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ANTIBIOTIC RESISTANCE IN SWINE-MANURE-IMPACTED ENVIRONMENTS

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11.1 INTRODUCTION

An increasing body of evidence demonstrating entry of antibiotics and antibiotic resistance genes from anthropogenic sources into natural soil and water environments has raised even more questions about the lasting and future impacts consequent to drug resistance development in bacteria. These concerns stem, in part, from emerging knowledge about increased incidences, persistence, and diversity of antibiotic resistance (ABR) genes in soil and water environments with still very limited knowledge about the molecular microbial ecology of ABR occurring in situ in these natural systems. The practice of subtherapeutic doses of antibiotics for use in disease prophylaxis and growth promotion in animal livestock production has been the subject of particularly intense scrutiny, prompting bans of such uses completely in the European Union since 2006 and causing mounting concerns in the United State for more judicious use of antibiotics in food animals. Numerous articles over the last two decades have discussed in detail the various health and environmental aspects concerning the routine use of subtherapeutic antibiotic treatment in animal production (Gustafson and Bowen, 1997; Khachatourians, 1998; USGAO, 1999; Isaacson and Torrence, 2002; Séveno et al., 2002; Kümmerer, 2004; Shea, 2004; Chee-Sanford et al., 2009).

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It has been well documented that the occurrence of ABR bacteria increases in environments where antibiotics are in frequent use such as clinical settings (Gomez-Lus, 1998; Poole and Srikumar, 2001; Walsh 2003) and in animal production or veterinary settings (Witte, 2001; McEwen and Fedorka-Cray, 2002). More than one-half of all antibiotics produced in the United States are used in animal production and up to 80% are estimated for use in growth promotion (Levy, 1998; Hileman, 2001; Graham et al., 2007). It is generally acknowledged that the widespread use of antibiotics in this manner selects for antibiotic-resistant bacteria in enteric systems (Haack and Andrews, 2000), often occurring first in commensals and later in pathogens if present (Sørum and Sunde, 2001; Salyers et al., 2004). The high bacterial load in the excreted manure is a consequent source for ABR bacteria and their resident ABR genes. Estimates of 30–90% of orally administered antibiotics may also be excreted unchanged (Elmund et al., 1971; Halling-Sørensen et al., 1998; Boxall et al., 2004; Chander et al., 2005), posing further potential for drug resistance selection to occur among the resident bacteria in environmental compartments downstream of the animal gut.

Since before the mid-1970s, livestock farms were small, integrated production systems that efficiently managed animal waste onsite, often by applying manure to cropland owned by the farmers. The shift toward fewer but large (thousands of head) concentrated animal feeding operations (CAFOs) precluded such management practices and necessitated a means by which to locally dispose of high quantities of manure year-round. The current most common method to dispose of swine and feedlot cattle waste in the United States is collection in storage holds such as lagoons or pits, followed by land application of the manure effluent to adjacent crop fields that are often leased to provide sufficient land area to support the waste loads. The manure not only introduces high bacterial and chemical loads to the soil, the changes in chemical (Siddique and Robinson 2003; Plaza et al. 2006), biological (de Freitas et al., 2003; Sun et al., 2004; Wienhold, 2005; Hartmann et al., 2006), and physical (Wienhold 2005) soil properties may act to affect microbial populations and their consequent activities (Frostegard et al., 1997; Sun et al., 2004; Wienhold, 2005; Hartmann et al., 2006). In concert, the changes in the soil factors may have various effects on the microbial and molecular ecology (i.e., molecular phylogenetics) specifically associated with antibiotic resistances.

11.2 DETECTION OF ABR GENES AND BACTERIA IN NATURAL ENVIRONMENTS

It has been well documented over the years that many microorganisms of enteric origin survive the transition from effluent pit or lagoon into soil (Kibbey et al., 1978; Chandler et al., 1981; Stoddard et al., 1998; Bolton et al., 1999; Lee and Stotzky, 1999; Jiang et al., 2002; Guan and Holley, 2003; Boes et al., 2005). Their survival, together with the notion that many of these bacteria may also harbor ABR genes, had generated increased interest in environmental epidemiological tracking of ABR bacteria to assess the possibility of reentry to human and animal populations. Moreover, the extent of genetic transfer and acquisition of ABR genes in these natural environments remains unclear. The role of native soil and water microbial populations in the overall molecular ecology of ABR genes remains largely unknown.

The advent of molecular-based methods based on ABR genetic sequences has resulted in numerous reports of ABR genes in manure-impacted soil and water environments. Numerous independent studies have reported ABR gene detection associated specifically with swine production facilities, where 88% of U.S. swine producers routinely administer antibiotics in feed to grower/finisher pigs (USDA, 2001). The macrolide antibiotic tylosin is exclusively used in swine livestock along with tetracyclines, hence tetracycline resistance (tet^R) and tylosin resistance (erm^R) genes presented useful targets in the evaluation of swine-manure-impacted environments. Detection based on polymerase chain reaction (PCR) has been used to reveal mobility, persistence, and distribution of tet^R and erm^R genes in swine manure, swine-manure-applied soils, and groundwater underlying and down gradient from swine waste lagoons (Aminov et al., 2001, 2002; Chee-Sanford et al., 2001; Hund-Rinke et al., 2004; Schmitt et al., 2006; Smith et al., 2004; Koike et al., 2009). While these methods yield important data that aid in detection, tracking, and quantifying specific determinants within the genetic pool of a environmental sample's metapopulation without the need to culture, the methods rely on sequences from known genes and allow only limited interpretation about the exact mechanisms of how these ABR genes came to be and the identities of the populations harboring them. Further, the functional expression of the ABR genes that are detected with these methods is not readily determined in the natural environments where they were harbored.

Cultivation-based approaches have yielded isolates of ABR bacteria grown under antibiotic selection (Cotta et al., 2003; D'Costa et al., 2006; Onan and LaPara, 2003) and provide a limited means of assessing genetic expression of ABR genes in environmental samples. However, practical considerations usually do not allow exhaustive strategies to be undertaken to obtain growth or to use wide-range testing of drug concentrations in selection. The approach is further hampered by the lack of knowledge and measurement of the biologically active state of drug residues in manure-impacted soil and water environments. Consequently, it is unknown what may elicit a true drug resistance response from a bacterium in these environments where concentrations of antibiotics at clinical dosages may not be ecologically relevant, yet are often selected as the starting point for laboratory cultivation-based strategies. This is particularly challenging in natural soil and water environments, where most bacterial species have not been cultivated, knowledge of the biological activity exerted by exogenous drug residues is lacking, and the nature and extent of the interactions involving background biotic and abiotic factors is virtually unknown under current technology.

Several reports employing a combinatorial approach including both culture- and molecular-based detection, were used to examine a limited range of bacterial species (Schmidt et al., 2001; Jensen et al., 2002; Miranda et al., 2003; Kim et al., 2004; Agersø and Sandvang, 2005; Nikolakopoulou et al., 2005). This type of approach provides not only information about the species harboring the drug resistance trait but also confirmed the phenotypic expression of the activity. The genetic sequence information obtained from individual cultivated strains provided some ability to analyze phylogenetic relationships, with clues toward the possible origin and flow of the ABR determinant.

An interesting study that characterized the corresponding tet^R determinants in a collection of chlortetracycline-resistant soil bacteria isolated from several

manure-impacted soils and nonagricultural soils represented, to date, one of the only such studies that involved a relatively diverse set of nonenteric bacteria (Ghosh and LaPara, 2007). The collection encompassed several bacterial phyla with members representing a variety of commonly associated soil species, including multiple isolates in the genera *Bacillus, Streptomyces, Variovorax, Chryseobacterium*, and *Pseudomonas*. Of the 14 genes targeted, the most common harbored overall were the efflux determinants *tet*(L) and *tet*(A), with several instances of two tet^R determinants detected within an isolate. The commonality of the tet^R determinants detected among the phylogenetically distinct isolates suggested that this was a shared trait quite possibly acquired by lateral transfer, and more prominent in soils following manure exposure from swine production farms with histories of antibiotic usage. It is noteworthy in this study that regardless of the source of the soil, isolates of *Streptomyces*, a genus made up of several known species that produce tetracyclines, did not harbor any of the tet^R determinants that were tested. The results may not have precluded other sources of tet^R besides manure, however, finding relatively diverse bacteria harboring homologous genes did suggest the potential for genetic mobility over a wide range of bacteria within the soil compartment. The study also presented some interesting notions regarding the frequency of some tet^R determinants over others in diverse bacteria, for example, *tet*(L) and *tet*(A), genes specifically encoding efflux mechanisms. More insight into the predominant tet^R determinants along with the genetic structure in conjunction with genes such as plasmid carriers, transposon elements, or gene cassettes would be quite valuable. Increasing the number of similar studies that assess a wide range of bacteria, including commensal species of both enteric and soil origins, and their corresponding ABR determinants would nevertheless provide a larger basis by which to more accurately evaluate the dynamics of ABR genes in natural environments.

11.3 CASE STUDY: tet^R AND erm^R GENE DISTRIBUTION IN BACTERIA ISOLATED FROM SWINE MANURE PITS AND SOILS WITH AND WITHOUT HISTORY OF MANURE EXPOSURE

To further examine the tet^R and erm^R genes, the frequency of determinants harbored by bacteria, and the possible links of these genes to specific phylogenetic groups, the sites of two large swine production farms were investigated. The approach used was a cultivation- and PCR-based analysis of bacteria isolated from the manure holding pits and soil from adjacent crop fields with long histories (25 years or more) of land-applied manure originating from their respective sites. Upslope of the manure-applied fields, soils with no known exposure to swine manure were used to assess bacterial populations with ABR genes that could be attributed to resistances acquired under similar edaphic factors, excluding manure influences. Bacterial isolates were grown both aerobically and anaerobically on MR2A agar (Fries et al., 1994) under either tetracycline or tylosin selection (both 20 mg/L) from the pits and soils. Each isolate was analyzed for the presence of tet^R determinants conferring tetracycline resistance mechanisms of efflux [*tet* (B), (C), (D), (E), (G), (H), (J), (S), (T), (Y), and (Z)], ribosomal protection [*tet* (M), (O), (Q), and (W)], and the macrolide resistance genes *erm* (B), (F), (G), and (Q) using methods described previously (Aminov et al., 2001, 2002; Koike et al., 2009).

11.3.1 Phylogenetic Distribution of Tetracycline- and Tylosin-Resistant Bacterial Isolates

In total, 124 distinct isolates were identified on the basis of nearly full-length 16S ribosomal ribonucleic acid (rRNa) gene sequences and analyzed for their corresponding suite of tet^R and erm^R determinants These isolates phenotypically expressed either tetracycline or tylosin resistance and were presumed to represent only a fraction of the ABR bacterial population present. Figures 11.1–11.4 show the results of the identifications based on nearly complete ($\sim$1500 bp) 16S rRNA gene sequences according to the closest genera matched using the national center for biotechnology information (NCBI) GenBank BLAST series of tools (Madden et al., 1996). The following denotes the identities and characteristics of each isolate: genus and unique strain designation, tetracycline (TcR) or tylosin (TIR) resistance phenotype, tet^R and erm^R genotype (if detected), and GenBank accession number. The sources of the isolates are denoted by black (pit), white (nonmanured soil), and gray (manured soil) text. Posterior probabilities of clades (shown as node labels) were calculated using a Bayesian Markov chain Monte Carlo method with the MrBayes v.3.1.1 program (http://mrbayes.csit.fsu.edu/index.php). The scale bar shows expected changes per site.

Overall, the isolates obtained comprised four major bacterial phyla, including Actinobacteria, Firmicutes, Bacteroidetes, and the α, β, and γ subclasses of the Proteobacteria (Fig 11.1-11.4), generally consistent with the phylogenetic distributions of chlortetracycline-resistant strains found in the study by Ghosh and LaPara (2007). Recurrence of these phyla is, at least in part, a function of similar cultivation strategies and bias, although many of these species are prevalent in natural soil environments and thus, could comprise a significant reservoir of the ABR genes being circulated.

Under the growth selection conditions employed, the manure pit environments were dominated by species of Firmicutes (Fig. 11.2), followed by the majority of Actinobacteria isolated (Fig.11.1). Of the Proteobacteria, most of the species originating from the pits were represented in the γ subclass, with several enteric species recovered as represented by such common species as *Escherichia coli*, *Shigella* sp., *Enterobacter* sp., and *Serratia* sp. (Fig. 11.4). The survivability in the environment of species such as *E. coli* and the common enteric Firmicutes *Enterococcus* has been well studied (Davies et al., 1995; Cools et al., 2001; Hartz et al., 2008), raising concerns that pathogenic strains of these species could circulate back to human and animal populations, along with exacerbating concerns that ABR traits have been found in these types of bacterial contaminants. The isolates from pits also comprised a large number of disparate species native to many natural soil and water environments, such as *Rhodococcus* and *Microbacterium*. Isolates from both untreated and manure-applied soils were largely comprised of a wide range of Proteobacteria (Fig. 11.1, 11.3, and 11.4), including many species that are frequently and easily cultivated from soil.

As possible vectors for the persistence and circulation of ABR genes, natural bacterial residents of soil may be highly significant due to their adaptive traits and probable growth in soil environments. Not unexpectedly, the relative higher diversity of ABR species was indeed obtained from environments where manure exposure occurred (pits and manured soils), but it is yet unknown if any existing concentrations of antibiotics may play a mechanistic role in drug resistance selection in these types of environments.

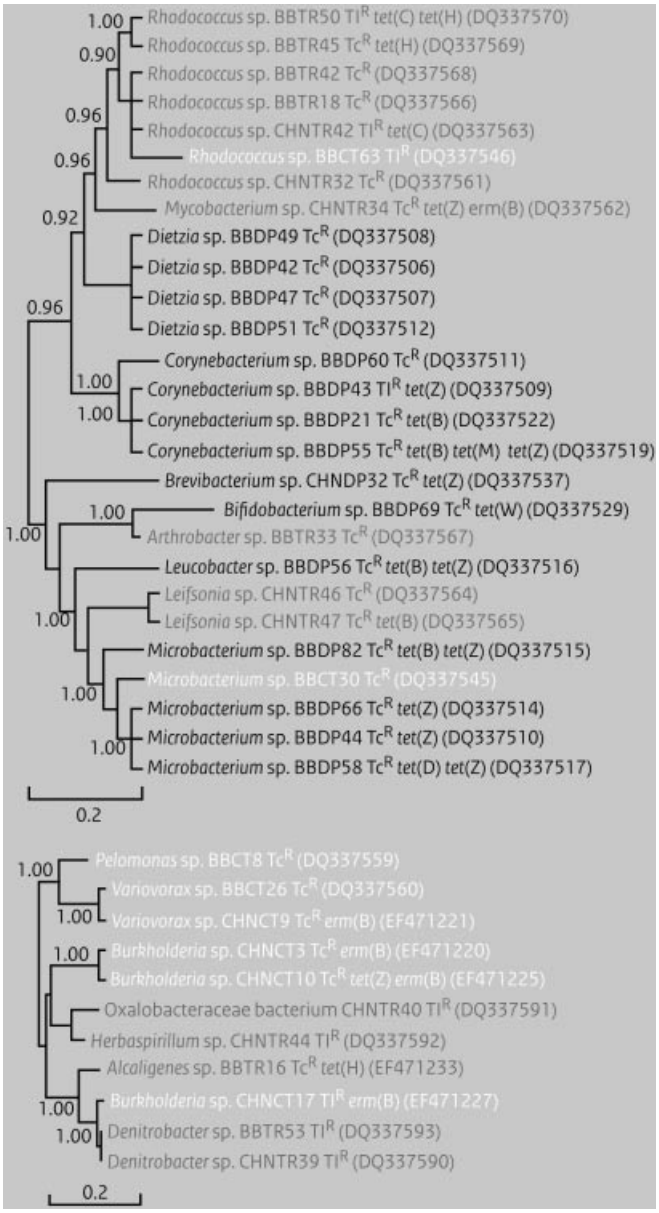

FIGURE 11.1 Phylogenetic relationships of tetracycline- or tylosin-resistant bacterial isolates belonging to the phylum Actinobacteria (upper image) and to the β subgroup of the phylum Proteobacteria (lower image) cultivated from manure holding pits and soils from two commercial swine production farms.

11.3.2 Distribution of *tet*^{R} and *erm*^{R} Genes among Isolates

The majority of the ABR genes targeted, namely *tet* (B), (C), (D), (E), (G), (H), (J), (M), (O), (Q), (W), (Y), (Z) and *erm* (B), (F), (G), were detected among the isolates, but numerous environmental species appeared to harbor unknown mechanisms of

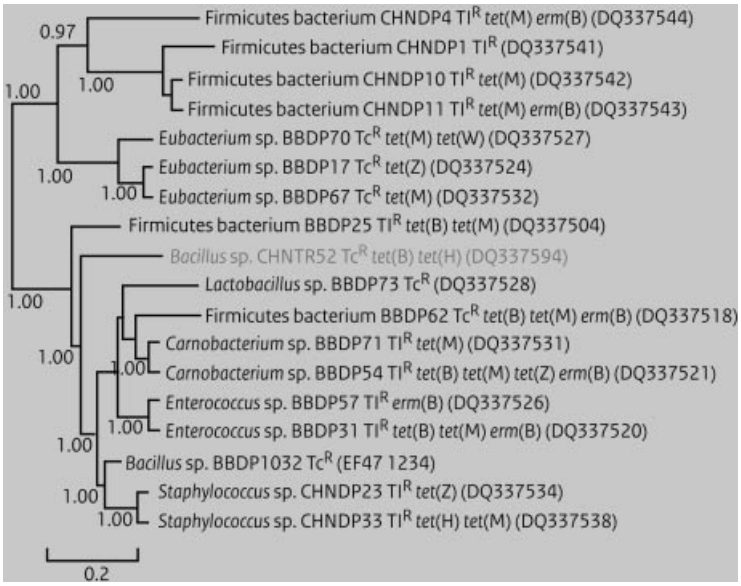

FIGURE 11.2 Phylogenetic relationships of tetracycline- or tylosin-resistant bacterial isolates belonging to the phylum Firmicutes cultivated from manure holding pits and soils from two commercial swine production farms.

tetracycline and tylosin resistances. Regardless of the history of manure exposure, 55–67% of the soil-derived isolates that exhibited either tetracycline- or tylosin-resistant phenotypes did not harbor any targeted tet^R or erm^R genes. These unknown determinants may comprise other genes found previously in other studies, such as *tet* (A), (K), (L), or [A(P)] but may also include novel resistance genes. Contrastingly, in pit environments, 72% of isolates selected on tetracycline and 93% of the isolates selected on tylosin did harbor known tet^R determinants that included *tet* (B), (D), (H), (M), (Q), (W), (Z). Pit-derived isolates, however, more frequently harbored multiple known tet^R genes, in some instances combinations of three or four different genes conferring both efflux and ribosomal protection mechanisms.

While incidences of multiple ABR strains within a single genus were isolated, also noteworthy were the different resistance determinants found harbored by closely related strains within a group. For example, *tet* (B), (G), and (H) were detected as individual determinants in closely related strains of the α-proteobacterium *Ochrobactrum* along with one additional strain harboring an unknown tet^R determinant (Fig. 11.3). The occurrence of different determinants suggested divergent processes leading to the circulation of these tet^R genes within this group.

Within the Actinobacteria, the three genera *Dietzia*, *Corynebacterium*, and *Microbacterium* appeared to comprise the main reservoir of ABR genes in the pits, harboring primarily *tet* (B) or *te*t (Z), with isolates expressing either tetracycline or tylosin resistances (Fig. 11.1). Aerobic coryneforms can be encountered in various ecosystems; in the human skin they harbor a considerable reservoir of tetracycline and erythromycin resistance determinants (Eady et al., 2000). Representatives of the other two genera, *Microbacterium* and *Dietzia*, are mostly isolated from environments such as soil and water, although the latter has been increasingly recognized as

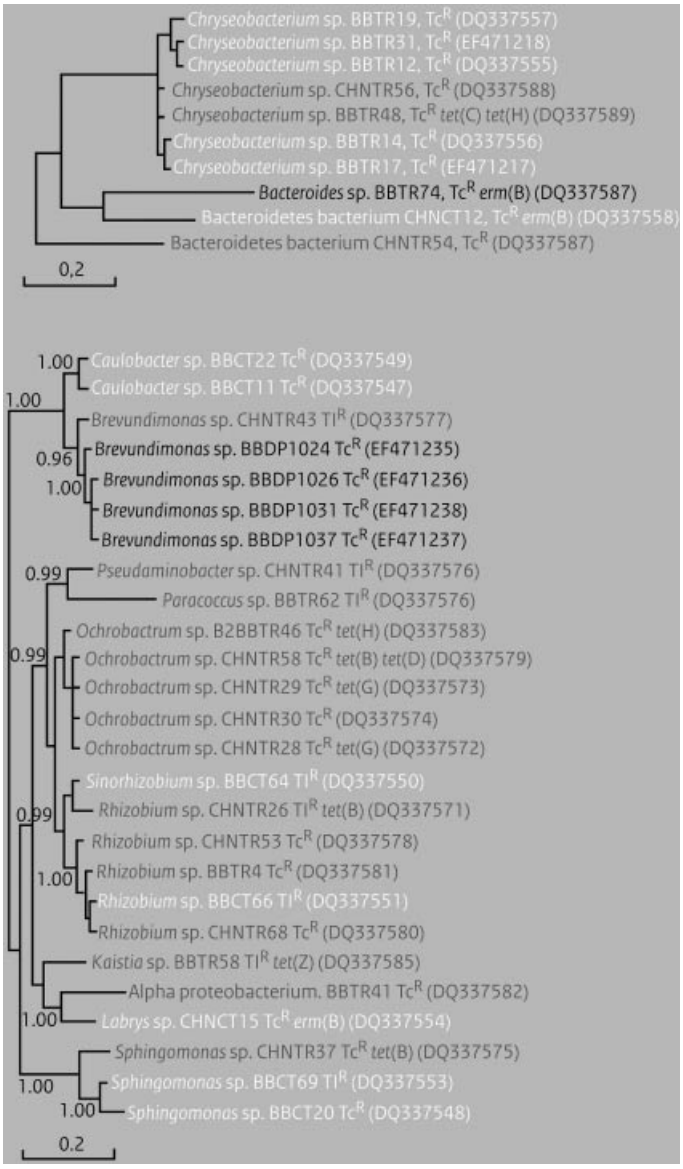

FIGURE 11.3 Phylogenetic relationships of tetracycline- or tylosin-resistant bacterial isolates belonging to the phylum Bacteroidetes (upper image) and the α subgroup of the phylum Proteobacteria (lower image) cultivated from manure holding pits and soils from two commercial swine production farms.

a potential human pathogen (Koerner et al., 2009). Very little is known about antibiotic resistance traits in these two genera, and these results are the first to describe tetracycline resistance determinants circulating in these bacteria.

In manured soil, the genus *Rhodococcus* dominated the isolates, with different strains exhibiting a variety of tet^R determinants (Fig. 11.1). It is interesting to note that *Rhodococcus*, common to soil and water environments, are known for their large

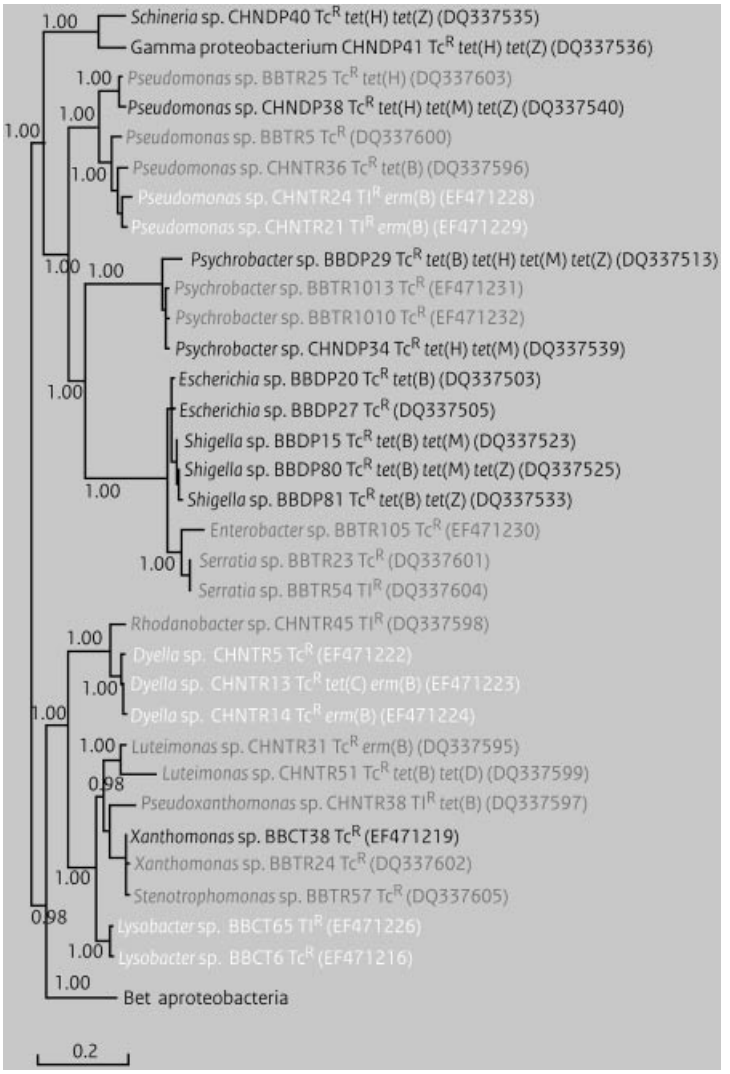

FIGURE 11.4 Phylogenetic relationships of tetracycline- or tylosin-resistant bacterial isolates belonging to γ subgroup of the phylum Proteobacteria cultivated from manure holding pits and soils from two commercial swine production farms.

genome size (e.g., *R. jostii* 9.7 Mb) and the presence of megaplasmids that carry large sets of genes (Martínková et al., 2009). Known strains of *Rhodococcus* can undergo a high frequency of recombination that confer new genes and metabolic adaptability, and, if the case is so with antibiotic resistance genes, this may be consistent with findings of multiple types of ABR determinants in these *Rhodococcus* isolates. The ABR genes, however, may not be readily expressed under laboratory conditions or confer resistance to high antibiotic concentrations tested (see, e.g., the tet^R genotype and phenotype of *Rhodococcus* sp. CHNTR42 in Fig. 11.1 and Table 11.1), but it cannot be excluded that they may do so in natural environments with modest antibiotic concentrations.

TABLE 11.1 Antibiotic Resistance Phenotypes[a] of Bacterial Isolates Cultivated from Swine Manure Pits, Manured-Soil, and Soil with No Known History of Manure Exposure

			Antibiotic[b]											
Isolate	Source[c]	Original Drug Selection	Tet	ChlTet	Cmp	Gent	Amp	Tyl	Ery	Bac	Pen	Van	Strp	Kan
Chryseobacterium sp. BBCT12	NMS	Tet	+	+	+	+	−	+	+	+	+	−	+	+
Chryseobacterium sp. BBTR48	MS	Tet	+	+	+	−	−	−	+	−	−	−	−	−
Burkholderia sp. CHNCT3	NMS	Tet	+	+	+	+	+	+	+	+	+	+	+	+
Burkholderia sp. CHNCT10	NMS	Tet	+	+	+/−	+	+	+	+	+	+	+	+	+
Serratia sp. BBTR23	MS	Tet	+	+	+	−	+	+	+	+	+	−	−	−
Ochrobactrum sp. CHNTR58	MS	Tet	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas sp. CHNTR36	MS	Tyl	+	+	+	−	+	+	+	+	+	+	+	+
Rhodococcus sp. CHNTR42	MS	Tyl	−	−	−	−	−	+	−	−	−	−	−	−
Leucobacter sp. BBDP56	PIT	Tet	+	+	−	−	−	+	−	−	−	−	−	+
Corynebacterium sp. BBDP55	PIT	Tet	+	+	−	−	−	+	+	−	−	−	+	−
Enterococcus sp. BBDP31	PIT	Tet	+	+	+	+	−	+	+	+	−	−	+	+
Staphylococcus sp. CHNDP33	PIT	Tet	+	+	−	−	−	−	+	+	−	−	+/−	−
Escherichia sp. BBDP20	PIT	Tyl	+	+	+/−	+/−	+	+	+	+	+	+	+/−	+
Psychrobacter sp. BBDP29	PIT	Tet	+	+	−	−	+	+	−	+	−	−	+	+
Shigella sp. BBDP80	PIT	Tet	+	+	−	−	−	+	−	−	−	+/−	−	−

[a] Bacterial isolates were tested on MR2A agar medium amended with 20 mg/L antibiotic compound. Growth in the presence of antibiotic was scored as (+) indicating resistance, (−) indicating non-resistance, and (+/−) indicating slight growth relative to growth on MR2A agar medium without antibiotic addition.

[b] Tetracycline (Tet), chlortetracycline (ChlTet), chloramphenicol (Cmp), gentamycin (Gent), ampicillin (Amp), tylosin (Tyl), erythromycin (Ery), bacitracin (Bac), penicillin (Pen), vancomycin (Van), streptomycin (Strp), and kanamycin (Kan).

[c] Isolate was originally obtained by growth under either tetracycline or tylosin selection from nonmanured soil (NMS), manured soil (MS), swine waste holding pit (PIT).

Within the pit environments, the reservoirs of *tet*^{R} genes resided primarily in isolates belonging to the Firmicutes phylum, including isolates of enteric origins such as *Lactobacillus*, *Eubacterium, Carnobacterium*, and *Enterococcus* (Fig. 11.2). Their remarkable survivability outside of the gut environment in pits may contribute to further dissemination of ABR genes preselected and amplified in antibiotic-fed animals. The Tn*916* family of mobile genetic elements is found widely among many commensal and pathogenic bacteria, and primarily found in many Firmicutes (Roberts and Mullany, 2009). These elements contain *tet*(M) but may often include other accessory genes and are thought to be important vectors in movement of genetic traits among many bacteria. Not only do the elements facilitate horizontal gene transfer, they also cause genome rearrangements that may lead to natural selection and gene evolution events. Another important factor contributing to these genetic processes may be the residual tetracycline in pits, which can enhance transposon-mediated conjugal transfer (Showsh and Andrews, 1992). Thus, Firmicutes may be a key bacterial group with members that facilitate the acquisition of ABR traits among both commensals and pathogens in a variety of environments.

The *tet*^{R} gene pool residing within the Bacteroidetes may likely be comprised in part of unknown genes represented in the cultivated *Chryseobacterium* spp. (Fig. 11.3). These unknown *tet*^{R} determinants may include one or more naturally occurring genes already present in the background bacterial populations, where the influence of manure may not be the dominant factor in the selection of the tetracycline resistance trait. This is supported by a broader range of antibiotic resistances found in *Chryseobacterium* sp. BBCT12 from a non-manured soil compared to the strain BBTR48 from manured soil (Table 11.1). In contrast, the *Bacteroides* sp. BBDP74 isolated from a manure pit harbored multiple ribosomal protection genes such as *tet* (M), (Q), (W), which would be consistent with the consequences of higher selective pressure within its origin in the gastrointestinal system and in the concentrated pit environment. During the original cultivation selection, none of the isolates from this phylum cross selected on tylosin, albeit *erm*(B) was detectable in two isolates (Fig.11.3). Also, *Chryseobacterium* sp. BBCT12 exhibited tylosin resistance in subsequent phenotypic testing but did not harbor any of the *erm*^{R} genes used in our study.

A wide range of α-Proteobacteria representatives was mostly isolated from both manure-treated and untreated soils (Fig. 11.3). Similar α-Proteobacteria were reported by Ghosh and LaPara (2007). The only genus recovered from pit samples was *Brevundimonas*, with multiple strains harboring a diversity of *tet*^{R} and *erm*^{R} genes beyond the range covered by our set of 19 primers. The *Caulobacter* and *Sphingomonas* isolates from non-manured soils also did not produce any PCR amplified signals with the primer set in use, suggesting that these strains may contain naturally occurring reservoirs of novel tetracycline and tylosin resistance genes. The occurrence of several isolates of *Ochrobactrum* and *Rhizobium* from the manure-treated soil might represent indigenous soil strains with ABR acquisition potential, harboring a variety of ABR determinants. Species of *Ochrobactrum* are found associated with the rhizosphere but may also be important opportunistic human pathogens (Berg et al., 2005a). Multiple antibiotic resistances have been found in rhizosphere isolates of this genus, where the localized environment of the plant root often hosts high microbial abundances and contain the presence of diverse

antibiotics produced by root-associated microbes. A species of *Ochrobactrum* obtained from manured soil in this study demonstrated phenotypic resistances to 12 antibiotics representing 7 drug classes (Table 11.1).

Rhizobia, in particular, are known for housing large plasmids (100 kb to 2 Mb) that encode genes of nitrogen fixation along with other genes necessary for symbiotic relationships with plants. The conjugal transfer of these large plasmids is thought to play a major role in the evolution of rhizobia (Ding and Hynes, 2009). Isolates of the genus *Rhizobium*, with the exception of one strain, did not contain the known tet^R or erm^R genes tested, yet represented one of the more frequent strains recovered in our study (Fig. 11.3). Enhanced genetic transfer rates and competition for resources contribute to the development of high levels of natural resistances found in the rhizosphere environment, and one can also speculate that adding exogenous sources of ABR genes and drug residues via manure inputs would create an even more conducive set of conditions for ABR traits to proliferate.

Concerns for soil bacteria emerging as opportunistic human pathogens along with their selection and acquisition of ABR genes have highlighted the need to better understand the ecological links that contribute to the evolution and dynamics of circulating ABR reservoirs. Such opportunistic pathogens are distributed over a wide phylogenetic range. Within the β-Proteobacteria, the genus *Burkholderia* is also thought to comprise some species of opportunistic pathogens found in the rhizosphere. Two *Burkholderia* isolates were recovered from nonmanured soil under tetracycline selection, with both strains found to harbor *erm*(B), but only one harbored a known tet^R determinant, *tet* (Z) (Fig. 11.1), suggesting a largely unknown set of determinants may comprise the natural ABR reservoir circulating among this particular genus. Many Gram-negative bacteria are intrinsically resistant to hydrophobic macrolides such as tylosin (Nikaido, 1996), and thus a physiological basis may also be a major mechanism conferring drug resistance for many of the isolates obtained in the absence of known erm^R genes. Further, however, the two strains of *Burkholderia* (CHNCT3 and CHNCT10) from nonmanured soils with no known antibiotic exposure, were also phenotypically resistant to antibiotics representing five other drug classes in addition to tetracyclines and macrolides (Table 11.1), thus suggesting the existence of a profound natural diversity of ABR genes circulating among this bacterial group.

The largest diversity of tetracycline- and tylosin-resistant bacteria in terms of genera and species isolated was from the γ-Proteobacteria (Fig. 11.4). Accordingly, these representatives were almost uniformly recovered from all three ecological niches sampled, ranging from the enterobacteria in pits to *Pseudomonas, Dyella*, and *Lysobacter* in nonmanured soils. The genus *Pseudomonas* was unique in that the species were isolated from all three ecological compartments. *Pseudomonas aeruginosa* is a well-studied example of a versatile bacterium encountered in many environments, as well as a recognized opportunistic pathogen, exhibiting very unique high intrinsic resistance to many antibiotics. The intrinsic resistance of *P. aeruginosa* has been attributed to an unusually impermeable outer membrane, along with the concerted action of several basic physiological genes and mechanisms of multidrug efflux pumps (Normark and Normark, 2002; Fajardo et al., 2008). Homologs of the efflux pump mechanisms in *P. aeruginosa* have been found in species of *Burkholderia* and *Stenotrophomonas maltophilia*, the latter also belonging to the γ-Proteobacteria and thought to be an opportunistic pathogen found in a broad range

of environments. With the exception of gentamicin, *Pseudomonas* sp. CHNTR36 was resistant to all other antibiotics shown in the panel (Table 11.1).

In the background metagenome [i.e., genomic deoxyribonucleic acid (DNA) pool] of both farm manure pits, all 15 tet^R and 4 erm^R determinants used in the study were detected. Finding a wide array of resistance genes is consistent with many other reports of ABR gene diversity in waste-holding compartments such as swine lagoons and pits where the most concentrated inputs of bacteria and drug residues occur. These pits were monitored frequently over a 3–year period, and at any instance of sampling, the same 15 tet^R and 4 erm^R determinants were always detected, suggesting the stable maintenance of these genes over time. Not all 19 determinants were, however, represented in the collection of isolates obtained.

Of the known tet^R determinants in the pit metagenome, only *tet* (B), (D), (H), (M), (Q), (W), and (Z) and *erm* (B) were represented among the pit-derived isolates. In the metagenomic pool of manured soils, 8 of the 15 tet^R determinants were detected, including *tet* (B), (C), (H), (M), (O), (Q), (W), (Z) while all 4 tylosin resistance genes *erm* (B), (F), (G), (Q) were present. A nearly similar diverse range of known tet^R genes were present in nonmanured soils, including *tet* (C), (H), (M), (O), (W), and (Z), along with the presence of *erm* (B), (F), and (G). The presence of tet^R and erm^R genes typically thought to be of clinical relevance but found in soil environments that were presumed to have no impact from agricultural wastes raises the notion that these particular determinants may also be part of the natural background reservoir of ABR. These types of studies do bring to further light that accurate knowledge of a soil's prior history is critical for meaningful interpretation in such studies. The tet^R and erm^R genes that were unaccounted for in isolated strains could have resided in the uncultivated proportion of the bacterial populations. Further, there may be exogenous copies of these genes outside of living cells packed in transducing phage particles (Zeph et al., 1988) that were maintained in the genetic pool within these environments.

11.3.3 Molecular Ecology of tet^R and erm^R Genes in Manure Pits and Soils

The known genes encoding ribosomal protection proteins (RPP) are widely disseminated, occurring in both Gram-positive and Gram-negative bacteria (Chopra and Roberts, 2001; Roberts, 2005). The lateral transfer of these genes is evidenced by the broad phylogenetic distribution presently seen among bacteria harboring RPP genes as the content of RPP genes suggested the genes originated in Gram-positive bacteria. Consistent with a broad host range, *tet* (M) was detected among a number of isolates belonging to the Firmicutes, Actinobacteria, and γ-Proteobacteria, with one Bacteriodetes isolate, and all were from pit environments. Other genes encoding RPP genes such as *tet* (W) and *tet* (Q) had limited host ranges among the isolates obtained, with one notable *Bacteroides* sp., which harbored all three RPP genes detected, *tet* (M), (W), and (Q).

Among the genes encoding efflux, *tet* (B), (H), and (Z) were the most widely distributed among all the phyla represented, including Gram-negative and Gram-positive bacteria. The determinants *tet* (B), (C), (D), and (H) have previously been reported in association with Gram-negative organisms but were detected in both Gram-negative and Gram-positive organisms from both pit and soil environments. The tylosin resistance gene *erm* (B), a gene coding for methylase, was detected and

was distributed across all phylogenetic groups, however, most of the isolates exhibiting tylosin resistance did not harbor any of the four erm^R tested.

Many isolates were found to harbor multiple determinants for tetracycline resistance, of which 29% of pit-derived isolates were found to carry multiple tetracycline resistance determinants with as many as four different genes in one *Psychrobacter* sp. (Fig. 11.4). In manure-applied soils, 10% of the isolates carried multiple tet^R determinants, and none of the isolates from nonmanured soils exhibited multiple tet^R determinants. The major mechanisms of tetracycline resistance originated in the tetracycline-producing genus *Streptomyces* common to soil, and the number of new genera harboring known tet^R determinants has broadened presumably through genetic transfer events, with many found to contain more than one determinant (Roberts, 2005). Multiple tetracycline resistance genes have been found in *Streptomyces*, including clinically significant species, (Pang et al., 1994; Petković et al., 2006), yet despite its prevalence in soil, surprisingly, no tetracycline-resistant *Streptomyces* isolates were obtained from either pits or soils. This result may be attributed to the cultivation strategy used in our study, and perhaps a different isolation approach may have yielded *Streptomyces*.

The selection pressure within a concentrated environment containing drug residues, high cell numbers, and ABR genes such as an animal gut or waste storage compartment would likely be conducive to acquisition of multiple determinants. It is not known if the multiple genes harbored by a single cell are functional, nor is it known if one or more determinants are expressed at once or that these genes can be expressed in the host carrying the genes. For example, despite the detection of *tet* (B) in *Rhodococcus* sp. CHNTR42, this strain could not grow on 20 mg/L of tetracycline or chlortetracycline. Still, genes may be only weakly expressed in certain hosts and may provide adequate function in environments with low concentrations of antibiotics. Differential expression of genes may also be possible, conferring the ability of a species to express resistance depending on type of drug and drug concentrations present.

Concentrations of drug residues in soil following land application of manure are not well established, however, studies have shown tetracyclines can persist in biosolids following storage (Wu et al., 2009) along with the accumulation of tetracycline in soils from repeated land application of manure liquids (Hamscher et al., 2002). It is difficult to ascertain whether environmentally relevant concentrations of these compounds exert a selective pressure on bacteria, especially under in situ field conditions. Tetracycline concentrations ranging from 0.2 to 4 mg/L have been reported for manure-treated soils and lagoons (Campagnolo et al., 2002; Hamscher et al., 2002), and soil-bound residues have been shown to be biologically active (Halling-Sørensen et al., 2002; Aga et al., 2005; Chander et al., 2005). Tylosin, in contrast, breaks down rapidly in both manure and lagoon slurries (Kolz et al., 2005) and soil (Sassman et al., 2007), thus limiting its persistence. There may be selective advantage to possessing additional mechanisms of resistances to the same drug in environments containing higher drug concentrations. Perhaps selection of ABR traits due primarily to the effect of drug exposure is significant only in the concentrated manure environment of the pit, while following entry into soil, other mechanisms further leading to acquisition of resistances come to play.

Low levels of tetracycline have been shown to stimulate the transfer of tet^R genes (Torres et al., 1991; Showsh and Andrews, 1992; Clewell et al., 1995). Conjugal transfer of ABR determinants has been shown in both soil microcosms and native

soil environments (Lee and Stotzky, 1999; Andrews et al., 2004). Gotz and Smalla (1997) reported a 10-fold increase in plasmid transfer in soils receiving manure application relative to those that had not and suggested that elevated nutrient levels, often associated with repeated land application, may be a significant factor. Other soil conditions in the manure-applied soils, such as increased metal concentrations, greater cation exchange capacity, and organic matter sorption may also facilitate the acquisition of ABR determinants (Perron et al., 2004; Berg et al., 2005b; Kong et al., 2006).

Many of the isolates from this study that expressed phenotypic resistances did not appear to harbor any of the known determinants tested. These isolates, as well as others that harbored the targeted genes, may carry other tet^R or erm^R genes that were not tested such as *tet* (L) or the class of tetracycline resistance genes encoding enzymatic degradation such as *tet* (X) (Roberts, 2005). On the basis of several studies, determinants such as *tet* (M), *tet* (B), *tet* (Z), *tet* (L), and *erm* (B) may be genes that are more readily mobilized with potential for distribution across widespread bacterial phyla. It is not yet known what new, or as yet undiscovered, genes may explain the drug resistance abilities of many of the isolates.

The two swine farms used in this study were managed similarly with regard to antibiotic usage. The study conducted here evaluated only a single time point in decades-long histories of large animal production and manure land application. There were clearly differences in the ABR characteristics of bacteria obtained from pit environments in contrast to the soils at both sites. While the diversity of tet^R and erm^R determinants was lower in the nonmanured soils relative to the manured soils, the isolates exhibiting tetracycline resistance from the "unimpacted" soil environments occurred over a similar diverse range of phyla as the manured soils, and represented 17% of the total number of strains recovered. Finally, it is also noteworthy that several isolates representing disparate phyla and sources exhibited multiple drug resistances over a wide range of drug classes (Table 11.1). Interestingly, two closely related strains of *Chryseobacterium* exhibited very different drug resistance phenotypes. One isolate (*Chryseobacterium* sp. BBTR48) was from manured soil and was resistant to 4 of the 12 drugs, while the isolate from nonmanured soil (*Chryseobacterium* sp. BBCT12) was resistant to 10 of the 12 drugs. Such instances serve to demonstrate the possible myriad mechanisms of ABR occurrence, acquisition, and persistence that can occur in soil environments, with some that may not be directly linked to manure effects.

11.4 CONCLUDING REMARKS

Such cultivation- and molecular-based studies remain too few yet to speculate on the exact nature of ABR gene flow in natural environments. Ultimately, the original reservoirs of ABR genes are the natural ecosystems, where the genes have been involved in a number of functions not necessarily associated with resistance to antibiotics per se (Aminov, 2009). Due to continuous gene exchange between different ecological compartments, the subset of these genes was acquired and amplified in microbial ecosystems subjected to intensive antibiotic selection. This pool residing in commensal and pathogenic bacteria is reasonably well characterized. It is becoming more apparent, however, that the diversity of bacteria harboring ABR

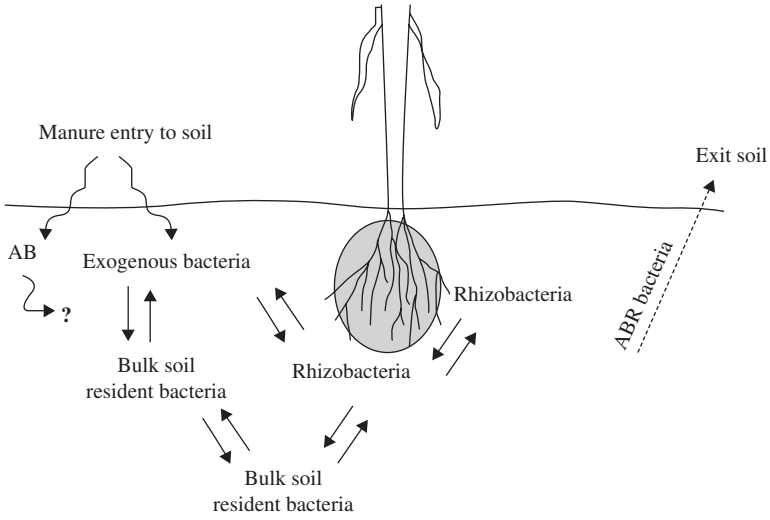

FIGURE 11.5 Schematic view showing potential for interactions among bacteria that may affect the dynamics of ABR development within a soil. An important point of entry into soil of nonnative (exogenous) ABR bacteria is shown as a consequent effect of manure land application. Antibiotic residues (AB) also enter the environment with unknown effects on the extant population of bacteria. The compartment of the rhizosphere (shaded) represents a unique environment of ABR development and exchange. Exit of ABR bacteria to other environments may occur via common mechanisms such as water runoff, leaching, and harvested plant associations.

genes may be much higher than imagined, even in environments with no known land application histories and many may exhibit multiple drug resistances. The unknown nature of determinants ABR in many isolates may also point to soil bacteria as important reservoirs of new antibiotic resistance genes (Riesenfeld et al., 2004; D'Costa et al., 2006). The types of bacterial species that became evident in cultivation studies also raise the importance of rhizobacteria in the overall molecular ecology of ABR genes in soil environments. As a soil subcompartment where gene transfer events may occur readily, rhizospheres have become apparent as significant reservoirs of resistance genes and gene movement (Fig. 11.5).

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