ATP solution, and 1μl of T4 ligase. The ligation mix was incubated overnight at room temperature (25 ^{o}C) prior to use.

A total 2μl of the above ligation mix was used to electroporate 50 μl of ONE-SHOT® TOP10 Electrocompetent cells (Epicentre). Electroporation was conducted using MicroPulser Electroporation system (Bio-Rad) using pre-programmed setting Ec1 (for *E. coli* in 0.1 cm cuvette, 1.8kV, 1 pulse). Cells were then recovered in 1 ml of SOC medium by shaking at 250 rpm for 1 hr at 37 °C.

For each transformation mix, 10 μl of the recovered cells were used for determining the library size. Cells were serially diluted in LB broth and plated on LB agar plates containing 50μg/ml of kanamycin for determination of total colony forming units (CFUs) of the 1ml recovered cells. The size of the library was then estimated by multiplying the total CFU of recovered cells and the average size of the inserted fragment. The rest of the transformation mixes were inoculated into 10ml LB broth supplemented with 50 μg/ml of kanamycin and grown overnight at 37 ^{o}C. Overnight cultures were mixed with glycerol (final concentration of 15%), and stored in 5-ml aliquots at -80 ^{o}C prior to screening. If needed, the 5-ml aliquot was inoculated into 20 ml LB broth with 50 μg/ml of kanamycin and grown overnight to maintain the library.

Screening and characterization of AR genes

Approximately 150 µl of stored culture from each library were spread on a single LB agar plate containing 50 ug/ml of kanamycin combined with the antibiotic of interest at theappropriate concentration (Table 3), which is equal to 2× the minimum inhibitory concentration (MIC) of TOP10 containing empty vector pZE21-MCS for the corresponding antibiotic. A total of 3-15 ml of stored culture from each library was used for screening transformants with resistance to a specific selected antibiotic. Plates were incubated at 37 °C for 16 hrs.

Colonies grown on agar plates containing combined antibiotics were selected randomly and inoculated into 5ml of LB broth supplemented with the same combination of two antibiotics. The culture was grown overnight and the plasmid was extracted from the culture using QIAprep® spin miniprep kit (Qiagen). The inserted fragment in the plasmid was then sequenced bidirectionally using primers pZE-F and pZE-R (Table 2). Plasmids from false-positive colonies (no insertion) and plasmids from the potential redundant colonies (same size of insertion within a library) were not subjected to sequencing.

To analyze the sequence, the open reading frame (ORF) of each inserted fragment was identified using ORFfinder (www.ncbi.nlm.nih.gov/projects/gorf/). Identified ORFs were compared to Genbank database using tblastx, which computes alignment based on the amino acid sequence of the gene, translated in all 6 frames. For each query, the gene of the top scoring hit in the database was recorded for their origin, annotation, and percentage sequential similarity.

Construction of *E. coli-Campylobacter* shuttle vector bearing a strong *Campylobacter* promoter

An *E. coli-Campylobacter* shuttle vector carrying a strong *Campylobacter* promoter was constructed according to the procedure described by Larsen *et al*. (2004). Briefly, the sigma 28 promoter of the *fla*A gene was PCR amplified from genomic DNA of *C. jejuni* NCTC 11168 (Tabel 1) using the primer pair Sig28-F/Sig28-R (Table 2). PCR reaction mix was prepared by mixing 1 µl of each primer (5 pmol/ml), 1 µl of template genomic DNA (50 ng/µl), 14.5 µl of ddH_2O and 17.5 µl of 2x GoTaq PCR mix into a total volume of 25 µl. The templates and primers were denaturized at 95°C for 180 seconds. Then a 3-step PCR was performed with 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 90 seconds. The PCR product was purified and digested by BamHI and XbaI (Promega), followed by ligation to the pRY111 vector that was digested with the same enzymes. Five µl of the ligation mix was then applied to 50µl of ice-cold *E. coli* DH5α chemical competent cells. The mixture was put on ice for 35 minutes before being heat shocked at 42 °C for 30 seconds. The mixture was then put on ice for 2 minutes, mixed with 500 µl SOC medium and shaked for 1 hour at 37 °C and 250 rpm. The transformation mix was spread on LB agar plates supplemented with 20 µg/ml of chloramphenicol and grown for 16 hours at 37 °C. Colonies were randomly picked and inoculated into 5 ml LB broth with 20 µg/ml chloramphenicol and grown overnight. The plasmids were extracted by using QIAprep® spin miniprep kit (Qiagen) according to the recommended procedure, and the correct recombinant plasmids were

determined by PCR amplification of the inserted sigma 28 promoter sequence. The plasmid containing the desired sigma 28 promoter was designated as pZW and the *E. coli* DH5α transformed with pZW was named JL850 (Table 1).

Cloning and expression of selected AR genes in *C. jejuni* 81-176.

To determine if the identified novel AR genes are functional in *Campylobacter*, two of the novel AR genes (*FRAmp1.1* and *FRSpe1.1*) were cloned into the newly constructed expression vector pZW. The kanamycin resistance gene *KAN-2* served as a positive control because this gene was from EZ::TN <KAN-2> Transposon and was functional in *Campylobacter* (Lin, Wang *et al*. 2009).

Promoterless *KAN-2* gene was PCR amplified from genomic DNA of a mutant with EZ::TN <KAN-2> Transposon insertion (Lin, Wang *et al*. 2009) using primer pair CKn-F and CKn-R (Table 2). The amplified fragment was digested by BamHI and XbaI (Promega) for directional coloning. The digested fragment was ligated into the pZW digested with the same restriction enzymes. The ligation mix was used to transform *E. coli* DH5α for selection of chloramphenicol resistant transformants. Plasmids from the selected transformants were extracted and the recombinant plasmid containing desired *KAN-2* gene was determined by PCR amplification of the plasmid using primer pair CKn-F and CKn-R (Table 2). The pZW containing a *KAN-2* gene that was inserted downstream of sigma 28 promoter was named pZW3 (Table 1).

Promoterless *FRAmp1.1* and *FRSpe1.1* were PCR amplified from plasmids

pZE21-MCS-*FRAmp1.1* and pZE21-MCS-*FRSpe1.1* (Table 1), respectively, using primers pairs Cam-BamHIF/Cam-EcoRIR for *FRAmp1.1* and CSp-BamHIF/CSp-EcoRIR for *FRSpe1.1* (Table 2). PCR, ligation and transformation were the same as above for constructing pZW3. The new plasmids pZW1 and pZW2 were generated, in which the novel *FRAmp1.1* and *FRSpe1.1* genes were inserted downstream of sigma 28 promoter in pZW, respectively.

To conjugatively transfer the above plasmids into *C. jejuni* 81-176 (JL242, Table 1), JL48 was used as the helper strain and the new construct JL850, JL851, JL852, or JL853 (Table 1) was used as donor strain for conjugation as described previously (Zeng, Xu *et al*. 2009). Briefly, late log-phase of *C. jejuni* 81-176 was spread onto MH agar and grown overnight. Then *C. jejuni* cells were harvested and resuspended in MH broth to an optical density of 1.0 at 600 nm (OD_{600}). Both donor strain and helper strain were grown in LB to an OD_{600} of approximate 1.0. Three types of cells were mixed at a ratio of 1:1:10 (donor/helper/recipient), resuspended in 100 μl MH broth and spotted onto MH agar plates. The plates were incubated for 5 hours at 42°C under microaerophilic conditions. The mating spots were then resuspended in MH broth and plated onto MH agar supplemented with 20 μg/ml chloramphenicol, 1 μg/ml polymyxin B and 10 μg/ml trimethoprim for selection of desired *C. jejuni* transformants. Plates were examined after 3 days of incubation and the colonies were randomly picked for confirming the presence of the desired recombinant plasmid. The resulting *C. jejuni* strains were referred to as JL854 (JL242 with pZW), JL855 (JL242 with pZW1), JL856 (JL242 with pZW2) and JL857 (JL242 with pZW3) (Table 1).

Susceptibility of JL854, JL855, JL856, and JL857 to ampicillin, kanamycin and spectinomycin were determined by a standard microtiter broth dilution method with an inocula

of 10^6 bacterial cells/ml as described previously (Lin, Michel *et al*. 2002). Minimum inhibitory concentrations (MICs) were determined by the lowest concentration of specific antibiotic showing complete inhibition of bacterial growth after two days of incubation at 42^0C.

Generation of a phylogenetic tree for different beta-lactamases.

Phylogenetic analysis of FRAmp1.1 was performed together with 25 presently identified beta-lactamases representing 21 different families. Amino acid sequences were aligned in ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) using the GONNET proteinweight matrix. Unrooted phylogenetic trees were generated from ClustalW2 alignments, using the neighbor-joining algorithm based on the principle of minimum evolution. Tree branch lengths are proportional to relative sequence identity, and the scale bar was in fixed amino acid substitutions per sequence position. Pairwise scores were calculated as the number of identities in the best alignment divided by the number of residues compared.

RESULTS

High-quality genomic DNA was extracted from chicken fecal samples.

Total genomic DNA was successfully extracted from fecal samples of two free range chickens and two conventionally raised chickens (FIG. 1A). For free range chickens, 5 ml of genomic DNA with a concentration about 24 ng/µl were obtained from 5 g of each cecal sample; the A_{260}/A_{280} of both samples was about 1.70. However, the yield (2.0 ng/µl) and purity (A260/A280 = 1.34) of the DNA extracted from the ileal content were lower than those from cecal content (Fig. 1A). For conventionally raised chickens, 5 ml of genomic DNA were obtained from two 5g cecal samples with a final concentration of 52.3 ng/µl and 99.5 ng/µl, respectively. The A_{260}/A_{280} ratio of the two DNA samples were 1.83 and 1.93.

Construction of the metagenomic libraries

Cecal genomic DNA (Fig. 1A) was sheared to small fragments ranging from 1,000 to 4,000 bp (Fig. 1B). Four libraries corresponding to cecal content samples from two free-range chickens and two conventionally raised chickens were successfully constructed. The randomly selected transformants contained plasmids bearing the inserted fragment, leading to increased plasmid size as compared to parent plasmid pZE21-MCS (FIG. 1C). Libraries were hereafter referred to as FR1 (from #1 Free Range chicken), FR2 (from #2 Free Range chicken), CR1 (from #1 Conventionally Raised chicken) and CR2 (from #2 Conventionally Raised chicken).

The total number of transformants obtained in the 1 ml SOC recovered cell culture was 2.0×10^4 for FR1, 7.0×10^4 for FR2, 1.2×10^5 for CR1 and 8.0×10^4 for CR2. Given the average fragment size estimated to be 2,000 bp (Fig. 1B), the total sizes of each of the four metagenomic libraries ranged from 0.4×10^8 to 2.4×10^8 bp.

Screening AR genes from the metagenomic libraries

Colonies grew on ampicillin-containing agar plates for cultures from all four libraries (Table 4). In terms of tetracycline, only cultures from library CR2 contained tetracycline-resistant transformants. The chloramphenicol-resistant colonies were observed only from libraries CR1 and CR2 while spectinomycin-resistant colonies were observed only from libraries FR1 and CR2 (Table 4). No single resistant colony was selected on the plates supplemented with norfloxacin or ciprofloxacin from any of the four libraries (Table 4). The AR colonies were randomly picked for plasmid extraction (the number of colonies picked from each library for all six antibiotics is listed in Table 4), and the successful insertion of fragments was confirmed by gel electrophoresis as shown in FIG. 1C. Inserted fragments in plasmids from selected clones were then sequenced bidirectionally (number of clones subjected for sequencing is listed in Table 5).

ORF was identified from the inserted sequence and compared to the sequence available in public database in Genbank (http://www.ncbi.nlm.nih.gov/genbank/). A total of 15 AR genes were identified from the four libraries with 3 from two free-range chicken libraries (Table 6) and 12 from conventionally raised chicken libraries (Table 7). Among the 15

identified genes, 8 shared low sequence similarities (58% - 76% at amino acid level) with the corresponding AR genes previously identified using culture-dependent approaches (Table 6 & 7). Notably, among the 8 novel genes, 4 were unique in this study, which shared low sequence similarities (59% - 76% at amino acid level) with recently identified novel AR genes in human gut (Sommer, 2009) (Table 6 & 7).

Each gene identified in this study was named with a two letter code corresponding to the source (FR – Free-Range chickens and CR – Conventionally Raised chickens) followed by a three letter code referring to the three initial letter of the antibiotic used for screening (Amp-ampicillin, Spe-spectinomycin, Chl-chloramphenicol, Tet-tetracycline), and then the digit corresponding to the number of the library (1 or 2 for free range chickens and 1 or 2 for conventionally raised chickens), and a final digit used to distinguish each unique gene against the specific antibiotic and from the specific chicken.

FRAmp1.1 was distantly related with identified beta-lactamases.

Phylogenetic analysis of different beta-lactamases showed high divergence between amino acid sequences of beta-lactamase from different groups (FIG. 2). The beta-lactamases identified by metagenomic-based functional cloning from the gut microflora, including FRAmp1.1 identified in this study, showed high divergence and tended to branch off early from the ancestor, while the common beta-lactamase groups well-characterized by culture-dependent methodologies were grouped into a major cluster (FIG. 2). The pairwise scores for all sequences range from 1.0 to 100.0 (the higher pairwise score the closer

evolutionary relationship; lower pairwise score the more distant phylogenetic relationship). Specifically, FRAmp1.1 was distantly related to all the beta-lactamases identified from human gut microflora (Sommer *et al*. 2009) using functional metagenomics (pairwise scores ranging from 4.0 to 36.0) (FIG. 2). In addition, FRAmp1.1 was also distantly related with all the well-characterized beta-lactamases in cultivated microorganisms, regardless of their particular origin (pairwise scores ranging from 2.0 to 34.0) (FIG. 2). Notably, FRAmp1.1 was very distantly related with the beta-lactamases characterized in *Campylobacter* (pairwise scores equal to 2.0).

The identified novel AR genes confer increased resistance in *C. jejuni*.

The expression vector pZW that was functional in *C. jejuni* was constructed correctly, which was confirmed by PCR amplification of the inserted *flaA* promoter (Fig 3A). Plasmids containing control *KAN-2* gene and two novel AR genes (*FRAmp1.1* and *FRSpe1.1*) were also successfully constructed using pZW as a parent plasmid; the correct insertion of desired AR gene was also confirmed by PCR amplification (FIG. 3B). All the new plasmids were conjugatively transferred into *C. jejuni* 81-176, generating strains JL854, JL855, JL856, and JL857 (Table 1).

Antibiotic susceptibility test showed that JL857 (*C. jejuni* 81-176 with a *KAN-2* containing plasmid) displayed a dramatically enhanced resistance to kanamycin (MIC>128 μg/ml) compared to the control strain JL854 (MIC=8 μg/ml). This finding strongly indicated that the cloned *flaA* promoter in pZW was functional and the plasmid pZW was an effective

expression vector for *Campylobacter*. When the novel *FRAmp1.1* was inserted in the pZW and transferred to *C. jejuni* 81-187, the derivative JL855 displayed an enhanced resistance to ampicillin (MIC=8 μg/ml) compared to control strain JL854 (MIC=1 μg/ml). Similarly, JL856, the strain containing plasmid bearing *FRSpe1*.1 gene showed enhanced resistance to spectinomycin (MIC=32 μg/ml) compared to control strain JL854 (spectinomycin MIC= 8 μg/ml).

DISCUSSION

In this study, novel AR genes were identified from the chicken gut microflora using a culture-independent functional cloning approach. Similar to what was observed in the human gut microflora (Sommer, 2009), novel AR genes constitute a substantial proportion (8 of 15) of all AR genes identified in this study. This confirmed our hypothesis that the animal gut microflora contains a large and diverse AR gene reservoir. Previous conventional culture-dependent work may only reveal a small subset of AR genes, leaving the majority underexplored. Notably, this study was the first to demonstrate that the selected novel AR genes can be expressed in *C. jejuni*, consequently leading to enhanced antibiotic resistance in this significant foodborne human pathogen. This finding suggests that these novel AR genes, although highly divergent from previously identified AR genes using culture-dependent approaches, are indeed utilizable by important zoonotic pathogens and may pose a significant threat to public health.

The likelihood of identification of AR genes using the metagenomic-based functional cloning method is primarily affected by following two factors. First, the abundance of particular AR genes in the gut microflora will define their probability to be identified. The particular subsets of AR genes may be selected and enriched due to selective pressure through the usage of specific antibiotics (van den Bogaard 1997). In accord with this, in this study we identified more AR genes from conventionally raised chickens that were exposed to antibiotics than those from free-range chickens that were never treated with antibiotics. The number of AR genes identified in human gut microflora (Sommer *et al*. 2009) was also larger than what we identified in free range chicken gut microflora in this study, which could be due to the

selective effect of antibiotic usage in humans. Although the human subjects from whom feces were sampled (Sommer *et al*. ,2009) had not been treated by antimicrobial drugs in the past one year prior to sampling, the selected AR flora may be "resistant to elimination" (Salyers and Amabile-Cuevas 1997). As a result, it was questionable to which extent the reshaping affect of earlier antibiotic treatment on gut microflora could be eliminated in one year. Second, besides the abundance of AR genes in the reservoir, the size of the library is also a limiting factor of the likelihood of identifying AR genes. Each of our metagenomic libraries, which has a size about 10^8 bp covered only a small proportion of the metagenome of the gut microflora (Qin, Li *et al*. 2010). The difference in size of the libraries used in the human study (~10^9 bp per library) (Sommer *et al*. 2009) and in this study (~10^8 bp per library) may explain the difference between the number of AR genes identified from the two sources (90 for human, 12 for conventionally raised chickens).

The AR genes identified in this study encode beta-lactamases, aminoglycoside adenylyltransferases, ribosome protection proteins, and chloramphenicol acetyltransferases. Notably, among the 15 AR genes identified in this study, 4 genes are novel and displayed low similarity to AR genes discovered either using culture-dependent methodologies or using similar culture-independent approach for human gut microflora (Sommer *et al*. 2009). These four genes include one beta-lactamase, two spectinomycin adenylyltransferase and one chloramphenicol acetyltransferase. Specifically, a phylogenetic tree was constructed to analyze phylogenetic relationship of the novel beta-lactamase identified in this study (FRAmp1.1) to other diverse beta-lactamases identified to date. The FRAmp1.1 was distantly related with all the other beta-lactamases included in the analysis including isolates from human and animal

commensals (FIG. 2). Considering the fact that our library only covered a small proportion of the whole metagenome in chicken gut microflora, it is reasonable to speculate that more AR genes are harbored by the animal gut microflora that are not present in human gut microflora. These "heterologous" AR genes could pose a potential threat to public health because it is possible that, after transmission to zoonotic human pathogens, they can confer resistance against antimicrobial drugs commonly applied to human patients, thus compromising effectiveness of clinical antibiotic usage

Horizontal gene transfer (HGT) that facilitates the spreading of AR genes has three major mechanisms including transfection, transformation, and conjugation. Because HGT occurs more frequently in densely packed cells like commensal bacteria in the intestinal tract (Marshall 2009), novel AR genes harbored by animal gut microflora have the potential to be transmitted into zoonotic pathogens through HGT. As a result, it is of concern if these novel AR genes can function in zoonotic pathogens when HGT actually happens. In this study, we tested the functionality of two sequentially novel AR genes (FRAmp1.1 conferring ampicillin resistance and FRSpe1.1 conferring spectinomycin resistance) in the zoonotic human pathogen *C. jejuni*. Concerning sequential difference, the two selected genes shared low sequential similarity to the major determinants of ampicillin resistance (Cj0229) (Griggs, Peake *et al*. 2009) and spectinomycin resistance (AadE) (Pinto-Alphandary, Mabilat *et al*. 1990) in *Campylobacter*. The Cj0229 and all other beta-lactamases identified in *Campylobacter* are the most distantly related with FRAmp1.1 among all the beta-lactamases used for phylogenetic analysis (FIG 2). With respect to FRSpe1.1, this putative adenylyltransferase shared only 67% similarity at the amino acid level with O6-adenylyltransferase AadE. Despite their significant

sequence difference with the identified AR gene products in *Campylobacter*, both FRAmp1.1 and FRSpe1.1 could express in *Campylobacter* and confer increased resistance to ampicillin and spectinomycin, respectively. The successful function of both AR genes demonstrated in this study strongly supports the concept that, if successfully transferred into a zoonotic pathogen, the novel AR genes may be expressed and subsequently confer enhanced AR resistance in the pathogen. This may pose a significant challenge for antibiotic therapy and diagnosis of antibiotic resistance. Particularly, because the resistance phenotype and spectrum of the novel AR genes are still largely unknown, we should be aware of the possibility that antibiotics presently used may become ineffective when novel AR genes are acquired by pathogens.

Characterization of novel AR genes from animal microbiota is important in several ways. First, the sequentially divergent genes can help to assemble a more complete image of the evolutionary history of AR genes. Second, further functional and structural study of novel AR genes would improve our understanding of the relationship between sequence diversity and the spectrum and level of resistance (Wolter, Kurpiel *et al*. 2009). Third, since the selected novel AR genes can express and function in zoonotic pathogens, thorough examination of novel AR genes in food animals and even other agriculture systems (e.g. manure management) will greatly enhance the power of molecular diagnostic tools for AR in zoonotic pathogens (Shamputa, Rigouts *et al*. 2004) and provide insights into the development and transmission of AR. Finally, considering the fact that genes for antibiotic biosynthesis were sometimes clustered with AR genes, identification of novel AR genes may lead to discovery of potentially novel antibiotics (Riesenfeld, Goodman *et al*. 2004).

In this study, a culture-independent functional cloning approach was used to identify AR genes in the gut microflora of both free-range and conventionally raised chickens. Despite the significant findings from this study, following limitations should be addressed for future large-scale identification of AR genes using a similar approach. First, restricted by the efficiency of molecular cloning, the size of the libraries constructed in this study was not large enough to cover genetic diversity of the whole gut microbial community. Thus, improving cloning efficiency would greatly increase library size and provide a greater chance to identify more novel AR genes. For instance, Shrimp Alkaline Phosphatase (SAP) may be used as an alternative to Calf Intestinal Alkaline Phosphatase (CIAP) to dephosphorylate the cloning vector, since it can be easily inactivated and will less likely affect downstream cloning procedures. Second, samples from four chickens may be inadequate to infer correlations between the uses of antibiotics with the change of prevalence and abundance of AR genes. On one hand, the antibiotics used in poultry were not uniform (Gyles 2008). Results from a small subset of chickens are not suitable to be used to draw solid conclusions regarding the relationship between antibiotic usage and profiles of AR reservoir in the gut. On the other hand, the effects of antibiotics on microbial communities are complex. Besides direct selection of corresponding AR strains, antibiotics may also affect the microbiota indirectly, or even function as signaling molecules that further complicate the system (Yim, Wang *et al*. 2007). More chickens treated with different combinations of antibiotics need to be sampled to better elucidate the consequences of the application of particular antibiotics on the emergence of AR genes in the chicken gut microflora. Finally, as a pilot study, we only used chickens as a model to analyze gut AR reservoir in this work. As mentioned above, the functional cloning method can be extended into the investigation of AR gene reservoir in other habitats, including gut

microbiota in other food animals, microbiota in other parts of the animal body, and microbiota in other targeted ecosystems of agriculture, such as soil, water, lagoon, etc. Similar functional cloning approaches have been used to identify specific AR genes, and these studies further demonstrated the power of the methodology in revealing natural AR gene reservoir that may exhibit much higher level of diversity than previously expected (Riesenfeld, Goodman *et al*. 2004; Allen, Moe *et al*. 2009; Donato, Moe *et al*. 2010).

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APPENDIX

Table 1. Bacterial strains and plasmids used in this study.

Strain or Plasmid		Description	Source
E.coli	DH5α	*E.coli*, F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen
	TOP10	*E.coli*, F– *mcr*A Δ(*mrr*-*hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen
	JL519	DH5α containing pZE21-MCS	This study
	JL48	Conjugation helper strain, DH5α containing pRK2013	(Zeng, Xu *et al.* 2009)
	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	JL241	NCTC 11168, human isolate	(Parkhill, Wren *et al.* 2000)
	JL242	*C. jejuni* 81-176, human isolate	(Black *et al.* 1988)
	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	Lutz 1997
	pRY111	*E. coli*-*C. jejuni* shuttle vector, Cm^r	(Yao, Alm *et al.* 1993)
	pRK2013	a helper plasmid for triparental mating	(Miller, Bates *et al.* 2000)
	pZE21-MCS-*FRAmp1.1*	pZE21-MCS with metagenomic fragment containing *FRAmp1.1*	This study
	pZE21-MCS-*FRSpe1.1*	pZE21-MCS with metagenomic fragment containing *FRSpe1.1*	This study
	pZW	pRY111 containing *flaA* promoter	This study
	pZW1	pZW containing ampicillin resistant gene *FRAmp1.1*	This study
	pZW2	pZW containing spectinomycin resistant gene *FRSpe1.1*	This study
	pZW3	pZW containing kanamycin resistant gene *KAN-2*	This study

Table 2. PCR primers used in this study.

Name	Sequence
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
CAm-EcoRIR	C-3’(BamHI)
	5’-CGG AAT TCC TCC TTA ACT CCT AAA ATT TAA CTT C-3’(EcoRI)
CSp-BamHIF	5’-CGG GAT CCC CCG AAT GTG AAT ATA TGT AC-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)

*: Restriction sites are underlined in the primer sequences and the corresponding names are in parentheses.

Table 3. Antibiotics used for functional screening.

Antibiotic	Concentration (μg/ml)
Ampicillin	50
Tetracycline	10
Chloramphenicol	25
Spectinomycin	75
Norfloxacin	12.5
Ciprofloxacin	10

Table 4. Number of colonies selected from various metagenomic libraries for plasmid extraction.

Antibiotic	Library[a]	Number of colonies selected for plasmid extraction
Ampicillin	FR1	18
	FR2	12
	CR1	10
	CR2	9
Tetracycline	FR1	0
	FR2	0
	CR1	0
	CR2	14
Chloramphenicol	FR1	0
	FR2	0
	CR1	2
	CR2	4
Spectinomycin	FR1	7
	FR2	0
Spectinomycin	CR1	0
	CR2	12

[a] FR1: free-range chicken #1; FR2: free-range chicken #2; CR1: conventionally raised chicken #1; CR2: conventionally raised chicken #2.

Table 5. Number of clones in which plasmids were subjected to sequencing.

Antibiotic	Library	Number of clones selected for sequencing analysis
Ampicillin	FR1	12
	FR2	6
	CR1	7
	CR2	6
Tetracycline	FR1	0
	FR2	0
	CR1	0
	CR2	7
Chloramphenicol	FR1	0
	FR2	0
	CR1	2
	CR2	2
Spectinomycin	FR1	3
	FR2	0
Spectinomycin	CR1	0
	CR2	5

Table 6. Antibiotic resistance genes identified in the cecal contents of free-range chickens.

Antibiotic	Gene name	Chicken (FR)	Length (bp)	Annotation:	% aa similarity with the genes from cultivated organisims*	% aa similarity with genes from metagenomic study**
Ampicillin	FRAMP1.1	1	902	Beta-lactamase	59	
	FRAMP2.1	2	861	Ampicillin resistant gene	100	-
Tetracycline	-	-	-		-	-
Chloramphenicol	-	-	-		-	-
Spectinomycin	FRSPE1.1	1	459	Adenylyltransferase	67	-
Norfloxacin	-	-	-		-	-
Ciprofloxacin	-	-	-		-	-

-: No genes identified or parameter not available.

*: similarity comparing with the AR genes discovered in studies using culture-dependent approach.

**: similarity comparing with the AR genes discovered by Sommer *et al.* (2009) using the same culture-independent, functional cloning approach.

Table 7. Antibiotic resistance genes identified in the cecal contents of conventionally raised chickens.

Antibiotic	Gene name	Chicken (CR)	Length (bp)	Annotation :	% aa similarity with the genes from cultivated organisms*	% aa similarity with genes from metagenomic study**
Ampicillin	CRAMP1.1	1	345	TEM	98	
	CRAMP1.3	1	525	TEM	91	-
	CRAMP2.1	2	513	CbIA	58	100
	CRAMP2.2	2	642	HGF-1	63	99
	CRAMP2.3	2	510	CbIA	67	98
	CRAMP1.4	1	861	TEM	100	-
	CRAMP1.5	1	423	Drug efflux transporter	77	97
Tetracycline	CRTET2.1	2	936	Tetw	99	-
	CRTET2.2	2	660	Tetw	99	-
Chloramphenicol	CRCHL2.1	1	804	CAT	92	-
	CRCHL2.2	1	639	CAT	76	-
Spectinomycin	CRSPE2.1	2	774	Adenylyl transferase	99	-
Norfloxacin	-	-	-	-	-	-
Ciprofloxacin	-	-	-	-	-	-

-: No genes identified or parameter not available.

*: similarity comparing with the AR genes discovered in studies using culture-dependent methodology.

**: similarity comparing with the AR genes discovered by Sommer *et al.* (2009) using the same culture-independent, functional cloning approach.

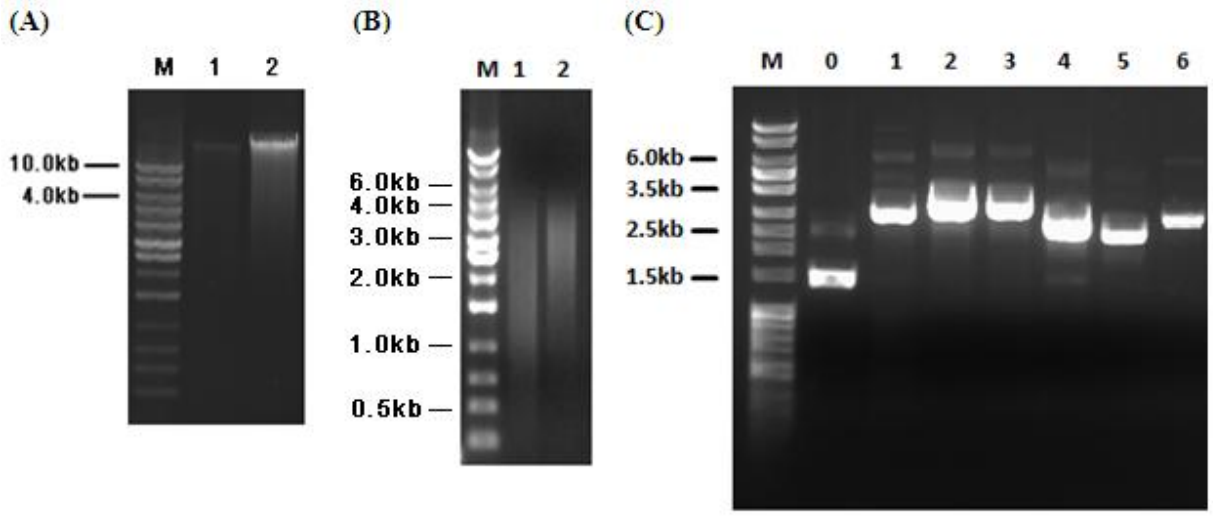

FIG. 1. Extraction, shearing and cloning metagenomic DNA into the expression vector pZE21-MCS. (A) Extraction of total genomic DNAs from chicken fecal contents. Lane M, 1kb DNA ladder (Promega); Lane 1, 5 µl of extracted DNA from ileal content of a free range chicken; Lane 2, 5 µl of extracted DNA from cecal content of a free range chicken B) Shearing of the extracted genomic DNA from cecal contents. Lane M, 1kb DNA ladder (Promega); Lane 1 and 2, 7 µl of sheared genomic DNAs of cecal samples from two different conventionally raised chickens; (C) Cloning of sheared metagenomic DNA fragments into pZE21-MCS. Lane M, 1kb DNA ladder (Promega); Lane 0, 5 µl of pZE21-MCS; Lane 1-6, 5 µl of plasmids extracted from different transformants randomly selected from one library.

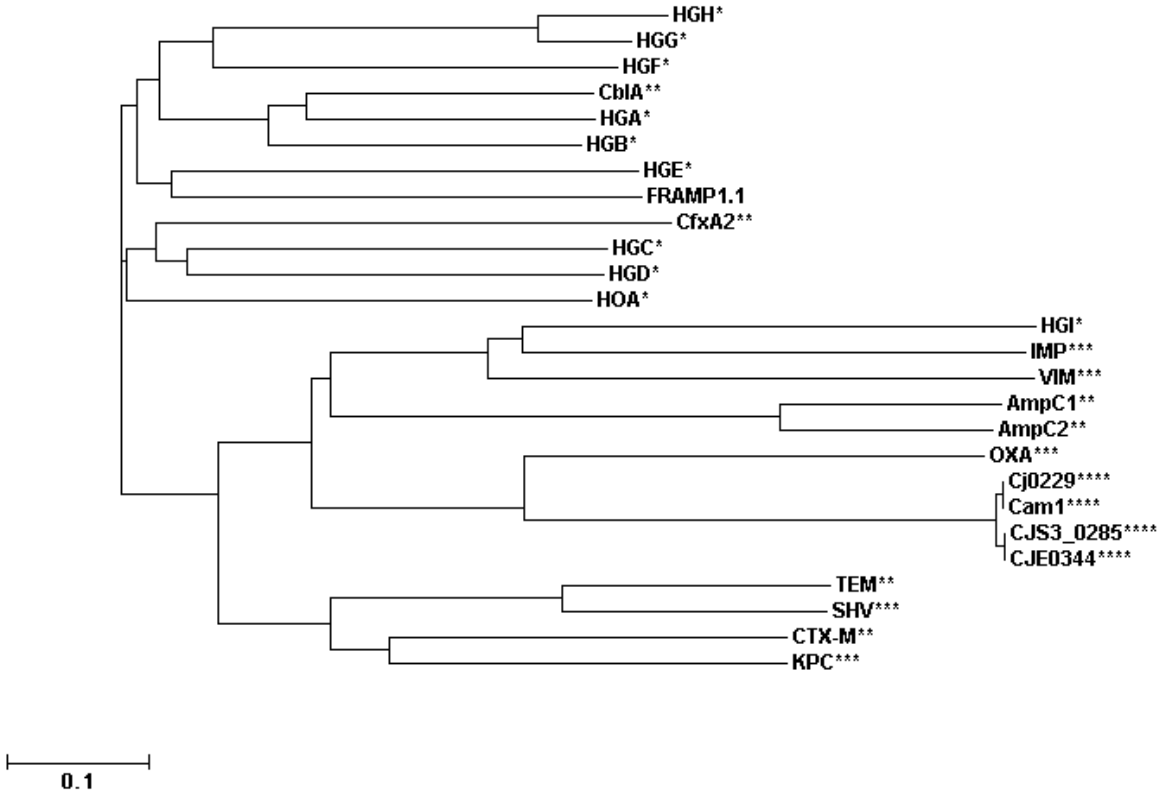

FIG. 2. Phylogenetic relationship of beta-lactamases from different sources. Unrooted phylogenetic trees were generated from ClustalW2 alignments using the neighbor-joining algorithm. FRAmp1.1 discovered in this study was analyzed together with 25 identified beta-lactamases. *: the beta-lactamases representing the novel beta-lactamase families (HOA and HGA, HGB, HGC, HGD, HGE, HGF, HGG, HGH, HGI) discovered by Sommer *et al.* using culture-independent approach (2009); **: the beta-lactamases discovered by Sommer *et al.* (2009) representing previously characterized beta-lactamase families (TEM, CfxA, CblA, CTX-M, two for AmpC); ***: the beta-lactamases representing previously characterized common beta-lactamase families not analyzed by Sommer *et al* (2009) [SHV (Veras, Alves *et al*. 2011), OXA-10 (Huang), IMP (Osano, Arakawa *et al.* 1994), VIM (Lauretti, Riccio *et al.* 1999), KPC (Yigit, Queenan *et al.* 2001)]; ****: the beta-lactamases identified in different *Campylobacter jejuni* strains, which include Cj0299 (Accession # YP_002343737), CJE0344 (Accession# AAW34933), CJS3_0285 (Accession # ADT72049), and Cam1 (Accession #AAT01092).

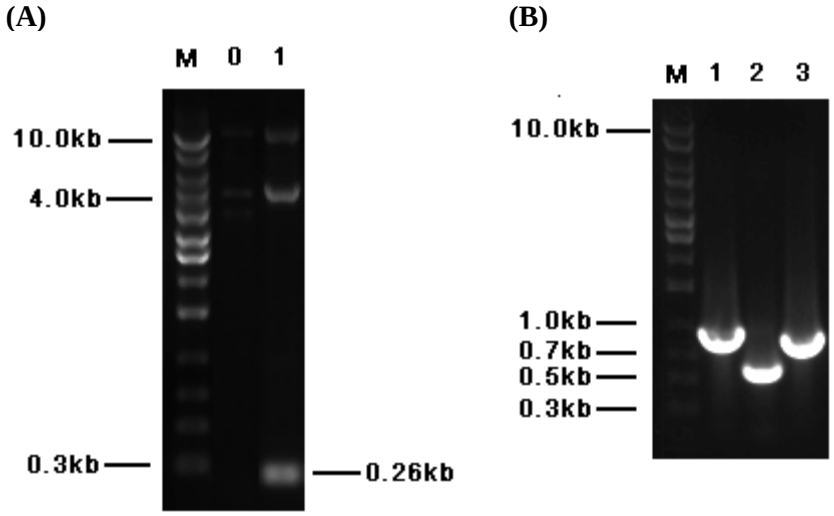

FIG. 3. PCR confirmation of the recombinant plasmids pZW, pZW1, pZW2 and pZW3. (A) PCR amplification of the inserted 0.26 kb *flaA* gene promoter from pZW. Lane M, 5 µl 1kb DNA ladder; Lane 0, 25 µl PCR negative control using pRY111 as the PCR template and sig28-F and sig28-R as primers; Lane 1, 25 µl PCR products amplified from pZW using primers sig28-F and sig28-R; (B) PCR amplification of inserted AR genes from pZW1, pZW2 and pZW3. Lane M, 5 µl 1kb DNA ladder; Lane 1, 25 µl PCR products amplified from pZW1 using primers of CAm-BamHIF and CAm-EcoRIR; Lane 2, 25 µl PCR products amplified from pZW2 using CSp-BamHIF and CSp-EcoRIR; Lane 3, 25 µl PCR products amplified from pZW3 using CKa-F and CKa-R.

Vita

Wei Zhou was born in Shanghai, P.R. China, to the parents of Naixin Zhou and Yanfang Wo. He attended Waigaoqiao Experimental Elementry School before accepted by Shanghai Foreign Language School where he continued as middle school and high school student. After graduation, He was enrolled in Fudan University where he finished his undergraduate study in Biological Sciences, and got the Bachelors of Science degree in July 2009. He then attended The University of Tennessee, Knoxville as a master student, in the Department of Animal Science, under the mentorship of Dr. Jun Lin.