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Presence of Multidrug-Resistant Enteric Bacteria in Dairy Farm Topsoil

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ABSTRACT

In addition to human and veterinary medicine, antibiotics are extensively used in agricultural settings, such as for treatment of infections, growth enhancement, and prophylaxis in food animals, leading to selection of drug and multidrug-resistant bacteria. To help circumvent the problem of bacterial antibiotic resistance, it is first necessary to understand the scope of the problem. However, it is not fully understood how widespread antibiotic-resistant bacteria are in agricultural settings. The lack of such surveillance data is especially evident in dairy farm environments, such as soil. It is also unknown to what extent various physiological modulators, such as salicylate, a component of aspirin and known model modulator of multiple antibiotic resistance (*mar*) genes, influence bacterial multidrug resistance. We isolated and identified enteric soil bacteria from local dairy farms within Roosevelt County, NM, determined the resistance profiles to antibiotics associated with *mar*, such as chloramphenicol, nalidixic acid, penicillin G, and tetracycline. We then purified and characterized plasmid DNA and detected *mar* phenotypic activity. The minimal inhibitory concentrations (MIC) of antibiotics for the isolates ranged from 6 to >50 μg/mL for chloramphenicol, 2 to 8 μg/mL for nalidixic acid, 25 to >300 μg/mL for penicillin G, and 1 to >80 μg/mL for tetracycline. On the other hand, many of the isolates had significantly enhanced MIC for the same antibiotics in the presence of 5 m*M* salicylate. Plasmid DNA extracted from 12 randomly chosen isolates ranged in size from 6 to 12.5 kb and, in several cases, conferred resistance to chloramphenicol and penicillin G. It is concluded that enteric bacteria from dairy farm topsoil are multidrug resistant and harbor antibiotic-resistance plasmids. A role for dairy topsoil in zoonoses is suggested, implicating this environment as a reservoir for development of bacterial resistance against clinically relevant antibiotics.
(**Key words:** antimicrobial resistance, bacteria, dairy soil, zoonosis)

Abbreviation key: Cm = chloramphenicol, ***mar*** = multiple antibiotic resistance operon, **Nal** = nalidixic acid, **PnG** = penicillin G, **Tc** = tetracycline.

INTRODUCTION

Bacterial antibiotic resistance is an emerging and serious public health concern due to the compromised efficacy of antimicrobial agents used in the treatment of infectious diseases (Cohen, 1992; Neu, 1992; Martínez and Baquero, 2002). Members of the Enterobacteriaceae family of bacteria are medically important as infectious agents, exhibit antibiotic resistance, and are present in large numbers in the animal gut (Paterson, 2002; Rupp and Fey, 2003). Antibiotics are extensively used in human and veterinary medicine, and in agricultural settings, for the treatment of infections, growth enhancement, and prophylaxis in food animals, potentially leading to selection of drug- and multidrug-resistant bacteria (Aarestrup, 1999; Barbosa and Levy, 2000b). In addition, antibiotic-producing microorganisms are found naturally in soil, suggesting intrinsic chromosomal antibiotic resistance originated in the soil in response to inhibitory environments generated by such antibiotic-producing microorganisms (George and Levy, 1983; Randal and Woodward, 2001). Whether commercially made or naturally occurring, stable antibiotics accumulate in soil inhabited by food animals and where antibiotics are used, thus selecting for multidrug resistance, which can be chromosomally (intrinsic) or plasmid-encoded (acquired) (George and Levy, 1983; Hanzawa et al., 1984; Bradford et al., 1999; Davies et al., 1999; Owens et al., 2001; Randal and Woodward, 2001).

Uncontrolled use of antibiotics in medicine and in farm animals has led to selection of multidrug-resistant bacteria in humans and cattle, respectively (Bradford et al., 1999; Davies et al., 1999). Consequently, enteric bacteria such as *Escherichia coli*, *Enterococcus faecalis*, and *Salmonella* spp. are not only resistant to multiple antibiotics given to animals but also to antibiotics made available to humans (Hanzawa et al., 1984; Bradford et al., 1999; Owens et al., 2001). These enteric bacteria are found in the intestinal tract of humans and cattle (Hanzawa et al., 1984; Bradford et al., 1999), providing a potential reservoir for these microorganisms in medi-

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cine and agriculture. Regarding agriculture in particular, if humans come in direct contact with infected fecal matter, transmission of antibiotic-resistant bacteria is possible (Levy, 1992; van den Bogaard and Stobberingh, 1999). Consequently, antibiotic resistance genes, if transferred to human microflora, may reduce efficacy of treatment for infectious diseases (Winokur et al., 2001; Ferber, 2002).

Although it is not completely understood how widespread the frequency of antibiotic-resistant bacteria is in the non-nosocomial community, the lack of surveillance data is especially evident in important agricultural environments, such as dairy farm soil. It remains unclear to what extent dairy soil harbors multidrug-resistant bacteria. Furthermore, it is unknown to what extent various modulators of enteric multiple antibiotic resistance (*mar*) genes (Cohen et al., 1993; Alekshun and Levy, 1997; Moken et al., 1997), such as salicylate, influence multidrug resistance among agriculturally derived soil bacterial isolates (Barbosa and Levy, 2000a). Other *mar* modulators include organic solvents, oxidative stress agents, and household disinfectants (White et al., 1997). Once surveillance data are known from the standpoint of intrinsic and inducible bacterial multidrug resistance in agriculture, it should be possible to invoke measures to reduce the conditions that lead to antibiotic resistance, and thus limit conditions that foster the spread or fixation of resistant infectious microorganisms in dairy farm environments.

Therefore, the objectives of our study were (i) to understand how widespread antibiotic-resistant enteric bacteria are in dairy soil, and (ii) to determine to what extent salicylate influences bacterial antibiotic resistance. We examined bacterial soil isolates from dairy farm soil for multiple resistance to antibiotics that are relevant in human clinical (nalidixic acid, penicillin, and chloramphenicol), veterinary (nalidixic acid, penicillin), and agricultural (tetracycline) settings. The work suggests that enteric bacteria from dairy farm topsoil contain inducible chromosomal elements that confer multiple antibiotic resistances and which harbor plasmids encoding resistance genes. This implies a role for dairy farm topsoil in zoonoses as well as in providing a reservoir for multidrug resistant bacteria.

MATERIALS AND METHODS

Soil Sample Collection and Bacterial Isolation

Topsoil was sampled from 11 randomly selected dairy farms in Roosevelt County, NM, from September 2001 to February 2002. The sampling and randomization strategies were as follows. For each dairy, 3 corrals were randomly selected, and 3 soil samples from each corral were taken. At the outer boundaries of the corral, base lines at right angles to each other were established through 2 axes of the area (corral), followed by pairs of randomly selected numbers to serve as coordinates for locating quadrats with references to the base lines. The apparatus, or quadrat, was a square device measuring 0.5 m × 0.5 m. The device was placed on the ground based on the randomly selected coordinates and a sample was taken from the middle of the quadrat. Nine samples were obtained from each dairy farm (3 samples from 3 randomly chosen corrals), plus 9 samples from adjacent roadsides as controls. About 5 g of topsoil was obtained per sample, transferred into 20 mL of LB broth, shaken, and placed on ice for 20 min. Each soil sample was centrifuged at 4°C and 13,000 × g for 30 s. Then, 20 μL of the supernatant was plated onto MacConkey agar plates containing 1% lactose, and incubated at 37°C for 24 h to select for enteric bacteria. Bacterial clones were colony-purified from each sample first by plating on LB agar and incubating at 37°C for 24 h. Colonies were picked, grown in LB broth at 37°C for 24 h, and stored in 25% glycerol at –20°C. MacConkey agar plates containing 1% lactose were inoculated using frozen bacterial stocks or fresh overnight cultures. Isolates were incubated at 37°C for 24 h and identified using the BBL Crystal Identification System as specified by the manufacturer (Becton Dickinson Microbiology Systems, Sparks, MD). Briefly, isolates were suspended in BBL Crystal Enteric/Stool Inoculum Fluid, vortexed for 10 to 15 s, inoculated, and incubated for 24 h at 37°C. After incubation, the kits were scored for identification. After identification, 22 of the isolates were designated as the working set according to their medical interest or to the amount of bacteria available in each genus.

Antibiotic Susceptibility Assays

Four antibiotics were chosen because of their association with *mar* or their widespread use in dairy cattle: chloramphenicol (**Cm**), nalidixic acid (**Nal**), penicillin G (**PnG**), and tetracycline (**Tc**). The level of isolate resistance to the antimicrobial agents was determined by the gradient plate method as described by Hachler et al. (1991) and George and Levy (1983). Isolates were scored for susceptibilities and resistances according to the National Committee for Clinical Laboratory Standards (2000). Briefly, isolates were grown to midlog phase in L broth (10 g of tryptone; 5 g of NaCl; 5 g of yeast extract; 2 g of glucose) at 30°C and streaked with sterile cotton swabs onto 1- to 3-h-old Luria agar antibiotic gradient plates. The plates were incubated for 40 h at 30°C. Minimum inhibitory concentrations were determined by interpolation of the relative lengths of growth, assuming a linear gradient across the plate.

Table 1. Bacterial isolates and strains utilized in the study.

Microorganism	Strain number(s) or relevant properties	Source or reference
Citrobacter braakii	26[1]	This study
Citrobacter freundii	41[1]	This study
Citrobacter koseri	32[1]	This study
Enterobacter gergoviae	81[1]	This study
Enterobacter taylorae	55,[1] 108[1]	This study
Escherichia coli	12[1]	This study
E. coli AG100/Kan	AG100 with *kan*^{R} replacing the *mar* locus	White et al., 1997
E. coli AG100/Kan/pRU600	AG100 with *kan*^{R} replacing the *mar* locus; harbors plasmid conferring chloramphenicol resistance (Cm^R)	Aslanidis et al., 1989
E. coli AG112	Δ1481–1485 in *mar*R locus	Oethinger et al., 2000
E. coli DW2 pNOEC73	Contains plasmid pNOEC73 encoding *amp*^{R}	Okazaki et al., 1997; Wilson and Wilson, 1987
Klebsiella pneumoniae	3,[2] 4,[2] 6,[1] 8,[1] 90,[1] 94[1]	This study
Proteus mirabilis	28,[1] 33,[1] 36[1]	This study
Proteus vulgaris	148[1]	This study
Pseudomonas aeruginosa	31,[1] 84[1]	This study
Pseudomonas fluorescens	85[1]	This study
Shigella spp.	78[1]	This study
Serratia plymuthica	21[1]	This study

[1]Isolates were obtained from corrals of dairies.

[2]Isolates were from adjacent roads.

Phenotypic Analysis of *mar* Locus Activity

Induction of *mar* operon activity was phenotypically assayed using modified methods of Hachler et al. (1991) and George and Levy (1983). Bacteria in midlog phase were streaked onto 1- to 3-h-old Luria agar antibiotic gradient plates supplemented with 5 m*M* salicylate. The inoculated plates were incubated for 40 h at 30°C, and the MIC were determined as described above.

Analysis of Bacterial Isolate Plasmids

Plasmid DNA was prepared from the isolates as described by Maniatis et al. (1982). Briefly, overnight cultures were harvested by centrifugation at 14,000 × *g* for 30 s, and the bacterial pellets were resuspended in 25 m*M* Tris•Cl buffer (pH 8.0) containing 50 m*M* glucose and 10 m*M* EDTA. Then, 0.2 *N* NaOH containing 1% SDS was added and incubated on ice for 15 min, followed by the addition of 5 *M* potassium acetate in glacial acetic acid. The mixture was centrifuged at room temperature and 14,000 × *g* for 5 min, and an equal volume of phenol:chloroform was added to the supernatant, which was then gently vortexed, and centrifuged at room temperature and 14,000 × *g* for 2 min. The DNA was precipitated with 2 volumes of 100% ethanol at –20°C and centrifuged for 5 min as above. The supernatant was decanted, and the resulting pellet was air dried and rinsed with ice cold 70% ethanol. The supernatant was removed, and the DNA pellet was air dried for 10 min followed by resuspension in 50 μL of 1.0 *M* Tris buffer (pH 8.0).

Plasmid DNA was analyzed, after restriction endonuclease digest, by gel electrophoresis and ethidium bromide staining using a 1-kb DNA ladder (New England Biolabs, Boston, MA). Plasmid DNA from selected isolates were used for transformation of competent *E. coli* strain AG100/Kan (Table 1). Transformants were prepared by transferring 300 μL of *E. coli* AG100/Kan competent cells and 5 μL of plasmid DNA into sterile, prechilled, microfuge tubes. The cells were incubated on ice for 40 min, heat shocked at 42°C for 45 s, and incubated with gentle shaking in LB broth at 37°C for 1 h. Transformants were added to 1-d-old plates containing either PnG (70 μg/mL) or Cm (10 μg/mL) and incubated for 24 h at 37°C. Transformant colonies were picked and grown in Luria broth for 24 h at 37°C and stored at –20°C as before. Transformant susceptibility to Cm and PnG was assessed as described above. *Escherichia coli* DW2 pNOEC73 and AG100/Kan pRU600, 2 positive controls, were grown at 37°C in Luria broth and at 30°C when plated onto gradient plates.

Data Analysis

To test for data (MIC in the presence of salicylate) normality, the D'Agostino test was conducted as described by Zar (1974) utilizing the grand mean MIC obtained in the absence and presence of salicylate for 11 isolates. The data were non-normal ($P < 0.05$). Transformation did not change these results, thus nonparametric analysis was used. To test the effects of salicylate on the antibiotic resistance to chloramphenicol, nalidixic acid, penicillin, and tetracycline, the nonparametric one-tail Wilcoxon-Paired sample test was used as described by Zar (1996). The one-tailed test was used, as the priori hypothesis was that salicylate increased

Table 2. Susceptibility of soil isolates to chloramphenicol (Cm), nalidixic acid (Nal), penicillin G (PnG), and tetracycline (Tc) and effects of salicylate (SAL) on antibiotic resistance.

	MIC,[1] μg/mL							
	Cm		Nal		PnG		Tc	
Soil isolate	SAL–	SAL+[2]	SAL–	SAL+[2]	SAL–	SAL+[2]	SAL–	SAL+[2]
Escherichia coli AG100/Kan	5 ± 0.4	≥9×	2 ± 0.2	≥15×	18	≥15.8×	2 ± 0.3	≥30×
E. coli AG112	20	≥2.3×	4	5×	53 ± 3	≥5.3×	≥5[3]	1.4×
Citrobacter braakii 26	22 ± 4	≥2.5×	5 ± 1	≥6×	≥300[3]	ND[4]	3 ± 0.1	≥3.7×
Citrobacter freundii 41	9 ± 0.8	≥5×	2 ± 0.2	≥7.5×	≥300[3]	ND	2 ± 0.7	≥5.5×
Citrobacter koseri 32	8 ± 0.7	≥5.6×	ND	ND	45 ± 13	≥1.5×	≥80[3]	ND
Enterobacter gergoviae 81	14 ± 3	≥6.4×	2 ± 0.3	≥7.5×	≥300[3]	ND	1 ± 0.3	≥11×
Enterobacter taylorae 55	8 ± 0.6	≥5.6×	<5[3]	ND	≥300[3]	ND	2 ± 0.2	≥5.5×
E. taylorae 108	6 ± 2	≥5×	<5[3]	ND	≥300[3]	ND	≤4[3]	ND
E. coli 12	8 ± 0.8	≥5.6×	2 ± 0.4	3.5×	34 ± 5	1.8×	2 ± 0.4	≥5.5×
Klebsiella pneumoniae 3	32	1.6×	8 ± 1	1.6×	51 ± 11	≥4.1×	10	0.8×
K. pneumoniae 4	6	4.2×	2	3×	48 ± 3	≥5.9×	2 ± 0.2	≥5.5×
K. pneumoniae 6	6 ± 0.7	≥5×	3 ± 0.5	≥5×	45 ± 6	≥6.3×	2 ± 0.2	≥5.5×
K. pneumoniae 8	9 ± 0.8	≥3.3×	2	≥7.5×	50 ± 7	≥4.8×	2 ± 0.4	≥5.5×
K. pneumoniae 90	7 ± 0.2	3.1×	3	18.3×	≥300[3]	ND	≥80[3]	ND
K. pneumoniae 94	32 ± 1	≥4.6×	6 ± 1	≥5×	≥300[3]	ND	≥80[3]	ND
Proteus mirabilis 28	10 ± 2	≥6×	3 ± 0.6	≥5×	≥300[3]	ND	13	≥4.6×
P. mirabilis 33	13 ± 1	1.1×	6 ± 1	≥3×	≥300[3]	ND	ND	ND
P. mirabilis 36	≥50[3]	ND	7 ± 2	≥2.1×	25	ND	≥80[3]	ND
Proteus vulgaris 148	12	1.9×	6 ± 2	1×	49	≥4.3×	6	≥5×
Pseudomonas aeruginosa 31	≥50[3]	ND	8 ± 2	ND	≥300[3]	ND	≥80[3]	ND
P. aeruginosa 84	6	3.8×	3 ± 0.6	≥5×	≥300[3]	ND	≥80[3]	ND
Pseudomonas fluorescens 85	≥30[3]	≥3.3×	3 ± 0.5	≥5×	46	≥4.6×	3 ± 0.8	≥3.7×
Shigella spp. 78	8 ± 0.7	≥5.6×	2 ± 0.2	≥7.5×	44 ± 7	≥4.8×	2 ± 0.2	≥5.5×
Serratia plymuthica 21	11	1.9×	4 ± 0.2	≥7.5×	37 ± 8	≥7.7×	3	≥5×

[1]Results were obtained using the gradient plate method. The overlay was constructed by supplementing Luria agar in the absence (SAL–) or presence (SAL+) of 5 m*M* salicylate with antibiotics, Cm, Nal, PnG, and Tc. Bacteria in log phase were inoculated 1 to 3 h after addition of the overlay and incubated for 40 h at 30°C. Minimum inhibitory concentrations were determined for growth between 40 and 70% along the gradient. Results are average values from at least 5 independent experiments with 15 repetitions.

[2]Data indicate the fold increase (×) in MIC in the presence of 5 m*M* salicylate.

[3]Results are values from 3 independent experiments each in triplicate.

[4]ND = Not determined.

antibiotic resistance in the isolates. Results were considered significant at $P \leq 0.05$.

RESULTS

Isolation of Bacterial Strains

One hundred forty-nine isolates were obtained from the soil of 11 randomly sampled dairy farms, and 9 isolates were obtained from adjacent roadsides (nondairy soil). Of the 102 isolates that were definitively identified, 22 were chosen for further study based on medical importance or high frequency of occurrence, and they were selected from a wide range of dairy farm locations (Table 1).

Antibiotic Susceptibility Profiles of Soil Isolates

Low levels of antibiotic resistance were observed in control isolates of *Klebsiella pneumoniae* (isolates # 3 and 4) obtained from a road adjacent to a dairy farm (Table 2). Slightly more than one-third of the isolates from dairy corrals showed relatively low levels of resistance to the antibiotics Cm, Nal, PnG, and Tc (Table 2). Most of the isolates demonstrated the least resistance to Nal, with MIC ranging from 2 to 8 μg/mL, and, in most cases, Tc, with MIC ranging from 1 to >80 μg/mL. Higher resistance levels were observed to Cm, with MIC from 9 to >50 μg/mL. However, *Citrobacter braakii* (isolate # 26), *Citrobacter freundii* (isolate # 41), *Enterobacter gergoviae* (isolate # 81), *Enterobacter taylorae* (isolates # 55 and 108), *K. pneumoniae* (isolates # 90 and 94), *Pseudomonas aeruginosa* (isolates # 31 and 84), and *Proteus mirabilis* (isolates # 28 and 33) showed high-level resistance (MIC >50 μg/mL) to PnG. Furthermore, *Citrobacter koseri* (isolate # 32), *E. coli* (isolate # 12), *K. pneumoniae* (isolates # 3, 4, 6, and 8), *Pseudomonas fluorescens* (isolate # 85), *P. mirabilis* (isolate # 36), *Proteus vulgaris* (isolate # 148), *Shigella* spp. (isolate # 78), and *Serratia plymuthica* (isolate # 21), although they did not express high levels of antibiotic resistance,

showed higher resistance to PnG than to Cm, Nal, and Tc (Table 2). With the exception of resistance to PnG, the isolates were, in most cases, more resistant than the *mar*-deleted negative control, AG100, and less resistant than the constitutive *mar* mutant positive control, AG112 (Table 2).

Analysis of *mar* Phenotype

Significantly increased resistances to Cm, Nal, PnG, and Tc were observed ($P \le 0.01$) in the presence of 5 m*M* salicylate compared with controls without salicylate (Table 2). In fact, the antibiotic resistance levels were generally doubled or tripled. Moreover, certain isolates, e.g., *Shigella* spp. (isolate # 78) and *E. gergoviae* (isolate # 81), showed more than a 5- and 6-fold increase in resistance to Cm, respectively. The same effect was observed with Tc, PnG, and Nal, to which the isolates were previously least resistant in the absence of salicylate (Table 2). As seen in Table 2, *K. pneumoniae* (isolate # 3) from roadside control soil was not induced by salicylate, as its MIC for Tc was reduced from 10 to 8 μg/mL. *Escherichia coli* AG100/Kan, the negative control, was induced by salicylate, thereby expressing equal or higher MIC than AG112, the positive control. Both controls demonstrated high-level resistance to PnG in the presence of salicylate (Table 3).

Table 3. Susceptibility of plasmid transformants (AG100/Kan pJB1-35) to chloramphenicol and penicillin G.

Cell type/plasmid[1]	MIC,[2] μg/mL	
	Cm	PnG
AG100/Kan	5 ± 0.9	21 ± 3
AG112	9 ± 0.8	61
pJB-1	11 ± 1	ND[3]
pJB-3	9 ± 1	30 ± 6
pJB-5	6 ± 0.6	30 ± 7
pJB-6	12 ± 2	32 ± 7
pJB-11	10 ± 1	ND
pJB-12	10 ± 0.8	ND
pJB-20	10 ± 1	36
pJB-21	10 ± 0.9	ND
pJB-22	13 ± 2	43 ± 11
pJB-28	ND	40
pJB-31	9 ± 0.8	32
pJB-32	11 ± 1	30 ± 7
pJB-35	6 ± 0.8	26 ± 4
DW2/pNOEC73	ND	≥80
AG100/Kan/pRU600	≥35	ND

[1]Numbers in the plasmid designations indicate the particular isolate from which the plasmid originated.

[2]Results were obtained using the gradient plate method. The overlay was constructed by supplementing L agar with chloramphenicol (Cm) or penicillin G (PnG) in the absence or presence of 5 m*M* salicylate. Transformant bacteria in log phase were inoculated 1 to 3 h after addition of the overlay with sterile cotton swabs. The plates were incubated for 40 h at 30°C. Minimum inhibitory concentrations were determined for growth between 40 and 70% along the gradient. Results are average values from 5 independent experiments with 15 replications.

[3]ND = Not determined.

Involvement of Plasmid DNA in Antibiotic Resistance

The presence of plasmid DNA was analyzed to assess whether elements other than the possible *mar* operon were contributing to the antibiotic resistances seen in the dairy soil isolates. Extracted plasmids ranged in size from 7.2 to 11.9 kb according to restriction endonuclease analysis (data not shown). *Escherichia coli* strain AG100/Kan transformants harboring the new plasmids demonstrated low-level resistances to Cm and PnG, with MIC from 6 to 13 μg/mL and 26 to 43 μg/mL, respectively (Table 3). Strain AG100/Kan containing plasmids pJB-3 or pJB-31 showed lower resistance to Cm than the wild-type, whereas AG100/Kan harboring pJB-6, pJB-12, or pJB-32 showed higher Cm resistance than plasmid-free AG112 wild-type cells (Table 3). Likewise, resistance to PnG was lower in AG100/Kan cells containing pJB-3, pJB-6, pJB-12, pJB-31, or pJB-32 than in plasmid-free wild-type cells.

DISCUSSION

In this study, a phenotypic analysis of the *mar* operon-like activity using salicylate was examined in dairy farm soil bacterial isolates to address the problem of emerging antibiotic resistance in dairy cattle. Low-level resistance was demonstrated in *K. pneumoniae* (isolates # 3 and 4) obtained from an adjacent dairy farm road (control isolates) and suggested a dissemination of antibiotic resistance from the corrals. With the exception of *K. pneumoniae* (isolate # 3), a 2-, 3-, or 5-fold increase in resistance to Cm, Nal, PnG, or Tc was observed with salicylate. Antibiotic resistance levels were significantly increased in the presence of salicylate ($P < 0.01$) in many of the isolates, such as *K. pneumoniae* (isolate # 4) and *S. plymuthica* (isolate # 21), compared with controls lacking salicylate (see Table 2). Although a *mar* phenotype was observed with salicylate induction, possession of a *mar* operon was not studied in this report. Cohen et al. (1993) has shown that the *mar* operon is conserved within members of the Enterobacteriaceae family, such as *Shigella flexneri*, *Salmonella* spp., and *Enterobacter aerogenes*, (Rajakumar et al., 1997; Kunonga et al., 2000; Chollet et al., 2002). *Klebsiella oxytoca* has a partially identified *mar* sequence (Chollet et al., 2002).

The low-level antibiotic resistance shown in Table 2 and the salicylate induction in Table 3 suggest involvement of *mar*, or other *mar*-like chromosomal elements in the topsoil isolates. Unlike high-level resistance, low-

level antibiotic resistance has been shown to be the result of mutational events in housekeeping genes (Baquero, 2001). Furthermore, other chromosomal elements have been implicated in eliciting low-level multiple antibiotic resistance, including the *pqr*A gene in *P. vulgaris*, *aar*P in *Providencia stuartii*, and *ram*A in *Enterobacter cloacae* (Ishida et al., 1995; Macinga et al., 1995; Alekshun and Levy, 1997; Lee et al., 2000). Moreover, the observed salicylate induction in Table 3 might be the result of induction of genes/operons independent of *mar*, as induction with salicylate is not limited to the *mar* operon (Cohen et al., 1993). Salicylate-inducible antibiotic resistance resulted in decreased porin channel expression in *Burkholderia cepacia* (formerly *Pseudomonas cepacia*) and increased fluoroquinolone resistance in *Staphylococcus aureus* (Burns and Clark, 1992; Gustafson et al., 1999). Unidentified *mar*-independent pathways have been induced with 5 m*M* salicylate in *E. coli* (Cohen et al., 1993). This is supported by our observation of salicylate induction in AG100/Kan, the negative control containing an interrupted *mar* locus (Table 3). Although this suggests a lack of a true negative control in the experimental design, it nonetheless indicates the presence of *mar*-independent elements capable of modulation by salicylate, and perhaps other putative modulators. Future studies are aimed at identifying the *mar*-independent mechanism(s) observed in the soil isolates in this study.

Considering the prevalence of plasmids in soil bacteria, it was not unexpected that we found higher resistance to Cm in AG100/Kan host cells containing plasmids pJB-1, pJB-6, pJB-11, pJB-12, pJB-20, pJB-21, pJB-22, and pJB-32 (Table 3) compared with wild-type cells without plasmid (Table 2). This may be due to an increase in copy number, as plasmid copy number has been shown to affect the general phenotype expressed by plasmids (Snyder and Champness, 1997), although we have not tested this possibility directly. Conversely, low copy number plasmids might be implicated in conferring lower resistances than the wild type to both Cm and PnG as seen in AG100/Kan cells with plasmids pJB-3, pJB-5, pJB-31, and pJB-35 (Tables 2 and 3). In any case, the presence of plasmids that confer Cm resistance (MIC 10 to 13 μg/mL) in these dairy soil bacteria is striking, as the plasmids might also harbor other pathogenic genes that could in turn lead to zoonoses upon contact with dairy farm topsoil. Consequently, a soil-borne mode of transmission is implied for bacteria harboring genes encoding virulence factors and other antibiotic resistances. Plasmid gene transfer between food animals and humans was demonstrated by detection of *amp*C in *Salmonella* and in *E. coli* isolates (Winokur et al., 2001). Future studies are aimed at characterizing the acquired plasmids and assessing types of genes encoded in these potentially mobile elements. For instance, low-level antibiotic resistance pumps such as Cmr could be involved in the presence of Cm resistance (Desomer et al., 1992). Resistances to other commonly used antibiotics in dairy farms are a focus of future studies.

The observed high-level resistances to Tc and PnG by *K. pneumoniae* (isolates # 90 and 94), *P. aeruginosa* (isolates # 31 and 84), and *E. taylorae* (isolates # 55 and 108) (Table 2) implies a step-wise evolution of resistance from low to high levels, although the exact mechanisms of resistances were not established in this study. Speculatively, this process could occur through a combination of bacterial resistance mechanisms (i.e., efflux pumps and porin mutations) that result in a phenotype higher than that of the single gene or by the facilitation of a second mechanism responsible for eliciting high levels of antibiotic resistance (Martínez and Baquero, 2000; Baquero, 2001). Alternatively, it could be argued that the high resistance to PnG and Tc are due to plasmid acquisition, as it has been shown that plasmids harboring *tet*(*M*) and β-lactamase genes are not only responsible for conferring high-level resistance but are also ubiquitous in soil particles (Chee-Sanford et al., 2001; Chopra and Roberts, 2001). However, the step-wise evolution process is more likely to have occurred due to the chronic soil exposure to excreted PnG and Tc, as supported by the observation that few isolates elicited high level resistances.

CONCLUSIONS

Concerns with the increased use of antibiotics in veterinary medicine have prompted a closer analysis of resistance mechanisms in zoonotic pathogens, primarily in the soil of food animal environments. It is possible that *mar* or *mar*-like genetic elements exist in dairy soil bacteria. It is striking that plasmids conferring chloramphenicol and (possibly) penicillin G resistance were detected in the soil isolates, suggesting compromised efficacy of chemotherapy. In addition to supporting the hypothesis that dairy farm topsoil can serve as a mode of zoonotic transmission, the observed high-level antibiotic resistances suggest that dairy farm topsoil serves as an environment in which clinically relevant resistance can develop. It is noteworthy that, due to poor absorption of drugs by food animals, there is a possibility that low levels of antibiotic resistance might persist on dairy farm topsoil even if prudent use of antibiotic takes place, suggesting the need for topsoil analysis of antibiotic residues as well as the establishment of surveillance programs for antibiotic resistant bacteria in the feces or intestine of food animals.

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