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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. In order to help circumvent the problem of bacterial antibiotic resistance, it is first necessary to understand the scope of the problem. However, is it 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 salycilate, 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 (MICs) of antibiotics for the isolates ranged between 6 - >50 μg/mL for chloramphenicol, 2–8 μg/mL for nalidixic acid, 25- >300 μg/mL for penicillin G and 1- > 80 μg/mL for tetracycline. On the other hand, the many of the isolates had significantly enhanced MICs for the same antibiotics in the presence of 5 mM salycilate. Plasmid DNA extracted from 12 randomly chosen isolates ranged in size between 6 and 12.5kb and in several cases conferred resistances to chloramphenicol and penicillin G. It is concluded that enteric bacteria from dairy farm topsoil are multi-drug resistant and harbor antibiotic resistance plasmids. A role for dairy topsoil in zoonosis is suggested, thus implicating this environment as a reservoir for bacterial resistance development against clinically relevant antibiotics.

Keywords

antimicrobial resistance; plasmids; bacteria; dairy soil; zoonosis

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 (Martínez and Baquero, 2002; Neu, 1992; Cohen, 1992). Members of the Enterobacteriaceae family of bacteria are medically important as infectious agents, exhibit antibiotic resistances 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, such as for the treatment of infections, growth enhancement and prophylaxis in food animals, potentially leading to selection of drug and multidrug resistant bacteria (Aarestrup, 1999;

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Barbosa *et al*., 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 resistant bacteria, which can be chromosomally-(intrinsic) or plasmid-encoded (acquired) (Bradford *et al*., 1999, Davies *et al*., 1999; Hanzawa *et al*., 1984; Owens *et al*., 2001; George and Levy, 1983; Randal and Woodward, 2001).

Uncontrolled use of antibiotics in medicine and in farm animals has led to selection of multiple antibiotic 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; Owens *et al*., 2001; Bradford *et al*., 1999). These enteric bacteria are found in the intestinal tract of humans and of cattle (Hanzawa *et al*., 1984; Bradford, 1999), providing a potential reservoir for these microorganisms in medicine 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 Bogard and Stobberingh, 1999). Consequently, antibiotic resistance genes, if transferred to human microflora, may reduce efficacy of treatment for infectious diseases (Ferber, 2002, Winokur *et al*., 2001).

Although it is not completely understood how widespread the frequency of antibiotic resistant bacteria are in the non-nosocomial community, the lack of surveillance data is especially evident in important agricultural environments, such as dairy farm soil. Whether antibiotics are improperly used or naturally occurring, it nonetheless 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 (Alekshun and Levy, 1997, Cohen, *et al*., 1993, Moken, *et al*., 1997), such as salycilate, a component of aspirin and known model modulator of *mar*, 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 resistances in agriculture, such as dairy farms, it could then become 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 are (i) to understand how widespread antibiotic resistant enteric bacteria are in an agricultural setting, i.e., dairy soil, and (ii) to determine to what extent salycilate influences bacterial antibiotic resistance. Here, we examined bacterial soil isolates from dairy farm soil for multiple resistances 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 zoonosis 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 during September 2001–February 2002. The sampling and randomization strategies were as follows. For each dairy, three corrals were randomly selected, and three soil samples from each of the

three corrals were taken. At the outer boundaries of the corral, base lines at right angles to each other were established through two 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 in length. The device was placed on the ground based on the randomly selected coordinates and a sample was taken from the middle of the quadrat. A total of 9 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 were obtained per sample, transferred into 20 mL of LB broth, shaken, and placed in ice for 20 min. Each soil sample was centrifuged at 4 °C at 13,000 ×g for 30 s. Then, 20 μL of the supernatant were 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 the isolates were stored in 25 % glycerol at −20 °C. MacConkey agar plates containing 1% lactose were inoculated using either 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 Dickenson Microbiology Systems, Sparks, Maryland). Briefly, isolates were suspended in BBL Crystal Enteric/Stool Inoculum Fluid, vortexed for 10–15 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 either of their association with *mar* or their widespread use in dairy cattle: chloramphenicol (Cm), nalidixic acid (Nal), penicillin G (Pn G), and tetracycline (Tc). The levels of isolate resistance to the antimicrobial agents were determined by the gradient plate method as described by Hachler *et al* (1991) and George and Levy (1983) and scored for susceptibilities and resistances according to the National Committee for Clinical Laboratory Standards (2000). Briefly, isolates were grown to mid-log phase in L broth (10 g tryptone; 5g NaCl; 5 g yeast extract; 2 g glucose) at 30 °C and streaked with sterile cotton swabs onto 1–3 hour-old L agar antibiotic gradient plates. The plates were incubated for 40 h at 30 °C. MICs were determined by interpolation of the relative lengths of growth, assuming a linear gradient across the plate.

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 mid-log phase were streaked onto 1–3 hour-old L agar antibiotic gradient plates supplemented with 5 mM salycilate. The inoculated plates were incubated for 40 h at 30 °C, and the MICs 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 mM Tris Cl buffer (pH 8.0) containing 50 mM glucose, 10 mM 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 mildly vortexed and centrifuged at room temperature and 14,000 ×g for 2 min. 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 DNAs from selected isolates were used for transformation of competent *E. coli* strain AG100/Kan (Table 1). Transformants were prepared by transferring 300 μL of AG100/Kan competent cells and 5 μL of plasmid DNA into sterile pre-chilled 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-day-old plates containing either Pn G (70 μg/mL) or Cm (10 μg/mL) and incubated for 24 h at 37 °C. Transformant colonies were picked and grown in L 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. *E.coli* DW2 pNOEC73 and AG100/Kan pRU600, two positive controls, were grown at 37 °C in L broth and at 30 °C when plated onto gradient plates.

Data Analysis

To test for data (MIC in the presence of salycilate) normality, the D'Agostino test was conducted as described by Zar (1974) utilizing the grand mean MICs obtained in the absence and presence of salycilate for 11 isolates. The data were non-normal ($P < 0.05$). Transformation did not change these results, thus non-parametric analysis was utilized. To test the effects of salycilate on the antibiotic resistance to chloramphenicol, nalidixic acid, penicillin, and tetracycline, the non-parametric 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 salycilate increased antibiotic resistance in the isolates. Results were considered significant at $P \leq 0.05$.

RESULTS

Isolation of Bacterial Strains

A total of 149 isolates were obtained from the soil of 11 randomly sampled dairy farms, and 9 isolates were obtained from adjacent roadsides (non-dairy soil). Of the 102 isolates that were definitively identified, 22 were chosen for further study on the basis of medical importance or high frequency of occurrence, and they were also selected from a wide range of dairy farm locations (Table 1).

Antibiotic Susceptibility Profiles of Soil Isolates

Low levels of antibiotic resistances were observed in control isolates *K. pneumoniae* (isolates # 3 and 4) obtained from an adjacent dairy farm road (Table 2). Slightly more than a third of the isolates from dairy corrals showed relatively low-levels of resistance to the antibiotics chloramphenicol, nalidixic acid, penicillin, and tetracycline (Table 2). Most of the isolates demonstrated the least resistance to nalidixic acid, with MICs ranging from 2 – 8 μg/mL, and, in most cases, tetracycline, with MICs ranging from 1 - > 80 μg/mL. Higher resistance levels were observed to chloramphenicol, with MICs 9 – >50 μg/mL. However, *Citrobacter braakii* (isolate # 26), *C. freundii* (isolate # 41), *Enterobacter gergoviae* (isolate # 81), *E. taylorae* (isolates # 55 and 108), *Klebsiella pneumoniae* (isolates # 90 and 94), *Pseudomonas aeruginosa* (isolates # 31 and 84), and *Proteus mirabilis* (isolates #28 and 33) showed high-level resistance (MICs > 50 μg/mL) to penicillin. Furthermore, *C. koseri* (isolate # 32), *E. coli* (isolate # 12), *K. pneumoniae* (isolates # 3, 4, 6, and 8), *P. fluorescens* (isolate # 85), *P. mirabilis* (isolate #36), *P. 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 penicillin than to chloramphenicol, nalidixic acid and tetracycline

(Table 2). With the exception of resistance to penicillin, 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 chloramphenicol, nalidixic acid, penicillin and tetracycline were observed ($P \leq 0.01$) in the presence of 5 mM salycilate compared to controls without salycilate (Table 2). In fact, the antibiotic resistance levels were doubled or tripled on average. Moreover, certain isolates, e.g., *Shigella* spp. (isolate # 78) and *E. gergoviae* (isolate # 81), showed more than a five and six-fold increase in resistance to chloramphenicol, respectively. The same effect was observed with tetracycline, penicillin and nalidixic acid, to which the isolates were previously least resistant in the absence of salycilate (Table 2). As seen in Table 2, *K. pneumoniae* (isolate # 3) from roadside control soil was not induced by salycilate, as its MIC for tetracycline was reduced from 10 to 8 μg/mL. *E. coli* AG100/Kan, the negative control, was induced by salycilate, thereby expressing equal or higher MICs than AG112, the positive control. Both controls demonstrated high-level resistance to penicillin in the presence of salycilate (Table 3).

Involvement of Plasmid DNA on 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 – 11.9 kbp according to restriction endonuclease analysis (data not shown). *E. coli* strain AG100/Kan transformants harboring the new plasmids demonstrated low-level resistances to chloramphenicol and penicillin, with MICs from 6 – 13 μg/mL and 26 – 43 μg/mL, respectively (Table 3). *E. coli* AG100/Kan containing plasmids pJB-3 or pJB-31 showed lower resistance to chloramphenicol than the wild-type, while AG100/Kan harboring pJB-6, pJB-12 or pJB-32 showed higher chloramphenicol resistance than plasmid-free AG112 wild-type cells (Table 3). Likewise, resistance to penicillin 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 salycilate was examined in dairy farm soil bacterial isolates in order to address the potential problem of emerging antibiotic resistance in dairy cattle. Low-level resistances were demonstrated in *K. pneumoniae* (isolates # 3 and 4) obtained from an adjacent dairy farm road (control isolates) and suggests 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 chloramphenicol, nalidixic acid, penicillin and tetracycline were observed with salycilate. Antibiotic resistance levels were significantly increased in the presence of salycilate compared to controls lacking salycilate ($P < 0.01$), in many of the isolates, such as in *K. pneumoniae* (isolate # 4) and *S. plymuthica* (isolate # 21), (see Table 2). Although a *mar* phenotype was observed with salycilate 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 *S. flexneri*, *Salmonella* spp., and *E. aerogenes*, (Rajakumar *et al.*, 1997;Kunonga *et al.*, 2000;Chollet *et al.*, 2002). *K. oxytoca* has a partially identified mar sequence (Chollet *et al.*, 2002).

The low-level antibiotic resistance seen in Table 2 and the salycilate 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 chromosomal housekeeping genes (Baquero, 2001). Furthermore, other chromosomal elements have been implicated in eliciting low-level multiple antibiotic resistance, including the *pqrA* gene in *P. vulgaris*, *aarP* in *Providencia stuartii*, and *ramA* in *E. cloacae* (Ishida *et al.*, 1995;Macinga *et al.*, 1995;Lee *et al.*, 2000;Alekshun and Levy, 1997). Moreover, the observed salycilate induction observed in Table 3 might be a result of induction of genes/operons independent of *mar*, as induction with salycilate has been shown to not be limited to the *mar* operon (Cohen *et al.*, 1993). Salycilate-inducible antibiotic resistance resulted in decreased porin channel expression in *Burkholderia cepacia* (formerly *P. cepacia)* and increased fluoroquinolone resistance in *Staphylococcus aureus* (Burns and Clark., 1992;Gustafson *et al.*, 1999). Unidentified *mar*-independent pathways have also been induced with 5mM salycilate in *E. coli* (Cohen *et al.*, 1993). This is supported by our observation of salycilate induction in AG100/Kan, the negative control containing an interrupted *mar* locus, in Table 3. Although this suggests a lack of a true negative control in the experimental design, it nonetheless, and more importantly, indicates the presence of *mar*-independent elements capable of modulation by salycilate, and perhaps by 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 chloramphenicol 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 to wild-type cells without plasmid (Table 2). This may possibly 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 chloramphenicol and penicillin 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 chloramphenicol resistance conferring plasmids (MICs 10–13 μg/mL) in these dairy soil bacteria is striking, as the plasmids might also harbor other pathogenic genes that could in turn lead to zoonosis 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 *ampC* 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 chloramphenicol resistance (Desomer *et al*, 1992). In addition, resistances to other commonly utilized antibiotics in dairy farms are a focus of future studies.

The observed high-level resistances to tetracycline and penicillin 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 of antibiotic resistance, 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) which result in a phenotype that is higher than that of the single gene or by the facilitation of a second mechanism responsible for eliciting high-levels of antibiotic resistance (Baquero, 2001;Martinez and Baquero, 2000). Alternatively, it could be argued that the high resistances to penicillin and tetracycline 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 (Chopra and Roberts, 2001;Chee-Sanford *et al.*, 2001). However, the step-wise evolution process is more likely to have occurred due to the chronic soil exposure to excreted penicillin and tetracycline and 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 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 resistances were detected in the soil isolates, suggesting compromised efficacy. 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 also serves as an environment in which clinically relevant resistance can develop. Thus, this study suggests the involvement of antibiotic resistance genes in zoonosis from dairy farm topsoil bacteria. 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 or any food environment even if prudent use of antibiotic takes place, suggesting the need for topsoil analysis of antibiotic residues in addition to establishment of surveillance programs for antibiotic resistant bacteria in the feces or intestine of food animals.

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Abbreviation key

mar
: multiple antibiotic resistance operon

MIC
: minimal inhibitory concentration

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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, 108[11]	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 pNOE73	Contains plasmid pNOEC73 encoding *amp*^{R}	Okazaki, *et al*, 1997; Wilson and Wilson, 1987
Klebsiella pneumoniae	3[2], 42, 61, 81, 901, 941	This study
Proteus mirabilis	28, 33[111]	This study
Proteus vulgaris	148[1]	This study
Pseudomonas aeruginosa	31, 84[11]	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.

Table 2

Susceptibility of Soil Isolates to Chloramphenicol, Nalidixic Acid, Penicillin G, and Tetracycline and Effects of Salycilate on Antibiotic Resistance.

	MIC[1] µg/mL (−Sal) and Fold MIC Increase in the Presence of Salycilate[2] (+Sal)							
	Cm		Nal		Pn G		Tc	
Cell	−Sal	+Sal[2]	−Sal	+ Sal[2]	−Sal	+ Sal[2]	−Sal	+ Sal[2]
E. coli AG100/Kan	5 ± 0.4	≥ 9x	2 ± 0.2	≥ 15x	18	≥ 15.8x	2 ± 0.3	≥ 30x
E. coli AG112	20	≥ 2.3x	4	5x	53 ± 3	≥ 5.3x	≥ 5[b33]	1.4x
C. braakii 26	22 ± 4	≥ 2.5x	5 ± 1	≥ 6x	≥ 300[3]	-[4]	3 ± 0.1	≥ 3.7x
C. freundii 41	9 ± 0.8	≥ 5x	2 ± 0.2	≥ 7.5x	≥ 300[3]	-[4]	2 ± 0.7	≥ 5.5x
C. koseri 32	8 ± 0.7	≥ 5.6x	-[4]	-[4]	45 ± 13	≥ 1.5x	≥ 80[3]	-[4]
E. gergoviae 81	14 ± 3	≥ 6.4x	2 ± 0.3	≥ 7.5x	≥ 300[3]	-[4]	1 ± 0.3	≥ 11x
E. taylorae 55	8 ± 0.6	≥ 5.6x	< 5[3]	-[4]	≥ 300[3]	-[4]	2 ± 0.2	≥ 5.5x
E. taylorae 108	6 ± 2	≥ 5x	< 5[3]	-[4]	≥ 300[3]	-[4]	≤ 4[3]	-[4]
E. coli 12	8 ± 0.8	≥ 5.6x	2 ± 0.4	3.5x	34 ± 5	1.8x	2 ± 0.4	≥ 5.5x
K. pneumoniae 3	32	1.6x	8 ± 1	1.6x	51 ± 11	≥ 4.1x	10	0.8x
K. pneumoniae 4	6	4.2x	2	3x	48 ± 3	≥ 5.9x	2 ± 0.2	≥ 5.5x
K. pneumoniae 6	6 ± 0.7	≥ 5x	3 ± 0.5	≥ 5x	45 ± 6	≥ 6.3x	2 ± 0.2	≥ 5.5x
K. pneumoniae 8	9 ± 0.8	≥ 3.3x	2	≥ 7.5x	50 ± 7	≥ 4.8x	2 ± 0.4	≥ 5.5x
K. pneumoniae 90	7 ± 0.2	3.1x	3	18.3x	≥ 300[3]	-[4]	≥ 80[3]	-[4]
K. pneumoniae 94	32 ± 1	≥ 4.6x	6 ± 1	≥ 5x	≥ 300[3]	-[4]	≥ 80[3]	-[4]
P. mirabilis 28	10 ± 2	≥ 6x	3 ± 0.6	≥ 5x	≥ 300[3]	-[4]	13	≥ 4.6x
P. mirabilis 33	13 ± 1	1.1x	6 ± 1	≥ 3x	≥ 300[3]	-[4]	-[4]	-[4]
P. mirabilis 36	≥ 50[3]	-[4]	7 ± 2	≥ 2.1x	25	-[4]	≥ 80[3]	-[4]
P. vulgaris 148	12	1.9x	6 ± 2	1x	49	≥ 4.3x	6	≥ 5x
P. aeruginosa 31	≥ 50[3]	-[4]	8 ± 2	-[4]	≥ 300[3]	-[4]	≥ 80[3]	-[4]
P. aeruginosa 84	6	3.8x	3 ± 0.6	≥ 5x	≥ 300[3]	-[4]	≥ 80[3]	-[4]
P. fluorescens 85	≥ 30[3]	≥ 3.3x	3 ± 0.5	≥ 5x	46	≥ 4.6x	3 ± 0.8	≥ 3.7x
Shigella spp. 78	8 ± 0.7	≥ 5.6x	2 ± 0.2	≥ 7.5x	44 ± 7	≥ 4.8x	2 ≧ 0.2	≥ 5.5x
S. plymuthica 21	11	1.9x	4 ± 0.2	≥ 7.5x	37 ± 8	≥ 7.7x	3	≥ 5x

[1] Results were obtained by using the gradient plate method. The overlay was constructed by supplementing L agar either in the absence (− Sal) or presence (+Sal) of 5 mM salycilate with chloramphenicol (Cm), nalidixic aid (Nal), penicillin G (Pn G), or tetracycline (Tc). Bacteria in log phase were inoculated 1–3 hours after addition of the overlay and incubated 40 h at 30°C. MICs were determined for growth between 40–70% along the gradient. Results are average values from at least five independent experiments with 15 repetitions.

[2] Data indicate the fold increase in MIC in the presence of 5 mM Salycilate.

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

[4] Not determined

Table 3

Susceptibility of Plasmid Transformants (AG100/Kan pJB1–35) to Chloramphenicol and Penicillin G

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

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

[2] MIC units are in μg/mL. Results were obtained by using the gradient plate method. The overlay was constructed by supplementing L agar with chloramphenicol (Cm) or penicillin G (Pn G) in the absence or presence of 5 mM salycilate. Transformant Bacteria in log phase were inoculated 1–3 hours after addition of the overlay with sterile cotton swabs. The plates were incubated for 40 hours at 30°C. MIC's were determined for growth between 40–70% along the gradient. Results are average values from five independent experiments with 15 replications.

[3] Not determined