Applied and Environmental Microbiology, Sept. 2009, p. 5814–5820 Vol. 75, No. 18
0099-2240/09/$08.00+0 doi:10.1128/AEM.00977-09

Macrolide Resistance in Microorganisms at Antimicrobial-Free Swine Farms[▽]

Zhi Zhou,† Lutgarde Raskin,‡ and Julie L. Zilles*

Department of Civil and Environmental Engineering, University of Illinois at Urbana-Champaign, 205 North Mathews Avenue, Urbana, Illinois 61801

Received 29 April 2009/Accepted 20 July 2009

To investigate the relationship between agricultural antimicrobial use and resistance, a variety of methods for quantification of macrolide-lincosamide-streptogramin B (MLS_B) resistance were applied to organic swine farm manure samples. Fluorescence in situ hybridization was used to indirectly quantify the specific rRNA methylation resulting in MLS_B resistance. Using this method, an unexpectedly high prevalence of ribosomal methylation and, hence, predicted MLS_B resistance was observed in manure samples from two swine finisher farms that reported no antimicrobial use (37.6% ± 6.3% and 40.5% ± 5.4%, respectively). A culture-based method targeting relatively abundant clostridia showed a lower but still unexpectedly high prevalence of resistance at both farms (27.7% ± 11.3% and 11.7% ± 8.6%, respectively), while the prevalence of resistance in cultured fecal streptococci was low at both farms (4.0%). These differences in the prevalence of resistance across microorganisms suggest the need for caution when extrapolating from data obtained with indicator organisms. A third antimicrobial-free swine farm, a breeder-to-finisher operation, had low levels of MLS_B resistance in manure samples with all methods used (<9%). Tetracycline antimicrobials were detected in manure samples from one of the finisher farms and may provide a partial explanation for the high level of MLS_B resistance. Taken together, these findings highlight the need for a more fundamental understanding of the relationship between antimicrobial use and the prevalence of antimicrobial resistance.

Clinical data have documented a substantial rise in the levels of antimicrobial resistance (reviewed in reference 22). In response to this alarming rise, national and international initiatives have been developed to limit the use of antimicrobials in both human and veterinary medicine, with some successes. However, some of the data suggest a more complicated relationship between the patterns of antimicrobial use and the resulting prevalence of resistance. For both avoparcin and chloramphenicol, a ban was not effective in reducing the prevalence of resistance to the respective antimicrobial in pig isolates (2, 9). This may be due to coselection by the continued use of other types of antimicrobials (1, 15, 16, 33). Coselection by other antimicrobials, however, cannot explain the persistence of antimicrobial resistance for years after all use of antimicrobials was stopped, as documented in other studies of swine (13, 25). A better understanding of this complex relationship is needed to provide a basis for developing more-effective measures to control the prevalence of antimicrobial resistance. One means for investigating the factors influencing the prevalence of resistance is through comparisons between conventional farms and organic, antimicrobial-free farms (12, 13, 18, 25) or the wilderness (14, 19).

The current study focused on macrolide antimicrobials, for which the most clinically relevant resistance mechanisms are efflux and target site modification (20). Resistance via modification of the target site on the ribosome may be achieved either through point mutations in rRNA or proteins or through acquisition of an *erm* gene catalyzing a site-specific mono- or dimethylation of the 23S rRNA (37). The point mutations confer various levels of resistance and degrees of cross-resistance (35), and their known distribution is currently limited, although this may simply reflect the historical experimental focus (20, 35). Dimethylation of A2058 (*Escherichia coli* numbering), on the other hand, consistently results in high-level resistance (for antimicrobial concentrations above 1 mg/ml) for three structurally unrelated classes of antimicrobials, macrolides, lincosamides, and streptogramin Bs, or macrolide-lincosamide-streptogramin B (MLS_B) antimicrobials, because of their shared target site (37). Constitutive expression of an *erm* dimethylase can also confer resistance to the newer ketolides, which are erythromycin (macrolide) derivatives developed for use on macrolide-resistant pathogens, and the degree of resistance correlates with the degree of methylation (11). The ribosomal methylation resistance mechanism is of particular concern for this work for the following three reasons. (i) It confers a high level of resistance. (ii) It can be acquired through horizontal gene transfer and thus has the potential for rapid spread. (iii) It is relevant to swine production environments in the United States because all three classes of MLS_B antimicrobials are used there. A variety of methods have been used to quantify macrolide resistance, including traditional culture-based methods (for an example, see reference 9), PCR (for examples, see references 27 and 32), or fluorescence in situ hybridization (FISH) (for an example, see reference 34) detection of specific point mutations known to result in resis-

* Corresponding author. Mailing address: 3204 Newmark Civil Engineering Laboratory, MC250, 205 North Mathews Avenue, Urbana, IL 61801. Phone: (217) 244-2925. Fax: (217) 333-6968. E-mail: jzilles@illinois.edu.

† Present address: Carollo Engineers, 2700 Ygnacio Valley Road, Suite 300, Walnut Creek, CA 94598.

‡ Present address: Department of Civil and Environmental Engineering, University of Michigan, 1351 Beal Ave., Ann Arbor, MI 48109.

▽ Published ahead of print on 24 July 2009.

TABLE 1. Oligonucleotide probes used for quantification of unmethylated ribosomes and microbial community analysis

Probe	Systematic name	Label (5′)	Target organisms	Target molecule	% Formamide[a]	Specificity (%)[b]	Coverage (%)[c]	Probe sequence (5′–3′)	Reference
MLS_B	L-*-MLSS-2053-a-S-13	Cy3	MLS_B-sensitive bacteria[d]	23S rRNA	12.5	NA	97.5	GGG TCT TTC CGT C	This study
ClostXIVa	S-*-Clos-0129-a-A-15	FAM[e]	*Clostridium* cluster XIVa	16S rRNA	30	99.8[f]	9.4[f]	CTG TAT GAG GCA GGT	36
ALF1B	S-Sc-aProt-0019-a-A-17	Cy3	*Alphaproteobacteria*	16S rRNA	20	52.2	68[g]	CGT TCG YTC TGA GCC AG	24
BET42a	L-C-bProt-1027-a-A-17	FAM	*Betaproteobacteria*	23S rRNA	35	NA	92.6	GCC TTC CCA CTT CGT TT	24
GAM42a	L-C-gProt-1027-a-A-17	FAM	*Gammaproteobacteria*	23S rRNA	35	NA	90.8	GCC TTC CCA CAT CGT TT	24
Bact0338	S-D-Bact-0338-a-A-18	Cy3	*Bacteria*	16S rRNA	20	99.9	90[g]	GCT GCC TCC CGT AGG AGT	3

[a] % Formamide, the percentage of formamide in the hybridization buffer for optimal hybridization conditions in FISH experiments.
[b] Specificity, the percentage of sequences matching the probe within the target group over the total number of sequences matching the probe. Source, Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp, accessed on 11 November 2008). NA, not available.
[c] Coverage, the percentage of sequences matching the probe within the target group over the total number of sequences in the target group. Source, Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp, accessed on 11 November 2008), except where noted.
[d] For the calculations presented here, MLS_B-sensitive bacteria are defined as all bacteria that contain G2057 and are unmethylated at position A2058.
[e] FAM, 6-carboxyfluorescein.
[f] Specificity and coverage were calculated for the target order *Clostridiales*.
[g] Source, probeBase (23; http://www.microbial-ecology.net/probebase/default.asp).

tance in the targeted microorganisms, using PCR to detect *erm* and *mef* (efflux) genes (for examples, see references 6 and 31) and using membrane hybridizations to detect the degree of methylation at A2058 (5, 18).

In our previous study of swine production, a discrepancy was observed between culture-based measurements of resistance to the macrolide tylosin and membrane hybridizations quantifying the ribosomal methylation leading to MLS_B resistance (18). Cultured fecal streptococci showed a low prevalence of tylosin resistance (4.0%) in manure samples from an organic farm, as expected in the absence of the selective pressure imposed by the use of antimicrobials. However, membrane hybridizations quantifying the ribosomal methylation leading to MLS_B resistance in all bacteria in the swine waste samples suggested the presence of a much higher level of resistance (approximately 50%). One explanation for this discrepancy is that the prevalence of resistance in the fecal streptococci was not representative of the overall prevalence of resistance in this community. However, the high level of resistance measured with the molecular method was surprising in the absence of antimicrobial use and could also be explained as an artifact of the membrane hybridization methodology.

The primary objectives of this paper were to resolve this discrepancy between culture-based and molecular methods and, if the unexpectedly high prevalence of antimicrobial resistance was confirmed, to investigate possible explanations. To accomplish the first objective, we developed a variation of FISH to indirectly quantify the specific rRNA methylation resulting in MLS_B resistance and provide insight into the identity of the putative resistant microorganisms. The major group identified, *Clostridium* cluster XIVa, was targeted with a culture-based method to provide an independent quantification of resistance. The results presented here have confirmed an unexpectedly high prevalence of MLS_B resistance at two organic farms. They also support the hypothesis that the prior discrepancy resulted from differences in the prevalence of resistance across groups of microorganisms.

MATERIALS AND METHODS

Sample collection and fixation. Four Illinois swine farms were sampled during this study. Antimicrobial use, initial measurements of the prevalence of antimicrobial resistance, and antimicrobial residues in manure samples obtained from a conventional farm (called LF) have been reported previously (18, 41), as have resistance levels from samples obtained from one organic farm (called OF) (18). Two additional farms reporting no antimicrobial use were included in the current study. One of these organic farms (OF2) operated similarly to OF, i.e., as a finisher operation, and purchased animals from the same supplier. The other organic farm (OF3) was a breeder-to-finisher operation. The swine manure samples from the organic farms were collected from the floors of the buildings that housed the pigs, which were straw-covered soil, and homogenized. Swine manure was collected from LF and OF in May 2002, OF2 in February 2003 and December 2005, and OF3 in October 2006. For the molecular analysis, samples were fixed by incubation in 100% ethanol (Aaper Alcohol & Chemical, Shelbyville, KY) for 2 h on ice, as described previously (10). Ethanol fixation was used because it resulted in higher Bact0338 counts and lower levels of background fluorescence than when using paraformaldehyde fixation (data not shown). Following fixation, the samples were resuspended in 1:1 (vol/vol) phosphate-buffered saline (PBS)/ethanol and stored at −20°C. Samples for antimicrobial analysis were stored without treatment at −20°C.

FISH methods and probes. The fixed samples were diluted in 1× PBS buffer, sonicated (5-s pulse on/off, output of 250 W, model 500; Fisher Scientific, Pittsburgh, PA), and filtered through 0.22-μm-pore-size polycarbonate membranes (diameter, 25 mm; Osmonics, Minnetonka, MN). Sonication times were optimized to break up clumps of cells without decreasing total cell counts as described previously (39), using 15 s for the organic farm samples and 60 s for the LF sample. Cells were transferred from the filters to gelatin-coated slides (3) by manually pressing the filters onto the slides for at least 10 s (39).

FISH was performed as previously described, using hybridization and washing temperatures of 46 and 48°C, respectively, except for with the MLS_B probe, which was hybridized and washed at 37°C (10, 39). The probes used are listed in Table 1. FISH slides were observed under ×630 magnification on a Zeiss Axioskop 40 or a Zeiss Axiovert 100 inverted microscope equipped for both light and fluorescence microscopy (Carl Zeiss, Oberkochen, Germany) under appropriate filter sets (Chroma Technology Corp., Rockingham, VT). FISH experiments were repeated between two and six times. Images were acquired from 15 random locations, with each location averaging 286 cells. The maximum and mean fluorescence intensities of each hybridized cell were analyzed in an automated fashion using the image analysis software Visilog (version 6; Noésis, Les Ulis, France) for expedience and consistency (39).

For the quantification of MLS_B resistance, the formamide concentration was optimized using three pure cultures, as follows: MLS_B-sensitive (positive) *Enterococcus faecalis* JH2-2, MLS_B-resistant (negative) *E. faecalis* JH2-2 pAMβ1,

and *Rhodococcus coprophilus*, which has one mismatch in position 2057. Under the optimized hybridization conditions used, the fluorescence intensity of the MLS_B probe was comparable to those of the other probes in this study, resulting in typical exposure times of 1 s. The prevalence of ribosomal methylation and presumed MLS_B resistance was calculated as follows: 1 − (number of MLS_B-hybridized cells/number of Bact0338-hybridized cells). To assess the prevalence of resistance in specific groups of microorganisms, this method was combined with differentially labeled phylogenetic probes in sequential hybridization dual-labeling FISH experiments.

Quantification of tylosin resistance with culture-based methods. Tylosin resistance among fecal streptococci was assessed using m-*Enterococcus* agar (274620; Becton Dickinson, Sparks, MD), as described previously (4, 18). The culture-based method targeting clostridia was conducted with reinforced clostridial medium (CM0149; Oxoid, Lenexa, KS) (17). Manure samples were diluted to the appropriate concentrations with sterile PBS (130 mM NaCl, 10 mM sodium phosphate [pH 7.2]) and filtered in triplicate through 0.45-μm filters (09-719-1B; Fisher Scientific, Pittsburgh, PA). These filters were then transferred to agar plates and incubated anaerobically for 1 week. The MLS_B antimicrobial tylosin and/or tetracycline (Sigma Chemical, St. Louis, MO) were added at 20 mg/liter for the quantification of resistance. Standard deviations were calculated from the results of three to five replicate dilution series.

Quantification of MLS_B antimicrobials with liquid chromatography-tandem mass spectrometry. Manure samples were shipped on ice to the Institute of Agriculture and Natural Resources at the University of Nebraska (Lincoln, NE) for antimicrobial analysis. The manure samples were diluted in a 0.5 M potassium phosphate/citric acid buffer, pH 2.5 (tetracyclines), or a neutral phosphate solution (macrolides) and extracted using Oasis HLB cartridges (Waters Corporation, Milford, MA). Following solid-phase extraction, the concentrations of tetracycline, macrolide, and lincosamide antimicrobials were quantified using triple quadruple liquid chromatography-tandem mass spectrometry, as previously described (30, 40).

Statistics. All reported data are shown as average values of measurements. The values after each "±" are standard deviations, except for with the FISH quantification of ribosomal methylation on OF2 manure, for which the half-range of duplicate analyses is reported. Correlation was considered significant at a P value of <0.05. Statistical analyses were carried out using the software R (http://www.r-project.org/).

RESULTS AND DISCUSSION

Development of a FISH method for quantifying the A2058 ribosomal methylation. To allow for culture-independent quantification and identification of the methylated (and presumed MLS_B-resistant) microorganisms, we developed a FISH protocol that uses a fluorescently labeled oligonucleotide probe targeting the methylation site (Table 1). Methylation of the 23S rRNA at position A2058 interferes with hybridization with the probe, allowing for quantification of unmethylated strains. This indirect detection of methylation was required because the 5-nitro-indole-substituted probe used in previous work (5, 18) for direct detection of methylated rRNA did not have a sufficient signal in FISH experiments. All results from the MLS_B probe were normalized to hybridization results with Bact0338 to limit the number of false positives resulting from poorly fixed or otherwise unresponsive cells.

The 23S rRNA sequence in the region targeted by this probe is highly conserved, except at position 2057, where about 10% of available 23S rRNA sequences have an adenine rather than the more common guanine. Although not all of the organisms containing A2057 have been evaluated, this substitution results in macrolide resistance in multiple genera (reviewed in reference 35). Single-base mutations in A2058 also frequently result in macrolide resistance (35). Because these sequence variations typically result in resistance, while our intent was to hybridize only with sensitive strains, the MLS_B probe was synthesized with a mismatch to these sequences.

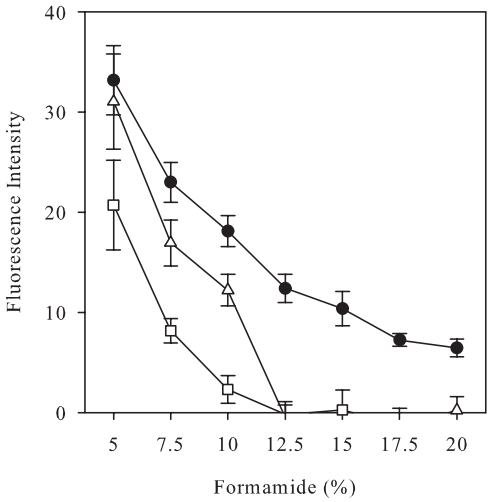

FIG. 1. Optimization of hybridization stringency for the MLS_B probe. ●, sensitive strain (*E. faecalis* JH2-2); Δ, resistant strain (*E. faecalis* JH2-2 pAMβ1); □, mismatch strain (*R. coprophilus* containing G2057). The optimal formamide concentration used to differentiate a methylated or mismatch strain from an unmethylated (sensitive) strain was 12.5%.

The hybridization conditions were optimized using unmethylated, methylated, and mismatch strains (Fig. 1). The methylated strain contained the *ermB* gene, while the *Rhodococcus coprophilus* mismatch strain contained the A2057 23S rRNA sequence. At a formamide concentration of 12.5%, sensitive strains can be differentiated from resistant strains but still have reasonable fluorescence intensities. Under the optimized conditions, the A2057 mismatch strain did not hybridize with the MLS_B probe and, therefore, will be correctly classified as MLS_B resistant. The optimized method was validated by measuring hybridization in defined mixtures containing sensitive and resistant strains. A significant correlation ($P = 7.9 \times 10^{-14}$) between the predicted prevalence of MLS_B resistance and the observed percentage of hybridization-negative cells was observed (data not shown).

The newly developed FISH method for assessing methylation of A2058 provides an estimate of MLS_B resistance in environmental samples and is compatible with the identification of resistant populations, filling a critical gap between commonly used culture-based and PCR-based methods. Prior to use in clinical situations, a more comprehensive evaluation of the FISH method with strains exhibiting various levels of resistance would be required. By the nature of the approach, cells containing silent resistance genes or cells with multiple copies of the resistance gene will be reported as sensitive and resistant, respectively, and will not result in an overestimate of resistance.

Microbial community analysis of swine manure samples. To test the hypothesis that the prevalence of resistance in the fecal streptococci was not representative of the entire microbial community, it was important to identify the bacteria that were abundant in the swine manure samples analyzed. The microbial communities in manure samples obtained from two farms (LF and OF2) were therefore analyzed with a set of phylogenetic probes targeting 16S rRNA (Table 1). Probe sequences

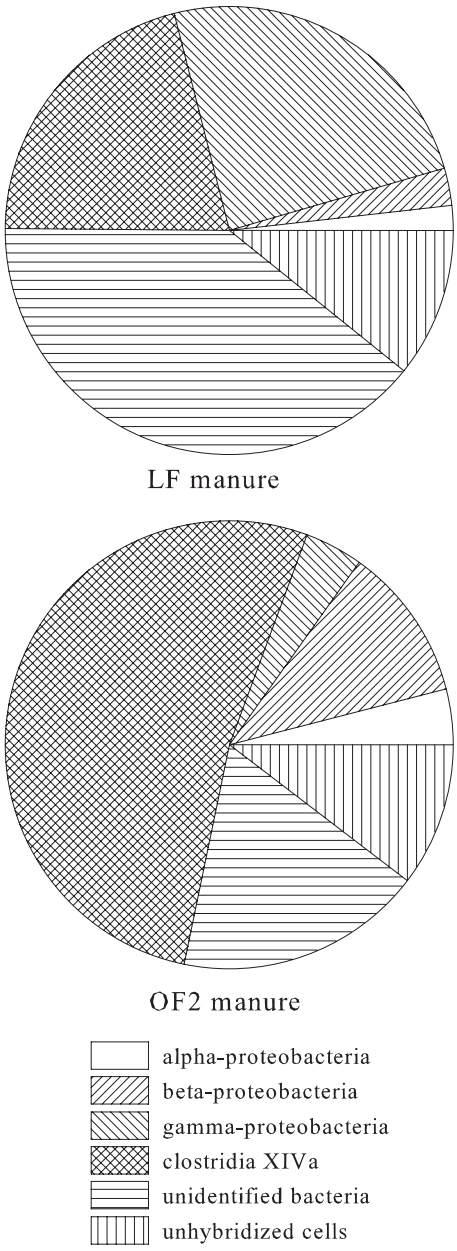

FIG. 2. Microbial community analysis of swine manure samples obtained from LF and OF2. The average half-range for duplicate FISH analysis was 1.8%.

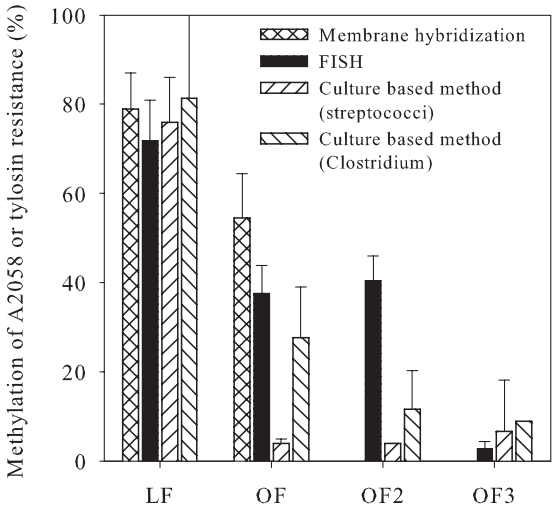

FIG. 3. Prevalence of ribosomal methylation and tylosin resistance in building samples obtained from LF and manure samples obtained from three organic farms (OF, OF2, and OF3), quantified using different methods. Membrane hybridizations and FISH quantified the prevalence of the specific ribosomal methylation leading to MLS_B resistance and were normalized to hybridization results obtained with a general bacterial probe, while the culture-based methods quantified resistance to the macrolide tylosin and were normalized to results under the same conditions in the absence of tylosin. All of the membrane hybridization data and the culture-based fecal streptococci results for LF were previously reported (18). Membrane hybridization data are not available for OF2 and OF3. Error bars show standard deviations, except for with the FISH quantification of ribosomal methylation on OF2 manure, for which the half-range of duplicate analyses is reported.

for *Bacteria* (8) and *Alphaproteobacteria* (26) with more-complete coverage than those used in this work have been reported (23). However, for the manure samples in this work, the bacterial counts were over 70% of the DAPI (4′,6-diamidino-2-phenylindole) counts, suggesting that the bacterial coverage was not a major limitation. The impact of the choice of the *Alphaproteobacteria* probe is more difficult to assess; the results presented here may underestimate the abundance of these organisms.

Clostridia are abundant in swine manure storage pits (7, 21, 29, 38). Based on clostridial sequences retrieved from Illinois swine manure pit samples (38), a probe for *Clostridium* cluster XIVa was included in our study (Table 1). The results indicated that microorganisms in *Clostridium* cluster XIVa were also abundant in the manure at LF and OF2, representing 20.9% and 52.4% of the total cells stained with DAPI, respectively (Fig. 2). In contrast, the cultured fecal streptococci were present at levels below 0.03% of total cells in both samples. We therefore concluded that the prevalence of resistance in clostridia was more likely to be representative of the resistance in the entire microbial community than that of the fecal streptococci.

Macrolide resistance in swine manure samples. To better define the scope of the discrepancy observed by Jindal et al. (18), macrolide resistance levels were compared in swine manure samples from four farms and with four different methods. The methods included membrane hybridization (5, 18), FISH, and culture-based methods targeting fecal streptococci (4) and clostridia (17). Two of the farms (LF and OF) had been included in a previous study (18). Because our aim was to investigate the unexpectedly high resistance previously observed at OF, two additional farms that did not use antimicrobials were sampled. One farm (OF2) purchased piglets from the same supplier as OF and used a similar production process, while the other farm (OF3) was a breeder-to-finisher operation.

For samples from LF, all four methods showed good agreement and confirmed the expected high prevalence of resistance (Fig. 3). Farms OF and OF2 both exhibited a discrepancy between the prevalence of ribosomal methylation (e.g., 37.6% ± 6.3% and 40.5% ± 5.4% by FISH, respectively) and

the prevalence of tylosin resistance in the cultivated fecal streptococci (4.0% ± 1.0% and 4.0% ± 0.0%, respectively). In contrast, OF3 had a low prevalence of methylation and presumed resistance even with the FISH method (2.9% ± 1.5%). When a culture-based method targeting the more abundant clostridia was applied, the tylosin resistance at OF was also relatively high (27.7% ± 11.3%), while at OF2 and OF3, the prevalence of tylosin resistance was lower (11.7% ± 8.6% and 9.0%, respectively).

In OF samples, and potentially also in OF2 samples, the culture-based method targeting fecal streptococci severely underestimated the overall prevalence of MLS_B resistance. This result emphasizes the need for caution in extrapolation from data obtained with indicator organisms. Current surveillance programs focus on specific microorganisms, many of which represent a direct risk to public health. However, if only select (and often relatively rare) microorganisms are monitored, then reservoirs of antimicrobial resistance such as those at OF will not be detected. Incorporation of community-level measurements of resistance, a more fundamental understanding of the variability of the prevalence of resistance across different types of microorganisms, or both are needed to improve the detection of environmental reservoirs of antimicrobial resistance.

Macrolide resistance in clostridia. The prevalence of macrolide resistance in clostridia was evaluated using the culture-based method (Fig. 3) and by combining the detection of unmethylated rRNA with phylogenetic analysis in dual-labeling FISH. These methods differ in both their target microorganisms and the types of resistance that are quantified. The culture-based method is less specific, enriching for clostridia in general and monitoring resistance to tylosin, regardless of the resistance mechanism, while the FISH method targets only *Clostridium* cluster XIVa and resistance via ribosomal methylation. Total clostridium counts differed between the two methods, with 9% of the total cell count cultured from OF2 samples on the clostridium medium, while 52% of the cells were classified as *Clostridium* cluster XIVa by FISH. However, in comparison to the recoveries of fecal streptococci, which accounted for less than 0.03% of the total microscope cell count in the same samples, the culture-based method targeting clostridia should allow for a more accurate assessment of the overall prevalence of resistance in the microbial community.

Considering the differences in their target microorganisms and the resistance mechanisms quantified, the tylosin resistance and methylation/point mutation results were relatively consistent. At OF, using the culture-based and dual labeling FISH methods, the prevalence of resistance in clostridia was 27.7% ± 11.3% and 34.7% ± 0.1%, respectively, while at LF, it was 81.3% ± 20.2% and 54.5% ± 0.5%, respectively. The consistency of these data suggests that ribosomal modification is the dominant tylosin resistance mechanism in the samples analyzed. Based on the FISH data, MLS_B-resistant clostridia accounted for approximately 1/4 of the resistant organisms in LF manure and for 1/5 of those in OF2 manure.

Factors influencing macrolide resistance at organic farms. While reducing the use of antimicrobials is an important objective for controlling the spread of antimicrobial resistance, cessation of use does not always result in low prevalence of resistance, as demonstrated by the results obtained from the organic farms in this study. Why is the prevalence of resistance unexpectedly high at OF and OF2? One simple explanation for elevated levels of antimicrobial resistance is the presence of antimicrobials.

TABLE 2. Concentrations of antimicrobials in swine manure

Antimicrobial	Detection limit	Concentration of antimicrobials in manure obtained from[a]:		
		LF[b]	OF2	OF3
Tiamulin	5	BD	BD	BD
MLS_B antimicrobials				
Lincomycin	2	4.4 (1.4)	BD	BD
Erythromycin A	5	BD	BD	BD
Spiramycin	5	BD	BD	BD
Oleandomycin	2	BD	BD	BD
Tilmicosin	5	BD	BD	BD
Tylosin	2	BD	BD	BD
Tetracycline antimicrobials				
Tetracycline	1	19.8 (1.4)	60.1 (6.0)	BD
Chlortetracycline	1	5.3 (1.5)	723.9 (100.9)	BD
Oxytetracycline	1	2,630.0 (836.0)	126.5 (12.5)	BD
Minocycline	10	BD	BD	BD
Anhydrotetracycline	2	BD	20.9 (14.9)	BD
Anhydrochlortetracycline	2	BD	BD	BD
Beta-apo-oxytetracycline	2	1,230.5 (15.5)	45.8 (2.8)	BD

[a] Values are given in ng of antimicrobial per g of wet manure. Values in parentheses represent the half-range for duplicate measurements. BD, below detection limit.

[b] Antimicrobial concentrations in manure obtained from LF were previously published (41).

To investigate this possibility, the levels of MLS_B and tetracycline antimicrobials were quantified in swine manure samples obtained from OF2 and OF3 (Table 2). Antimicrobial levels in the LF manure were previously reported (41). MLS_B antimicrobials were not detected in any of the samples analyzed, except that low levels of lincomycin were found in LF manure. Tetracyclines were detected in LF and OF2 manure but not in OF3 manure.

The tetracycline antimicrobials detected in manure from an organic farm (Table 2) are likely to contribute directly to tetracycline resistance and perhaps indirectly to MLS_B resistance via coselection. Such coselection is reasonable based on previous reports of genetic linkage between the *erm* and *tet* genes (28). The coselection hypothesis predicts that the tylosin-resistant microorganisms will also be resistant to tetracyclines. We used the culture-based method targeting clostridia to test this hypothesis. The prevalence of tetracycline resistance was high but variable in OF and OF2 manure (44.3% ± 33.2% and 61.9% ± 50.7%, respectively), while dual resistance (tylosin/tetracycline) was observed at low frequency in the same samples (5.8% ± 4.1% and 6.3% ± 6.7%, respectively). Samples from OF3 were not available for comparison. This frequency of dual resistance explains 13% to 14% of the observed tylosin resistance; factors other than coselection must also be contributing to the prevalence of resistance. These factors could include contamination of the feed with antimicrobial resistance genes (18), maternal transmission or exposure to selective pressures prior to arrival at the finisher operation, or persistence from prior usage of the site. The source of the tetracycline antimicrobials detected in the manure samples obtained from OF2 is not known.

This work has advanced our understanding of antimicrobial resistance in the environment in the following ways. First, a new molecular method has been developed that allows for culture-independent estimation of the prevalence of the specific ribosomal methylation, leading to MLS_B resistance and identification of the presumed resistant microorganisms in complex environmental samples. Second, this work demonstrates an unexpectedly high prevalence of MLS_B resistance in the absence of antimicrobial usage at selected farms and provides support for the hypothesis that some of this resistance is due to the presence of tetracycline antimicrobials. This resistance probably would not be detected by current antimicrobial resistance surveillance programs. The potential risk associated with this resistance depends on the persistence and pathogenicity of the resistant microorganisms and the mobility of the resistance genes. Third, the identification of *Clostridium* cluster XIVa as a major class of resistant microorganisms in this environment provides a basis for evaluating the risk in future studies. Together, these results illustrate the complex relationship between antimicrobial use and resistance and suggest the need for caution in extrapolating resistance levels across microorganisms.

ACKNOWLEDGMENTS

We thank the farmers for their cooperation, Matt Robert for assistance with the sample collection, and Terry Whitehead and Roberta Fulthorpe for helpful discussions. The *E. faecalis* strains were graciously provided by Nadja Shoemaker and Abigail Salyers.

We acknowledge funding from the U.S. Department of Agriculture under cooperative agreement AG 58-3620-1-179 and the National Pork Board under project 05-068. Zhi Zhou was also supported by a John W. Page fellowship from the University of Illinois at Urbana-Champaign.

REFERENCES

1. **Aarestrup, F. M.** 2000. Characterization of glycopeptide-resistant *Enterococcus faecium* (GRE) from broilers and pigs in Denmark: genetic evidence that persistence of GRE in pig herds is associated with coselection by resistance to macrolides. J. Clin. Microbiol. **38:**2774–2777.
2. **Akwar, H. T., C. Poppe, J. Wilson, R. Reid-Smith, M. Dyck, J. Waddington, D. Shang, and S. A. McEwen.** 2008. Associations of antimicrobial uses with antimicrobial resistance of fecal *Escherichia coli* from pigs on 47 farrow-to-finish farms in Ontario and British Columbia. Can. J. Vet. Res. **72:**202–210.
3. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
4. **American Public Health Association, American Water Works Association, and Water Environment Federation.** 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, American Water Works Association, and Water Environment Federation, Washington, DC.
5. **Angenent, L. T., M. Mau, U. George, J. A. Zahn, and L. Raskin.** 2008. Effect of the presence of the antimicrobial tylosin in swine waste on anaerobic treatment. Water Res. **42:**2377–2384.
6. **Arthur, M., C. Molinas, C. Mabilat, and P. Courvalin.** 1990. Detection of erythromycin resistance by the polymerase chain reaction using primers in conserved regions of *erm* rRNA methylase genes. Antimicrob. Agents Chemother. **34:**2024–2026.
7. **Cotta, M. A., T. R. Whitehead, and R. L. Zeltwanger.** 2003. Isolation, characterization and comparison of bacteria from swine faeces and manure storage pits. Environ. Microbiol. **5:**737–745.
8. **Daims, H., A. Brühl, R. Amann, K.-H. Schleifer, and M. Wagner.** 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. **22:**434–444.
9. **Danish Integrated Antimicrobial Resistance Monitoring and Research Programme.** 2006. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark. http://www.danmap.org/pdfFiles/Danmap_2006.pdf.
10. **de los Reyes, F. L., W. Ritter, and L. Raskin.** 1997. Group-specific small-subunit rRNA hybridization probes to characterize filamentous foaming in activated sludge systems. Appl. Environ. Microbiol. **63:**1107–1117.
11. **Douthwaite, S., J. Jalava, and L. Jakobsen.** 2005. Ketolide resistance in *Streptococcus pyogenes* correlates with the degree of rRNA dimethylation by Erm. Mol. Microbiol. **58:**613–622.
12. **Gebreyes, W. A., S. Thakur, and W. E. M. Morrow.** 2006. Comparison of prevalence, antimicrobial resistance, and occurrence of multidrug-resistant *Salmonella* in antimicrobial-free and conventional pig production. J. Food Prot. **69:**743–748.
13. **Gellin, G., B. E. Langlois, K. A. Dawson, and D. K. Aaron.** 1989. Antibiotic resistance of gram-negative enteric bacteria from pigs in three herds with different histories of antibiotic exposure. Appl. Environ. Microbiol. **55:**2287–2292.
14. **Ghosh, S., and T. M. LaPara.** 2007. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J. **1:**191–203.
15. **Hammerum, A. M., O. E. Heuer, H.-D. Emborg, L. Bagger-Skjot, V. F. Jensen, A.-M. Rogues, R. L. Skov, Y. Agerso, C. T. Brandt, A. M. Seyfarth, A. Muller, K. Hovgaard, J. Ajufo, F. Bager, F. M. Aarestrup, N. Frimodt-Moller, H. C. Wegener, and D. L. Monnet.** 2007. Danish integrated antimicrobial resistance monitoring and research program. Emerg. Infect. Dis. **13:**1632–1639.
16. **Harada, K., T. Asai, A. Kojima, K. Ishihara, and T. Takahashi.** 2006. Role of coresistance in the development of resistance to chloramphenicol in Escherichia coli isolated from sick cattle and pigs. Am. J. Vet. Res. **67:**230–235.
17. **Hirsch, A., and E. Grinsted.** 1954. Methods for the growth and enumeration of anaerobic sporeformers from cheese, with observations on the effect of nisin. J. Dairy Res. **21:**101–110.
18. **Jindal, A., S. Kocherginskaya, A. Mehboob, M. Robert, R. I. Mackie, L. Raskin, and J. L. Zilles.** 2006. Antimicrobial use and resistance in swine waste treatment systems. Appl. Environ. Microbiol. **72:**7813–7820.
19. **Kozak, G. K., P. Boerlin, N. Janecko, R. Reid-Smith, and C. Jardine.** 2009. Antimicrobial resistance in *Escherichia coli* isolates from swine and wild small mammals in the proximity of swine farms and in natural environments in Ontario, Canada. Appl. Environ. Microbiol. **75:**559–566.
20. **Leclercq, R.** 2002. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin. Infect. Dis. **34:**482–492.
21. **Leung, K., and E. Topp.** 2001. Bacterial community dynamics in liquid swine manure during storage: molecular analysis using DGGE/PCR of 16S rDNA. FEMS Microbiol. Ecol. **38:**169–177.
22. **Levy, S. B., and B. Marshall.** 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nat. Med. **10:**S122–S129.
23. **Loy, A., F. Maixner, M. Wagner, and M. Horn.** 2007. probeBase—an online resource for rRNA-targeted oligonucleotide probes: new features 2007. Nucleic Acids Res. **35:**D800–D804.
24. **Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer.** 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. Syst. Appl. Microbiol. **15:**593–600.
25. **Mathew, A. G., M. A. Beckmann, and A. M. Saxton.** 2001. A comparison of antibiotic resistance in bacteria isolated from swine herds in which antibiotics were used or excluded. J. Swine Health Prod. **9:**125–129.
26. **Neef, A.** 1997. Anwendung der in situ Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen. Technische Universität München, Munich, Germany.
27. **Occhialini, A., M. Urdaci, F. Doucet-Populaire, C. M. Bébéar, H. Lamouliatte, and F. Mégraud.** 1997. Macrolide resistance in *Helicobacter pylori*: rapid detection of point mutations and assays of macrolide binding to ribosomes. Antimicrob. Agents Chemother. **41:**2724–2728.
28. **Roberts, M. C., J. Sutcliffe, P. Courvalin, L. B. Jensen, J. Rood, and H. Seppala.** 1999. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob. Agents Chemother. **43:**2823–2830.
29. **Snell-Castro, R., J. J. Godon, J. P. Delgenes, and P. Dabert.** 2005. Characterisation of the microbial diversity in a pig manure storage pit using small subunit rDNA sequence analysis. FEMS Microbiol. Ecol. **52:**229–242.
30. **Snow, D. D., D. A. Cassada, S. J. Monson, J. Zhu, and R. F. Spalding.** 2003. Tetracycline and macrolide antibiotics: trace analysis in water and wastewater using solid phase extraction and liquid chromatography-tandem mass spectrometry. *In* I. Ferrer and E. M. Thurman (ed.), Liquid chromatography/mass spectrometry, MS/MS, and time-of-flight MS analysis of emerging contaminants. American Chemical Society, Washington, DC.
31. **Sutcliffe, J., T. Grebe, A. Tait-Kamradt, and L. Wondrack.** 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrob. Agents Chemother. **40:**2562–2566.
32. **Szczebara, F., L. Dhaenens, P. Vincent, and M. O. Husson.** 1997. Evaluation of rapid molecular methods for detection of clarithromycin resistance in *Helicobacter pylori*. Eur. J. Clin. Microbiol. Infect. Dis. **16:**162–164.
33. **Travis, R. M., C. L. Gyles, R. Reid-Smith, C. Poppe, S. A. McEwen, R.**

Friendship, N. Janecko, and P. Boerlin. 2006. Chloramphenicol and kanamycin resistance among porcine *Escherichia coli* in Ontario. J. Antimicrob. Chemother. **58:**173–177.
34. **Trebesius, K. H., K. Panthel, S. Strobel, K. Vogt, G. Faller, T. Kirchner, M. Kist, J. Heesemann, and R. Haas.** 2000. Rapid and specific detection of *Helicobacter pylori* macrolide resistance in gastric tissue by fluorescent in situ hybridisation. Gut **46:**608–614.
35. **Vester, B., and S. Douthwaite.** 2001. Macrolide resistance conferred by base substitutions in 23S rRNA. Antimicrob. Agents Chemother. **45:**1–12.
36. **Weber, S., S. Stubner, and R. Conrad.** 2001. Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. Appl. Environ. Microbiol. **67:**1318–1327.
37. **Weisblum, B.** 1995. Erythromycin resistance by ribosome modification. Antimicrob. Agents Chemother. **39:**577–585.
38. **Whitehead, T. R., and M. A. Cotta.** 2001. Characterization of microbial populations in swine feces and waste handling pits by 16S rDNA sequence analysis. Anaerobe **7:**181–187.
39. **Zhou, Z., M. N. Pons, L. Raskin, and J. L. Zilles.** 2007. Automated image analysis for quantitative fluorescence in situ hybridization with environmental samples. Appl. Environ. Microbiol. **73:**2956–2962.
40. **Zhu, J., D. D. Snow, D. A. Cassada, S. J. Monson, and R. F. Spalding.** 2001. Analysis of oxytetracycline, tetracycline, and chlortetracycline in water using solid-phase extraction and liquid chromatography-tandem mass spectrometry. J. Chromatogr. A **928:**177–186.
41. **Zilles, J., T. Shimada, A. Jindal, M. Robert, and L. Raskin.** 2005. Presence of macrolide-lincosamide-streptogramin B and tetracycline antimicrobials in swine waste treatment processes and amended soil. Water Environ. Res. **77:**57–62.