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Class 1 Integrons and Tetracycline Resistance Genes in *Alcaligenes*, *Arthrobacter*, and *Pseudomonas* spp. Isolated from Pigsties and Manured Soil

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The presence of tetracycline resistance (Tc^r) genes and class I integrons (in-1), and their ability to cotransfer were investigated in Tc^r gram-negative (185 strains) and gram-positive (72 strains) bacteria from Danish farmland and pigsties. The isolates belonged to the groups or species *Escherichia coli*, *Enterobacter* spp., *Arthrobacter* spp., *Alcaligenes* spp., *Pseudomonas* spp., and *Corynebacterium glutamicum*. The 257 isolates were screened for in-1. Eighty-one of the gram-negative isolates were also screened for the Tc^r genes *tet*(A), *tet*(B), and *tet*(C), and all ($n = 72$) gram-positive isolates were screened for *tet*(33). Fourteen (7%) of the soil isolates and eleven (25%) of the pigsty isolates contained in-1. All isolates that contained *tet* genes also contained in-1, except one gram-negative isolate from a pigsty that contained *tet*(B). All gram-positive isolates with in-1 also contained *tet*(33). No isolates contained more than one *tet* gene. The in-1-positive isolates were tested for resistance to selected antimicrobial agents and showed resistance to three to nine drugs. Filter-mating experiments showed cotransfer of Tc^r and class I integrons from soil isolates to *Escherichia coli* and/or *Pseudomonas putida*. We conclude that soil bacteria in close contact to manure or pigsty environment may thus have an important role in horizontal spread of resistance. Use of tetracyclines in food animal production may increase not only Tc^r but also multidrug resistance (caused by the presence *tet* genes and in-1) in bacteria.

In Danish food animal production, large amounts of tetracyclines are used therapeutically in the pig production (4). Tetracyclines are excreted in active form in urine and feces (23) and can also be detected in animal manure slurry and manured soil (6, 13, 29). Therefore, the use of tetracyclines may exert selective pressure not only on bacteria within the intestinal system but also on bacteria in close contact with the environment of a pigsty or with animal manure. The presence of tetracyclines may also increase horizontal transfer of tetracycline resistance (Tc^r) genes (24, 30). Indigenous soil bacteria may exchange resistance genes with bacteria in animal manure and thereby contribute to the transfer of antimicrobial resistance to bacterial populations associated with humans and animals via crops.

Class I integrons (in-1) contribute to the spread of antimicrobial resistance genes and have been found in *Enterobacteriaceae* and other gram-negative bacteria (7), but they have also been found in a few gram-positive bacteria. Martin et al. were the first to report integrons in gram-positive bacteria; these authors found transposon-borne integrons in *Mycobacterium fortuitum* (18). An *int-1*-like gene truncated by the insertion sequence IS*6100* located on the 27.8-kb plasmid pTET3, which also carries a newer Tc^r gene, *tet* (32), has been found in *Corynebacterium glutamicum* (31). Clark et al. found integrons with *aadA1* gene cassettes on an 80-kb transferable plasmid in *Enterococcus faecalis* (5). Recently, Nandi et al. found that gram-positive bacteria present in poultry litter were a major reservoir for in-1, especially among *Corynebacterium* spp. (20).

Tc^r genes have not been found as gene cassettes in class I integrons, but have been found associated with class I integrons on self-transferable plasmids in both gram-positive and gram-negative bacteria such as *Salmonella*, *Aeromonas*, and *Corynebacterium* spp. (10, 11, 16, 31).

We investigated the presence of commonly found Tc^r genes and class I integrons in bacteria isolated from manured soil and pigsty environment. Our purpose was to investigate whether environmental bacteria constitute a reservoir that could contribute to the spread of antimicrobial resistance between the animal and human populations.

MATERIALS AND METHODS

Farms. The eight farms included in this investigation were located on the island Zealand in Denmark. The sampling took place from September 1998 to September 2000. One farm (farm 5) was a test farm for the Royal Veterinary and Agricultural University; this farm did not use animal manure as fertilizer. The seven other farms were conventional pig farms at which manure was spread on the fields once a year. The crops grown in these fields included winter wheat, beets, and spring barley. The soil samples from the seven conventional farms used in our investigation came from fields that were treated with slurry of pig manure once per year for more than 10 years.

Farm 1 kept animal waste as a dunghill, and this manure was spread on fields once a year in April. Farm 2 also kept animal waste as a dunghill, and manure was spread once a year in August. The capacity of farm 1 and 2 was 700 and 1,800 pigs/year, respectively. Pig manure on farms 3, 4, A, B, and C was kept in large slurry containers. This manure slurry was spread in the fields of the respective farms once a year around May. The capacities of these five farms were 2,200, 900, 1,350, 1,100, and 1,250 pigs per year, respectively. Use of tetracyclines, aminoglycosides, and sulfonamides/trimethoprim has been documented for farms A, B, and C. None of the three farms used aminoglycosides. Farms A, B, and C used 285, 345, and 6,229 g of tetracycline/year and 96, 169, and 1,136 g of sulfadiazin and trimethoprim (5:1)/year, respectively.

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Bacterial isolates from farms and fields. Soil samples were collected on the eight farms as previously described (15, 29). Ten to twenty-five soil samples were collected from each farm, and bacterial isolates were recovered from the soil as previously described (29).

Bacteria from pigsty environments were isolated from fecal samples collected on three Danish pig farms (12 samples on farm 2 and 1 sample each on farms A and C). Approximately 100 g of samples were collected 1 m apart. Two grams of samples were added to 20 ml of 0.9% saline and shaken on a water bath at 120 rpm and 25°C for 1 h. The samples were serially 10-fold diluted in 0.9% saline and plated by surface spreading onto Luria-Bertani (LB) agar (25) and MacConkey agar (Oxoid, Greve, Denmark), both supplemented with tetracycline (8 μg/ml; Sigma-Aldrich, Brøndby, Denmark). The *Escherichia coli* isolates were isolated from the MacConkey plates and identified by using API 20E (bioMérieux, Marcy l'Etoile, France) and serotyped (32). The other pigsty isolates were isolated on LB agar plates.

From each soil or fecal sample one to three isolates were included in this investigation; none of the isolates from the same sample were from the same genus.

All isolates (except *E. coli*) were Gram stained and tested for oxidase, catalase, mobility, and for their oxidative or fermentative activity. The in-1-positive bacteria were identified by 16S rRNA sequencing by using primers 16S 10FX and 16S 519R (Table 1) from positions 13 to 545 (GenBank accession no. X80745). DNA sequencing was performed as described below. The obtained sequences were compared by use of the BLAST program (version 2.2.9) with sequences submitted to GenBank. The identification of *Pseudomonas* and *Alcaligenes* spp. was verified by use of API20NE and for *Enterobacter* sp. by use of API 20E (bioMérieux).

Susceptibility testing. All positive in-1 isolates and transconjugants were tested for susceptibility to a series of antimicrobial agents in Mueller-Hinton broth. The antimicrobial agents were supplied on commercially prepared dehydrated Sensititre panels (Trek Diagnostic Systems, West Sussex, England). The antimicrobial agents in the tests included ampicillin (0.5 to 32 μg/ml; *Arthrobacter* sp. not tested), ciprofloxacin (0.03 to 8 μg/ml), nalidixic acid (2 to 128 μg/ml), chloramphenicol (1 to 64 μg/ml), florfenicol (0.5 to 32 μg/ml), gentamicin (0.5 to 32 μg/ml), streptomycin (2 to 128 μg/ml), spectinomycin (128 to 4 μg/ml), sulfonamethoxazole (8 to 512 μg/ml), trimethoprim (0.5 to 32 μg/ml), and tetracycline (0.5 to 32 μg/ml). The susceptibility testing was preformed according to NCCLS guidelines (21). The panels were incubated aerobically for 23 to 24 h at 25°C except for the inoculates with *E. coli* and *P. putida* isolates, which were incubated for 18 to 20 h at 37°C. The MIC results were interpreted by use of the NCCLS breakpoints for *Enterobacteriaceae* for *E. coli*, *Pseudomonas* sp., *Alcaligenes faecalis*, and *Enterobacter* sp. (21). For *Arthrobacter* isolate breakpoints for *Streptococcus pneumoniae* were used (21). For spectinomycin and florfenicol, no NCCLS breakpoints are currently available, so for spectinomycin the recommended breakpoint from Rosco (Denmark) was used (*Rosco Diagnostica Users Guide* [updated 052203]) and for florfenicol the breakpoint was set to ≥32 μg/ml. Since not all of the species have standardized and well-characterized breakpoints, approximations were used in the present investigations. The measured MICs for the resistant isolates were higher by a factor of four or more over the used breakpoints for all antimicrobials, except in some cases involving streptomycin and spectinomycin, in which the term "reduced in susceptibility" was used.

PCR detection of in-1 and Tc^r genes. The primers used in the present study are listed in Table 2. Tc^r gram-negative (n = 164) and gram-positive (n = 49) soil isolates and gram-negative (n = 21) and gram-positive (n = 23) pigsty isolates were screened for in-1 by use of three primer sets: qacEΔ1-F and qacEΔ1-B, qacEΔ1-F and Sul-1B, and Att1-F and 3′CS-B (Table 2). Sixty of the gram-negative soil isolates (including the in-1-positive isolates, resulting in six to eight isolates from each farm) and all of the gram-negative pigsty isolates (20) were also screened for the Tc^r genes *tet*(A), *tet*(B), and *tet*(C). All gram-negative in-1-positive isolates that did not contain *tet*(A-C) (n = 3) were screened for *tet*(D), *tet*(E), *tet*(G), *tet*(31), and *tet*(34). Six *Arthrobacter* isolates were screened for *tet*(M), *tet*(O), *tet*(S), *tet*(L), *tet*(K), *tet*(Z), and *tet*(33). Since *tet*(33) was found in all six isolates and none of the other *tet* genes were found, the remaining gram-positive isolates (n = 66) were screened for *tet*(33). Transconjugants were screened for in-1 and the same *tet* genes that the respective donors contained.

Sequencing of gene cassettes. DNA sequencing (28) of PCR products were preformed on an ABI 373A automatic sequencer by using the Prism BigDye terminator kit (Applied Biosystems, Foster City, CA) by use of the primers listed in Table 2 as previously described (2).

Mating experiments with in-1-positive soil isolates. Bacteria from the soil environment, intestinal system, and a pathogen were chosen as recipients. Mating experiments with gram-negative donors were done with *E. coli* [1005(R)] and *Pseudomonas putida* [KT2442(N)] as recipients. For gram-positive matings, *E. faecalis* (JH2-2) was used as the recipient. Three *Arthrobacter* isolates (R343, R344, and R345) were also used as donors to the recipients: *E. coli* [1005(R)], *P. putida* [KT2442(N)], *E. faecium* (BM4105), and *Staphylococcus aureus* (8794RF) (Table 1) as described by Hammerum et al. (12). Exponentially growing donor and recipient cultures were washed twice in 0.9% saline and mixed 1:1 at an optical density at 450 nm of 0.5. A volume of 500 μl of donor-recipient mixture was added to a filter placed on an LB agar plate for the gram-negative recipients and on a calf blood agar plate for the gram-positive recipients. The agar plate with the mixed cell suspension was incubated at 25°C for 20 to 24 h. The cells were serially diluted, and appropriate dilutions were plated onto selective media in order to determine the extent of horizontal transfer. Transconjugants of *E. coli* and *P. putida* were selected on LB agar supplemented with 8 μg of tetracycline and 100 μg of rifampin (Sigma-Aldrich, Brøndby, Denmark)/ml. Transconjugants for *E. faecalis*, *E. faecium*, and *S. aureus* were selected on brain heart infusion agar (Difco, Detroit, MI) supplemented with 8 μg of tetracycline, 25 μg of rifampin, and 25 μg of fusidic acid (Sigma-Aldrich)/ml. The transconjugants were restreaked on selective plates.

Plasmid isolation and Southern blotting. All donors and gram-negative transconjugants with a transferable integron and Tc^r were screened for plasmids by use of an alkaline lysis method (9), followed by gel electrophoresis on a 0.8% Tris-borate-EDTA gel at 80 V for 5 h. Southern blots on undigested and BamHI-digested plasmids were performed. The blots were hybridized with two digoxigenin-labeled DNA probes for the presence of the 3′ segment of class 1 integrons using the PCR product (225 bp) amplified by the primers qacEΔ1-F and qacEΔ1-B and for *tet*(A) (956 bp) by using the PCR products amplified by tet(A)-1 and tet(A)-2, respectively (Table 2).

RESULTS

Screening for class 1 integrons and *tet* genes. A total of 213 Tc^r gram-negative (n = 164) and gram-positive (n = 49) bacteria from farmland soil and 44 Tc^r gram-negative (n = 21) and gram-positive (n = 23) bacteria from a pigsty environment were isolated from 130 soil samples and 15 fecal samples, respectively.

Class 1 integrons were found in 5% (8 of 164) of the gram-negative soil isolates, 33% (7 of 21) of the gram-negative pigsty isolates, 12% (6 of 49) of the gram-positive soil isolates, and 17% (4 of 23) of the gram-positive pigsty isolates. The soil isolates belonged to species of *Pseudomonas*, *Alcaligenes*, *Corynebacterium*, and *Arthrobacter* The pigsty isolates were identified as *E. coli*, *Enterobacter* spp., *Arthrobacter* spp., and one unidentified gram-positive species with 96% homology to 16S RNA of *Leucobacter komagatae* (GenBank accession no. AJ746337, positions 10 to 750) (Table 1).

Sixty of the Tc^r soil isolates, including the in-1-positive isolates and all 21 gram-negative pigsty isolates, were screened for *tet*(A), *tet*(B), and *tet*(C). *tet*(A) was found in three *Alcaligenes* spp. from soil, seven *E. coli* strains, and one *Enterobacter* sp. from a pigsty, *tet*(B) was found in one *E. coli* pigsty isolate, and *tet*(C) was found in two *Pseudomonas* spp. from soil. The remaining 67 gram-negative isolates did not contain any of the three *tet* genes [*tet*(A), *tet*(B) or *tet*(C)]. All *tet*(A) or *tet*(C) genes were found among in-1-positive isolates except one *tet*(A)-positive *E. coli*. Three in-1-positive soil isolates did not contain *tet*(A-C) or any of the other *tet* genes for which we screened.

Ten isolates were positive for *tet*(33), and all isolates had, in addition, in-1 (Table 1). The PCR product of *tet*(33) was sequenced in seven isolates (R70, R344, R345 [GenBank accession no. DQ077487], R1809, R1811, R1554, and R2139) and had 99 to 100% homology to *tet*(33) from *C. glutamicum* (GenBank accession no. AJ420072, positions 23085 to 23840). The remaining 62 gram-positive isolates did not contain *tet*(33).

TABLE 1. Bacterial strains and class 1 integron-positive isolates used in this study

Strain	Source	Identification (genus, species, and/or serotype)	Resistance phenotype[a]	Class I integron gene cassette(s)[b]	*tet* class
R6	Farm 2, pigsty	*E. coli* O:8	AMP, TET, STR, SPE, SMX, TMP	*aadA1, dfrA1*	*tet*(A)
R8	Farm 2, pigsty	*E. coli* O:9	AMP, TET, STR, SPE, SMX, TMP	*aadA1, dfrA1*	*tet*(A)
R9	Farm 2, pigsty	*E. coli* O:85	AMP, TET, STR, SPE, SMX, TMP	ND[d]	*tet*(A)
R10	Farm 2, pigsty	*E. coli* O:85	AMP, TET, STR, SPE, SMX, TMP	*aadA1, dfrA1*	*tet*(A)
R11	Farm 2, pigsty	*E. coli* O:85	AMP, TET, STR, SPE, SMX, TMP	ND	*tet*(A)
R57	Farm 2, pigsty	*E. coli*	AMP, TET, STR, SPE, SMX, TMP	*aadA1*	*tet*(A)
R72	Farm 2, pigsty	*Enterobacter* sp.	TET, STR, SPE, SMX	ND	*tet*(A)
R2139	Farm C, pigsty	Unidentified, gram positive[c]	ND	*aadA2*	*tet*(33)
R70	Farm 2, pigsty	*A. nicotianae*	TET, STR, SPE, SMX	*aadA9*	*tet*(33)
R55	Farm 2, pigsty	*A. protophormiae*	TET, STR, SPE, SMX	*aadA9*	*tet*(33)
R66	Farm 2, pigsty	*A. protophormiae*	TET, (STR), SPE, SMX	*aadA2*	*tet*(33)
R343	Field 3, farm 2	*A. protophormiae*	TET, STR, SPE, SMX, TMP	*aadA2*	*tet*(33)
R344	Field 3, farm 2	*A. protophormiae*	TET, STR, SPE, SMX, TMP	*aadA2*	*tet*(33)
R345	Field 3, farm 2	*A. protophormiae*	TET, STR, SPE, SMX	*aadA9*	*tet*(33)
R1809	Soil, farm B	*A. protophormiae*	ND	*dfrB2a*	*tet*(33)
R1811	Soil, farm B	*A. protophormiae*	ND	*dfrA1, aadA2*	*tet*(33)
R1554	Soil, farm 3	*C. glutamicum*	ND	*aadA2*	*tet*(33)
R156	Field 2, farm 2	*Pseudomonas* sp.	CHL, FFN, AMP, TET, STR, SPE, SMX, TMP	*aadA2*	*tet*(C)
R164	Field 1, farm 2	*Pseudomonas* sp.	CHL, FFN, AMP, TET, STR, SPE, SMX, TMP	*aadA2*	–[f]
R347	Field 3, farm 2	*Pseudomonas* sp.	NAL, CHL, FFN, AMP, **TET, STR, SMX, TMP, SPE**	*aadA2, dfrA1*	*tet*(C)
R956A	Soil, farm 4	*Pseudomonas* sp.	**TET, STR, SMX**	*aadA11*	–
R194*	Soil, farm 2	*A. faecalis*	NAL, **TET, STR, SMX, TMP, SPE**, CIP	*aadA11, dfrA1*	*tet*(A)
R214	Field 1, farm 2	*A. faecalis*	NAL, **TET, STR, SMX, TMP, SPE**, CIP	*aadA11, dfrA1*	*tet*(A)
R811	Soil, farm 3	*Alcaligenes* sp.	TET, STR, SMX, TMP, SPE, CIP	*aadA11, dfrA1*	*tet*(A)
R813	Soil, farm 3	*Alcaligenes* sp.	TET, STR, SPE, SMX, TMP, CIP	*aadA2*	–
1005(R)	Recipient	*P. putida*	RIF, NAL, AMP, CHL, FFN	–	–
KT2442(N)	Recipient	*E. coli*	RIF, NAL	–	–
JH2-2	Recipient	*E. faecalis*	RIF, FUS	–	–
BM4105	Recipient	*E. faecium*	RIF, FUS	–	–
8794RF	Recipient	*S. aureus*	RIF, FUS	–	–
R347-P	TC[e] of R347	*P. putida*	RIF, NAL, AMP, CHL, FFN, **TET, SMX, TMP, STR, SPE**	*aadA2, dfrA1*	*tet*(C)
R956A-E	TC of R956A	*E. coli*	RIF, NAL, **TET, SMX, (STR)**	ND	–
R194-P	TC of R194	*P. putida*	RIF, NAL, AMP, CHL, FFN, **TET, SMX, TMP, (STR), SPE**	*aadA11, dfrA1*	*tet*(A)
R194-E	TC of R194	*E. coli*	RIF, NAL, **TET, SMX, TMP, (SPE)**	*aadA11, dfrA1*	*tet*(A)
R214-P	TC of R214	*P. putida*	RIF, NAL, AMP, CHL, FFN, **TET, SMX, TMP, (STR), SPE**	*aadA11, dfrA1*	*tet*(A)
R214-E	TC of R214	*E. coli*	RIF, NAL, **TET, SMX, TMP, SPE**	*aadA11, dfrA1*	*tet*(A)

[a] Resistances: TET, tetracycline; ERM, erythromycin; CHL, chloramphenicol; FFN, florfenicol; AMP, ampicillin; STR, streptomycin; NAL, naldixic acid; FUS, fusidic acid; SPE, spectinomycin; SMX, sulfonamethoxazole. Boldfacing indicates transferable resistance. Resistances in parentheses indicate reduced susceptibility to the drug.
[b] 99% homology at the nucleic acid level and the amino acid level to *addA11*.
[c] Showed 96% homology to 16S rRNA of *Leucobacter komagatae* (AJ746337).
[d] ND, not determined.
[e] TC, transconjugant.
[f] –, found to be negative.

None of the soil isolates from farms A, 1, and 5 (field not treated with animal manure) contained in-1 or any of the *tet* genes for which we tested.

Mating experiments and plasmids. Eleven integron-positive Tc^r soil isolates were used as donors in mating experiments with the recipients *E. coli* and *P. putida*. The two *A. faecalis* isolates showed cotransfer of *tet*(A) and in-1 to both *P. putida* and *E. coli*. A *Pseudomonas* sp. showed cotransfer of *tet*(C) and in-1 to *P. putida*. One *Pseudomonas* sp. with unidentified Tc^r showed cotransfer of Tc^r and in-1 to *E. coli* (Table 3). None of the *A. protophormiae* isolates showed transfer to any of the recipients chosen in the mating experiments.

In the two *A. faecalis* donors and transconjugants, the in-1 was located on a plasmid of approximately 36 kb. Hybridization with a *tet*(A) probe showed that *tet*(A) was located on the same plasmid as in-1 in both donors and transconjugants (Fig. 1A and B). Plasmids from both donors and transconjugants digested with BamHI showed two BamHI restriction sites and the *tet*(A) probe hybridized to the same size band in both donors and their transconjugants (Fig. 1C). A plasmid approx-

TABLE 2. Primers used in this study for detection of Tc^r genes, class 1 integrons, and 16S RNA by PCR and DNA sequencing

Primer	Sequence (5′-3′)	Amplicon size (bp)	Source or reference
Tet(A)-1	5′-GTAATTCTGAGCACTGTCGC-3′	956	33
Tet(A)-2	5′-CTGCCTGGACAACATTGCTT-3′		33
Tet(B)-1	5′-CTC AGT ATT CCA AGC CTT TG-3′	414	29
Tet(B)-2	5′-ACT CCC CTG AGC TTG AGG GG-3′		29
Tet(C)-1	5′-GGT TGA AGG CTC TCA AGG GC-3′	505	29
Tet(C)-2	5′-CCT CTT GCG GGA TAT CGT CC-3′		29
Tet(D)-1	5′-GGA TAT CTC ACC GCA TCT GC-3′	435	19
Tet(D)-2	5′-CAT CCA TCC GGA AGT GAT AGC-3′		19
Tet(E)2-1	5′-TGATGATGGCACTGGTCA-3′	262	This study
Tet(E)2-2	5′-GCTGGCTGTTGCCATTA-3′		This study
Tet(G)-1	5′-GCAGCGAAAGCGTATTTGCG-3′	662	This study
Tet(G)-2	5′-TCCGAAAGCTGTCCAAGCAT-3′		This study
Tet(31)-1	5′-GCTCTATCTAGGGAGAATGA-3′	652	This study
Tet(31)-2	5′-GCTAACCATGATACCTTGTA-3′		This study
Tet(34)-1	5′-ATACGGGGATGCAAACTTCA-3′	729	This study
Tet(34)-2	5′-ACGAGTGAGCTCTGATGTCTCTT-3′		This study
Tet(M)-1	5′-GTTAAATAGTGTTCTTGGAG-3′	657	1
Tet(M)-2	5′-CTAAGATATGGCTCTAACAA-3′		1
Tet(O)-1	5′-GATGGCATACAGGCACAGAC-3′	614	1
Tet(O)-2	5′-CAATATCACCAGAGCAGGCT-3′		1
Tet(S)-1	5′-TGGAACGCCAGAGAGGTATT-3′	660	1
Tet(S)-2	5′-ACATAGACAAGCCGTTGACC-3′		1
Tet(L)2-1	5′-CATTTGGTCTTATTGGATCG-3′	488	1
Tet(L)2-2	5′-ATTACACTTCCGATTTCGG-3′		1
Tet(K)-1	5′-TTAGGTGAAGGGTTAGGTCC-3′	718	1
Tet(K)-2	5′-GCAAACTCATTCCAGAAGCA-3′		1
Tet(Z)-1	5′-CCCACTGCACGTCGGACTAC-3′	753	This study
Tet(Z)-3	5′-GGCGATACCGACGAGGATC-3′		This study
Tet(33)-1	5′-ATGCGGTTCCGCTGAA-3′	784	This study
Tet(33)-2	5′-GGAAAATGCGTCAGTGACAA-3′		This study
qacEΔ1-F	5′-ATC GCA ATA GTT GGC GAA GT-3′	226	26
qacEΔ1-B	5′-CAA GCT TTT GCC CAT GAA GC-3′		26
Sul-1 B and qacEΔ1-F	5′-GCA AGG CGG AAA CCC GCG CC-3′	798	26
Att-1-F	5′-CGG GCA TCC AAG CAG CAA-3′	V[a]	26
3′CS-B	5′-CGA TTA TGA CAA CGG CGG AAG GGG C-3′		26
16S 10FX	5′-AGA GTT TGA TCC TGG CTN AG-3′	447	This study
16S 519R	5′-GTA TTA CCG CGG CTG CTG G-3′		This study

[a] V, variable.

imately 30 kb was also found in *Pseudomonas* sp. strain R347 that contained transferable *tet*(C) and in-1. No plasmid could be extracted from any of the strains by the method used.

Sequencing of gene cassettes and susceptibility testing. The gene cassettes were sequenced in 22 in-1-positive isolates from soil and pigsty. The 22 isolates had gene cassettes with *aadA* genes encoding streptomycin-spectinomycin resistance in all isolates except one *Arthrobacter* isolate that contained a *dfrB2a* gene encoding trimethoprim resistance. *aadA1* was present in *E. coli. aadA9* was found in *Arthrobacter* spp., and *aadA2* was found in *Arthrobacter* sp., *Alcaligenes* sp., *C. glutamicum*, *Pseudomonas* spp., and an unidentified gram-positive bacterium. The *aadA* genes had 100% homology with known *addA9*, *addA1*, or *addA2* genes. Two pseudomonads and three *Alcaligenes* isolates (R194, GenBank accession no. DQ074759) contained a variant of *aadA11* that had 99% nucleotide and amino acid homology to the streptomycin resistance gene *aadA11* (GenBank no. AJ567827 from bp 173 to 792) (Table 1). Three *E. coli*, one *Pseudomomas*, one *Arthrobacter*, and three *Alcaligenes* isolates had, in addition to *aadA*, the trimethoprim resistance gene *dfrAI* (Table 1).

All in-1-positive isolates, recipients, and transconjugants

TABLE 3. Filter-mating experiments with gram-negative donors and *E. coli* and *P. putida* recipients

Donor	Recipient	Tc^r gene	Frequency[a] (tetracycline resistance and integrons)
R156, *Pseudomonas* sp.	*E. coli*	*tet*(C)	–
	P. putida		–
R164, *Pseudomonas* sp.	*E. coli*	Not identified	–
	P. putida		–
R347, *Pseudomonas* sp.	*E. coli*	*tet*(C)	–
	P. putida		10^{-4}
R956A, *Pseudomonas* sp.	*E. coli*	Not identified	2×10^{-5}
	P. putida		–
R194, *A. faecalis*	*E. coli*	*tet*(A)	2×10^{-3}
	P. putida		4×10^{-8}
R214, *A. faecalis*	*E. coli*	*tet*(A)	2×10^{-3}
	P. putida		5×10^{-4}
R811, *Alcaligenes* sp.	*E. coli*	*tet*(A)	–
	P. putida		–
R813, *Alcaligenes* sp.	*E. coli*	Not identified	–
	P. putida		–

[a] That is, the number of transconjugants/number of donors. –, no transfer observed.

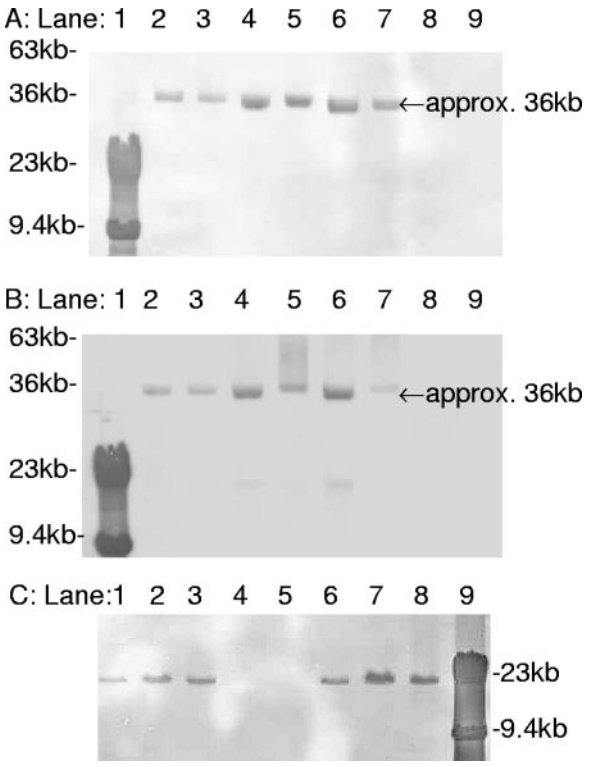

FIG. 1. Southern blot of plasmid extractions. (A) Hybridized with class 1 integron probe (qacEΔ1). Lane 1, HindIII-cut λ marker; lane 2, *A. faecalis* R194; lane 3, transconjugant R194-P; lane 4, transconjugant R194-E; lane 5, *A. faecalis* R214; lane 6, transconjugant R214-P; lane 7, transconjugant R214-E; lane 8, recipient *P. putida*; lane 9, recipient *E. coli*. (B) Hybridized with Tc^r *tet*(A) probe. Lane 1, HindIII-cut λ marker; lane 2, *A. faecalis* R194; lane 3, transconjugant R194-P; lane 4, transconjugant R194-E; lane 5, *A. faecalis* R214; lane 6, transconjugant R214-P; lane 7, transconjugant R214-E; lane 8, recipient *P. putida*; lane 9, recipient *E. coli.* (C) BamHI-restricted 36-kb plasmids hybridized with *tet*(A). Lane 1, strain R194; lane 2, transconjugant R194-P; lane 3, transconjugant R194-E; lane 4, recipient *P. putida*; lane 5, *E. coli*; lane 6, strain R214; lane 7, transconjugant R214-P; lane 8, transconjugant R214-E; lane 9, HindIII-digested λ marker. The size markers were based on HindIII-digested λ marker and plasmids extracted from *E. coli* R39.

were tested for antimicrobial resistance and showed resistance to three to nine drugs (Table 1). All in-1-positive isolates were resistant to tetracycline, streptomycin, spectinomycin (except one pseudomonad), and sulfonamethoxazole. The *E. coli* isolates were in addition resistant to ampicillin and trimethoprim. Of the pseudomonads, one was resistant to nine antimicrobial agents, and the *Alcaligenes* isolates were resistant to six or seven drugs (Table 1).

Transconjugants from mating experiments differed from the respective recipients in being resistant to tetracycline and sulfonamethoxazole and resistant or reduced in susceptibility to streptomycin and spectinomycin. *P. putida* transconjugants R347-P, R194-P and R214-P were also resistant to trimethoprim like their respective donors (Table 1).

DISCUSSION

Class 1 integrons have been found widely distributed among gram-negative bacteria, especially among *Enterobacteriaceae* (7) but, to our knowledge, this class of integrons have not been described in the genus *Alcaligenes*. In the present study, in-1 was found among *A. faecalis* and *Alcaligenes* spp. The in-1 from *A. faecalis* was found on conjugative plasmids of approximately 36 kb capable of interspecies transfer to both *E. coli* and *P. putida*. The Tc^r gene *tet*(A) was located on the same plasmid. It is therefore likely that in-1 and tetracycline resistance are spread to other gram-negative species by this plasmid. Earlier studies have shown *Alcaligenes* spp. from marine environments to harbor *tet*(E) (3), but *tet*(A) has not been described in *Alcaligenes* before.

Pseudomonas isolates showed cotransfer of Tc^r and in-1 to either *P. putida* or *E. coli*. One *Pseudomonas* isolate contained *tet*(C), and one isolate did not contain any of the *tet* genes for which it was screened but might contain a new class of *tet* genes.

Horizontal cotransfer of *tet*(A) and in-1 have been observed among *Aeromonas* species from freshwater (16, 27). This demonstrates once more how a common gene pool can be shared between organisms belonging to different environments.

Thus far, no published studies have described Tc^r genes or integrons in the genus *Arthrobacter* probably because of a limited focus of antimicrobial resistance in these bacteria. The six *Arthrobacter* spp. in the present study all contained the Tc^r gene *tet*(33), which was previously described for *C. glutamicum* (31). In earlier studies, integrons were found in gram-positive isolates of *C. glutamicum*, whose genus is closely related to *Arthrobacter* (22). gram-positive bacteria, especially *Corynebacterium* spp., were found to be a reservoir for in-1 in poultry litter (20). Coryneform bacteria such as *Arthrobacter* and *Corynebacterium* spp. seem to be important reservoirs for in-1 and not only *Enterobacteriaceae* and other gram-negative bacteria (20).

Even though no element containing in-1 and *tet*(33) was found to exist in the gram-positive strains, a very clear pattern existed among the gram-positive bacteria since all in-1-positive isolates also contained *tet*(33). It is therefore likely that these bacteria contain in-1 and *tet*(33) on the same genetic element and may be related to the pTET3 from *C. glutamicum* (31). The bacteria were isolated from two different pigsties and three farmland soils and belonged to four species: *A. nicotianae*, *A. protophormiae*, *C. glutamicum*, and an unidentified gram-positive bacterium. The presence of *tet*(33) in different soil bacteria indicates the gene to be horizontally spread in the soil environment rather than clonally spread, although none of the *Arthrobacter* isolates or other gram-positive bacteria could cotransfer integron and Tc^r to any of the gram-negative or gram-positive recipients. Intergenic transfer of Inc plasmids between *E. coli* and *Arthrobacter* has been observed before (17), so the lack of transfer of Tc^r in the present study may have been due to the choice of recipients.

The presence of *Arthrobacter* or other indigenous soil bacteria with multiple resistances within a pigsty environment may lead to the transfer of resistance genes from soil bacteria to bacteria of animal origin. *Alcaligenes*, *Pseudomonas*, and *Arthrobacter* spp. are very common bacteria in soil and water and are therefore likely to be in close contact with humans and animals via crops and drinking water. The fact that these bacteria are soil bacteria means that they can survive and grow in the environment for a long period of time. *Arthrobacter* species

are now recognized as opportunistic pathogens (8, 14). *Arthrobacter* may therefore be an important reservoir for both in-1 and Tc^r genes.

The presence of *aadA* gene cassettes in all in-1 resulted in resistance to streptomycin and/or spectinomycin, in addition to sulfonamide and tetracycline resistance in these isolates (Table 1). This indicates that soil bacteria can contribute to the spread of multidrug resistance. One *Pseudomonas* and three *Alcaligenes* isolates from soil contained a variant of *aadA11* from a clinical isolate of *E. coli*, recently submitted to GenBank (accession no. AJ567827). This gene cassette may be more frequently present in the soil environment than in the human reservoir and could have been exchanged between the different reservoirs.

Whether the soil environment acts as a reservoir for multidrug resistance (encoded by in-1 and tetracycline resistance genes) or whether these genes are present in the soil environment as a result of spreading animal manure to the farmland soil was not determined here, but it is interesting that none of the isolates from the soil not treated with animal manure (farm 5) contained in-1 or *tet* genes. The results may indicate that multidrug resistance caused by *tet* genes and in-1 can be transferred from the soil environment to animals and humans via crops or drinking water.

Our overall conclusion is that soil bacteria in close contact with manure or pigsty environments seem to have an important role in horizontal spread of multidrug resistance (especially plasmid-mediated resistance encoded by class 1 integron gene cassettes and *tet* genes). The increased use of tetracyclines in food animal production may contribute to increased multidrug resistance in bacteria.

GenBank submissions. The sequence of the gene cassettes (*aadA11*, *dfrA1*) from *A. faecalis* R194 and *tet*(33) *Arthrobacter protophorniae* described in the present study have been submitted to GenBank (GenBank submission no. DQ074759 and DQ077487, respectively).

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