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Prevalence of streptomycin-resistance genes in bacterial populations in European habitats

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Abstract

The prevalence of selected streptomycin (Sm)-resistance genes, i.e. *aph* (3″), *aph* (6)-1d, *aph* (6)-1c, *ant* (3″) and *ant* (6), was assessed in a range of pristine as well as polluted European habitats. These habitats included bulk and rhizosphere soils, manure from farm animals, activated sludge from wastewater treatment plants and seawater. The methods employed included assessments of the prevalence of the genes in habitat-extracted DNA by PCR, followed by hybridisation with specific probes, Sm-resistant culturable bacteria and exogenous isolation of plasmids carrying Sm-resistance determinants. The direct DNA-based analysis showed that *aph* (6)-1d genes were most prevalent in the habitats examined. The presence of the other four Sm-modifying genes was demonstrated in 58% of the tested habitats. A small fraction of the bacterial isolates (8%) did not possess any of the selected Sm-modifying genes. These isolates were primarily obtained from activated sludge and manure. The presence of Sm-modifying genes in the isolates often coincided with the presence of IncP plasmids. Exogenous isolation demonstrated the presence of plasmids of 40–200 kb in size harbouring Sm-resistance genes from all the environments tested. Most plasmids were shown to carry the *ant* (3″) gene, often in combination with other Sm-resistance genes, such as *aph* (3″) and *aph* (6)-1d. The most commonly found Sm-modifying gene on mobile genetic elements was *ant* (3″). Multiple Sm-resistance genes on the same genetic elements appeared to be the rule rather than the exception. It is concluded that Sm-resistance genes are widespread in the environmental habitats studied and often occur on mobile genetic elements and *ant* (3″) was most often encountered.

Keywords: Streptomycin resistance; Natural habitat; Exogenous plasmid isolation; Polymerase chain reaction

1. Introduction

Streptomycin (Sm) has been widely applied for prophylactic and therapeutical purposes in human and veterinary medicine and as a phytosanitary agent [1,2]. Although the use of Sm has been restricted in many countries, applications at small scale still continue. For example, in 1998 apple trees in Germany were treated for fireblight with 72 kg of Sm ha^{-1} applied in one growth season. There is concern that the input of antibiotics into open environments might lead to enhanced selective pressure for resistance genes which may ultimately lead to antibiotic-resistant pathogens [3]. Indeed, to date most reports on Sm resistance in bacteria have emerged from isolates of clinical and agricultural origin [3,4]. It is now well accepted that a key route to the acquisition of antibiotic-resistance genes is horizontal gene transfer [5], but the origin of these genes is as yet unexplored.

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The molecular mechanisms leading to bacterial Sm resistance can be divided in either the alteration of the cellular target site for Sm or the detoxification of the antibiotic by modification. Detoxification can occur via either adenylylation or phosphorylation of the Sm molecule [6]. The genes that encode the modifying enzymes can reside on the chromosome or on mobile genetic elements such as plasmids and transposons [6]. Antibiotic-resistance genes on mobile elements are readily transferable from cell to cell and even between different bacterial species [7].

There is an overwhelming body of knowledge from clinical and agricultural research with respect to the genes that encode Sm-modifying enzymes, as well as the mobile genetic elements carrying these genes [6]. Four structurally different genes coding for Sm phosphotransferases, i.e. *aph* (3″) and *aph* (6), and Sm adenylases, i.e. *ant* (3″) and *ant* (6), have been described [6]. These genes were found in both Sm-producing strains (Actinomycetes) and non-producing strains. Two genes encoding Sm phosphotransferases, i.e. *aph* (6)-1a and *aph* (6)-1b, have been observed, on large plasmids, in different *Streptomyces* producer strains [8]. Two other Sm phosphotransferases, i.e. *aph* (3″) and *aph* (6)-1d, were often found to be linked on transposon Tn*5393* [9] and on relatives of plasmid RSF1010 [10]. A fifth Sm phosphotransferase gene, *aph* (6)-1c, has so far only been found on transposon Tn*5* originating from a *Klebsiella* strain [11]. Genes encoding the Sm-adenylylating enzymes *ant* (3″) and *ant* (6) have been recovered from both Gram-positive and Gram-negative species. The *ant* (6) gene has only been detected in plasmid pS194 in *Staphylococcus aureus* [12] and in pK214 of *Lactococcus lactis* [13].

Bacterial resistance to Sm in different natural habitats may, at least partially, be explained by the presence of known genes such as the ones described above. However, still unknown Sm-modifying genes may exist, whereas genes encoding enzymes that modify aminoglycosides other than Sm may also exert activity with Sm [6]. However, there is a paucity of information about the prevalence of Sm-resistance genes in natural environments. Following early reports on the occurrence of such determinants in plant-associated bacteria [14–17], it has been convincingly shown that both the *aph* (3″) and *aph* (6)-1d genes carried on IncQ plasmids can be prevalent among plant-pathogenic bacteria that occur in association with Sm-treated plants [10,18]. However, this finding obviously does not shed light on the occurrence of such genes in pristine environments.

In this study, we assessed the prevalence of selected Sm-resistance genes, i.e. *aph* (3″), *aph* (6)-1c, *aph* (6)-1d, *ant* (3″) and *ant* (6), in bacterial populations in a range of habitats throughout Europe. A polyphasic approach, consisting of a combination of total community DNA extraction followed by PCR hybridisation screening, exogenous plasmid isolation and direct isolation, was applied. This included as yet unknown Sm-modifying genes as well as Sm-modifying genes in non-culturable cells in our assessment.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli CV601 Rp^r, *Ralstonia eutropha* JMP228 Rp^r and *Pseudomonas putida* UWC1 Rp^r were used as recipients in filter matings, whereas *P. putida* UWC1 Rp^r was used in exogenous plasmid isolation (Table 1). *E. coli* CV601 Rp^r and *P. putida* UWC1 Rp^r were grown in Luria–Bertani broth (LB: Difco tryptone, 10 g; Difco yeast extract, 5 g; NaCl, 5 g; H_2O, 1 l; pH 7.2), whereas *R. eutropha* JMP228 Rp^r was grown in one-tenth strength tryptone soy broth (0.1×TSB: 10% strength TSB Oxoid, Unipath Ltd, Basingstoke, UK) amended with 50 μg ml^{-1} of rifampicin (Rp). *E. coli* CV601 Rp^r was grown at 37°C, whereas the other two strains were grown at 27°C. All three strains were *gfp*-marked using the *gfp*- and *npt*II- (conferring resistance to kanamycin (Km)) containing vector pAG508 [19]. *Gfp*-marked derivatives of all three strains were selected in the presence of 50 μg ml^{-1} of Km. Transconjugants, either obtained by biparental mating or by exogenous plasmid isolation (see later) were cultured in their respective media supplemented with 50 μg ml^{-1} of Rp and Sm, either with or without 50 μg ml^{-1} of Km.

Probes were obtained from *E. coli* strains JM101 (RSF1010), S17-1 (pSUP 1021), 935 (pUC8::Tn*1826*), DH5α (pK214) and *Pseudomonas aeruginosa* ST44 (pSA1701) (Table 1). All strains were grown in LB with their appropriate antibiotics and grown either at 37°C (*E. coli* strains) or 27°C (*P. aeruginosa* ST44 containing pSA1701). Plasmids were extracted from these cultures according to the plasmid mini-preparation method of Sambrook et al. [20].

2.2. Primer design and testing

Sequences of Sm-modifying genes were obtained from the NCBI database (NCBI; http://www.ncbi.nlm.nih.gov). In total, 25 sequences belonging to five distinct classes of genes, i.e., *aph* (3″), eight sequences, *aph* (6)-1c, one, *aph* (6)-1d, three, *ant* (3″), 11, and *ant* (6), two, were aligned. Consensus regions consisting of at least 20 base pairs within each class of Sm-modifying genes were selected. To design PCR systems, a maximum of 560 base pairs was allowed between two consensus regions which could serve as annealing sites for forward and reverse primers. For determination of their specificity, the sequences of selected primers were compared with all available sequences in the database using the BLAST-N search algorithm [21]. For each class of Sm-resistance genes, two 20-mer oligonucleo-

tides were, thus, synthesised (Amersham Pharmacia, Roosendaal, The Netherlands; Table 2).

The primer pairs targeting *aph* (3″), *aph* (6)-1c, *aph* (6)-1d, *ant* (3″) and *ant* (6) (Table 2) were assessed for amplification of respective targets (plasmid or chromosomal DNA extracts from bacterial strains containing the target Sm-modifying genes; Table 1). They were also tested for the absence of any signal after PCR with plasmid or chromosomal DNA containing non-homologous Sm-modifying genes as targets.

PCR reactions were performed in an MJ Research PTC-200 machine (Biozyme, Landgraaf, The Netherlands) with the following temperature cycling programme: 1 min at 94°C, 1 min at 66°C (*aph* (6)-1c), 55°C (*ant* (3″), *aph* (3″), *aph* (6)-1d), or 46°C (*ant* (6)), 2 min at 72°C, for 30 cycles followed by a final chase (10 min) at 72°C. These conditions for PCR amplification were used for testing the presence of Sm-modifying genes in total community DNA and plasmid and chromosomal DNA obtained from Sm-resistant isolates.

The limit of detection was determined in soil (from Emmeloord, The Netherlands) and activated sludge (sewage treatment plant in Ede, The Netherlands), spiked with *E. coli* strains S17-1 (pSUP 1021) and JM101 (RSF1010), *P. aeruginosa* ST44 (pSA1701) and *E. coli* XL1-blue (pVP1) at densities of Log 3, 4, 5, 6 or 8 CFU g^{-1} dry soil or ml^{-1} activated sludge. Following incubation (overnight), total community DNA was extracted from these samples as described [22]. One-microlitre aliquots were used for PCR amplification using the primers described in Table 2.

2.3. Sampling of different habitats

In total, 26 experimental sites in the UK, The Netherlands, Germany, Belgium and Greece were sampled (Table 3). Four samples from different sources of bulk and rhizosphere soils, manure, activated sludge and seawater were taken and sampling was repeated in time with time intervals of 6 months or more (including a summer and a winter period). However, the manure, white radish and cauliflower rhizosphere samples were taken at only one time point.

2.4. Sample processing

2.4.1. Total community DNA extraction

Total community DNA was extracted using different procedures as follows: bulk soil [23], rhizosphere soil [24], manure [22], activated sludge [25] and seawater [26]. All procedures for total community DNA extraction included a final Wizard® DNA clean-up (Promega, Madison, WI, USA) step. The DNA obtained was dissolved in 100 μl ultrapure water and kept at −20°C. The DNA preparations were checked for quality (molecular size, purity and amount) by standard 0.6% agarose gel electrophoresis [20] whereas DNA yield was estimated from the intensity of the DNA band in the gel as compared to a standard of known concentration.

2.4.2. Isolation of Sm-resistant colonies

Bulk and rhizosphere soil and manure suspensions, as well as samples from activated sludge and seawater, were either serially (10-fold) diluted in 0.1% sodium pyrophosphate (NaPPi) or directly plated onto R2A agar (Difco Laboratories Inc., Detroit, MI, USA) supplemented with 100 μg ml^{-1} cycloheximide and nystatin either without (non-selective plating) or with 50 μg ml^{-1} of Sm (selective plating). Plates were incubated at 27°C for 10 days, after which colonies were counted. Eight individual colonies per habitat sampled were randomly selected from the Sm-selective plates, purified and stored in 20% glycerol at −70°C.

2.4.3. Exogenous plasmid isolation

Suspensions obtained from bulk and rhizosphere soils, manure, activated sludge and seawater were centrifuged (7000×*g*, 10 min) and the bacterial cell pellets were washed twice in LB broth after which they were resuspended in this medium. Final levels of washed *P. putida* UWC1 Rp^r and *P. putida* UWC1 Rp^r (*chr*::*gfp*) cells (both acting as recipient strains) approximated 10^9 ml^{-1}. Mating was performed by mixing, in a 1:1 (v/v) ratio, the suspensions from each habitat with the washed cells of strain UWC1 Rp^r (first sampling) or of strain UWC1 Rp^r (*chr*::*gfp*) (second sampling) and applying 100 μl of these mixtures to sterile 0.45 μm nitrocellulose filters (Millipore) on LB agar (LBA: LB broth with 1.2% agar) amended with 0.2% glucose and 5 mM of both $CaSO_4$ and $MgSO_4$. Plates were incubated at 27°C (overnight). Following incubation, bacterial cells were resuspended in 2 ml of LB broth by vortexing filters for 1 min. Then, aliquots of 100 μl were plated either directly, or following serial dilution in saline, onto LBA supplemented with 50 μg ml^{-1} of each Sm and Rp and 100 μg ml^{-1} of CH, either without (*P. putida* UWC1 Rp^r as the recipient) or with 50 μg ml^{-1} of Km (UWC1 Rp^r (*chr*::*gfp*) as recipient). Plates were incubated for 5 days at 27°C. Sm-resistant colonies of strain UWC1 Rp^r (first sampling) were streaked onto King's B agar (peptone, 20 g; K_2HPO_4, 1.5 g; $MgSO_4.7H_2O$, 1.5 g; glycerol, 10 g; H_2O, 1 l; pH 7.2) and incubated at 27°C for 3 days. Colonies were analysed by ERIC-PCR genetic profiling [27] and selected colonies from matings with putative strain UWC1 Rp^r (*chr*::*gfp*) transconjugants (second sampling) were checked under UV to single out genuine recipients from background growth, using green fluorescence.

2.5. DNA extraction from Sm-resistant isolates and putative transconjugants

Total genomic and plasmid DNA was extracted from

putative *E. coli* CV601 Rp^r (with and without *gfp*) transconjugants obtained by filter matings (see later), using standard techniques [20]. DNA from putative *P. putida* UWC1 Rp^r (with and without *gfp*) transconjugants and from selected Sm-resistant isolates was extracted according to the genomic DNA extraction procedure [28] and the modified Ish–Horowitz plasmid extraction protocol [29].

2.6. Molecular methods

DNA extracts and PCR products were analysed in ethidium bromide-stained agarose gels [20]. Gels containing PCR products were Southern blotted onto nylon membranes (Boehringer, Mannheim, Germany) for analysis by hybridisation [20]. For that purpose, digoxigenin (Dig)-labelled probes were prepared according to the manufacturer's procedure (Boehringer, Mannheim, Germany) using as DNA templates: RSF1010 (containing *aph* (3″) and *aph* (6)-1d), Tn*1826* and pSA1701 (*ant* (3″)-1 and *ant* (3″)-2 respectively), Tn*5* (*aph* (6)-1c), and pVP1 (*ant* (6)). Colony hybridisation was performed according to [30] using these probes. Hybridisation signals were detected using the chemoluminescence detection kit (Amersham Pharmacia, Uppsala, Sweden), using the protocol supplied by the manufacturer.

2.7. Identification of Sm-resistant isolates

To identify bacterial isolates, PCR of 16S rDNA was applied with primers F984 and R1378 [31]. Amplicons were cloned using the pGEM-T Easy vector system (Promega, Leiden, The Netherlands) and clones were subjected to sequence analysis. Similarity of DNA sequences (Table 6) to sequences of the database was obtained using the BLAST-N algorithm [21] provided by NCBI.

2.8. Plasmid characterisation

2.8.1. Plasmid transfer

Transfer of Sm-resistance plasmids from Sm-resistant isolates and transconjugants obtained via exogenous isolation into *P. putida* UWC1 Rp^r (*chr*::*gfp*), was performed by filter matings (see before) with three recipient strains; *R. eutropha* JMP228 Rp^r (*chr*::*gfp*), CV601 Rp^r (*chr*::*gfp*) and *P. putida* UWC1 Rp^r (*chr*::*gfp*). Transconjugants of *R. eutropha* JMP228 Rp^r (*chr*::*gfp*) were selected on 0.1×TSBA, and transconjugants of *E. coli* CV601 Rp^r (*chr*::*gfp*) and *P. putida* UWC1 Rp^r (*chr*::*gfp*) on LBA, with 50 μg ml^{-1} of both Km and Sm. Incubation was at 37°C (*E. coli* CV601 Rp^r (*chr*::*gfp*)) or 27°C (*R. eutropha* JMP228 Rp^r (*chr*::*gfp*) and *P. putida* UWC1 Rp^r (*chr*::*gfp*)).

2.8.2. Plasmid replicon-specific PCR

Plasmids from Sm-resistant isolates as well as from putative *P. putida* UWC1 Rp^r transconjugants were tested with plasmid IncP and IncQ group-specific PCR systems [29]. Plasmid DNA extracts were, thus, subjected to four different PCR reactions, i.e. those specific for the backbone genes *korA* and *trfA2* (both IncP) and *oriT* and *oriV* (both IncQ), according to Götz et al. [29]. PCR products were analysed on ethidium bromide-stained agarose gels [20].

3. Results

3.1. Design and testing of PCR primers for detection of Sm-modifying genes

All primer sequences (Table 2) were compared to sequences in the database provided by NCBI. Some primers showed homology with varying (eukaryotic) genes up to a level of 85% (17/20 bp). However, no similarities with potential targets were found in either primer of each pair (Table 2).

All PCR primers designed to target Sm-modifying genes produced amplicons with their respective templates (Table 2) as shown by the appearance of single bands with the expected sizes in agarose gels (data not shown). Moreover,

Table 1
Strains used as recipients and genetic elements used for probe construction

Recipient strain (R) or genetic element (P)[a]	Selectable markers[b]/Sm-resistance gene	Source or Ref.
R: *Escherichia coli* CV601 Rp^r	Sm^s Rp^r	[49]
R: *Pseudomonas putida* UWC1 Rp^r	Sm^s Rp^r	[49]
R: *E. coli* CV601 Rp^r (*chr*::*gfp*)	Sm^s Rp^r Km^r	[48]
R: *P. putida* UWC1 Rp^r (*chr*::*gfp*)	Sm^s Rp^r Km^r	[48]
R: *Ralstonia eutropha* JMP228 Rp^r (*chr*::*gfp*)	Sm^s Rp^r Km^r	[48]
P: *E. coli* JM101 (RSF1010)	*aph* (3″) and *aph* (6)-1d	[50]
P: *E. coli* S17-1 (pSUP 1021)	*aph* (6)-1c	[51]
P: *E. coli* 935 (pUC8::Tn*1826*)	*ant* (3″)-1	[52]
P: *Pseudomonas aeruginosa* ST44 (pSA1701)	*ant* (3″)-2	[53]
P: *E. coli* DH5α (pK214)	*ant* (6)	[13]

[a]R, used as recipient strain for biparental filter matings and exogenous plasmid isolation. P, used for probe construction using primers described in Table 2.
[b]Sm, streptomycin; Rp, rifampicin; Km, kanamycin; r, resistant; s, sensitive.

Table 2
Primers designed for PCR-based detection of Sm-modifying genes in different habitats

Genotype	DNA sequence of primers[a]		Fragment size (bp)	Template used in PCR
	Forward	Reverse		
ant (3″)[b]	5′-CAGCGCAATGACATTCTTGC-3′	5′-GTCGGCAGCGACA(C/T)CCTTCG-3′	295	Tn*1826*, pSA1701
aph (3″)[c]	5′-GCTCAAAGGTCGAGGTGTGG-3′	5′-CCAGTTCTCTTCGGCGTTAG-3′	515	RSF1010
aph (6)-1d[d]	5′-GACTCCTGCAATCGTCAAGG-3′	5′-GCAATGCGTCTAGGATCGAG-3′	560	RSF1010
aph (6)-1c[e]	5′-GAGCGCACCTTCGACTATGC-3′	5′-GCCATGGCGTTTACGGCCAG-3′	248	Tn*5*
ant (6)[f]	5′-CCATTCATCTCAAAAGTAGC-3′	5′-CTCAACGTATGTAGCAAAGG-3′	218	pK214

[a]Accession numbers used for primers directing *ant* (3″): X12870, X03043, U90945, Y14748, M95287, D43625, X68227, U12338, M55547, M86913, AF071555; *aph* (3″): M28829, U20588, M83717, U57647, L23118, M77502, M96392, AF024602; *aph* (6)-1d: M28829, M96392, AF024602; *aph* (6)-1c: U00004; *ant* (6): X92946, X06627. Position of primers matching target DNA: *ant* (3″), 337–357 and 612–632 (X12870), *aph* (3″), 169–189 and 664–684 (M28829), *aph* (6)-1d, 106–126 and 646–666 (M28829), *aph* (6)-1c, 534–554 and 762–782 (U0004), *ant* (6), 145–165 and 343–363 (X92946).
[b]Based on sequences from: Tn*21*, Tn*7*, pACM1, pCG4, pR46, pSA1700, pSA, R1033, Tn*1331*, Tn*2426*, DT104.
[c]Based on sequences from: RSF1010, pIG1, pLS88, Tn*5393*.
[d]Based on sequences from: RSF1010, Tn*5393*.
[e]Based on sequence from: Tn*5*.
[f]Based on sequences from: pK214, pS194.

no signal was observed in PCR reactions with the non-homologous Sm-modifying genes as templates, indicating the absence of cross-reactivity with unrelated Sm-modifying genes.

PCR reactions performed on total community DNA extracts from soil collected at Emmeloord (The Netherlands) and activated sludge from a sewage plant at Ede (The Netherlands) gave weak signals after analysis by gel electrophoresis and Southern hybridisation using primers and the appropriate probes directing *ant* (3″), *aph* (3″) and *aph* (6)-1d whereas they gave no signal with those targeting *ant* (6) and *aph* (6)-1b. Spiked soil and activated sludge samples with 10^3 amplicons g^{-1} dry soil or ml^{-1} activated sludge, respectively, gave weak signals with primers and probes directing *ant* (3″), *aph* (3″) and *aph* (6)-1d whereas in samples with 10^4 and higher, strong signals with all

Table 3
Origin and treatment of the habitats investigated

Type	Label	Description and number of samplings in parentheses	Ref. for DNA extraction procedure used
Bulk soil	BS1-Sm	Dossenheim (G); plantomycin (active compound streptomycin sulfate, 154 g ha^{-1})-treated soil (2)	[23]
	BS2-U	Dossenheim; untreated soil (2)	
	BS3-Se	Droitwich; sewage sludge (111 m^3 ha^{-1})-treated soil (2)	
	BS4-U	Cotswolds; limestone-based soil, fallow (sparse vegetative cover) (2)	
Rhizosphere soil	RS5-Cu	Ens; white radish (*Raphanus sativus* L. rettich) grown in $CuSO_4$ (11 kg ha^{-1})-treated soil (1)	[24]
	RS6-U	Ens; white radish (*R. sativus* L. rettich) grown in untreated soil (1)	
	RS7-Cu	Ens; cauliflower (*Brassica oleracea* var. *botrytis*) rhizosphere grown in $CuSO_4$ (11 kg ha^{-1})-treated soil (1)	
	RS8-U	Ens; cauliflower (*B. oleracea* var. *botrytis*) rhizosphere grown in untreated soil (1)	
	RS9-Sm	Dossenheim (G); grass grown in plantomycin (active compound streptomycin, 154 g ha^{-1})-treated soil (2)	
	RS10-U	Dossenheim (G); grass grown in untreated soil (2)	
Manure	M11-Fm	Broiler chicken grown on flavomycin-treated food (1)	[22]
	M12-U	Broiler chicken grown on untreated food (1)	
	M13-Ba	Layer chicken grown on Zn-bacitracin-treated food (1)	
	M14-U	Layer chicken grown on untreated food (1)	
	M15-Mn	Cattle grown on monensin-treated food (1)	
	M16-U	Cattle grown on untreated food (1)	
	M17-Nm	Pigs grown on neomycin-treated food (1)	
	M18-U	Pigs grown on untreated food (1)	
Activated sludge	AS19-Ho	Brussels; hospital wastewater treatment facility (Erasmus Hospital) (2)	[25]
	AS20-Ho	Ghent; hospital wastewater treatment facility (Maria Middelares Hospital) (2)	
	AS21-U	Wavre; treatment plant of the Dyle valley (population, industry, university) (2)	
	AS22-U	Rosière; treatment plant of Lasne (population, industry) (2)	
Seawater	S23-Tc	Volos, Pagasitikos Gulf; fish farm, tetracycline administered at regular base (2)	[26]
	S24-Ww	Athens; wastewater outflow (2)	
	S25-U	Fleves island; pristine (2)	
	S26-U	Maliakos Gulf; pristine (2)	

Table 4
Presence of Sm-modifying genes in total community DNA extracts, as determined by PCR (Table 2) followed by hybridisation with specific probes

Origin	Sample[a]	Reaction with PCR system targeting Sm-modifying genes[b]				
		aph (3″)-1	*aph* (6)-1d	*aph* (6)-1c	*ant* (3″)-1	*ant* (6)-1
Bulk soil	BS1-Sm	+−	++	+−	++	++
	BS2-U	+−	++	−−	++	−−
	BS3-Se	++	+−	+−	++	++
	BS4-U	+−	++	−−	+−	−−
Rhizosphere soil	RS5-Cu	+	+	+	+	+
	RS6-U	+	+	+	+	+
	RS7-Cu	+	+	+	+	+
	RS8-U	-	+	+	−	−
	RS9-Sm	+−	++	+−	−−	−−
	RS10-U	+−	++	+−	+−	−−
Manure	M11-Fm	+	+	+	+	+
	M12-U	+	+	+	+	+
	M13-Ba	+	+	+	+	+
	M14-U	+	+	+	+	+
	M15-Mn	+	+	+	+	+
	M16-U	+	+	+	+	+
	M17-Nm	+	+	−	+	+
	M18-U	+	+	−	+	+
Activated sludge	AS19-Ho	++	++	++	++	++
	AS20-Ho	+−	++	+−	++	−−
	AS21-U	++	++	+−	++	++
	AS22-U	++	++	+−	++	++
Seawater	S23-Tc	+−	++	+−	++	+−
	S24-Ww	++	++	+−	++	+−
	S25-U	++	++	++	++	+−
	S26-U	+−	++	−−	++	++

[a]See Table 3 for description of samples.
[b]+, positive PCR/hybridisation signal; −, negative; ++, −−, +−, signals from two samplings.

primer systems were obtained (data not shown). This established limits of detection in soil and activated sludge for all five Sm-modifying genes between 10^3 and 10^4 amplicons g^{-1} dry soil or ml^{-1} activated sludge, respectively.

3.2. PCR screening of Sm-modifying genes in total community DNA extracts

High-quality total community DNA was obtained from all 26 habitats as shown by the appearance of high molecular mass bands ($\geq$20 kb) in agarose gels (not shown). PCR amplification of these DNA samples followed by hybridisation with the relevant probes showed positive signals with multiple, sometimes up to all five, PCR systems (Table 4). In one habitat (RS9-Sm), a signal with only one PCR system, *aph* (6)-1d, was found. This indicated the presence of multiple Sm-modifying genes in most of the habitats investigated. *Aph* (6)-1d was the gene which was most abundantly present in all habitats; 98% of the samples produced positive signals with the PCR system for *aph* (6)-1d. Evidence for the presence of the other Sm-modifying genes in the habitats investigated indicated the following incidence: *ant* (3″) – 88%, *aph* (3″) – 78%, *ant* (6) – 65%, and *aph* (6)-1c – 58%.

As Sm-modifying genes were abundantly present in most habitats, it was not possible to assess any effect caused by putative selective pressure in the samples investigated. In the locations that were sampled twice (Table 3), several of the Sm-modifying genes were not detected on either occasion (Table 4). Thirty percent of the signals obtained were different between the first and second samplings, i.e. gave positive versus negative signals at either sampling. This indicated that fluctuations in the number of targets around the detection limits occurred in these habitats.

3.3. Sm-resistance quotients and analysis of Sm-resistant colonies in the habitats studied

The numbers of Sm-resistant CFUs, expressed as percentages of total CFUs (Table 5) varied between 0.02% (M17-Nm) and 52.9% (S26-U). The percentage of Sm-resistant CFUs varied over 2500-fold between lowest and highest frequency and no trend was observed between samples originating from the different habitats.

In total, 268 Sm-resistant colonies were analysed for the presence of Sm-modifying genes by colony filter hybridisation using the probes. Only 22 showed signals with one or more probes (Table 6). Of these 22 isolates, eight originated from manure of untreated animals (M14-U), three from community sewers which were presumably unaffected by antibiotics (AS21-U and AS22-U) and 11 from

Table 5
Resistance quotients and Sm-transfer frequencies, via exogenous plasmid isolation, to *P. putida* UWC1 Rp^r (sampling 1) or *P. putida* UWC1 Rp^r (*chr*::*gfp*) (sampling 2)

Origin of sample[a]	Values obtained for sampling periods[b]			
	Sm^r quotient (%)		Transfer frequency to *P. putida* UWC1 Rp^r (*chr*::*gfp*)	
	1	2	1	2
BS1-Sm	0.36	0.90	3.6×10^{-11}	3.6×10^{-11}
BS2-U	0.15	0.40	BD[c]	1×10^{-11}
BS3-Se	0.30	1.1	2.7×10^{-11}	1.8×10^{-11}
BS4-U	0.10	0.76	4.5×10^{-11}	BD
RS5-Cu	11.1	ND	2.5×10^{-5}	ND
RS6-U	4.11	ND	1.4×10^{-7}	ND
RS7-Cu	ND[d]	16.0	ND	2.9×10^{-4}
RS8-U	ND	21.5	ND	BD
RS9-Sm	12.5	5.02	BD	1.3×10^{-5}
RS10-U	1.41	5.86	BD	1.1×10^{-5}
M11-Fm	0.50	ND	BD	ND
M12-U	0.40	ND	7.2×10^{-11}	ND
M13-Ba	2.70	ND	BD	ND
M14-U	0.10	ND	4.5×10^{-11}	ND
M15-Mn	ND	2.50	ND	BD
M16-U	ND	4.80	ND	3.3×10^{-8}
M17-Nm	ND	0.02	ND	BD
M18-U	ND	0.10	ND	3.4×10^{-7}
AS19-Ho	0.08	4.89	BD	1.1×10^{-10}
AS20-Ho	0.10	0.23	1.8×10^{-11}	BD
AS21-U	0.80	1.33	BD	1×10^{-11}
AS22-U	0.02	0.77	BD	1.1×10^{-10}
S23-Tc	44.2	18.5	BD	BD
S24-Ww	1.50	5.10	6.3×10^{-11}	BD
S25-U	2.50	31.2	3.6×10^{-11}	1×10^{-11}
S26-U	52.9	18.3	7.2×10^{-11}	BD

[a]Origin of sample, see Table 3.
[b]1, 2, first and second sampling; Sm^r quotient = Sm-resistant CFU/total CFU.
[c]BD, below detection; limit of detection: 10^{-11}.
[d]ND, not determined.

the manure of treated animals and activated sludge from hospitals (M11-Fm, M13-Ba, AS19-Ho and AS20-Ho), indicating that the presence was not dependent on identifiable selective pressure. The presence of known Sm-modifying genes in the 22 isolates was confirmed by specific PCR (Table 2) using genomic DNA extracts. Only seven isolates appeared to possess a single Sm-modifying gene, whereas the other 15 possessed two or more genes. Furthermore, *aph* (3″) was the most dominant gene (present in 17 isolates), followed by *ant* (3″) and *aph* (6)-1d (both present in 13 isolates), whereas the incidence of the *aph* (6)-1c and *ant* (6) genes was lowest (present in one and zero isolates, respectively).

Plasmid extraction from the 22 isolates, followed by agarose gel electrophoresis, revealed clear plasmid bands in 14 isolates (Table 6) with sizes between about 40–50 kb and 200 kb. Transfer of the Sm-resistant phenotype to suitable recipient strains was observed for nine isolates. Determinants for IncP-type plasmids were observed in nine isolates and the presence of these determinants only coincided with Sm-resistance transfer in five isolates (3-32, 3-34, 4-64, 5C6 and 4-71). Evidence for the presence of IncQ-type plasmids was not found.

Identification of the selected isolates by 16S rDNA sequence analysis (Table 6) revealed that they belonged to phylogenetically distinct species. Thirty-two percent of the isolates were Gram-positive. Remarkably, one isolate, denoted 4-60 and affiliated with *Corynebacterium glutamicum* (100%), possessed IncP plasmid-type determinants, whereas no plasmid was observed. *Proteus mirabilis* was the most abundantly observed species (five out of 22 isolates). All five strains originated from the same habitat, i.e. manure from untreated chickens. Although all *P. mirabilis* isolates were able to transfer Sm resistance to one or more recipients and also possessed *ant* (3″), some differences were present, like an extra Sm-resistance gene – *aph* (6)-1d – in strain 3-31 and different signals with IncP-directed primers between strains.

3.4. Exogenous Sm-resistance plasmid isolations

P. putida UWC1 Rp^r was used as the recipient strain to acquire mobile Sm-resistance determinants of the first samplings, whereas *P. putida* UWC1 Rp^r (*chr*::*gfp*) was the recipient for all second samplings. Spontaneous resistance to Sm for both recipients was observed at a frequency of 10^{-11}. Transfer frequencies with the first samples ranged from 2.5×10^{-5} to below the limit of detection (10^{-11}), whereas the frequencies obtained with the second samples ranged from 2.9×10^{-4} to below detection (Table 5). With both recipients, putative transconjugants could be discriminated from indigenous Sm- and Rp- resistant organisms by their fluorescence on King's B agar in combination with ERIC-PCR. However, the use of *P. putida* UWC1 Rp^r (*chr*::*gfp*) as the recipient was clearly less laborious for the selection of genuine recipients with acquired Sm resistance.

Transferable Sm resistance was observed in samples from all habitats, either in one or both of the samplings. The highest frequencies (between 2.9×10^{-4} and 1.4×10^{-7}) of Sm-resistance transfer were observed in matings with the microflora from rhizosphere soils (white radish, cauliflower and grass), whereas in all other habitats these frequencies were at least 1000-fold lower.

Plasmids were observed in 133 *P. putida* UWC1 recipients with acquired Sm resistance. Plasmid sizes varied in accordance with the environment the exogenous isolates were obtained from. In general, small plasmids (sizes 40–80 kb) were obtained from manure, activated sludge and seawater samples, whereas large plasmids (200 kb) were found in extracts from bulk and rhizosphere soil isolates. Characterisation of the Sm-resistance genes present on all plasmids by PCR/hybridisation indicated that most plasmids (93%) possessed one or more of the Sm-modifying genes (Table 7). Of the plasmids carrying these Sm-mod-

Table 6
Sm-resistant bacterial isolates obtained from different habitats that probed positive with Sm^r genes

Isolate	Habitat[a]	Closest match (16S rDNA)[b]	Phylum[c]	Sm^r genes	Transfer of Sm^r to[d]	Plasmid detected[e]	IncP determinant[f]
3-32	M13-Ba	*Proteus mirabilis* (100%)	γ	*ant* (3″)	P, E	+	*korA*
3-34	M13-Ba	*P. mirabilis* (100%)	γ	*ant* (3″)	P, E	+	*trfA2*
3-38	M13-Ba	*P. mirabilis* (100%)	γ	*ant* (3″)	P, E	+	
4-64	M13-Ba	*P. mirabilis* (100%)	γ	*ant* (3″)	P	+	*trfA2*
3-41	M13-Ba	*Rhodococcus globerulus* (100%)	HGC	*ant* (3″), *aph* (3″)		−	
4-69	M14-U	*Bacillus silvestris* (99%)	LGC	*ant* (3″), *aph* (3″)		−	
3-10	M13-Ba	*Ochrobactrum* sp. (99%)	α	*ant* (3″), *aph* (3″), *aph* (6)-1d		−	
3-18	M11-Fm	*Dietzia maris* (99%)	HGC	*ant* (3″), *aph* (3″), *aph* (6)-1d	P, E, R	+	
4-73	M14-U	*Brevibacterium liquefaciens* (99%)	HGC	*ant* (3″), *aph* (3″), *aph* (6)-1d	P	+	
4-76	M14-U	*Moraxella phenylpyruvica* (99%)	γ	*ant* (3″), *aph* (3″), *aph* (6)-1d		+	*korA*
5B1	AS19-Ho	*Aeromonas media* (99%)	γ	*ant* (3″), *aph* (3″), *aph* (6)-1d		+	*korA*, *trfA2*
5C6	AS22-U	*Pseudomonas putida* (99%)	γ	*ant* (3″), *aph* (3″), *aph* (6)-1d	P, E, R	+	*korA*, *trfA2*
5D4	AS20-Ho	*Aeromonas salmonicida* (100%)	γ	*ant* (3″), *aph* (3″), *aph* (6)-1d		+	*korA*, *trfA2*
3-31	M13-Ba	*P. mirabilis* (100%)	γ	*aph* (3″), *aph* (6)-1d	P, E	+	
4-59	M14-U	*M. phenylpyruvica* (99%)	γ	*aph* (3″), *aph* (6)-1d		+	
4-60	M14-U	*Corynebacterium glutamicum* (100%)	HGC	*aph* (3″), *aph* (6)-1d		−	*korA*, *trfA2*
4-70	M14-U	*Brevundimonas diminuta* (100%)	α	*aph* (3″), *aph* (6)-1d		+	
4-71	M14-U	*B. diminuta* (100%)	α	*aph* (3″), *aph* (6)-1d	P, E, R	+	*korA*, *trfA2*
3-36	M13-Ba	*Alcaligenes faecalis* (98%)	β	*aph* (3″)		−	
5A1	AS21-U	*Pseudomonas aeruginosa* (100%)	γ	*aph* (3″)		−	
5A2	AS21-U	*P. aeruginosa* (99%)	γ	*aph* (3″)		−	
4-77	M14-U	*B. liquefaciens* (99%)	HGC	*aph* (6)-1d, *aph* (6)-1c		−	

[a]Explanation: see Table 3.
[b]Isolates were identified by closest match of their 16S rDNA regions, in parentheses degree of sequence similarity.
[c]α, β, γ, α, β and γ subdivision of Proteobacteria; LGC, HGC, low and high G+C Gram-positive bacteria.
[d]Transfer of Sm resistance to *P. putida* UWC1 Rpr (*chr*::*gfp*) (P), *E. coli* CV601 Rpr (*chr*::*gfp*) (E) or *R. eutropha* JMP228 Rpr (*chr*::*gfp*) (R). Blank, no transfer.
[e]Plasmid band in agarose gel.
[f]Inc group determined using primers directed against *korA*, *trfA2* (both IncP), *oriT* and *oriV* (both IncQ) [48].

Table 7
Genotypic characterisation of exogenously isolated plasmids obtained from different habitats encoding Sm resistance

Sm-resistance genotype of plasmid	Number of plasmids in Sampling period		Occurrence in (habitat)[a]:
	1	2	
'No'[b]	5	4	BS3-Se, BS4-U, RS6-U, S25-U, S26-U
ant (3″)	9	35	BS1-Sm, RS5-Cu, M18-U, AS22-U, S25-U
ant (3″), *aph* (3″)	6	1	M12-U, M18-U, S26-U
ant (3″), *aph* (3″), *aph* (6)-1c	1	1	BS2-U, M12-U
ant (3″), *aph* (3″), *aph* (6)-1c, *aph* (6)-1d	5	3	RS10-U, M12-U, M14-U
ant (3″), *aph* (3″), *aph* (6)-1c, *aph* (6)-1d, *ant* (6)	3	2	M18-U
ant (3″), *aph* (3″), *aph* (6)-1c, *ant* (6)	2	1	M18-U
ant (3″), *aph* (3″), *aph* (6)-1d	10	0	BS1-Sm, RS9-Sm, S24-Ww
ant (3″), *aph* (3″), *aph* (6)-1d, *ant* (6)	1	5	BS1-Sm, RS9-Sm, S24-Ww
ant (3″), *aph* (3″), *ant* (6)	0	1	RS5-Cu
ant (3″), *aph* (6)-1c	0	9	AS19-Ho, AS21-U, AS22-U
ant (3″), *aph* (6)-1c, *aph* (6)-1d	1	2	BS2-U, M14-U
ant (3″), *aph* (6)-1c, *ant* (6)	2	1	RS10-U, M14-U, S26-U
ant (3″), *aph* (6)-1d	3	2	BS1-Sm, RS7-Cu, M18-U, AS20-Ho
ant (3″), *aph* (6)-1d, *ant* (6)	0	2	RS8-U
ant (3″), *ant* (6)	1	4	BS1-Sm, M18-U, AS19-Ho, S25-U
aph (3″), *aph* (6)-1c, *aph* (6)-1d	2	1	BS2-U, BS4-U, M18-U
aph (3″), *aph* (6)-1d	4	0	BS4-U
aph (6)-1c	0	1	BS4-U
aph (6)-1c, *aph* (6)-1d, *ant* (6)	0	1	RS9-Sm
aph (6)-1d	0	1	M18-U
ant (6)	1	0	S25-U
Total number of plasmids isolated	56	77	

[a]Explanation: see Table 3.
[b]'No', undefined, i.e. not belonging to the five classes of Sm^r genes screened with PCR and hybridisation.

ifying genes, 62% possessed at least two, and even up to five, resistance determinants, indicating an abundant presence of multiple Sm resistances on these plasmids.

The Sm-modifying gene most commonly present on all plasmids was *ant* (3″), which was present in 85% of the plasmids obtained by exogenous plasmid isolation. Other Sm-modifying genes were present in 37% (*aph* (3″)), 36% (*aph*(6)-1d), 29% (*aph*(6)-1c) and 20% (*ant* (6)) of all plasmids. In nine plasmids obtained by exogenous plasmid isolation (7%), no PCR/hybridisation response with either of the primer/probe combinations was observed, indicating that the selected Sm-modifying genes were not present on these elements.

A vast majority of the plasmids obtained by exogenous plasmid isolation (87%) showed signals in PCR reactions with primers directed against plasmid 'backbone' genes specific for IncP and IncQ plasmids. Twenty-eight percent were positive with either one of the two IncP-specific primers (*korA*, *trfA2*), 35% with either one or both primers specific for IncQ plasmids (*oriT*, *oriV*), and 24% to primers from both Inc groups. Seven percent of the plasmids did not show a signal with any PCR system and no further attempt was made to characterise these. Five selected *P. putida* UWC1 Rp^r (*chr*::*gfp*) derivatives (DB69, DO57, DO59, EB-A and EB-B), containing plasmids that produced different signals with IncP- and IncQ-specific PCR, were mated with recipient strains *R. eutropha* JMP228 Rp^r (*chr*::*gfp*) and *E. coli* CV 601 Rp^r (*chr*::*gfp*). Transfer of Sm resistance, at frequencies of at least 10^{-6}, was observed for strains DB69, DO57, DO59 and EB-A in biparental matings with both recipients, but not for EB-B. Transconjugants DB69 and DO57 reacted positively with both IncP-specific PCR systems, DO59 only with *korA* (IncP), EB-A with none of the four tested primers, and EB-B with both IncQ-directed PCR systems. Transfer of Sm resistance was expected when IncP plasmids were present but not in the case of IncQ plasmids. Transfer from strain EB-B indicates that other, possibly co-isolated, plasmids, different from the IncP type, may have been involved in the transfer.

4. Discussion

The incidence of Sm resistance in pathogenic and commensal bacteria in clinical and agricultural habitats is often high and this has most likely been caused by the use of Sm in these environments [3,32,33]. However, the spread and distribution of these genes in pristine habitats, i.e. habitats which are not influenced by anthropogenic activities, is less well known. For instance, these habitats might serve as reservoirs for Sm-resistance genes translo-

cated from 'Sm-affected' locations [32]. Pristine habitats might even be the sources of most commonly detected resistance genes in clinical, veterinary and agricultural habitats [5,8] and the sources of Sm-resistance genes may lie in the natural microflora. Irrespective of their origin, however, the mobility of Sm-resistance genes is likely to play a key role in their spread. Horizontal transfer of mobile elements might even provide the engine by which Sm-resistance genes self-perpetuate [5]. To provide baseline knowledge on the occurrence of Sm resistance, this study focussed on the distribution of Sm-modifying genes in habitats with and without selective pressure.

PCR primer systems targeting Sm-modifying genes were developed to screen for the presence of these genes. These primer systems turned out to be excellent tools to discriminate the different Sm-modifying genes. The limit of detection by PCR followed by hybridisation in total community DNA extracts was estimated to be between 10^3 and 10^4 target molecules g^{-1} of soil or ml^{-1} activated sludge, which is about 100–1000-fold lower than by direct hybridisation using total community DNA [30]. Although only parts of the Sm-modifying genes are detected in total community DNA samples, the signals obtained are likely to represent intact genes as the primer systems were specific, the sequence alignments of Sm-modifying genes from databases always revealed high homologies and PCR signals on gels could always be confirmed with hybridisation using the appropriate probes.

The screening for Sm-modifying genes in total community DNA from different habitats revealed that several genes were abundant in almost all samples, irrespective of sampling time or the selective pressure presumed to be present. The *aph* (3″), *aph* (6)-1d and *ant* (3″) genes (Table 4) represented the most frequently occurring Sm-modifying genes in the habitats studied. All three genes have been reported to reside on mobile genetic elements in isolates from clinical [34,35], plant [10] and soil settings [36,37], or from other habitats [38]. This illustrates their ubiquity in the natural environment. Although less abundant, the *aph* (6)-1c and *ant* (6) genes were also present in more than 50% of the habitats investigated. Reports on the occurrence of the latter genes are less frequent and they have only so far been described in isolates from clinical environments [39,40]. In this study, their wider distribution is revealed by the presence of signals in DNA extracts from different bulk and rhizosphere soils, manure, activated sludge and seawater.

The fact that no effect of selective pressure by Sm or copper treatment [38,41] was found can be explained by several hypotheses: (1) dilution or inactivation of the selective compounds, (2) a possible effect was obscured by rapid decay of the Sm-resistant populations, or (3) the effect was too small to be detected at all. Sm-resistant populations in natural habitats may fluctuate in time and space, occasionally dropping to below the limit of detection, and the populations targeted, which were possibly favoured by selection, may have followed the same trends.

The majority (66%) of the habitats that were sampled twice were unambiguous in their reaction in the PCR, i.e. they either showed evidence for the presence of a particular gene at both occasions or not at all. This indicated that dramatic shifts in the numbers of Sm-modifying genes over time did not occur in these habitats. Furthermore, the absence of enhanced abundance of Sm-resistance genes in the habitats under putative selective pressure indicated that the distribution of Sm-resistance genes may be more influenced by factors other than selective pressure.

As the majority of the bacterial populations, including those possessing Sm-resistance genes, might remain in a dormant state in many of the habitats investigated [42], they may not actively contribute to the spread of resistance genes, which would limit their abundance. In addition, outgrowth of the Sm-resistant populations may only take place under strong selection, and it is uncertain if initially dormant populations are able to proliferate under these conditions. Thus, the active microflora in natural habitats might be mainly involved in the spread of Sm-resistance genes; hence, Sm-resistant isolates and their plasmids were further investigated. The R2A medium allows the growth of a broad spectrum of bacteria, thus allowing for fair sampling of the Sm-resistant 'diversity'. Surprisingly, only a small fraction (8%) of the Sm-resistant bacteria showed signals with probes and primers that targeted the selected Sm-modifying genes. On the other hand, the finding of a high variety of species, including different bacterial taxa, indicated that Sm-modifying genes are probably widely spread among different prokaryotic groups. Reactions with, mostly, three Sm-modifying genes, *aph* (3″), *aph* (6)-1d and *ant* (3″), and, on one occasion, with *aph* (6)-1c, were observed. The presence of these genes often coincided with the presence of IncP plasmids. Furthermore, transfer of several Sm-resistance determinants to suitable recipients was shown, which indicates the ease of their dissemination among different prokaryotes. IncP plasmids are commonly observed in many different habitats. They have been isolated from, for instance, veterinary [43], activated sludge [44] and rhizosphere (unpublished results) samples. The plasmids involved often carried Sm-modifying genes.

The exogenous plasmid isolation into *P. putida* UWC1 showed the presence of all selected Sm-modifying genes on various mobile elements. One gene in particular, *ant* (3″), dominated in this horizontal gene pool. This gene was also abundantly found in total community DNA as well as in Sm-resistant isolates. Thus, of all Sm-modifying genes, *ant* (3″) is most commonly associated with mobile elements. The *ant* (3″) family is the best described Sm-resistance gene family, it is observed in many different habitats [45–47], and the observations made in this study emphasise their wide dissemination in habitats throughout Europe.

The vast majority of the plasmids obtained possessed at least one Sm-modifying gene, which further established their occurrence on mobile genetic elements. A minor fraction (7%) of the plasmids obtained by exogenous isolation gave no positive signal with the detection systems used. Thus, these plasmids carry either aberrant forms of the selected Sm-resistance genes or hitherto unknown Sm-resistance genes.

The finding of multiple Sm-resistance genes in a majority (62%) of the plasmids obtained via exogenous plasmid isolation indicated that such genes may accumulate on plasmids, possibly via transposable elements. Carriage of multiple Sm-resistance genes may seem redundant, as simultaneous expression of these genes might be costly in terms of cellular energy usage. However, the presence of multiple Sm resistance on mobile elements is commonly reported. For instance, *aph* (3″) and *aph* (6)-1d reside together on both Tn*5393* [9] and RSF1010 [10]. Two hypotheses may explain the co-existence of multiple Sm resistances on plasmids from an evolutionary point of view. (1) Not all genes may be functional, and some may even be degenerated and non-functional. (2) Mobile elements carrying multiple Sm resistance may be ecologically favoured, because not all genes are expressed in different hosts and, thus, selection for the Sm resistance-carrying plasmid will be extended to a wider host range.

Surveys of the prevalence of gentamicin [48] and tetracycline [25] resistance genes were performed in the same habitats investigated here. From both these studies, it was concluded that the respective antibiotic-resistance genes are often located on mobile elements which promote their spread in different habitats. It is obvious that the dispersion of Sm-modifying genes, as shown in this study, is not particularly related or restricted to this antibiotic. Rather, the mechanism for spread and distribution of Sm-resistance genes in different habitats might be gene transfer mediated by plasmids from common or currently unknown incompatibility groups. The impact of selective pressure on the enrichment of particular Sm-resistance genes may be lower than expected, whereas the role of environmental factors like nutrient availability is possibly underestimated. Gene transfer events in nature are complex, and factors other than direct selective pressure, such as co-selection and plasmid stability, may play an essential role in the persistence of antibiotic-resistance genes in natural bacterial assemblages.

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