

Fate and Transport of Antibiotic Residues and Antibiotic Resistance Genes following Land Application of Manure Waste

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Antibiotics are used in animal livestock production for therapeutic treatment of disease and at subtherapeutic levels for growth promotion and improvement of feed efficiency. It is estimated that approximately 75% of antibiotics are not absorbed by animals and are excreted in waste. Antibiotic resistance selection occurs among gastrointestinal bacteria, which are also excreted in manure and stored in waste holding systems. Land application of animal waste is a common disposal method used in the United States and is a means for environmental entry of both antibiotics and genetic resistance determinants. Concerns for bacterial resistance gene selection and dissemination of resistance genes have prompted interest about the concentrations and biological activity of drug residues and break-down metabolites, and their fate and transport. Fecal bacteria can survive for weeks to months in the environment, depending on species and temperature, however, genetic elements can persist regardless of cell viability. Phylogenetic analyses indicate antibiotic resistance genes have evolved, although some genes have been maintained in bacteria before the modern antibiotic era. Quantitative measurements of drug residues and levels of resistance genes are needed, in addition to understanding the environmental mechanisms of genetic selection, gene acquisition, and the spatiotemporal dynamics of these resistance genes and their bacterial hosts. This review article discusses an accumulation of findings that address aspects of the fate, transport, and persistence of antibiotics and antibiotic resistance genes in natural environments, with emphasis on mechanisms pertaining to soil environments following land application of animal waste effluent.

ANTIBIOTICS are routinely used in the livestock industry to treat and prevent disease. In addition, subtherapeutic concentrations of antimicrobials are commonly added to animal feed and/or drinking water sources as growth promoters, and have been a regular part of swine (*Sus scrofa*) production since the early 1950s (Cromwell, 2001). When used in this manner, antibiotics can select for resistant bacteria in the gastrointestinal tract of production animals, providing a potential reservoir for dissemination of drug resistant bacteria into other animals, humans, and the environment (Andremont, 2003). Bacteria have been shown to readily exchange genetic information in nature, permitting the transfer of different resistance mechanisms already present in the environment from one bacterium to another (Salyers and Amábile-Cuevas, 1997; Amábile-Cuevas and Chicurel, 1992; Stewart, 1989). Transfer of resistance genes from fecal organisms to indigenous soil and water bacteria may occur (Nielsen et al., 2000; Daane et al., 1996; DiGiovanni et al., 1996; Lorenz and Wackernagel, 1994), and because native populations are generally better adapted for survival in aquatic or terrestrial ecosystems, persistence of resistance traits may be likely in natural environments once they are acquired. Antibiotic resistance has received considerable attention due to the problem of emergence and rapid expansion of antibiotic resistant pathogenic bacteria.

The potential for long-term, cumulative inputs of antibiotics and correspondingly, their potential effects on acquisition and maintenance of antibiotic resistance mechanisms in bacteria, collectively suggest a degree of impact on the occurrence, persistence, and mobility

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Abbreviations: CAFOs, concentrated animal feeding operations; MICs, minimum inhibitory concentrations; OTC, oxytetracycline; PCR, polymerase chain reaction; RPPs, ribosomal protection proteins; SCP, sulfachlorpyridazine; UCS, Union of Concerned Scientists.

of resistance genes in natural environments. A number of reviews, reports, and opinion papers have emerged to address the possible link between antibiotic use and the impact on antibiotic resistance development (e.g., Kümmerer, 2004; Shea, 2004; Isaacson and Torrence, 2002; Séveno et al., 2002; USGAO, 1999; Khachatourians, 1998; Gustafson and Bowen, 1997). These papers have highlighted various issues related to antibiotic use in agriculture, often focusing on the link to emerging antibiotic resistant bacteria, gene transfer mechanisms, and consequent risks to human and animal health.

In the following review, we seek to provide a comprehensive overview of the dissemination and fate of antibiotic residues, and the environmental persistence, mobility, and transferability of antibiotic resistance determinants and their bacterial hosts following the practice of land application of livestock waste (Fig. 1). The significance of these issues pertains to continuing efforts in determining the true ecological impact of antibiotics and antibiotic resistance genes on entry into natural environments.

Antibiotic Use in Animal Agriculture

In commercial livestock production, antibiotics are used: (i) therapeutically to treat existing disease conditions, (ii) prophylactically at subtherapeutic doses to mitigate infection by bacterial pathogens of livestock animals undergoing high stress situations, and (iii) subtherapeutically to enhance growth. A survey of members of the Animal Health Institute reported that overall, the ionophores/arsenicals and tetracycline classes of antibiotics were the most commonly used antimicrobials in animal production (Table 1; AHI, 2001). Among the antibiotics commonly used in swine, poultry, and beef cattle (*Bos taurus*), penicillins, macrolides, polypeptides, streptogramins, and tetracyclines are used not only for purposes of disease treatment and disease prevention, but for growth promotion too (Table 2). Other classes, such as quinolones, lincosamides, and aminoglycosides are primarily used only in disease treatment or prevention.

The Animal Health Institute (AHI, 2001) and Union of Concerned Scientists (UCS) (Union of Concerned Scientists, 2001) recently reported two different estimates of antibiotic usage in agriculture. The AHI reported a total of 20.5 million pounds of antibiotics sold for all animal use in 1999. Of the 20.5 million pounds, 17.7 million pounds were used for treatment and prevention of disease and only 2.8 million pounds were used for improving feed efficiency and enhancing growth. In contrast, the UCS reported 24.6 million pounds of antibiotics were used for nontherapeutic purposes alone in the swine, poultry, and cattle industries. According to the UCS report, livestock use accounts for the major share of total antimicrobials used in the United States, estimated at 50 million pounds annually, based on extrapolation from a 1989 Institute of Medicine report (Institute of Medicine, 1989). Despite the discrepancy over usage, it is clear that the amount of antibiotics used in agriculture is large.

Management of Animal Waste from Production Agriculture

Historically, until the mid- to late 1970s, livestock operations were usually part of larger integrated farming operations

that produced crops. Manure management practices differ depending on number of head, the type of livestock and operation, and production stage of the animals. The direction toward large operations using total animal confinement facilities has led to major issues of waste storage and disposal in livestock (poultry, beef, and swine) production. Swine production, in particular, has seen a trend toward specialized large production facilities (e.g., farrow to weaning, farrow to feeder, nursery, finishing, farrow to finish). Over the last 25 yr swine production has largely shifted from such integrated farming systems to concentrated animal feeding operations (CAFOs) that may house thousands of animals. In 1984, there were approximately 690,000 U.S. producers producing 20 billion pounds of pork. By 2000, about 95,000 producers were producing 26 billion pounds of pork (USDA NASS, 2002). Due to geographic patterns of feed grain production and other market forces, swine CAFOs have become concentrated in certain geographic regions in the United States, primarily North Carolina and several Midwest states, with Iowa, Minnesota, and Illinois among the largest producers. United States Department of Agriculture surveys performed in 2000 found that 28.3% of swine facilities were located within a half mile of another swine production site and 53.9% were within one mile of another site (USDA, 2001a, 2001b). While the following review derives much of the information from the large number of studies with swine waste, antibiotics are administered in all the major animal production industries (Table 1, Table 2).

Under the earlier integrated system of production, producers typically owned large tracts of land necessary for agronomic activity. Waste and effluent from a modest number of animals was applied rotationally over different fields, effectively diluting nutrients and recycling waste for fertilizer use. Swine each typically produce approximately 1.5 tonnes of fresh manure in the 5 to 6 mo it takes to grow them to a market weight of 114 kg (ca. 250 lbs) (Richert et al., 1995). The National Agricultural Statistics Service (NASS) estimated that in 2002, 185 million head of swine were sold in the United States, generating approximately 2.8×10^8 tonnes of fresh manure annually. Chicken (*Gallus gallus*) production in the United States in 2006 was estimated at nearly 9 billion head, generating approximately 4.6×10^8 tonnes of manure. Beef cattle estimates in the United States in 2007 were 33.3 million head (Nebraska Beef Council, 2007), producing approximately 3.6×10^6 tonnes of manure (USDA-NASS, 2002; USDA-NRCS, 1995). With the advent of CAFOs, large quantities of waste are concentrated in a single location and/or region, and producers may not own or access sufficient tracts of land suitable for disposal of manure through land application.

Methods of waste storage vary among operations, but particular to the confined operations of the swine industry, these usually follow one of three primary types: (i) a slatted floor over a deep concrete pit, (ii) a slatted floor over a shallow pit with outdoor areas for slurry storage, and (iii) a slatted floor over a shallow pit with outdoor lagoon treatment. Additional land is often required to house secondary waste storage systems. In lagoon systems, manure solids are partially degraded and organic N is converted to inorganic forms and released from the lagoon pri-

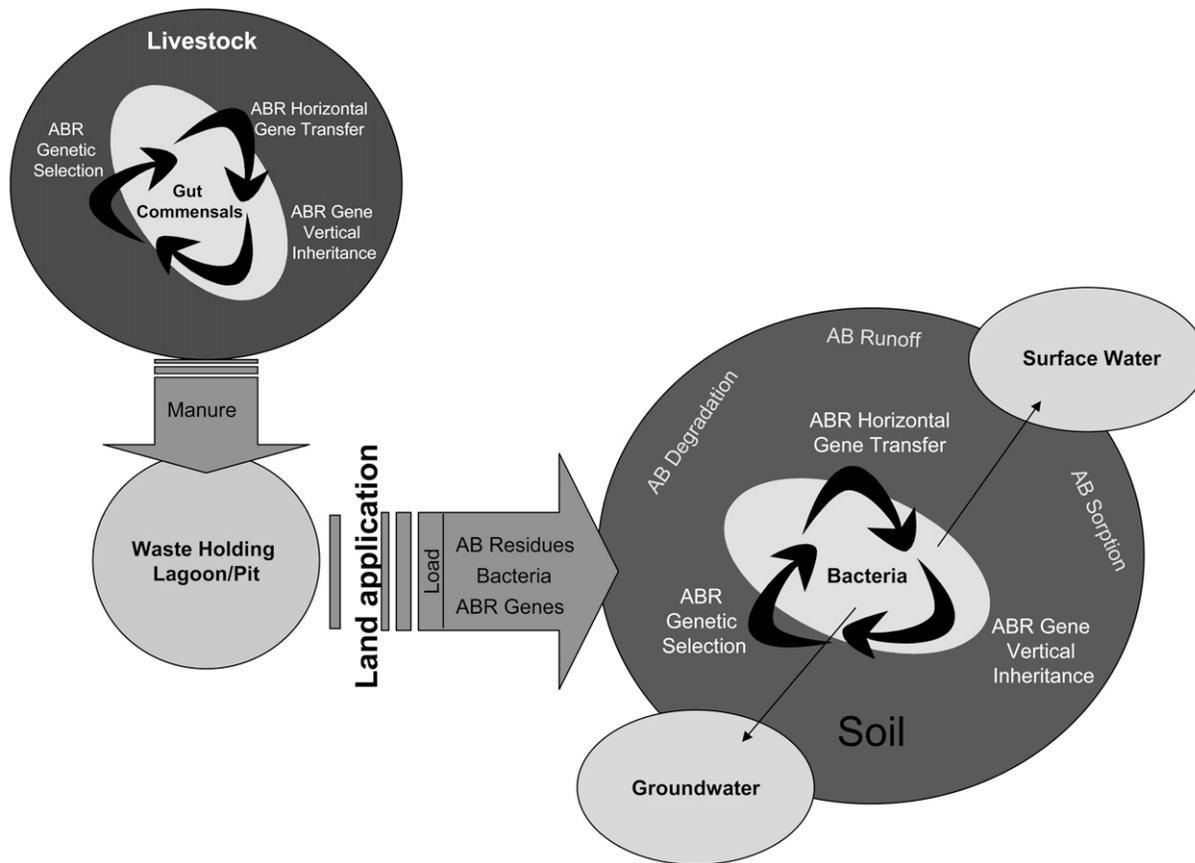


Fig. 1. Conceptualized view showing the possible fates of antibiotic residues and mechanisms of antibiotic resistance gene acquisition and dissemination by bacteria, beginning with land application of animal waste as the source of entry of drugs, bacteria, and resistance genes into the soil environment. AB = antibiotic, ABR = antibiotic resistance.

Table 1. Survey of the most commonly used antibiotics in animal production (AHI, 2001).

| Antibiotic class | Amount |
|---|---------------|
| | metric tonnes |
| Ionophores/Arsenicals | 3520 |
| Tetracyclines | 3239 |
| Other antibiotics-includes macrolides, lincosamides, polypeptides, streptogramins, cephalosporins | 1937 |
| Penicillins | 821 |
| Sulfonamides | 269 |
| Aminoglycosides | 117 |
| Fluoroquinolones | 16 |

marily through ammonia volatilization and as N_2 or N_2O gases (Harper et al., 2004; Rotz, 2004). The loss of N and the sequestering of much of the P in wastes in lagoon sludge can reduce the amount of land required for waste disposal to meet agronomic guidelines for best management practices (Beegle, 1997).

The most common method to dispose of swine and feedlot cattle waste effluent in the United States following lagoon or pit storage is through land application, where application of liquid manure at agronomic rates can produce crop yields that equal those obtained with chemical fertilizers (Schmitt et al., 1995; Sarmah et al., 2006). To use and dispose of the manure effluent, CAFO operators often contract with neighboring growers to apply effluent to their land or apply it to land surrounding the

Table 2. Antibiotics commonly used in swine, poultry, and beef cattle production industries (USGAO, 1999; USDA, 2007).

| Antibiotic class | Industry |
|-----------------------------------|-----------------------------|
| Aminoglycosides | Swine, poultry, beef cattle |
| β -Lactams | Swine, poultry, beef cattle |
| Chloramphenicol | Beef cattle |
| Ionophores | Poultry, beef cattle |
| Lincosamides | Swine, poultry |
| Macrolides | Swine, poultry, beef cattle |
| Polypeptides | Swine, poultry |
| Quinolones (and Fluoroquinolones) | Poultry, beef cattle |
| Streptogramins | Swine, poultry, beef cattle |
| Sulfonamides | Swine, poultry, beef cattle |
| Tetracyclines | Swine, poultry, beef cattle |
| Others: | |
| Glycolipids (Bambermycin) | Swine, poultry, beef cattle |
| Carbadox | Swine |
| Aminocoumarins (Novobiocin) | Poultry |
| Aminocyclitols (Spectinomycin) | Swine, poultry |

facility. Because it is costly to transport liquid effluent any great distance, there is an incentive to apply effluent as close to the source as possible. In the United States the crop cycles coincide with seasonal cycles, with the application of manure occurring between crop cycles. For many locations, manure is stored for 6 mo to 1 yr before being applied to crop fields as fertilizer. Effluent differs from fresh manure in that it has a much greater

water volume. Fresh swine waste contains approximately 10% solids, while deep pit and lagoon effluents are 4 to 8% solids and <0.5 to 1%, respectively (Fulhage and Post, 2005). O'Dell et al. (1995) found the solids content ranged from 4 to 10 g/L in 18 separate tank loads of swine effluent that had been agitated for 24 h before application, suggesting effluent application rates can be highly variable. The practice of stockpiling fresh manure and applying directly to fields is also used in the beef cattle industry, however, little is known about the effects on the nutrient properties (Dolliver and Gupta, 2008; Larney et al., 2006). Poultry waste management differs somewhat from swine and cattle in that poultry litter is a dry mixture of excrement, bedding material, and feed, and the composition and disposal largely depends on the type of bird produced. Pit storage is often used in production of layer hens and for all types of poultry, direct land application of litter is the primary method of disposal, with a small percentage using composting (MacDonald, 2008).

It is clear that the amounts of manure generated by commercial livestock is high, and while the types of antibiotics in use may differ between industries, similar issues are raised concerning environmental exposure to animal waste that may relate broadly across the entire animal production industry. Confinement livestock production, especially large animal facilities, is increasingly a source of surface- and groundwater contamination, and elevated levels of antibiotic resistance in humans and animals have been linked to the practice of antimicrobial growth promotant use at poultry and swine farms (Gilchrist et al., 2007). The widespread practice of land application prompted the Environmental Protection Agency (EPA) in the 1990s to require nutrient management plans for CAFOs. Initially, nutrient management plans were N-based, requiring manure to be applied at a rate that would not exceed crop N requirements. Swine manure, however, has a high P content relative to N content; as excreted, swine manure contains a P_2O_5/N ratio of approximately 0.86:1 (Livestock and Poultry Environmental Stewardship, 2005). Applying effluent to meet the N requirements of a crop often leads to a buildup of P in the soil, in some instances to values in excess of 2000 mg/kg of total soil P (Lehmann et al., 2005).

The three primary methods used to apply effluent include: (i) surface application, (ii) surface application followed by incorporation, and (iii) direct soil injection. One primary reason to incorporate surface-applied effluent is to limit the loss of N by at least 50% compared to surface application alone (Rotz, 2004). Other reasons include odor reduction and minimization of surface runoff. The preferred method of application from a nutrient management standpoint is deep injection into the soil, which eliminates the N loss associated with other methods, reduces odor, and virtually eliminates the possibility of surface runoff. Due to cost or soil conditions, direct injection or incorporation of waste may not always be feasible options.

Because surface application has been associated with N loss, it is often considered “environmentally unfriendly,” yet it has merits as a method of managing pathogen loads. Hutchison et al. (2004) reported that the mean D value, or time needed to reduce the variable being measured by one order of magnitude, for four zoonotic pathogens, *Salmonella* sp., *Escherichia coli* 0157, *Listeria* sp., and

Campylobacter sp., was 1.42 d for unincorporated pig slurry and 2.48 d for slurry incorporated immediately after application. These pathogens also declined at similar rates regardless of season (summer vs. winter). Dessication may be an important factor in population decline because more intense UV radiation in the summer would be expected to accelerate cell mortality (Hoerter et al., 2005; Booth et al., 2001). A significant rainfall event immediately following surface application of effluent would likely result in vertical movement of bacteria and mobile compounds into the soil profile as well as off-site movement due to surface runoff (Saini et al., 2003). Surface applications to frozen soil are usually avoided because of the likelihood of significant runoff.

Entry of Antibiotics into the Environment

Antibiotics used in animal agriculture can enter the environment via a number of routes, including the drug manufacturing process, disposal of unused drugs and containers, and through the use and application of waste material containing the drugs (Buchberger, 2007; Utah Department of Health, 2007; Daughton, 2004). The excretion of waste products by grazing animals, atmospheric dispersal of feed and manure dust containing antibiotics, and the incidental release of products from spills or discharges are also potential pathways of antibiotic residue entry into the environment. Animal agriculture is only one potential source of entry of drug residues in the environment, and good estimates of the quantities contributed by various sources is not available.

Many antibiotics are not completely absorbed in the gut, resulting in the excretion of the parent compound and its breakdown metabolites (Boxall et al., 2004; Halling-Sørensen et al., 1998; Feinman and Matheson, 1978). Elmund et al. (1971) estimated that as much as 75% of the antibiotics administered to feedlot animals could be excreted into the environment. Feinman and Matheson (1978) suggested that about 25% of the oral dose of tetracycline is excreted in feces and another 50 to 60% is excreted unchanged or as an active metabolite in urine. Oral administration of the macrolide tylosin resulted in a maximum of 67% of the antibiotic excreted, mainly in the feces.

The practice of land application of livestock manure provides large area scale for introduction of antibiotics into the environment. Once released into the environment, antibiotics can be transported either in a dissolved phase or (ad)sorbed to colloids or soil particles into surface- and groundwater (Krapac et al., 2004; Yang and Carlson, 2003; Campagnolo et al., 2002; Kolpin et al., 2002). Manure and waste slurries potentially contain significant amounts of antibiotics and their presence can persist in soil after land application (Gavalchin and Katz, 1994; Donohoe, 1984).

Chemical Characteristics of Antibiotics and Behavior in Soil and Water

Veterinary antibiotics comprise a group of organic compounds that have a wide variety of functional groups that affect their chemical properties. The octanol-water partition coefficient (K_{ow}) is used as a general measure of hydrophobicity, and most antibiotics have $\log K_{ow}$ values <5 indicating that they are

Table 3. Chemical properties and fate of selected veterinary antibiotics (modified from Beausse, 2004; Boxall et al., 2004; Tolls, 2001).

| Antibiotic | Solubility in water | Log K_{ow} | Log K_{oc} | K_d | pK_a and chemical degradation | Mobility |
|---------------------------------|---------------------|--------------|--------------|-----------|---|--|
| | g/L | | | L/kg | | |
| Lincomycin (hydrochloride salt) | freely | ND† | ND | ND | pK_a 7.6 In spiked soil 10 mg/kg undetectable after 11 wk and 80% lost after 7 wk | Immobile especially in high organic matter/clay soil based on manufacturer column tests. |
| Sulfathiazole | 0.6 | 0.05 | 2.30 | 4.9 | pK_{a1} 2, pK_{a2} 7.24 | Medium mobility based on K_d |
| Sulfamethazine | 1.5 | 0.89 | 1.78–2.32 | 0.6–3.1 | pK_{a1} 2.65, pK_{a2} 7.65 Biodegradable but persistent in water phase | High to medium based on K_d |
| Tylosin | 5 | 3.5 | 2.74–3.90 | 8.3–240 | pK_a 7.1 Stable at pH 4 to 9, < pH 4 desmycosin is formed. | Low to immobile based on K_d |
| Virginiamycin | 0.054–0.080 | 1.5–1.7 | 2.7–2.8 | ND | $T_{1/2}$: 87–173 d 89% inactivated within 18 d and undetectable after 84 d. Activity decreases rapidly in water and increasing temperature. Degrades under alkaline pH. | Immobile due to low water solubility, high lipophilicity and rapid inactivation in soil. |
| Tetracycline | 1.7 | -1.19 | ND | >400–1620 | pK_{a1} -3.30, pK_{a2} -7.68, pK_{a3} -9.69 | Immobile based on K_d |
| Chlortetracycline | 0.6 | -0.62 | ND | 282–2608 | $T_{1/2}$ in manure 1 wk at 37°C & > 20 d at 4° or 28°C 85% of CTC added to soil was recovered. | Immobile based on K_d |
| Oxytetracycline | 1 | -1.22 | 1.2–5.0 | 0.3–1030 | pK_{a1} 3.27, pK_{a2} 7.32, pK_{a3} 9.11 Stable compared to CTC | Immobile based on K_d |
| Ciprofloxacin | 30 | 0.4 | 4.78 | 430 | pK_{a1} 5.9, pK_{a2} 8.89 | Immobile based on K_d |
| Enrofloxacin | 130 | 1.1 | 4.22–5.89 | 260–6310 | pK_{a1} 6.27, pK_{a2} 8.3 | Immobile based on K_d |
| Penicillin | 4 | 1.87 | ND | ND | pK_a 2.79 Unstable, rapidly degrades to penicilloic acid. $T_{1/2}$ < 7 d | Weakly sorbed to soils |

† ND = not determined or not found in the literature reviewed.

relatively nonhydrophobic (Tolls, 2001). Additionally, the water solubility for many antibiotics exceeds 1 g/L suggesting that they are relatively hydrophilic (Table 3). Tolls (2001) and Boxall et al. (2004) compiled sorption coefficients (K_d) for a variety of antibiotics, soils, and soil components measured over the course of many studies. Based on K_d values, antibiotics exhibit a range of affinities for the solid phase (K_d 0.2–6000 L/kg) with consequent effects on their mobility in the environment. Estimations of antibiotic organic carbon-normalized sorption coefficients (K_{oc}) made by using a compound's octanol-water partition coefficient (K_{ow}) generally results in underestimates of the K_{oc} value, suggesting that mechanisms other than hydrophobic partitioning occur. Cation exchange, surface complexation, and hydrogen bonding are included as likely mechanisms for antibiotic sorption to soils. Many of the acid dissociation constants (pK_a) for antibiotics are in the range of soil pH values, such that the protonation state of these compounds depends on the pH of the soil solution (Tolls, 2001).

Studies have shown that under a broad range of environmental conditions, tetracyclines (tetracycline, chlortetracycline, and oxytetracycline) can adsorb strongly to clays (Allaire et al., 2006; Sithole and Guy, 1987a, 1987b; Pinck et al., 1961a, 1961b), soil (Krapac et al., 2004), and sediments (Rabolle and Spliid, 2000). Sorption of chlortetracycline also occurred rapidly in sandy loam soil (Allaire et al., 2006). Macrolides such as tylosin have a weaker tendency to sorb to soil materials (Rabolle and Spliid 2000), however a sorption kinetic

study showed 95% of tylosin was sorbed within 3 h in both sandy loam and clay soils (Allaire et al., 2006). Sulfonamides exhibit weak sorption to soil, and likely are the most mobile of the antibiotics (Tolls, 2001). Pinck et al. (1962) determined that two macrolide antibiotics (carbomycin and erythromycin) sorbed significantly (231–263 mg/g) to montmorillonite and to a much lesser extent (0–39 mg/g) to vermiculite, illite, and kaolinite. In a literature review on the fate of antibiotics in the environment Huang et al. (2001) concluded that there was little information on the sorption of aminoglycoside and β -lactam antibiotics. Because aminoglycosides can be protonated under acidic conditions, they could be sorbed to clay minerals under certain conditions, while β -lactams are highly polar compounds and would not be expected to sorb readily to soil components. Because of the strong sorption of the tetracycline and macrolide antibiotics, their mobility in the environment may be facilitated by transport with manure and soil colloidal material (Kolz et al., 2005a). Interestingly, although most antibiotics do not require metal ion coordination to exert biological action, other compounds like bacitracin, streptonigrin, bleomycin, and tetracycline have prerequisites for binding of metals ions to function properly (Ming, 2003). Sorption of these drug compounds in clays, where intercalation of metal complexes occur, may provide suitable conditions for the drug to exert a biological effect.

Mechanisms of Antibiotic Degradation

Because antibiotics are generally introduced from livestock operations via water (effluent) into the environment, hydrolysis can be an important degradation pathway. Beta-lactams, macrolides, and sulfonamides appear to be the most susceptible classes of antibiotics to hydrolysis (Huang et al., 2001). At near neutral pH, tylosin A was found to have a hydrolysis half-life of 300 to 500 h at 60°C (Paesen et al., 1995). At more environmentally relevant temperatures, these half-lives are expected to be longer. Doi and Stoskopf (2000) determined that under relatively high temperatures (43°C) the half-life of oxytetracycline in deionized water was 0.26 d, but was relatively stable at 4°C. Beta-lactams are rapidly hydrolyzed under mild acidic and basic conditions (Huang et al., 2001; Hou and Poole, 1969).

Photolysis can be another abiotic transformation process affecting antibiotics introduced into the environment. The photodegradation of antibiotics in soil can occur at the soil-atmosphere interface and at the surface of liquid manure. Soils can provide a much different photodegradation environment than aqueous solutions and transformation rates can vary significantly in soils compared to those in water (Balmer et al., 2000). Quinolones and tetracyclines are susceptible to photodegradation (Huang et al., 2001), and photodegradation of oxytetracycline was three times more rapid under light than dark conditions (Doi and Stoskopf, 2000). Halling-Sørensen (1998) suggested that tylosin might be resistant to photolysis because it has only limited light absorbance in the visible spectrum, and Boxall et al. (2004) determined that sulfonamides would not be readily photodegraded. Beausse (2004) concluded that photodecomposition of antibiotics under field conditions were negligible when compared with other abiotic processes.

Limited numbers of studies to assess the biodegradation of antibiotics have been conducted. Depending on test conditions, biodegradation half-lives of organic compounds can widely vary. Studies using standard laboratory test assays have demonstrated limited or no degradation of antibiotics such as metronidazole and oxytetracycline (Kümmerer et al., 2000; Samuelsen et al., 1994; Jacobsen and Berglund, 1988). In another study of 18 antibiotics tested, none were found readily biodegraded (0–27% of the parent compound lost) after 28 d, and in some cases occurring only when additional nutrient supplement was made (Alexy et al., 2004). Penicillin G was found readily biodegradable along with some biodegradation of amoxicillin, imipenem, and nystatin (Gartiser et al., 2007a, 2007b). A study of aquaculture sediments showed bacterial mineralization of erythromycin A (Kim et al., 2004). Inherent to the process of biodegradation, the toxic effects of antibiotics on the resident bacteria have also been demonstrated. A range of antibiotic concentrations were found to inhibit activated sludge in waste water treatment (Gartiser et al., 2007a; Alexy et al., 2004), however it is yet unknown the exact effects of antibiotic entry in natural environments on microbial populations resident to these systems.

Plant uptake and bioaccumulation of antibiotics has received considerable interest due to issues of food safety and human health. A number of studies have shown this mechanism to oc-

cur with a variety of plant species (e.g., Dolliver et al., 2007; Boxall et al., 2006; Kumar et al., 2005), and further includes biotransformation of compounds through well-known plant detoxification mechanisms (Park and Choung, 2007; Sandermann, 1992). While significant to the fate of antibiotics, discussion of these processes is outside of the scope of this review.

Persistence of Antibiotics in Manure

Antibiotics excreted from animals are often concentrated in the solid phase because of sorption dynamics (Kolz et al., 2005a, 2005b; Loke et al., 2002; Tolls, 2001). Half-lives that have been reported for a variety of antibiotic classes in manure (Boxall et al., 2004) (Table 4) were less than the anticipated storage period of manure, suggesting the possibility that significant degradation of the parent compounds might occur before land application. Quinolones and tetracyclines were the most persistent with half-lives approaching 100 d. Kolz et al. (2005a) determined that 90% of tylosin, tylosin B, and tylosin D that were added at the start of the experiment were not detected in the extractable fraction of the slurry mixture within 30 to 130 h in anaerobic manure slurries at 22°C. Aerating the slurries reduced the time to achieve 90% loss of tylosin to 12 to 26 h. Although biodegradation and abiotic degradation occurred, the primary mechanism for tylosin loss was thought to be irreversible sorption to manure solids (Kolz et al., 2005a, 2005b). Residual tylosin and its breakdown product, dihydrodesmycosin, were also detected in the slurries after 8 mo. In several studies, tetracycline concentrations were found generally higher than macrolides, β -lactams, and sulfonamides (Table 5). Tetracycline concentrations in some swine lagoons were as great as 1 mg/L (Campagnolo et al., 2002). Gavalchin and Katz (1994) determined the persistence of seven antibiotics in a soil-feces matrix under laboratory conditions and found that the order of persistence was chlortetracycline > bacitracin > erythromycin > streptomycin \geq bambamycin \geq tylosin \geq penicillin with regard to their detection in the soil. The application of manure to agricultural fields also likely introduces breakdown products into the environment along with the parent compound, however, persistence data for degradation products were not found in the reviewed literature. The nature of these breakdown products pertains to the potential biological activities of these chemicals, along with the parent compounds. The lack of information on breakdown metabolites in natural environments can in large part be attributed to analytical difficulties and instability of suspected or unknown metabolites (O'Connor and Aga, 2007; Ingerslev and Halling-Sørensen, 2001).

Persistence of Antibiotics in Soil and Water

Until recently, information regarding the occurrence, fate, and transport of antibiotics under field conditions has been limited. In a sandy soil that had repeated swine liquid manure applications, tetracycline and chlortetracycline were detected down to a depth of 30 cm (Hamscher et al., 2002, 2005). The highest tetracycline and chlortetracycline concentrations, 198 and 7.3 $\mu\text{g}/\text{kg}$, respectively, were detected at soil depths of 10 to 20 cm and 20 to 30 cm, respectively. Sulfamethazine was generally not detected in soil samples, but was detected in ground-

Table 4. Persistence of antibiotics in manure (modified from Boxall et al., 2004).

| Antibiotic class | Half-life |
|------------------|-----------|
| | d |
| Aminoglycosides | 30 |
| β-Lactams | 5 |
| Macrolides | <2–21 |
| Quinolones | 100 |
| Sulfonamides | <8–30 |
| Tetracyclines | 100 |

Table 5. Antibiotic concentrations detected in manure from swine and poultry lagoons.

| Antibiotic | Concentration | Reference |
|----------------------------------|------------------|-------------------------|
| Lincomycin | 2.5–240, µg/L | Campagnolo et al., 2002 |
| Chlortetracycline | 68–1000, µg/L | Campagnolo et al., 2002 |
| | 0.1, mg/kg | Hamscher et al., 2002 |
| | <0.5–1.0, mg/kg | Hamscher et al., 2005 |
| Tetracycline/ Oxytetracycline | 25–410, µg/L | Campagnolo et al., 2002 |
| | 4.0, mg/kg | Hamscher et al., 2002 |
| Sulfamethazine | 14.1–41.2, mg/kg | Hamscher et al., 2005 |
| | 2.5–380, µg/L | Campagnolo et al., 2002 |
| | 0.13–8.7, mg/kg | Haller et al., 2002 |
| Sulfadimethoxine | 0.2–7.2, mg/kg | Hamscher et al., 2005 |
| | 2.5, µg/L | Campagnolo et al., 2002 |
| Erythromycin | 2.5, µg/L | Campagnolo et al., 2002 |
| Penicillin G | 2.1–3.5, µg/L | Campagnolo et al., 2002 |

water collected at a depth of 1.4 m. Oxytetracycline, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethoxypridazine, sulfamethoxazole, sulfadimethoxine, and tylosin were not detected in any soil or groundwater samples. While it appeared some of the tetracyclines could accumulate in soil, none of the antibiotics from the study were detected at soil depths >30 cm and only sulfamethazine was detected in groundwater suggesting limited transport, even in highly porous sandy soils.

In a field study with clay loam soil that received swine manure spiked with the sulfonamide, sulfachlorpyridazine (SCP), the antibiotic was found to be mobile and readily entered the field drain, with a maximum concentration of 590 µg/L detected 7 d after manure application (Boxall et al., 2002). In the same study conducted with sandy loam field soil, SCP concentrations in soil pore water were significantly lower (maximum concentration 0.78 µg/L) than the field with clay loam, and contrasted with laboratory sorption studies that predicted larger soil water concentrations. The lower concentrations detected in the field samples were hypothesized to be the result of SCP degradation.

In another soil transport study, SCP and oxytetracycline (OTC) were detected in soil at concentrations up to 365 and 1691 µg/kg, respectively (Kay et al., 2004). Similar to other investigations, these compounds were not detected below a depth of about 37 cm. The SCP and OTC were detected in tile drainage at peak concentrations of 613 and 36 µg/L, respectively. Only 0.004% of the OTC that was applied was in the particulate phase, and 23% of OTC moved to tile drainage. The investigators concluded that the antibiotics behaved similarly to pesticides under field conditions, and that tile drainage may be a significant route for these compounds to migrate to surface waters. The manure in this study

was surface-applied without incorporation into the soil and the authors suggested that tillage before or during manure application might limit transport of antibiotics. In a later study by the same authors, swine manure spiked with SCP, OTC, and tylosin was surface-applied to wheat (*Triticum aestivum* L.) stubble in a clay loam soil and mass recovery of SCP and OTC lost in surface runoff was 0.42 and 0.07%, respectively (Kay et al., 2005). While surface runoff did not appear to be a significant transport loss, the authors suggested that incorporation of manure into the soil would further limit loss from the soil. Tylosin was not detected in any samples suggesting its rapid degradation in the manure, supporting previous evidence that macrolides may more readily undergo microbial degradation processes.

In a study where swine manure was spiked with sulfadiazine and sulfathiazone and irrigated on to grassland, <5% of sulfonamide applied was lost to runoff (Burkhardt et al., 2005). The sulfonamide losses were 10 to 40 times greater on the manured plots when compared to control plots, the latter receiving only aqueous solutions of the compounds. The authors concluded that the manure formed a seal at the soil surface, creating conditions for more runoff. Also, the high manure pH may have caused deprotonation of the sulfonamides resulting in decreased sorption to the soil. These results suggested that repeated surface application of manure might yield a higher likelihood for runoff situations.

While detection of antibiotic residues poses a challenge in any environmental matrix, detection of low levels of compounds, particularly in natural waterways, are highly challenging. The U.S. Geological Survey (USGS) has a comprehensive stream-monitoring network throughout the United States and improved detection of compounds by developing state-of-the-art analytical techniques such as LC-MS-MS. A recent study by the USGS (Kolpin et al., 2002) conducted a reconnaissance of the occurrence of pharmaceuticals, hormones, and other organic wastewater contaminants in water resources. In 139 streams sampled across 30 states during 1999 and 2000, a number of antibiotics were detected (Table 6). Carbodox, doxycycline, enrofloxacin, sarafloxacin, sulfachlorpyridazine, sulfamerazine, sulfathiazole, and virginiamycin were not detected in any samples. Many of the compounds that were not detected are commonly used in livestock operations, suggesting limited transport of these compounds to surface waters in the aqueous phase. As analytical technologies improve, detection of compounds can provide a more accurate characterization of the quantities and occurrence of antibiotics in natural soil and water systems.

In a study to investigate the occurrence of five tetracyclines and six sulfonamides in water collected along the Cache la Poudre River, Colorado, no antibiotics were detected in a pristine mountain stretch of the river (Yang and Carlson, 2003). Few sulfonamides were detected along the entire river, however, the frequency of detection and concentration of tetracyclines increased as the river water quality became impacted by urban and agricultural sources. Tetracycline concentrations in filtered samples ranged from 0.08 to 0.30 µg/L. Photolysis, biodegradation, and sorption of the tetracyclines could have occurred in various reaches of the stream but the authors concluded that proximate

agricultural activity influenced tetracycline occurrence in the river. In a study to detect antitibiotics in surface- and groundwaters, Campagnolo et al. (2002) found 31 and 67% of the samples collected near swine and poultry confinement facilities, respectively, had detectable quantities of <10 µg/L, compared to 1 mg/L of the total antibiotics that could be detected in the more concentrated environment of swine manure storage lagoons. The study concluded that the presence of detectable quantities of antibiotics in water environments proximal to swine waste lagoons suggested the practice of land application of waste may serve as a source of contamination. Chlortetracycline, monensin, and tylosin were detected in runoff from cattle feedlot manure stockpiles, and in situ degradation of antibiotics in protected manure could result in a significant reduction of contamination risk due to runoff events (Dolliver and Gupta, 2008).

Few studies have determined the occurrence of veterinary antibiotics in groundwater. Krapac et al. (2004) collected shallow (<8 m) groundwater samples near two swine confinement facilities. Fewer than five percent of the samples contained any of the tetracyclines at either of the facilities. Parent tetracycline compounds were detected in a small number of groundwater samples collected from wells that had also been significantly impacted by manure seepage as evident by elevated chloride, ammonium, and potassium concentrations. Tetracycline breakdown products were detected in some groundwater samples even when the parent compound was not detected. When detected, antibiotic concentrations were <0.5 µg/L. Hirsch et al. (1999) collected more than 30 groundwater samples from agricultural areas in Germany containing large numbers of animal confinement facilities. Of the 18 antibiotics representing macrolides, sulfonamides, penicillins, and tetracyclines, only sulfonamide residues were detected in four samples, and none of the other antibiotics were detected in the groundwater samples. The authors concluded that sulfonamides in two of the samples were the result of sewage irrigation and sulfamethazine detected in the other samples was likely from veterinary use.

Occurrence of Bacteria and Development of Antibiotic Resistance in Animal Guts

Antibiotic resistance among commensal bacteria represents a major avenue for the development of resistance in bacterial pathogens, since resistances increase first in commensals and then transferred to pathogens later (Salysers et al., 2004; Sørsum and Sunde, 2001). First, commensal gut bacteria are likely to be highly efficient contributors to antibiotic resistance because the numbers of commensal bacteria in the intestinal ecosystem are large, often more than 10¹⁴ bacteria comprising several hundred species (Andremont, 2003). Anaerobic bacteria dominate this ecosystem and number 10¹¹ to 10¹² cells/g of intestinal content whereas enterobacteria and enterococci are relatively minor players ranging from 10⁶ to 10⁸ cells/g of intestinal content. Second, the commensal genetic pool is large and encompasses the potential for many different mechanisms conferring antibiotic resistance. Third, antibiotic-resistant commensal bacteria may be selected each time an antibiotic is

Table 6. Detection frequency and maximum concentrations of selected antibiotics in 139 filtered stream samples from 30 U.S. states (modified from Kolpin et al., 2002).

| Antibiotic | Frequency of detection | Maximum concentration |
|-------------------|------------------------|-----------------------|
| | % | µg/L |
| Trimethoprim | 27.4 | 0.30 |
| Erythromycin-H | 20.2 | 1.5–1.7 |
| Lincomycin | 21.5 | 1.7 |
| Sulfamethoxazole | 19.0 | 0.52 |
| Tylosin | 13.5 | 0.28 |
| Roxithromycin | 4.8 | 0.18 |
| Ciprofloxacin | 2.6 | 0.03 |
| Chlortetracycline | 2.4 | 0.69 |
| Oxytetracycline | 1.2 | 0.34 |

administered regardless of the health status of the animal. This microbial population is excreted in feces and stored as manure where it undergoes changes in the numbers and proportions of the dominant bacterial species. An analysis of stored swine manure indicated that the predominant culturable microorganisms from these environments were obligately anaerobic, low mol% G + C Gram-positive bacteria (Firmicutes) comprised of members of the Clostridial, Eubacterial, and Lactobacillus/Streptococcus phylogenetic groups (Cotta et al., 2003).

Although reports of the percentage of viable, culturable antibiotic-resistant bacteria in swine effluent vary, it is clear that antibiotic resistance is a common phenomenon. A study conducted in the 1980s of coliforms in swine waste found that 97% of *E. coli* were resistant to at least one of the following antibiotics: ampicillin, furatrizine, chloramphenicol, kanamycin, streptomycin, sulfonamides, or tetracycline (Hanzawa et al., 1984). Haack and Andrews (2000) found that 71% of *Enterococcus fecalis* isolates from swine farrowing house effluent was resistant to tetracycline. Cotta et al. (2003) found between 4 and 32% of the bacteria in swine manure were resistant to tylosin, depending on the depth from which the sample was collected in the manure holding pits.

Bacterial Survival and Transport in the Environment

Persistence of Bacteria during Manure Storage

Deep waste holding pits located beneath the slatted floors of hog barns have four to eight times the amount of solid material suspended in liquid relative to a lagoon holding system, where most of the solid fraction settles out as sediment. Little research has been conducted to determine the effects of the various manure storage techniques on overall bacterial populations and the corresponding genetic pool that includes antibiotic resistance genes. Investigations that have been done in this area generally involve human or livestock pathogens. Hutchison et al. (2004) studied zoonotic agents in fresh wastes, akin to a below barn holding pit, and stored wastes, analogous to the second stage of a two stage lagoon. A general trend was identified that suggested storage of waste without addition of fresh waste reduces pathogen numbers. Total bacterial populations

may also decrease in stored effluent, however, this hypothesis has yet to be adequately tested. Detailed correlations between bacterial populations and their corresponding antibiotic resistance determinants have not been comprehensively examined.

There is strong evidence that thermophilic aerobic or anaerobic digestion of swine manure in a reactor can reduce pathogen loads by more than four orders of magnitude (Sobsey et al., 2001), although the effects of the process specifically on antibiotic-resistant bacterial populations, pathogenic or not, is generally unknown. Anaerobic digestion of different swine slurries in bench scale sequencing batch reactors operating at a temperature of 20°C for 20 d reduced the colony forming units count (CFU/mL) of the total coliform populations to nondetectable limits or by 1.62 to 4.23 log CFU/mL, and *E. coli* populations to nondetectable limits or by 2.48 to 4.16 log CFU/mL (Côté et al., 2006). Although coliform bacteria from livestock typically exhibit some degree of antibiotic resistance, it is unknown what effect digestion treatments would have on the overall antibiotic resistance of swine waste bacteria.

Persistence of Bacteria Introduced to Soil

Land application of animal manure, with its high concentration of microbial biomass, is a significant route for the introduction of new bacteria into the terrestrial environment, including potential pathogens (e.g., *E. coli* O157:H7) and some human enteric viruses (e.g., rotavirus). The persistence and transport of these organisms in the environment continues to be a concern for environmental quality, food safety, as well as human and animal health. Gavalchin and Katz (1994) concluded that the longer an antibiotic persists in the soil in an active form, the greater the potential for native soil bacterial populations to be affected. Nutrient amendment via the application of animal waste to soil has been hypothesized to promote faster adaptation of the soil microbial community to antibiotic effects (Schmitt et al., 2005). In addition, biologically active antibiotics (or antibiotic breakdown products) introduced to the soil may confer a selective advantage for soil commensal bacteria carrying resistance genes, or exert selective pressure for acquisition of resistance genes in soil commensal populations.

It has been well documented over the years that many microorganisms survive the transition from effluent pit or lagoon into soil (Boes et al., 2005; Guan and Holley, 2003; Jiang et al., 2002; Bolton et al., 1999; Lee and Stotzky, 1999; Stoddard et al., 1998; Chandler et al., 1981; Kibbey et al., 1978). Most investigations have however, focused on pathogens of clinical interest. The length of time that introduced organisms can persist in the soil varies with temperature, moisture, pH, and the indigenous community present. The wide range of persistence times of four well-studied pathogens in different environments and at different temperatures has been reported (Table 7). A recent study examining the survival of *E. coli* and *Salmonella typhimurium* applied to a clay soil with swine effluent, however, found considerably shorter persistence times (21 d for *E. coli* and 7 d for *Salmonella typhimurium*) (Boes et al., 2005), highlighting the variation in survival times under different environmental conditions. Sengelov et al. (2003) studied the per-

sistence of cultureable aerobic, heterotrophic, tetracycline resistant bacteria in four Danish farm soils following variable rates of pig slurry application. An increase in numbers of resistant bacteria was seen following application, with greater increases occurring in the more heavily manured soils. Five months following application, the proportion of tetracycline resistant bacteria in all of the treated soils had returned to levels within the range of the nonmanured control samples. Andrews et al. (2004) found enterococci declined from 4.8×10^5 CFU/g soil to <10 CFU/g in soil microcosms over a 5-wk period. These studies suggest a certain rebound effect of bacterial populations following an initial spike, although there may be sufficient time and opportunity for mechanisms of resistance selection and gene transfer to occur.

Transport of Bacteria and Viruses into Groundwater

As hosts of genetic elements that may include antibiotic resistance genes, bacteria and viruses have great potential to move deep into the subsurface environment, and can even penetrate and reach confined aquifers. Several studies have focused on bacterial occurrence and movement in soil into groundwater following manure application. A study of the vadose zone at sites of manure application established that the potential for fecal bacteria to be transported to depth in soil was correlated with the water content of the manure (Unc and Goss, 2003). The investigators concluded that application of animal manure to soil can readily lead to groundwater contamination with fecal bacteria, especially under moist soil conditions, and that macropores, but not total porosity, are important in the transport of bacteria through soil. In a study of poultry manure, fecal coliform movement corresponded to preferential water movement in each soil block sampled, whether sod-covered or tilled (McMurry et al., 1998). The findings were consistent with those of Howell et al. (1995) where groundwater contamination by infiltration of fecal coliform through well-structured soil may be significant even during modest rainfall.

It has been estimated that 20 to 25% of groundwater sources in the United States are contaminated with microbial pathogens, which include more than 100 types of viruses (Macler, 1995). The extremely small size of viruses allows them passage through sediment pores that would trap much larger bacteria and protozoa. While the significance of viruses and their potential roles in the transfer of genetic material in soil and water systems is not well-known, numbers of bacterial viruses, or bacteriophages, are thought to be positively correlated with the ecological function as well as abundance, of heterotrophic bacteria (Weinbauer and Rassoulzadegan, 2004; Wommack and Colwell, 2000). Lytic phages are involved in normal bacterial turnover in natural environments. A single lytic phage incorporation into a bacterial cell can result in release of a high number of new phage particles, where the cycle can then continue. Many studies have shown that bacterial indicators do not accurately reflect the occurrence of viruses in aquatic system (Nasser and Oman 1999; Scandura and Sobsey, 1997; Nasser et al., 1993; Payment and Armon 1989; Goyal, 1983), and that the occurrence and diversity of bacteria and phage relationships may be much greater than what is currently known.

Transport of bacteria has not been as extensively studied as viruses. Viruses can be considered colloidal and may move substantially faster than dissolved solutes, like bromide, in subsurface environments as a result of preferential flow through the large soil apertures offered by fractures and root holes. Solutes have a higher probability than colloids of entering smaller pores, resulting in more tortuous, longer, and ultimately slower flow paths. In clay formations, fractures and root holes can be as large as 100 μm , 5000 times larger than the smallest viruses (Hinsby et al., 1996), and viruses can pass through fractured clay till in apertures as small as 3 to 5 μm (Sims, 1993). McKay et al. (1993) showed that approximately 50% of clay pore throats are smaller than 65 nm, which is about the same size as enteric viruses. The same study demonstrated that viruses PRD-1 and MS-2 move through fractured clay at velocities of 2 to 5 m/d, which is 100 to 200 times faster than bromide. Hinsby et al. (1996) measured PRD-1 and MS-2 virus velocities of 4 to 360 m/d through clay with fractures and root holes. Depending on sediment chemistry, viruses may not be completely attenuated by adsorption onto sediment grains.

Viruses have been found in groundwater at a depth of 67 m and are reported to move horizontally as far as 408 m in glacial till and 1600 m in fractured limestone (Robertson and Edberg 1997; Keswick and Gerba, 1980). Viruses may survive chlorination, sunlight, spraying and percolation through sandy soil (Wellings et al., 1974) and are capable of surviving at least 28 d in groundwater (Wellings et al., 1975). Bacteria and viruses are widely available for transport within groundwater systems due their common occurrence in drinking water wells (Gerba and Rose, 1990). The processes of viral transport are extremely complex. A host of major factors can affect subsurface viral transport, including temperature, moisture content, pH, hydraulic conditions, organic matter, adsorption and desorption, salt content, type of virus, virus inactivation (i.e., virus decay), soil properties, rainfall, source of virus and water table depth (Cherry et al., 2007; Azadpour-Keeley et al., 2003; Schijven and Hassanizadeh, 2000; Bitton and Harvey, 1992; Yates and Yates, 1988; Gerba and Bitton, 1984). The ability of viruses to mediate their effect on host bacteria (and their genetic complement) is highly dependent on site-specific conditions.

Attention to modeling the transport of bacteria, viruses, and antibiotic residues has arisen due to interest in predicting the fate and occurrence of pathogens and antibiotic resistance genes. Most virus transport models are based on the primary processes of viral transport: advection, dispersion, sorption, and inactivation (Loveland et al., 2003) and are beyond the scope of discussion in the present review. Computer models have been used, however, and the outcomes are highly heterogeneous at the field-scale, differ widely by virus type, and are associated with a great deal of uncertainty (Yates and Jury 1995). For example, Yates et al. (2000) compared predicted virus concentrations using two models, CANVAS (Park et al., 1994) and HYDRUS-2D (Simunek et al., 1999), in a septic system leach field. CANVAS predictions either overestimated or underestimated the field data. Although HYDRUS-2D accurately predicted the virus breakthrough curves, the model

Table 7. Persistence times of pathogenic bacteria in different environments.

| Environment | Temperature, °C | Estimation of survival time | | | |
|--------------|-----------------|--|---|--|--|
| | | <i>Salmonella</i> (Guo et al., 2002; Santo Domingo et al., 2000; Mitscherlich and Marth, 1984; Zibilske and Weaver, 1978) | <i>Campylobacter</i> (Buswell et al., 1998; Rollins and Colwell, 1986; Mitscherlich and Marth 1984; Blaser et al., 1980) | <i>Yersinia enterocolitica</i> (Karapinar and Gonul, 1991; Chao et al., 1988) | <i>Escherichia coli</i> 0157:H7 (Wang and Doyle, 1998; Tauxe 1997; Zhao et al., 1995; Cieslak et al., 1993) |
| Water | <0 ~5 | ~6 mo ~6 mo | ≤8 wk 1 wk–4 mo | >1 yr >1 yr | >300 d >300 d |
| Soil | ~30 <0 ~5 | ~6 mo >6 mo ≤28 wk | ~4 d ≤28 wk ~2 wk | ~10 d >1 yr >1 yr | ~84 d >300 d ~100 d |
| Slurry | ~30 | ~4 wk ≤75 d | ~1 wk <112 d | ~10 d ≥28 d | ~2 d ≤100 d |
| Dry surfaces | | ≤7 d | ~1 d | ~1 d | ~1 d |

required extensive data input and advanced expertise not readily available and costly to obtain. Moreover, development of a separate model would then be needed for predicting virus movement through an aquitard into a confined aquifer, but to our knowledge this type of model does not exist. Further, the estimation of groundwater travel time is complex in highly heterogeneous settings. Measurement of bulk fluid groundwater age (the time elapsed for water to travel to a given location) may not reflect contributions via preferential flow that, albeit small, may contain measurable infectious viruses (Powell et al., 2003). If the longest survival time for enteric viruses is reportedly one to 2 yr, then water taking 10 to 100 yr to reach a confined aquifer is unlikely to be a significant source of active viral particles. It is not definitively known, however, the time elapsed for water to reach a confined aquifer.

Mechanisms of Antibiotic Resistance Gene Transfer in the Environment

The entry of antibiotic resistant bacteria into soil and water via manure application yields a potentially significant reservoir of antibiotic resistance genes, however, the fate of these determinants and the extent of their transfer into commensal bacteria in natural systems is still relatively unknown. Once antibiotic resistant bacteria and their corresponding suite of resistance genes enter the soil and water, the persistence and fate of the introduced determinant is dependent on the nature and viability status of the host bacteria harboring the determinant(s), and the partitioning of free genetic material following cell lysis that may be subject to degradation, sorption, or uptake by new cells. As long as a resistance gene is present in the environment, the possibility for its transfer exists.

A number of studies have recently shown that horizontal transfer of antibiotic resistance genes between bacteria of different genera and species occurs readily and frequently in natural systems such as soil and groundwater (Onan and LaPara, 2003; Chee-Sanford et al., 2001; Salyers and Amabile-Cuevas, 1997). Virtually identical copies of the same resistance genes are found in distantly related bacteria. While studies have reported increased incidences of antibiotic-resistant enteric bacteria in surface water down-gradient or in close proximity to swine CAFOs (Sapkota et al., 2007; Sayah et al., 2005), it is not yet known the extent or potential of ABR gene acquisition in surface water systems. Furthermore, the existence of a naturally high level of antibiotic resistance among diverse soil-dwelling bacteria is supported in recent studies (D'Costa et al., 2006; Riesenfeld et al., 2004), suggesting myriad mechanisms of genetic transfer occurring well beyond the contemporary time frame of antibiotic use in industry.

Genetic mechanisms involved in lateral exchange of antibiotic resistance genes may include: (i) conjugative transfer (e.g., via plasmids, transposons, integrons, nonreplicating *Bacteroides* units [NBU]); (ii) transduction by bacteriophage; (iii) transformation, which is dependent on the native competent state of bacteria as well as cells acquiring induced competency (e.g., the presence of calcium, lightning event). More recently, novel

phage-like gene transfer agents (GTA) have been reported in diverse environmental isolates (Stanton, 2007), suggesting additional mechanisms of gene transfer that may also be significant in soil systems. These mechanisms of horizontal gene transfer have been reviewed at great length in the literature. The following section provides a brief overview of the processes and primarily focuses on the mechanisms of gene transfer in the context of soil environmental conditions.

Conjugation

Conjugation is the transfer of DNA between a donor and a recipient cell (for review, see e.g., Mazodier and Davies, 1991). Chromosomal and plasmid DNA can be transferred, and if the acquired DNA is stabilized, the transconjugant recipient can go on to act as a donor and the process repeats. Conjugation requires physical cell-to-cell contact via the donor by means of a pilin bridge. Because conjugation is dependent on direct cell contact, cell densities and the environment in which the bacteria reside plays a large role in the outcome frequency of conjugation events. For example, a high frequency of conjugation events occurs in the hydrated environments of the gastrointestinal tracts (Salyers et al., 1995) and biofilms (Hausner and Wuertz, 1999), both examples of high bacterial populations residing in close physical proximity to one another. In general, it is thought that conjugative mechanisms of gene transfer in the environment are important in the spread of genetic information, occurring over a broad host range of genera and species, and explains incidences of similar DNA sequences found among distantly related bacterial species. Triparental mobilization of DNA can occur when a conjugative plasmid is transferred from a parent cell to a recipient containing a nonconjugative plasmid, and both plasmids may be subsequently transferred to a recipient containing neither plasmid. While such triparental matings occur at lower frequencies than biparental matings, such a mechanism of DNA transfer has been shown to occur in soil bacteria (Trevors, 1999; Lesická-Hupková et al., 1996).

Many antibiotic resistance genes are harbored on mobile genetic elements such as transposons, integrons, or plasmids and can be readily transferred between members of the same species, and between bacteria of diverse genera. Numerous microcosm studies have documented plasmid transfer in soil environments, and plasmid transfer from introduced bacteria to soil native bacteria (Andrews et al., 2004; Heuer et al., 2002; Lee and Stotzky, 1999; DiGiovanni et al., 1996; Wellington et al., 1992). In particular, Andrews et al. (2004) examined the persistence of the conjugative transposon Tn916 associated with antibiotic resistance in autoclaved or native soil microcosms treated with swine effluent that contained large populations of enterococci carrying Tn916. In autoclaved microcosms, persistence of the transposon closely correlated with the persistence of its enterococci host. In native microcosms however, Tn916 was still detected at substantial levels 6 wk after treatment with effluent despite the fact that the introduced host enterococci were undetectable.

Plasmid transfer through conjugation among diverse genera has occurred in the soil rhizosphere under both field and simulated microcosm conditions (van Elsas et al., 1998). Higher rates

of gene transfer were found in rhizosphere soil relative to bulk soil (Lilley and Bailey, 1997). Daane et al. (1996) found that earthworm (*Lumbricus terrestris*) activity increased the mobilization of a mercury resistance plasmid from introduced organisms to indigenous bacteria because the worms aid in the dispersal of bacteria in the soil. It also appears that the presence of animal waste effluent itself may increase the likelihood of conjugal transfer in the soil. Gotz and Smalla (1997) found a 10-fold increase in plasmid transfer in soils receiving manure application relative to those that had not. The presence of antibiotics, even at very small concentrations, can stimulate conjugation and the transfer of resistance genes by as much as 10,000-fold in enteric systems (for reviews see Bahl et al., 2004; Salyers et al., 1995; Doucet-Populaire et al., 1991). It was noted more than two decades ago, that subinhibitory concentrations of β -lactams enhanced the transfer of tetracycline resistance plasmids in *Staphylococcus aureus* by up to 1000-fold (Barr et al., 1986). A number of studies with *Bacteroides* have demonstrated low concentrations of tetracycline promoted or accelerated conjugation events (Whittle et al., 2002; Stevens et al., 1993; Valentine et al., 1988). Subinhibitory concentrations of tetracycline were also shown to substantially enhance transposon-mediated conjugal transfer (Showsh and Andrews, 1992; Torres et al., 1991). Ohlsen et al. (2003) found that 0.1 $\mu\text{g}/\text{mL}$ of the antibiotic, gentamicin, increased plasmid transfer in *Staphylococcus aureus*. It is not yet known if environmentally relevant concentrations can stimulate plasmid transfer in natural environments, or if these concentrations pose a selective factor for acquisition of resistance mechanisms. Given the high concentrations of bacteria in manure and potentially significant time of survival of introduced bacteria, conjugation events may be especially likely during stages of waste storage and in early times immediately following land application. The magnitude of conjugation among commensal soil bacteria, however, is not yet clearly defined as the most significant lateral gene transfer mechanism.

Transduction

The transfer of DNA between bacterial cells can be mediated by bacteriophages in a mechanism referred to as transduction (for review see Brüssow et al., 2004; Miller, 2001). In generalized transduction, DNA from any part of a host bacterial genome is mispackaged into a phage capsid (protein coat) during replication, and on infection of a new host, the foreign DNA may be incorporated by homologous recombination into the host chromosome. In cases where the DNA is a replicon, such as a plasmid, it can be inherited directly. In specialized transduction, inaccurate excision of the prophage results in a hybrid of bacterial and phage DNA being packaged into phage particles, and replication can yield abundant copies of specialized transducing phages that contain specific bacterial genome fragments. Once the foreign DNA inserts into the host bacterium, maintenance and expression of genetic determinants may occur.

Bacteriophages are very common in all natural environments (Weinbauer and Rassoulzadegan, 2004) and transduction is thought to play a more significant role in the evolution of bacteria than considered previously (Brabban et al., 2005; Clokie et al., 2003; Miller, 2001; Trevors, 1999; Schicklmaier

and Schmieger, 1995). As described earlier, phages are thought to be important in microbial ecological function. In the last two decades, transduction has been demonstrated to occur in a range of environments such as sewer plants and natural systems that include swamps; marine, lake, and stream sediments; and waters. Jiang and Paul (1998) reported that in marine environments where abundant phage populations occur, transduction could be a significant mechanism of horizontal gene transfer. In a study by Ogunseitan et al. (1992), sewage contained the largest diversity and number of bacteriophages infecting *Pseudomonas aeruginosa*, in contrast to soil, which had between 2 and 37% of *P. aeruginosa* that harbored bacteriophages. The number of phage particles reported in a rhizosphere soil is $1.5 \times 10^8 \text{ g}^{-1}$, equivalent to 4% of the total bacterial populations (Ashelford et al., 2003), and up to nearly $1.1 \times 10^9 \text{ g}^{-1}$ dry wt. in agricultural soils (Williamson et al., 2005). While less is known about phage abundance and occurrence of natural transduction in soil, recent studies have suggested the potential for transduction as a means of significant gene transfer in soil systems (Ghosh et al., 2008; Williamson et al., 2007).

Transformation

Bacteria can acquire new genetic information through the process of natural transformation, which involves specific host genes in a complex process of transporting exogenous DNA molecules into the cell cytoplasm and the stable integration of the transforming DNA into the genome of recipient (for reviews see Chen and Dubnau, 2004; Lorenz and Wackernagel, 1994). Transformation requires cells to be genetically competent, or in a state whereby foreign DNA can bind and be uptaken in a form that is resistant to intracellular restriction nucleases. Many examples of naturally competent bacteria have been reported, including several genera native to soil environments (Demanèche et al., 2001c; Levy and Miller, 1989). Both Gram positive and Gram negative bacteria have been found to have natural transformation ability, including *Bacillus*, *Micrococcus*, *Agrobacterium*, *Pseudomonas*, and *Vibrio*, to name a few among genera found in natural environments.

Competence factors produced by some bacteria have also been shown to induce competence in other cells (Trevors, 1999; Solomon et al., 1995). For example, in a soil microcosm study, cell lysates of *Acinetobacter* sp., *Pseudomonas fluorescens*, and *Burkholderia cepacia* conferred natural transformation of *Acinetobacter* sp. strain BD413, with the activity declining by 31% after 1 h in nonsterile soil (Nielsen et al., 2000). A related study found that plant exudates in the soil rhizosphere also had a stimulatory effect on the transformation of *Acinetobacter* sp. strain BD413 (Nielsen and van Elsas, 2001).

While not all bacteria can undergo natural transformation, many more species can be artificially transformed in the laboratory. Artificial transformation involves chemical (e.g., CaCl_2) or electrical (e.g., electroporation) methods to alter the cell membrane and allow passive uptake of DNA and is considered distinct from the active process of natural transformation. While natural transformation has been thought to be more significant in situ, studies suggest that natural salt (e.g., Ca^{2+}) concentra-

tions in freshwater and soil, and lightning events can induce competence in bacteria (C er monie et al., 2004; Deman che et al., 2001a; Bauer et al., 1996; Lorenz and Wackernagel, 1994). The calcium content in swine manure is relatively high, with concentrations equivalent to the phosphate content (Luo et al., 2002). In soil environments, the increased calcium exposure following repeated history of effluent application, along with low nutrient concentrations and nonexponential growth characteristics, may be all contributing factors in inducing competence in the extant population of bacteria.

Transformation processes in nature depends on the stability and bioavailability of free DNA. While DNA has been shown to sorb to sand (Lorenz and Wackernagel, 1987), it is the clay fraction that is the primary constituent sorbing DNA in soil systems. Both chromosomal and plasmid DNA sorb to clay particles, particularly at neutral or lower pH values (Deman che et al., 2001b; Ogram et al., 1988) and in the presence of high concentrations of multivalent cations (Paget et al., 1992). Cai et al. (2006a) reported fine textured 2:1 swelling clays, for example, montmorillonite, bind DNA primarily through weak electrostatic forces allowing easier desorption of DNA. The coarser textured 1:1 nonswelling clays, for example, kaolinite, bind DNA primarily through ligand exchange and possibly hydrogen bonding, resulting in a much stronger bond with less likelihood of DNA desorption.

The stability of naked DNA in soil environments and the retention of its ability to transform cells has not been extensively studied, however several investigations have demonstrated that DNA adsorbed to surface active particles in soils are protected against nuclease activity (Cai et al., 2006b; Stotzky, 2000; Crecchio and Stotzky, 1998; Gallori et al., 1994). Blum et al. (1997) found a high capacity for binding of DNA to particulates in three agricultural soils (> 13µg/g soil), and found these interactions offered protection of the DNA against DNase activity. Bound DNA was found to persist longer than free DNA in the soil environment, ranging from months (Recorbet et al., 1993; Romanowski et al., 1993) to as much as 2 yr (Gebhard and Smalla, 1999). The mechanisms in action thought to protect soil-bound DNA include physical protection from contact with endonucleases, and sorption of endonucleases to clay, the latter resulting in physical separation and enzyme conformational changes that decrease nuclease activity (Deman che et al., 2001b; Khanna and Stotzky, 1992).

Both chromosomal and plasmid DNA bound to clay particles reportedly transformed bacteria in nonsterile soil (Gallori et al., 1994). The DNA bound to humic acids was also observed in transformation of *Bacillus subtilis* (Crecchio and Stotzky, 1998), and the addition of montmorillonite clay, known to bind both DNA and endonucleases, increased the transformation rate of *B. subtilis* (Lee and Stotzky, 1999). The likelihood of DNA persisting in soil, particularly ones higher in coarse clays and humic acids, and retention of ability for the DNA to transform cells, would suggest the possibility for native soil bacteria to acquire new genetic material, including genes that confer antibiotic resistances.

Detection of Antibiotic Resistance Genes in the Environment

Accurate and meaningful information on the persistence and dissemination of antibiotic resistance genes in bacteria is of fundamental importance in assessing potential health risks and environmental quality. The detection of specific genes and their bacterial hosts are important components, and recently developed techniques have been applied for detection of specific resistance genes and bacteria in natural environments. In particular, the use of molecular techniques provides rapid, sensitive, and specific detection without the requirement for bacterial growth and isolation, which often poses a major challenge given the vast unknown amounts and functions of environmental microbial species (Nocker et al., 2007). Commonly used molecular microbial techniques are based on unique sequence features of genes to allow detection and identification of microorganisms. Gene probes and the use of polymerase chain reaction (PCR) amplification of nucleic acids is now widely used to enable detection and quantitation of low levels of target sequences, and has become a key procedure in the detection and identification of bacteria and genes from a variety of environments including soil, water, and fecal material (Malik et al., 2008; Koike et al., 2007; Aminov et al., 2002; Chee-Sanford et al., 2001; Wang et al., 1996; Karch et al., 1995; Josephson et al., 1993). New approaches like microarray technology are being developed specifically to detect and identify antimicrobial resistances in clinical and environmental bacteria (Frye et al., 2006; Call et al., 2003; Volokhov et al., 2003). A recent study using a gene array approach simultaneously screened for the presence of 23 tetracycline resistance genes and 10 erythromycin resistance genes in soil and fecal samples from swine to find the most prevalent genes (Patterson et al., 2007). Molecular fingerprinting tools and robotic technology have facilitated more accurate and sensitive microbial characterization of complex environmental samples and has proven to be essential in providing more informative data in environmental monitoring studies (for reviews see: Harwood and Buckley, 2008; Wagner et al., 2007). The recent development of a number of probes that target specific antibiotic resistance genes has increased the number of studies that investigate the occurrence of these genes in natural environments. Such studies include detection of genes from antibiotic-producing bacteria, as well as genes resident in the background of natural populations. The following section highlights the application of molecular-based methods for detection and quantitation of antibiotic resistance genes in bacteria and environmental samples.

Specific classes of antibiotics can be characteristic of the industry in which they are used, and multiple antibiotic resistance phenotype profiles of bacteria have been used to identify sources of fecal pollution (e.g., human, poultry, cattle, swine) in environmental samples (Olivas and Faulkner, 2008; Parveen et al., 2006; Simpson et al., 2002; Wiggins et al., 1999; Pillai et al., 1997; Kaspar et al., 1990). Many of these studies focus on bacterial strains of clinical importance and do not fully address the characterization of populations that have acquired resistance genes in natural environments. To circumvent issues related to cultivation of bacteria, analysis of antibiotic resistance genes can be used to

characterize the genetic pool from an environment, with possibility of tracking the source of fecal contamination in surface- and groundwater. Similar to the strategy used in microbial diversity studies, the starting point in the design of probes and primers for detection of antibiotic resistance genes is a robust phylogenetic analysis. Specific gene sequences can be targeted for detection, and such an approach has been used to demonstrate the diversity of antibiotic-resistant genes present in swine lagoon and pit effluent. For example, Aminov et al. (2001, 2002) and Chee-Sanford et al. (2001) found the tetracycline resistance efflux genes (*tet* B, C, E, H, Y, Z) and the ribosomal protection protein (RPP) genes (*tet* W, O, Q, M, S, T, B(P), and *otr* A) were all present in a single swine waste lagoon. Koike et al. (2007) detected *tet* (M), (O), (Q), (W), (C), (H) and (Z) continually over a 3-yr period in groundwater underlying two swine farms. Further, *tet* (W) sequences detected in the groundwater were nearly identical (99.8%) to those found in the corresponding lagoon. In the same study, the application of the same PCR primers further allowed the detection of unique and novel tetracycline resistance gene sequences. Using molecular-based detection, agricultural soils were found to be a rich reservoir of genes closely related to the glycopeptide resistance gene *vanA* in enterococci (Guardabassi and Agersø, 2006).

Tetracycline resistance genes have been found in large numbers in lagoon effluent. In a study of a cattle feedlot lagoon, a real time PCR method was used to detect and quantify *tet* (O), (W), and (Q) genes, and correlated gene copy numbers to tetracycline levels (Smith et al., 2004). A recent study showed the persistent effects of manure and the presence of sulfadiazine on soil bacterial communities, where the numbers of cultureable resistant bacteria and sulfonamide resistance genes increased (Heuer and Smalla, 2007). A recent study suggested that manure storage and treatment have a large impact on persistence and decline of macrolide-lincosamides-streptogramin B resistance, where levels of *erm* gene abundances in composted swine manure were reduced by several orders of magnitude over levels found in manure (Chen et al., 2007).

Recent studies have reported isolation of a wide range of antibiotic-resistant bacteria recovered from soil and water environments (Dang et al., 2008; Onan and LaPara, 2003; Ash et al., 2002; Esiobu et al., 2002; Chee-Sanford et al., 2001). A number of soil samples used in these studies were directly exposed to animal waste. Furthermore, sequences of resistance genes detected in bacterial isolates were found to be identical to sequences found in lagoon or animal waste. Nikolakopoulou et al. (2005) screened tetracycline-resistant streptomycete isolates from a range of environmental samples for oxytetracycline resistance genes and found resistance genes in nontetracycline producing isolates. It is also noteworthy that cultivation strategies, particularly for populations from environmental samples, have thus far only provided an underestimate of bacteria, suggesting the possibility that a much higher diversity of antibiotic resistant bacteria may exist but are not yet accounted for. Further notable, archaea are now thought to be ubiquitous in many soil environments, including agricultural soils (Gattinger et al., 2006; Leininger et al., 2006). Archaeal multidrug-resistance MarR family proteins were found to have similar transcriptional regulation features to those counterparts found in bacteria (Miya-

zono et al., 2007). Further, mutations in the archaeon *Haloarcula marismortui* were found to confer resistance to anisomycin, a drug not known to be active against bacteria, but involve ribosomal binding sites similar for drugs such as chloramphenicol and clindamycin (Blaha et al., 2008). Far less is known about soil archaea and the extent of their resistance mechanisms or their contributions to genetic exchange within the soil metagenome.

Evolution and Ecology of Antibiotic Resistance Genes

As an important aspect of understanding the impact of land application of animal waste in soil, the final section of this review attempts to address complex issues of antibiotic resistances in an evolutionary and ecological context. While scientific evidence shows numerous incidences of dissemination of clonal lines of pathogenic bacteria with resistance mechanisms acquired by mutation and selection events, the majority of antibiotic resistances is thought to be acquired through the transfer of genes from other bacteria (Sørum and L'Abée-Lund, 2002; Roberts, 1998, 1998). In addition to natural resistances (e.g., lacking the drug target or drug transport system), antibiotic resistances in bacteria reside on genes that are either chromosomal- or plasmid-encoded, with the latter thought to encompass mechanisms to most of drugs currently in use today (Bennett, 2008). The range of action by antibiotic classes and the corresponding modes of resistances exerted by bacteria are summarized in Table 8. The following discussion details the lines of genetic evidence that supports the role of horizontal gene transfer in the natural history of antibiotic resistance genes leading into the more contemporary era of antibiotic use.

What is the Origin of Antibiotic Resistance Genes?

Actinomycetes are bacteria commonly found in soil, with members comprised of many well-known antibiotic-producing species. Correspondingly, antibiotic-producing strains also harbor resistance mechanisms to protect against the effects of the antibiotics being produced. Over three decades ago, it was reported that aminoglycoside-inactivating enzymes in actinomycetes were similar to those present in clinical isolates of antibiotic-resistant bacteria, suggesting an origin for resistance determinants (Benveniste and Davies, 1973). Many bacterial-derived antibiotic preparations were also reported to contain significant quantities of DNA and that this DNA could be transformed into bacteria (Chakrabarty et al., 1990). Webb and Davies (1993) confirmed the presence of antibiotic resistance gene sequences in a number of antibiotic preparations employed for human and animal use and hypothesized that the rapid development of multiple antibiotic resistance was due to the acquisition of the DNA residues by bacteria. Highly sensitive fluorescence detection technique confirmed quantities of DNA in many antibiotic preparations of both research and clinical grades (Woegerbauer et al., 2005). Moreover, in most of these preparations, the DNA was specific to the antibiotic producing strain used in the synthesis process, and contained the corresponding resistance genes. Attempts to demonstrate in vivo transformation by these antibiotic prepara-

Table 8. Mechanisms of antibiotic action and resistance (from Mathers et al., 2004; Sköld, 2000; Prescott et al., 1999; Roberts, 1998; Cocito et al., 1997).

| Antibiotic class (examples) | Mechanism of antibiotic action | Resistance mechanisms |
|--|--|--|
| Tetracyclines (Tetracycline, Chlortetracycline, Oxytetracycline) | Inhibition of protein synthesis (binds 30S ribosomal subunit); interferes with aminoacyl-tRNA binding | <ul style="list-style-type: none"> · Ribosomal protection protein · Efflux pumps · Enzymatic (drug alteration) |
| Macrolides (Erythromycin, Tylosin, Carbomycin A) | Inhibition of protein synthesis (binds 50S ribosomal subunit, inhibits translocation of peptidyl tRNA) | <ul style="list-style-type: none"> · Methylation of 23S rRNA · Efflux pumps · Enzymatic (drug inactivation) |
| Lincosamides (Lincomycin, Clindamycin) | Inhibition of protein synthesis (binds 50S ribosomal subunit, inhibits peptide elongation) | <ul style="list-style-type: none"> · Methylation of rRNA · Mutation · Enzymatic (drug inactivation) |
| Streptogramins (Virginiamycin) | Inhibition of protein synthesis (inhibits peptide elongation) | <ul style="list-style-type: none"> · Target modification (rRNA or ribosomal protein) · Efflux pumps · Drug inactivation (lactonases) |
| Aminoglycosides (Gentamicin, Neomycin, Streptomycin) | Inhibition of protein synthesis (binds 30S ribosomal subunit, inhibits translocation of peptidyl tRNA; misreading of mRNA) | <ul style="list-style-type: none"> · Reduced uptake or decreased cell permeability · Altered ribosomal binding sites · Production of aminoglycoside modifying enzymes |
| Chloramphenicol | Inhibition of protein synthesis (binds 50S ribosomal subunit; inhibits peptidyl transferase) | <ul style="list-style-type: none"> · Altered membrane permeability · Enzymatic (acyltransferase action to inactivate drug) · Mutation (50S ribosomal subunit) |
| β-Lactams (including Cephalosporins) (Penicillin, Ampicillin, Carbenicillin, Ceftiofur) | Inhibition of cell wall (peptidoglycan) synthesis, binds and inactivates PBPst | <ul style="list-style-type: none"> · β-lactamases (drug-modification enzymes) · Modified or low affinity PBPs · Efflux pumps |
| Sulfonamides (Sulfamethoxazole, Sulfamethoxypyridine, Sulfadimethoxine) | Inhibition of folic acid synthesis (competitively inhibits DHPS‡ by structural analogy with p-aminobenzoic acid) | <ul style="list-style-type: none"> · New enzyme (mutation or mosaic dhps, high affinity to PABA) · Insertion of nucleotides (new DHPS) |
| Fluoroquinolones (Ciprofloxacin, Difloxacin, Sarafloxacin) | Inhibition of DNA replication and transcription (inhibits DNA gyrase and topoisomerase IV) | <ul style="list-style-type: none"> · Efflux pumps · DNA gyrase-binding proteins · Mutations (DNA gyrase, topoisomerase IV) |
| Polypeptides (Actinomycin, Bacitracin, Polymyxin B) | Disruption of cell membrane structure and permeability Other (Axelsen, 2008) | <ul style="list-style-type: none"> · Altered membrane structure |
| Ionophores (Monensin, Nystatin, Gramicidin A) | Disruption of transmembrane ion concentration gradients | <ul style="list-style-type: none"> · Enzymatic (drug degradation) · Extracellular polysaccharides (excludes drug from cell membrane) |

† Penicillin-binding proteins (PBPs) are enzymes involved in the terminal stages of assembling the cell wall during growth and division.

‡ Dihydropteroate synthase catalyzes the condensation of p-aminobenzoic acid (PABA) and 7,8-dihydro-6-hydroxymethylpterin-pyrophosphate to form dihydropteroic acid.

tions, however, were unsuccessful (Woegerbauer et al., 2005). These collective reports supported the notion that antibiotic-producing strains were the original source of resistance genes to be later found in nonantibiotic producing bacteria.

Use of Phylogeny to Infer Evolutionary Origins of Antibiotic Resistance Genes

Molecular phylogeny is the comparative analysis of gene sequences, at the nucleotide or amino acid level, to gain information on genetic or protein evolutionary relationships. This analysis can be used to trace the evolutionary history of antibiotic resistance genes, and the organisms that host the genes. Such relationships have been investigated with genes encoding ribosomal protection proteins (RPPs) that function as alternative protein elongation factors and confer resistance to tetracyclines (Connell et al., 2002, 2003). A recent phylogenetic analysis suggested early branching events and independent lines of diversification of at least eight clusters of RPPs (Aminov et al., 2001). The divergence occurred well before the modern “antibiotic era” with

no indication of transfer of antibiotic resistance genes from antibiotic producing strains to pathogenic or commensal bacteria. A subsequent analysis with a larger database of sequences showed the monophyletic origin (i.e., descended from a single common ancestor) of the RPP genes, with an early branching event separating them from a separate group of elongation factors, EF-G, encoded by the *fusA* genes (Aminov and Mackie, 2007). Several incidences of closely-related species harboring distinctly different *tet* determinants were also evident. Several *tet* genes encoding RPPs, distinct from those found in known species, have been found in the environment but not correlated to known cultivars (Yu et al., 2005), demonstrating the potential for a wider range of RPP genetic diversity than what is currently known.

As another example of a phylogenetic approach, a similar analysis was conducted of the clinically relevant *erm* gene family. These genes encode enzymes that catalyze S-adenosyl-L-methionine-dependent methylation of a specific adenine residue in the 23S rRNA molecule, providing steric protection of the ribosomes from binding the macrolide, lincosamide, and streptogramin B classes of antibiotics, thereby preventing the inhibition of pro-

tein biosynthesis. Acquisition of these methylase genes confers bacterial resistance to erythromycin. The phylogeny of *erm* genes shows that the origin of this gene family is polyphyletic (i.e., having more than one line of evolution) (Aminov and Mackie, 2007) and, as with the RPP genes, there was no indication of gene exchange between antibiotic-producing strains and the majority of other commensal/pathogenic bacteria found with divergent *erm* genes. The more recent phylogenetic analyses of the RPP and *erm* gene families supported conclusions presented in studies that showed no evidence for transfer of antibiotic resistance genes from antibiotic producing bacteria to human- and animal-associated bacteria (Lau et al., 2004).

Phylogenetic analyses of antibiotic resistance genes also suggest a relatively recent time frame of rapid movement of antibiotic resistant genes between taxonomically divergent commensal and pathogenic bacteria. Tetracycline resistance genes like *tet(M)* can be found in both Gram-positive and Gram-negative bacteria and is known to be transferred by transposon *Tn916*, a common mobile genetic element. The *tet(W)* gene is virtually identical in a range of bacterial species (e.g., *Megasphaera elsdenii*, *Bifidobacterium longum*, *Roseburia hominis*, *Mitsuokella multacida*, and *Butyrivibrio fibrisolvens*) suggesting very recent lateral gene exchange events between bacteria from human, swine, and cattle digestive tracts (Kazimierczak et al., 2006; Barbosa et al., 1999). The precise genetic mechanisms responsible for the movement of *tet(W)* between the gut ecosystems of different species are presently unknown but presumably involves an unidentified mobile element. The vast majority of bacteria carrying *tet* genes are of intestinal or genital origin, and horizontal gene exchange between these, as well as transient bacteria, is considerable.

Lateral gene transfer of the *erm(B)* and *erm(C)* genes between Gram-positive and Gram-negative bacteria has been indicated (Aminov and Mackie, 2007). Analysis of the *erm* and *tet* genes in *Bacteroides* and other predominant intestinal bacteria suggested that these genes have been disseminated rapidly among human populations in hospitals and in the community over the past three decades (Shoemaker et al., 2001), which coincides with the “antibiotic era”. Molecular analysis of human, swine, and poultry *Enterococcus faecium* isolates and their *erm(B)* genes also suggests that horizontal exchange of antibiotic resistance genes is more important in dissemination of antibiotic resistance than direct transmission of resistant strains (De Leener et al., 2005).

Factors Contributing to Persistence and Dissemination of Antibiotic Resistance Genes

It has been generally thought that the maintenance of antibiotic resistance determinants imposes an additional metabolic cost on a bacterial cell and that resistance genes will be eliminated from the population once the selective pressure is removed. Increased incidences of antibiotic resistant bacteria are clearly evident, however, simply removing the selection pressure does not reverse the occurrence (Salysers and Amabile-Cuevas, 1997). A number of studies have shown adaptations by bacteria to ameliorate the metabolic

cost to maintain drug resistance (Enne et al., 2005; Ramadhan and Hegedus, 2005; Lenski, 1997). Moreover, the acquisition of an antibiotic resistance genotype may actually increase the fitness of certain bacteria in the absence of antibiotic selective pressure, possibly allowing rapid emergence and dissemination of antibiotic resistance on a worldwide scale (Luo et al., 2005; Enne et al., 2004). The amelioration of the fitness cost required to maintain antibiotic resistance may be one of the reasons why, for example, antibiotic resistance genes persist in wild animals in the absence of selection imposed by the presence of antibiotics (Gilliver et al., 1999). Antibiotic resistance can then be viewed as a self-perpetuating process, where the antibiotic-susceptible genotype becomes replaced by resistant genotypes in the absence of any antibiotic selective pressure. Thus, the release of antibiotic resistance genes into the environment is thought to be a critical control point. Areas with historically low levels of agricultural antibiotic use had lower frequencies of antibiotic resistance genes (Osterblad et al., 1995).

Another consequence of prolonged antibiotic usage could be selection of novel genetic variants or recombinants that may confer higher minimum inhibitory concentrations (MICs). Mosaic recombinant *tet(O/W/O)* variants were first isolated from *Megasphaera elsdenii* from swine (Stanton and Humphrey, 2003). The previously characterized *tet(32)* gene from a human commensal *Clostridium* sp. was also shown to be a mosaic recombinant, *tet(O/32/O)* (Stanton et al., 2005). Analysis of the published *tet(O)* gene sequence from *Campylobacter coli* suggested that this gene resulted from a double-crossover recombination and should be described as a *tet(O/M/O)* (Batchelor et al., 2004). The recombinant variants of the gene appeared to have higher MICs for tetracycline, which might explain the selection and persistence of these mosaic recombinants (Stanton et al., 2004). Thus, commensal soil bacteria may serve as both sinks and sources for existing and new variations of antibiotic resistance genes.

While the functional role of antibiotic resistance genes in antibiotic-producing bacteria is obvious (self-protection against the antibiotics synthesized), the presence and function of these genes in bacteria from other ecological niches is not as evident. Numerous incidences of antibiotic resistance genes in presumably antibiotic-free environments suggest other driving factors in place to maintain these functional genes within cells. One plausible explanation for harboring these genes may be attributed to other metabolic housekeeping functions before the need specifically for antibiotic resistance mechanisms. Certainly, in environments with no history of drug exposure, low levels of a natural genetic pool of antibiotic resistance genes may exist. These genes may persist, conferring a selective advantage for the host cell in naturally competitive environments such as soils, or co-selection resulted in resistance genes mobilizing along with genes for other selection pressures (Aminov and Mackie, 2007).

More recent studies have shed light on the role of small redox-active antibiotics, like phenazines, which are produced in the environment by many species of *Pseudomonas*, and serve a possible number of functions, including roles as electron shuttles in bacterial mineral reduction pathways for energy (Stams et al., 2006; Hernandez et al., 2004; Hernandez and Newman, 2001). The glycopeptide bleomycin was also found to stimulate dissimi-

latory iron-reduction in *Shewanella oneidensis* MR1 (Hernandez et al., 2004). The role of efflux pump mechanisms involved in protection of cells from antibiotics are thought to be required to mitigate the toxic effects of overaccumulation of the humic acid analog electron shuttle, anthraquinone-2,6-disulfonate (AQDS) (Shyu et al., 2002). AQDS, like other electron-shuttling compounds, has structural similarities to aromatic antibiotics like tetracycline, doxorubicin, and the phenazine pyocyanin (Hernandez and Newman, 2001). These lines of study suggests natural antibiotics produced in soils by many bacteria have likely evolved with bacterial cell metabolic functions, and suggests resistance genes have also evolved accordingly. The further effects on natural microbial functions from the added presence of antibiotics and resistance genes due to agricultural inputs are not known and can only be hypothesized.

Co-selection of Antibiotic Resistance Genes

Many antibiotic resistance genes reside on large self-transmissible genetic elements capable of carrying multiple genes, including those encoding antibiotic-, heavy metal-, and biocide-resistances. The physical link between the conjugative class of R plasmids and resistances to heavy metals was reported almost 40 yr ago (Smith, 1967). Ampicillin- and mercury-resistant *Bacillus* strains were found nearly six times more frequent in sediment with a long history of heavy metal exposure (Timoney et al., 1978). The genetic linkage between antibiotic resistance and mercury resistance in enterobacteria was shown to involve the genetic element *Tn21*, which encodes a mercury-resistance operon, transposition functions, and resistances to streptomycin/spectinomycin and sulphonamides (Liebert et al., 1999). The resistance genes are carried in bacteria on an integron, which is a portable, transferable, multi-gene cassette. Such mechanisms of co-selection suggest the potential for a single transfer event to result in multiple resistance acquisition by a bacterium. It has further been shown that cellular stress can induce the mobility of transposons and insertion sequences (Ilves et al., 2001; Levy et al., 1993). The DNA-damaging agents such as the antibiotic class of fluoroquinolones induce a major bacterial stress response mechanism, in turn causing an increase in the rate of horizontal transfer of antibiotic resistance genes by more than 300-fold (Beaber et al., 2004). Stress response-inducing antibiotics may also co-select for other antibiotic resistance genes that are physically linked on mobile genetic elements (Hastings et al., 2004).

Summary

The impacts resulting from agricultural use of antibiotics and the practice of land application of animal waste on environmental quality and health risk potential is not completely clear, albeit there are demonstrated links to increased and accelerated incidences of antibiotic resistant bacteria. The phenomenon, however, is not a simple relationship of cause and effect. What is evident is the myriad complexity of abiotic and biological mechanisms, and the ecological interactions that can occur at numerous points along the course of antibiotic use and disposal of livestock waste in soil environments, beginning

with entry of antibiotics into animal gut systems. Regulatory aspects related to continued use of land application for waste management in animal production has real current concerns for nutrient (N, P) loads in soil; the practical impact of loading antibiotic residues and resistance genes is not yet known. The collective examination of specific mechanisms that affect the fate of compounds, microorganisms, and the genetic pool (Fig. 1), will provide a better understanding of the true impacts of land application of effluent, as well as the general nature of the microbial and molecular ecology of antibiotic resistances.

Field information on the fate and transport of antibiotics is still limited, but in general, low amounts have been detected in soil and water environments, including the presence of breakdown metabolites. Predictive measures for solutes and bacteria transport in soil and water have relied on existing models, which do not adequately predict contamination, and indicate a clear need for a larger database to develop and to better inform models. The physicochemical characteristics of the soil environment are likely to influence compound persistence, bacterial survival, and genetic mechanisms at work. Trace amounts of antibiotics or other compounds (e.g., heavy metals) could act as selection pressures for maintenance and (co-) transfer of antibiotic resistance genes.

While the half-lives of antibiotics in manure are relatively short, it remains possible that drug residues may exert effects on biological functions within bacterial populations present in soils. Studies have shown application of animal manure to soil can readily lead to groundwater contamination with fecal bacteria. The acquisition of antibiotic resistances, however, appears to span a diverse phylogenetic range of bacteria, including those native to soil and water environments. Phylogenetic analyses of genes involved in tetracycline and erythromycin resistances demonstrate the evolution of these genes over time, and suggest that obtaining resistance genes from antibiotic-producing bacteria is not a major mechanism of resistance acquisition evident in a broad range of bacteria. Resistance genes have been maintained in bacteria before the modern antibiotic era, even though the origin and purpose of these genes is not yet clear. The exact mechanisms contributing to antibiotic resistance gene acquisition and maintenance in natural environments are not yet well established, however, an increasing number of studies support lateral gene transfer events. Acquisition of antibiotic resistances through mechanisms of selective mutations and lateral gene transfer may be acting in concert with other natural mechanisms of genetic adaptation among a diverse range of bacteria in natural soil and water environments.

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