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Prevalence of Sulfonamide Resistance Genes in Bacterial Isolates from Manured Agricultural Soils and Pig Slurry in the United Kingdom▽

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The prevalences of three sulfonamide resistance genes, *sul1*, *sul2*, and *sul3* and sulfachloropyridazine (SCP) resistance were determined in bacteria isolated from manured agricultural clay soils and slurry samples in the United Kingdom over a 2-year period. Slurry from tylosin-fed pigs amended with SCP and oxytetracycline was used for manuring. Isolates positive for the *sul* gene were further screened for the presence of class 1 and 2 integrons. Phenotypic resistance to SCP was significantly higher in isolates from pig slurry and postapplication soil than in those from preapplication soil. Of 531 isolates, 23% carried *sul1*, 18% *sul2*, and 9% *sul3* only. Two percent of isolates contained all three *sul* genes. Class 1 and class 2 integrons were identified in 5% and 11.7%, respectively, of *sul*-positive isolates. In previous reports, *sul1* was linked to class 1 integrons, but in this study only 8% of *sul1*-positive isolates carried the *intI1* gene. Sulfonamide-resistant pathogens, including *Shigella flexneri*, *Aerococcus* spp., and *Acinetobacter baumannii*, were identified in slurry-amended soil and soil leachate, suggesting a potential environmental reservoir. Sulfonamide resistance in *Psychrobacter*, *Enterococcus*, and *Bacillus* spp. is reported for the first time, and this study also provides the first description of the genotypes *sul1*, *sul2*, and *sul3* outside the *Enterobacteriaceae* and in the soil environment.

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Since their introduction in the 1930s, sulfonamides have been widely used in clinical and veterinary medicine to treat bacterial and protozoal infections. They act as a structural analogue of ρ-amino-benzoic acid and bind dihydropteroate synthase (DHPS), a catalytic enzyme in the folic acid biosynthesis pathway, resulting in the inhibition of dihydrofolic acid formation (26). Resistance is conferred by mutations in the DHPS gene (*folP*) (30) or from the acquisition of an alternative DHPS gene (*sul*) (18, 20, 29).

The first of the three known alternative DHPS genes, *sul1*, is usually located on the 3′ conserved region of a class 1 integron (25) and is frequently identified with this potentially mobile element in the slurry and soil environment (12, 22, 29). *sul2* was first identified on RSF1010 in *Escherichia coli* and has been found on small nonconjugative resistance plasmids (20). The *sul3* gene was identified during a study on sulfonamide resistance in pathogenic *E. coli* isolates from swine from Switzerland (18).

The prevalence of each of the sulfonamide resistance genes varies among published studies, depending on environments and bacterial species sampled. The majority of reports relate to *Enterobacteriaceae* isolates, specifically *E. coli* and *Salmonella* spp.

Previous investigations have screened for all three *sul* genes, but only Antunes et al. (2), in an investigation of *Salmonella enterica* strains, found all three genes. One previous study screened for all three genes in environmental isolates and soil; Heuer and Smalla (13) screened silt and loamy sand soils, known to have lower sorbance properties for antibiotics (especially for tetracyclines and sulfonamides) than clay soils (8, 10). This short-term study suggested that manure from treated pigs enhanced the spread of antibiotic resistance in bacterial communities in soil (13).

Here we report the prevalences of sulfonamide resistance genes in bacterial isolates from agricultural clay soil, where long-term (2-year) application of slurry from tylosin (TY)-fed pigs with the experimental addition of sulfachloropyridazine (SCP) and oxytetracycline (OTC) occurred. The study also aimed to investigate whether the prevalence of the sulfonamide resistance gene was due to dissemination of sulfonamide resistance in bacteria from the farm environment (manure) to indigenous soil bacteria.

MATERIALS AND METHODS

Field study. Triplicate clay soil cores were collected at predetermined time points from an agricultural field in Lincolnshire, United Kingdom, which received an application of TY-fed-pig slurry which had been amended with SCP and OTC at concentrations of 25.58 mg liter^{-1} and 18.85 mg liter^{-1}, respectively (8). The time points were as follows: preapplication; year 1, day 1 after application; year 1, day 21; year 1, day 90; year 2, day 289; year 1, day 1; year 2, day 90; and year 2, day 240. Preapplication soil cores were used as controls. No TY was detected in the slurry or soil samples preceding or following the slurry applications. SCP was detected in soil leachate at 590 μg liter^{-1} at day 7 postapplication, 64 μg liter^{-1} at day 10, and then at low levels of ≤1 μg liter^{-1} from day 20. SCP and OTC concentrations (365 to 1,691 μg kg^{-1}) through the soil profile were AQ: B

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TABLE 1. Primer sequences used for PCR amplification and sequencing

Gene	Primer	Sequence (5′ to 3′)	Annealing temp (°C)	Reference
16S rDNA	pA	AGA GTT TGA TCC TGG CTC AG	62	11
	pH	AAG GAG GTG ATC CAG CCG CA		
sul1	sul1bF	CTT CGA TGA GAG CCG GCG GC	63	29
	sul1bR	GCA AGG CGG AAA CCC GCG CC		
sul2	sul2F	TCG TCA ACA TAA CCT CGG ACA G	60	V. Enne
	sul2R	GTT GCG TTT GAT ACC GGC AC		
sul3	sul3F	GAG CAA GAT TTT TGG AAT CG	51	18
	sul3R	CAT CTG CAG CTA ACC TAG GGC TTT GGA		
intI1	intA	ACA GGG CAA GCT TAG TAA AGC C	67	22
	intB	CTC GCT AGA ACT TTT GGA AA		
intI2	int2F	CAC GGA TAT GCG ACA AAA AGG T	58.5	32
	int2R	GTA GCA AAC GAG TGA CGA AAT G		
qacE	KazamF1	GGGAATTCGCCCTACACAACAAATTGGGAGA	50	14
	KazamR1	TACTCGAGTTAGTGGGCACTTGCTTTGG		
qacEΔ1	KazamF2	GGGAATTCGCCCTACACAACAAATTGGGAGA	60	14
	KazamR2	GCTGCAGCTGCGGTACCACTGCCACAA		

[a] Sequence from bp 8 to 1522.

reported previously (8). Soil leachate samples were chosen from the three highest periods of rainfall and investigated separately, from year 1, day 15; year 2, day 49; and year 2, day 164 of the study. For ease of reporting, all results from the soil leachate samples were grouped together. The liquid from each sample was centrifuged, and the pellets were resuspended in 1 ml for serial dilutions, which were carried out in triplicate. The original volume was used in calculating the numbers of bacteria per sample per milliliter.

Viable plate counts. Counts were performed on three separate cores from slurry-amended agricultural soils from the United Kingdom at nine time points, samples of pig slurry which had been obtained from a catchment tank below TY-fed animals (pig slurry control), and three separate pig slurry samples from the slurry tank after antibiotic amendment (pig slurry amended) (8). One gram of soil from each 0- to 5-cm core or 1-ml slurry sample was resuspended in 9 ml of sterile distilled water. Serial dilutions were made and spread onto Iso-Sensitest agar (Oxoid, United Kingdom) containing different concentrations of SCP (5, 10, 25, and 50 μg ml^{-1}; Sigma, United Kingdom), OTC (0.2, 1, 5, 10, 25, and 50 μg ml^{-1}; Sigma), and TY (5, 10, 25, 50, and 100 μg ml^{-1}; Sigma). All plates contained 100 μg ml^{-1} cycloheximide (Sigma) to inhibit growth of fungi. Plates were incubated overnight and for 5 days aerobically at 28°C. Resistance quotients (RQs) were calculated by dividing the mean count from triplicate selective plates by the mean count from triplicate nonselective plates.

Bacterial isolation. Bacterial colonies were randomly picked from nonselective and selective plates containing different concentrations of SCP, OTC, and TY and streaked until pure cultures were obtained. The numbers of isolates obtained from each antibiotic concentration at each time point varied due to differences in the resistance of the sampled population (no growth was observed at higher antibiotic concentrations in some samples) and to the loss of viability of some glycerol stocks. To overcome the variable numbers of isolates from each selective media at different time points, comparisons of *sul* gene prevalences were made from a subset of the data. A standardized number of isolates were randomly selected from each antibiotic selective media, SCP, TY, or OTC (11, 10, and 8 isolates, respectively), for each of the following samples: pig slurry; preapplication year 1; year 1, day ●●●; year 1, day 90; and year 1, day 289.

DNA extractions. DNA was extracted using a DNeasy kit (Qiagen) according to the manufacturer's instructions from isolates grown in 5 ml of Iso-Sensitest broth (Oxoid) overnight at 28°C. PCR was performed, using 1 unit of *Taq* DNA polymerase and the manufacturer's buffer (Invitrogen, United Kingdom) with 4 mM $MgCl_2$. Thirty cycles were performed at 95°C for 1 min, at varying annealing temperatures (shown in Table 1) for 1 min, and at 72°C for 1 min. The PCR products were eluted from a 1% agarose gel (Helena Biosciences, United Kingdom), using a QIAquick gel extraction kit (Qiagen). All *sul*-positive isolates were identified by using *16S rDNA* sequences of approximately 800 bp in both directions. Sequencing reactions were performed with a Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) as described by the manufacturer, and electrophoresis and readout were performed on an ABI Prism 3100 genetic analyzer (Applied Biosystems).

Analysis of DNA sequences. The resulting DNA sequences were edited, using BioEdit (Isis Pharmaceuticals, Inc.), and were subsequently analyzed using the BLAST program, with a sequence similarity of ≥97% used for species identification (1).

Conjugal transfers. *Pseudomonas putida* UWC1 (Rif^r) and *E. coli* K-12 CV601 (Rif^r Thr^- Leu^- Thi^-) were used as recipients in conjugal transfers which were performed according to the method of Smalla et al. (27). The recipients had a MIC of SCP of 0.5 μg ml^{-1}. Transconjugants that had been involved in a transfer event were selected for on 50 μg ml^{-1} of rifampin (Sigma) and 8 μg ml^{-1} of SCP containing Iso-Sensitest agar plates for 48 h at 30°C. Colonies were screened by PCR to confirm *sul* gene transfer and disregard the possibility of spontaneous mutations. Transfer rates (transconjugants per donor) were calculated according to the method of Binh et al. (4), where the transfer event equaled the CFU ml^{-1} of transconjugants divided by the CFU ml^{-1} of recipients. This rate was then normalized by the number of positive colonies screened by PCR for the gene of interest. The limits on transfer frequencies were set by our ability to detect a single transconjugant cell but varied with experimental conditions, due to differences in the initial numbers of recipients. Control plates of donors only were included to investigate the rates of rifampin mutations; these plates were always negative.

MIC determination. MICs and antibiotic resistance breakpoints were determined on Iso-Sensitest agar plates, using an agar dilution method (21). The inoculum (100 μl) was adjusted to an optical density at 600 nm of 0.4 for each isolate to ensure consistency in the determination of the MIC. The antibiotics (Sigma) tested were streptomycin (at a concentration of 16 μg ml^{-1}), ampicillin (16 μg ml^{-1}), kanamycin (16 μg ml^{-1}), chloramphenicol (16 μg ml^{-1}), tetracycline (8 μg ml^{-1}), trimethoprim (16 μg ml^{-1}), neomycin (8 μg ml^{-1}), and nalidixic acid (16 μg ml^{-1}). Breakpoints were selected on the basis of identifying mechanisms of resistance that were likely clinically relevant. MIC determinations for SCP were performed, using the following concentrations: 0, 1, 2.5, 5, 25, 50, and 100 μg ml^{-1}.

Statistical analysis. RQs and prevalences were compared, using a chi-square test for the comparison of two proportions (from independent samples). Statistical analyses were performed, using MedCalc for Windows, version 9.3.7.0 (MedCalc Software, Mariakerke, Belgium).

Nucleotide sequence accession numbers. The newly sequenced *Psychrobacter* genomes have been deposited in GenBank under accession numbers AM086633 (*sul1*), AY360321 (*sul2*), and AY494779 (*sul3*).

RESULTS AND DISCUSSION

Antibiotic resistance. RQs calculated from SCP plate counts are shown for each sample in Fig. 1. Using a chi-square test for the comparison of two proportions (from independent samples), RQs for culturable bacteria were compared among samples and at different time points. SCP resistance was significantly higher in pig slurry than in preapplication soil, with selections of 10, 25, and 50 μg ml^{-1} ($P < 0.0001$). Resistance

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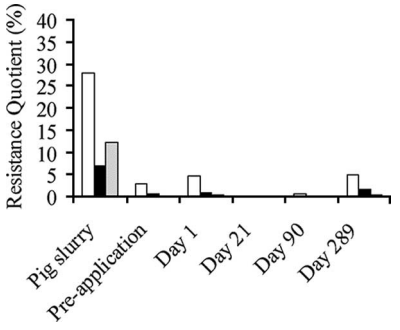

FIG. 1. SCP RQ values for soil and slurry samples collected over year 1; 10 μg ml^{-1}, white bars; 25 μg ml^{-1}, black bars; 50 μg ml^{-1}, gray bars.

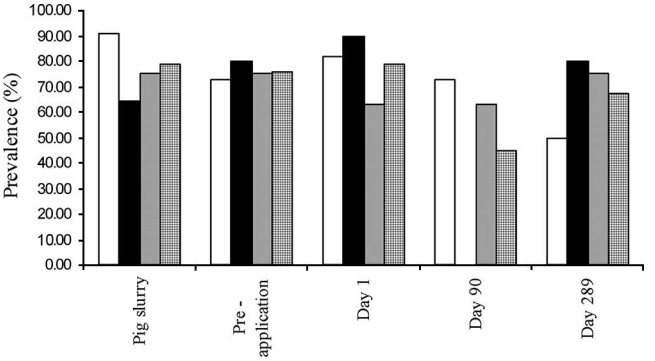

FIG. 2. Prevalences of *sul* gene-bearing bacteria, isolated on TY (white bars), SCP (black bars), OTC (gray-stippled bars), and all selective plates (cross-hatched bars). For TY, there were 11 isolates at each time point, for SCP, 10, and for OTC, 8. AQ: G

was also significantly higher at day 1 in postapplication soils than in preapplication soils ($P < 0.0001$) and remained higher at day 289 ($P < 0.0001$). This was not the case in day 21 and day 90 samples, possibly due to a patchy distribution of slurry and uneven retention of antibiotic residues. Clay soils are characterized by a network of cracks and fissures that allow localized mobilization of rainfall, dissolved compounds, and suspended particles. The resistance observed at day 289 cannot be attributed to continued selective pressure exerted by SCP after slurry application, as SCP was quickly washed out of the soil due to its high mobility and low K_d (15), where K_d is the sorption coefficient (28). Thus, the persistence of the resistant phenotype is likely due to the survival of bacteria carrying resistance determinants or transfer of the resistance gene to indigenous bacteria rather than selective pressure exerted by SCP in the environment.

***sul* gene prevalence.** All 531 bacterial isolates collected in this study were screened by PCR for *sul1*, *sul2*, and *sul3*. The most common genotypes were those of single genes; *sul1* had the highest prevalence, followed by *sul2* and subsequently by *sul3* (Table 2). A total of 67% ($n = 358$) of the isolates collected were PCR positive for one or more sulfonamide resistance genes, and 17.5% ($n = 93$) of these carried combinations of the three genes, *sul2* and *sul3* being the most frequent. The genotypes of *sul1*, *sul2*, and *sul3* occurred in 2.3% ($n = 12$) of isolates.

All genotypes were present in all samples, with the exception of the *sul2 sul3* genotype, which appeared to originate from the amended slurry and was present only in isolates collected from amended soil in year 1 postapplication, appearing again in isolates from year 2 after a second slurry application. Preapplication soil cores displayed a high number of *sul*-containing isolates (Table 2), possibly because of repeated pig slurry applications over the previous decade. While these previous slurry applications were not known to have included sulfonamides, they may have been used therapeutically.

When *sul* gene prevalences were compared over time in a subset of the data that included bacteria isolated on the same selective media, there were no significant differences in prevalences when all *sul* genes were considered together (Fig. 2). F2

Characterization of isolates. All *sul*-positive isolates were identified by *16S rDNA* typing, and the presence of class 1 and

TABLE 2. Summary of total numbers of isolates collected per sample during the study and number positive for each *sul* genotype

Sample site and time of sampling	No. of isolates with indicated *sul* genotype(s):								No. of isolates analyzed from each sample
	sul ve	*sul1*	*sul2*	*sul3*	*sul1 sul2*	*sul1 sul3*	*sul2 sul3*	*sul1 sul2 sul3*	
PS (amended)	52	7	18	10	4	0	11	2	78
PS (control)	4	1	1	2	0	0	0	0	18
Preapp (soil)	53	21	13	7	6	0	0	6	79
1,1 (soil)	53	6	14	12	7	1	12	1	71
1,21 (soil)	9	6	1	0	2	0	0	0	20
1,90 (soil)	15	13	0	0	1	0	1	0	39
1, 289 (soil)	23	6	8	6	3	0	0	0	41
2,1 (soil)	37	6	21	3	3	0	4	0	45
2,21 (soil)	35	13	7	6	3	1	3	2	39
2,90 (soil)	36	17	4	1	6	0	7	1	41
2,240 (soil)	19	16	1	1	0	0	1	0	29
Soil leachate	22	8	7	2	1	2	2	0	31
Total	358	120	95	50	36	4	41	12	531

AQ: I [a] Sample sites were as follows: PS (amended), pig slurry amended with 25.58 mg liter^{-1} SCP and 18.85 mg liter^{-1} OTC; PS (control), unamended pig slurry; preapp (soil), soil cores from year 1 before slurry application; 1,1 (soil), soil cores from year 1, day 1 time point; 1,289 (soil), soil cores from year 1, day 289 time point; 2,1 (soil), soil cores from year 2, day 1 time point; soil leachate (three combined samples collected over the sample period from large rainfall events).

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TABLE 3. Summary of prevalences and total numbers of *sul*-positive bacterial species isolated, their *sul* genotypes, and samples from which they were collected

Genus[a]	No. (percentage) of *sul*-positive isolates	Source of isolate[b]	*sul* genotype	No. of *sul*-positive isolates
Acinetobacter	127 (35.7)	PS, PSC, all soil samples, SL	*sul1*	20
			sul2	30
			sul3	31
			sul1 sul2	13
			sul1 sul3	3
			sul2 sul3	21
			sul1 sul2 sul3	7
Aerococcus	10 (2.8)	PS; 1,1	*sul1*	2
			sul2	8
Agrobacterium	2 (0.6)	2,90	*sul2 sul3*	2
Arthrobacter	16 (4.5)	PSC; PS; 1P; 1,90; 1,289; 2,21	*sul1*	6
			sul2	4
			sul3	1
			sul1 sul2	5
Bacillus	29 (8.2)	PSC; 1P; 1,289; 2,21; 2,240	*sul1*	7
			sul2	8
			sul3	9
			sul1 sul2	1
			sul2 sul3	3
			sul1 sul2 sul3	1
Brevibacterium	1 (0.3)	PS, PSC	*sul2 sul3*	1
Carnobacterium	3 (0.8)	1,289	*sul1*	3
Comamonas	3 (0.8)	2,1	*sul2*	3
Corynebacterium	3 (0.8)	2,1	*sul2*	3
Enterococcus	10 (2.8)	PS, PSC	*sul2*	4
			sul3	3
			sul2 sul3	3
Planococcus	1 (0.3)	PSC	*sul1 sul2*	1
Providencia	3 (0.8)	2,1	*sul3*	3
Pseudomonas	88 (24.7)	PS; all soil samples; 1P; 1,1; 1,21; 2,90	*sul1*	64
			sul2	12
			sul3	3
			sul1 sul2	5
			sul2 sul3	4
Psychrobacter	51 (14.3)	PS; 1P; 1,1	*sul1*	13
			sul2	18
			sul1 sul2	10
			sul1 sul3	1
			sul2 sul3	5
			sul1 sul2 sul3	4
Shigella	3 (0.8)	SL	*sul2*	3
Stenotrophomonas	5 (1.4)	2,21; 2,90	*sul1*	1
			sul2	1
			sul3	1
			sul2 sul3	2
Weissella	3 (0.8)	PSC	*sul1 sul2*	3

[a] As identified by 16S DNA.

[b] Sample sites were as follows: pig slurry amended with 25.58 mg liter^{-1} SCP and 18.85 mg liter^{-1} OTC (PS); unamended pig slurry (PSC); soil cores from year 1 before slurry application (1P); soil cores from year 1, day 1 time point (1,1); soil cores from year 1, day 289 time point (1,289); soil cores from year 2, day 1 time point (2,1); soil leachate (SL; three combined samples collected over the sample period from large rainfall events).

2 integrons was determined. Seventeen genera, including opportunistic pathogens and indigenous soil bacteria, were identified, as shown in Table 3. The most prevalent *sul*-positive species isolated in this study were *Acinetobacter* spp., which were collected from all soil/slurry samples. *Acinetobacter* spp. were reported to have developed resistance to a large number of antibiotic groups, including the sulfonamides, making them a serious problem in hospitals (5, 9, 31). *A. baumannii* and other species of *Acinetobacter* have previously been identified in diverse environments, now including an agricultural soil environment, a potential hot spot of gene acquisition from the vast gene pool found in soil and rhizosphere bacteria (16, 17, 19, 23). This is the first report of *sul3* in *Acinetobacter*. Table 3 demonstrates that *Acinetobacter* spp. carrying *sul* genes were present in the soil for up to a year after slurry application, as were *Arthrobacter*, *Bacillus*, *Carnobacterium*, and *Pseudomonas* spp. *Agrobacterium* and *Stenotrophomonas* spp. carrying *sul* genes were detected at 90 days post-slurry application. In contrast, *Aerococcus*, *Brevibacterium*, *Comamonas*, *Corynebacterium*, *Planococcus*, *Providencia*, *Psychrobacter*, and *Wiessella* spp. were present in pig slurry or amended soil only immediately after slurry application; these bacteria are therefore more likely to enter the human population via the food chain than by environmental-transport routes. *Enterococcus* and *Shigella* spp. were found only in pig slurry and soil leachate samples, suggesting that they were quickly washed out of the soil into ground water and drainage systems by rain.

Table 4 displays a number of representative isolates from the main genotypes and all isolates containing the three *sul* genes. A surviving enteric isolate and human pathogen, identified as *Shigella flexneri* (C506), with 96% similarity, was isolated from a soil leachate sample and contained *sul2* and *intI1* with a multiple-resistance phenotype. The *sul2* and *sul3* genotype was found in *Acinetobacter lwoffii*, *Enterococcus sulfureus*, and *Aerococcus viridans* isolates, all pig-associated pathogens/commensals entering the soil through the slurry applications. Isolates PGS21 and PGS22 from the antibiotic-amended slurry were both identified as *Aerococcus viridians* with 99% nucleotide similarity (*16S rDNA*, 800 bp), and a number of *Psychrobacter* spp. were also identified (Table 3). The *Psychrobacter* sp. isolates were resistant to tetracycline, streptomycin, chloramphenicol, trimethoprim, and nalidixic acid, with calculated SCP MICs of between 5 and 16 mg l^{-1}. The isolation of *Psychrobacter* spp. was unusual, as they are commonly isolated from cold marine environments and sediments (6, 7, 24). BLAST analysis (1) of the newly sequenced *Psychrobacter* genomes (www.jgi.doe.gov) did not reveal any sulfonamide resistance genes.

Isolate C361, identified with 99% nucleotide similarity to the *16S rDNA* sequence of *Arthrobacter arilaitensis*, was resistant to five antibiotics, including streptomycin, chloramphenicol, tetracycline, neomycin, and nalidixic acid, as well as to a low MIC of SCP of 5 mg l^{-1}.

Class 1 and class 2 integron carriage. Screening of the *sul*-positive isolates ($n = 358$) revealed that 5.0% ($n = 18$) carried class 1 integrons and 11.7% ($n = 42$) carried class 2 integrons (positive for the *intI1* and *intI2* genes, respectively). Of 173 *sul*-negative isolates, 8.7% ($n = 15$) carried *intI1* genes, and 5.2% ($n = 9$) carried *intI2* genes. There was no significant difference in *intI1* prevalences between *sul*-positive and *sul*-negative isolates (chi-square test, 0.5; $P = 0.5$), whereas the prevalence of *intI2* was significantly higher in *sul*-positive isolates (chi-square test, 57.6; $P < 0.0001$). Given the association of *sul1* with class 1 integrons and the fact that there was no known link between class 2 integrons and *sul* gene carriage, the observed prevalences of *intI1* and *intI2* in *sul*-positive and -neg-

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TABLE 4. Characterization of a number of cultured bacterial isolates encoding different *sul* genotypes

Isolate	Bacterial source[a]	16S rDNA identification	% BLAST[b] similarity	*sul* gene	Class of integron[c]	Antibiotic resistance phenotype of isolates[e]	MIC of SCP (mg liter^{-1})
C237	1, Preapp (soil)	*Bacillus sphaericus*	98	*1*		Sm^r Tmp^r Nal^r	1
C422	2,21 (soil)	*Stenotrophomonas maltophilia*	98	*1*	2	Sm^r	1
C131	2,240 (soil)	*Pseudomonas fluorescens*	97	*1*		Tet^r	32
C3	Pig slurry	*Acinetobacter lwoffi*	99	*1*	2	NG	1
PGS22	Pig slurry	*Aerococcus viridians*	99	*2*	1[d]	Sm^r Cm^r Tet^r Nm^r Nal^r	8
C506	Soil leachate	*Shigella flexneri*	99	*2*	1[d]	Sm^r Cm^r Tet^r Tmp^r Nm^r Nal^r Km^r Amp^r	8
C701	1, Preapp (soil)	*Pseudomonas lini*	99	*2*	2	Sm^r Amp^r Tmp^r Nal^r	1
C439	2,90 (Soil)	*Acinetobacter sp. N2*	97	*2*	2	Sm^r Tet^r Tmp^r Nal^r	16
C5	Pig slurry	*Pseudomonas borealis*	99	*3*	2	Cm^r Tet^r	32
PGS48	Pig slurry	*Enterococcus hirae*	97	*3*		Sm^r Tet^r	4
C167	2,1 (soil)	*Providencia stuart2*	97	*3*		Sm^r Tet^r	6
C2	Pig slurry	*Acinetobacter lwoffi*	97	*3*		Sm^r Cm^r Tet^r Tmp^r Nal^r	5
C231	1,21 (soil)	*Pseudomonas putida*	97	*1 + 2*	1[d]	Amp^r Cm^r Tet^r Nm^r Nal^r	8
C361	1,289 (soil)	*Arthrobacter arilaitensis*	99	*1 + 2*	1[d]	Sm^r Cm^r Tet^r Nm^r Nal^r	5
PGS49	Pig slurry	*Acinetobacter sp. An9*	99	*1 + 2*	2	Sm^r Nal^r	16
C410	1,1 (soil)	*Psychrobacter ikaite*	98	*1 + 2*	2	Tet^r	16
PGS47	Pig slurry	*Acinetobacter lwoffi*	97	*2 + 3*	2	Sm^r Nal^r	16
PGS61	Pig slurry	*Enterococcus sulfureus*	97	*2 + 3*		Sm^r Tet^r	4
PGS21	Pig slurry	*Aerococcus viridans*	99	*2 + 3*	2	Cm^r Tet^r	32
C15	Pig slurry	*Acinetobacter lwoffi*	99	*1 + 2 + 3*	2	Sm^r Cm^r Tet^r Tmp^r Nm^r Nal^r	5
C20	Pig slurry	*Psychrobacter ikaite*	98	*1 + 2 + 3*		Sm^r Cm^r Tet^r Tmp^r Nal^r	5
C711	1, Preapp (soil)	*Psychrobacter sp. DY9-2*	97	*1 + 2 + 3*		Sm^r Tet^r Tmp^r Nal^r	8
C712	1, Preapp (soil)	*Psychrobacter frigidicola*	96	*1 + 2 + 3*		Sm^r Tet^r Tmp^r Nal^r	8
C713	1, Preapp (soil)	*Psychrobacter ikaite*	97	*1 + 2 + 3*	2	Sm^r Cm^r Tet^r Tmp^r Nal^r	8
C35	1, Preapp (soil)	*Acinetobacter calcoaceticus*	97	*1 + 2 + 3*		Sm^r Cm^r Tet^r Tmp^r Nal^r	8
C36	1, Preapp (soil)	*Acinetobacter calcoaceticus*	97	*1 + 2 + 3*		Sm^r Cm^r Tet^r Tmp^r Nal^r	8
C37	1, Preapp (soil)	*Acinetobacter lwoffi*	97	*1 + 2 + 3*		Sm^r Cm^r Tet^r Tmp^r Nal^r	8
C44	1,1 (soil)	*Acinetobacter rhizosphaerae*	98	*1 + 2 + 3*		Sm^r Cm^r Tet^r Tmp^r Nm^r Nal^r	5
C141	2,21 (soil)	*Acinetobacter lwoffi*	98	*1 + 2 + 3*		Sm^r Km^r Cm^r Tet^r Tmp^{r}Nm^r Nal^r Amp^r	6
C328	2,21 (soil)	*Bacillus psychrodurans*	98	*1 + 2 + 3*		Sm^r Km^r Cm^r Tet^r Nm^r Nal^r	8
C442	2,90 (soil)	*Acinetobacter baumannii*	99	*1 + 2 + 3*		Cm^r Tet^r Nal^r	8

[a] 1, Preapp (soil), soil cores from year 1 before slurry application; 1,1, soil cores from year 1, day 1 time point; 1,289, soil cores from year 1, day 289 time point; 2,1, soil cores from year 2, day 1 time point.

[b] Percent nucleotide similarity of an approximately 800-bp *16S rDNA* sequence to bacterial strains submitted to databases and searched using the BLAST program (1).

[c] *qacEΔ1*/*qacE* was screened for in all *intI1*-positive isolates.

[d] *qacEΔ1* detected.

[e] Antibiotic resistance breakpoints: Sm^r, resistance to 16 μg ml^{-1} streptomycin; Amp^r, 16 μg ml^{-1} ampicillin; Km^r, 16 μg ml^{-1} kanamycin; Cm^r, 16 μg ml^{-1} chloramphenicol; Tet^r, 8 μg ml^{-1} tetracycline; Tmp^r, 16 μg ml^{-1}, trimethoprim; Nm^r, 8 μg ml^{-1} neomycin; Nal^r, 16 μg ml^{-1}, nalidixic acid. NG, no growth.

ative populations were surprising. Only 8.1% ($n = 11$) of *sul1*-positive isolates carried the *intI1* gene, whereas 9.4% ($n = 12$) were observed to carry the *intI2* gene (data not shown). Interestingly, no isolates were positive for both *sul3* and *intI1* (data not shown). Of the *sul2* isolates, 17.9% ($n = 18$) and 1.1% ($n = 2$), respectively, carried an *intI2* or *intI1* gene (data not shown). A low frequency of *sul1*-positive isolates contained *intI1*, despite *sul1* having only been found adjacent to *qacEΔ1* in the 3′ conserved region of class 1 integrons (3). This finding indicates that *sul1* is likely to be situated on non-class 1 integron mobile elements in most *sul1*-positive isolates identified in this study. Only one other published investigation has reported the prevalence of class 1 integrons in sulfonamide-resistant isolates from the environment, but it involved a brief temporal study in a different soil type (13).

Isolates encoding the three known *sul* genes. Twelve isolates positive for the three *sul* genes were identified by *16S rDNA* typing as members of the genera *Psychrobacter*, *Acinetobacter*, and *Bacillus* (Table 4). Of these, 10 isolates were cultured from agricultural soils which had undergone long-term applications of slurry from TY-fed pigs. Two isolates, *Acinetobacter lwoffii* (C15) and *Psychrobacter ikaite* (C20), were recovered from the antibiotic-amended slurry. The 12 isolates were negative for *intI1*, but one, *Psychrobacter ikaite* (C713), contained *intI2*. The 12 isolates displayed phenotypes with multiple-antibiotic resistance, with resistance to between three and eight antibiotics, including nalidixic acid, tetracycline, trimethoprim, and neomycin (Table 4). MIC tests indicated that the presence of the three sulfonamide resistance genes conferred only a low level of resistance to SCP of between 5 to 8 mg liter^{-1}.

Conjugal transfers. Conjugal transfers were performed with 11 of the 12 strains that contained, simultaneously, *sul1*, *sul2*, and *sul3* and had *P. putida* or *E. coli* recipients (isolate C36 failed to grow). The transfer rates of these three genes are

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TABLE 5. Conjugal transfer rates of the *sul1*, *sul2,* and *sul3* genes from the 12 bacterial host isolates carrying the three known *sul* genes into either an *E. coli* K-12 CV601 or *P. putida* UWC1 recipient

Donor isolate[a]	Transfer rates of indicated *sul* genes for recipients *E. coli* and *P. putida*[c]:					
	sul1		*sul2*		*sul3*	
	E. coli	*P. putida*	*E.coli*	*P. putida*	*E. coli*	*P. putida*
Pseudomonas sp. *DY9-2* (C711)	4.3×10^{-4}	7.3×10^{-3}	ND	ND	ND	ND
P. frigidicola (C712)	ND	ND	ND	ND	ND	ND
P. ikaite[b] (C713)	6.5×10^{-5}	1.9×10^{-3}	ND	ND	ND	ND
A. lwoffi (C15)	2.6×10^{-4}	7.5×10^{-3}	7.2×10^{-5}	2.5×10^{-3}	ND	ND
P. ikaite (C20)	ND	ND	ND	ND	ND	ND
A. calcoaceticus (C35)	9.5×10^{-4}	1.1×10^{-3}	7.5×10^{-4}	1.9×10^{-3}	ND	ND
A. lwoffi (C37)	ND	ND	ND	ND	ND	ND
A. rhizosphaera (C44)	9.0×10^{-4}	2.4×10^{-2}	2.5×10^{-4}	7.1×10^{-2}	ND	ND
A. lwoffi (C141)	4.2×10^{-4}	3.4×10^{-3}	4.2×10^{-4}	3.4×10^{-3}	ND	ND
B. psychrodurans (C328)	ND	ND	ND	ND	ND	ND
A. baumannii (C442)	3.5×10^{-4}	1.7×10^{-3}	ND	ND	ND	ND

[a] Isolate *A. calcoaceticus* (C36) failed to grow when tested for transfer.
[b] This isolate carried an *intI2* gene.
[c] Number of transconjugants per donor. ND, not detected.

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shown in Table 5. It was observed that in most isolates, *sul1* and *sul2* were transferred at different rates, indicating their presence on different mobile elements. The exception to separate transfers of *sul1* and *sul2* was an *Acinetobacter* sp. (C141) from which both genes transferred at a frequency of 3.44×10^{-3} transconjugants per donor cell into *P. putida* recipients, an equal transfer rate suggesting that the two genes are physically linked. *sul3* was not observed to transfer into any of the recipients used in this study. In all cases where genes transferred into *P. putida*, transfer also took place into *E. coli*, but at a lower frequency. The absence of transfer in a number of isolates may have been due to the carriage of *sul* genes on nonconjugative plasmids or on the chromosome, whereas in the *Bacillus* sp. (C328), the failure to transfer may have been due to the presence of a gram-positive specific mobile element.

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES 1

AQH—Please check the name of the department in the present address for K. G. Byrne-Bailey. Should it be "Department of Plant Microbiology"?

AQA—There were numerous errors in the references, and some have been realphabetized and renumbered. Original reference 4 is now 16, 5 through 7 are 4 through 6, 9 is 7, and 10 through 16 are 9 through 15. Please check renumbering throughout text.

AQB—In the sentence beginning "The time points were," is change of year from 1 to 2 in "year 2, day 289" correct? Please clarify.

AQC—Correct that "o/n" is abbreviation for "overnight"? If not, please spell out correctly here and in paragraph headed "DNA extractions." Per ASM style, abbreviations must be used 3 times to be allowed.

AQD—What day is meant for "year 1, day •••"? Please replace bullets with correct day.

AQE—If the sequences with the listed GenBank accession numbers were not determined in this study, please delete this paragraph.

AQF—In Table 3, periods between year and day were replaced with commas for consistency with Tables 2 and 4.

AQG—Correct that the numbers in parentheses in the last sentence of the legend to Fig. 2 refer to the numbers of isolates? If not, please clarify and reword sentence.

AQI—Ed: Pls. provide footnote link *a* in table body—Ptr.