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Gentamicin resistance genes in environmental bacteria: prevalence and transfer

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Abstract

A comprehensive multiphasic survey of the prevalence and transfer of gentamicin resistance (Gm^r) genes in different non-clinical environments has been performed. We were interested to find out whether Gm^r genes described from clinical isolates can be detected in different environmental habitats and whether hot spots can be identified. Furthermore, this study aimed to evaluate the impact of selective pressure on the abundance and mobility of resistance genes. The study included samples from soils, rhizospheres, piggery manure, faeces from cattle, laying and broiler chickens, municipal and hospital sewage water, and coastal water. Six clusters of genes coding for Gm-modifying enzymes (*aac*(*3*)-I, *aac*(*3*)-II/VI, *aac*(*3*)-III/IV, *aac*(*6′*)-II/Ib, *ant*(*2″*)-I, *aph*(*2″*)-I) were identified based on a database comparison and primer systems for each gene cluster were developed. Gm-resistant bacteria isolated from the different environments had a different taxonomic composition. In only 34 of 207 isolates, mainly originating from sewage, faeces and coastal water polluted with wastewater, were known Gm^r genes corresponding to five of the six clusters detected. The strains belonged to genera in which the genes had previously been detected (Enterobacteriaceae, *Pseudomonas*, *Acinetobacter*) but also to phylogenetically distant bacteria, such as members of the CFB group, α- and β-Proteobacteria. Gm^r genes located on mobile genetic elements (MGE) could be captured in exogenous isolations into recipients belonging to α-, β- and γ-Proteobacteria from all environments except for soil. A high proportion of the MGE, conferring Gm resistance isolated from sewage, were identified as IncPβ plasmids. Molecular detection of Gm^r genes, and broad host range plasmid-specific sequences (IncP-1, IncN, IncW and IncQ) in environmental DNA indicated a habitat-specific dissemination. A high abundance and diversity of Gm^r genes could be shown for samples from faeces (broilers, layers, cattle), from sewage, from seawater, collected close to a wastewater outflow, and from piggery manure. In the latter samples all six clusters of Gm^r genes could be detected. The different kinds of selective pressure studied here seemed to enhance the abundance of MGE, while an effect on Gm^r genes was not obvious.

Keywords: Gentamicin resistance gene; Exogenous isolation; Polymerase chain reaction analysis; Community DNA

1. Introduction

Application of antibiotics in human and veterinary medicine and in agriculture for more than 50 years has had a major impact on microbial communities [1], resulting in an enrichment of bacterial populations which were resistant to, or developed or acquired resistance to, the antibiotics used. Due to their enormous genetic flexibility bacteria rapidly adapted to the selective pressure. Mobile genetic elements (MGE) such as plasmids, phages, conjugative

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transposons, IS elements or gene cassettes are important for this genetic flexibility. These allow bacteria to acquire the genetic makeup required to flourish under changing environmental conditions [2,3].

Nowadays, the significant increase in both multiresistance and range of bacterial pathogens displaying resistance to a growing number of clinically important drugs is threatening the success of medical therapy. The majority of epidemiological studies have focused on the spread of resistance in human pathogens. Whilst the reservoir for such resistance genes within populations of clinical isolates has been, and is being documented in view of the importance for the treatment of infections, no such database exists for bacterial populations in soil and water. The incidence of bacteria which are resistant to one or to multiple antibiotics has been traditionally studied by plating on antibiotic-containing nutrient media, or by screening bacterial isolates for their antibiotic resistance patterns. Molecular tools which more and more became available in the 80s allowed scientists to make great progress in studying the epidemiology of antibiotic resistance genes and mobile genetic elements at the genetic level. DNA probes and PCR-based detection systems [4–9] enable us not only to analyse the dissemination of antibiotic resistance genes and mobile genetic elements in the culturable fraction of bacteria, but also to extend our knowledge to the majority of bacteria which are not accessible to traditional cultivation techniques [10,11]. Recently, exogenous isolation of MGE based on selectable markers or on the mobilising capacity was successfully applied to capture plasmids from a wide range of environments in Gram-negative recipient strains [12–20].

The major objective of this study was to survey the prevalence and transfer of genes conferring a resistance to gentamicin (Gm) in cultured and non-cultured bacteria from different environmental habitats such as soils, rhizosphere, manure, sewage, coastal waters and sediments. Samples originated from different geographic regions in Europe. To evaluate the impact of selective pressure, soil, rhizosphere and animal faeces or manure samples were taken from experimental sites with or without antibiotics, or copper treatment. They were analysed by cultivation-dependent (selective plating) and cultivation-independent methods. Primers and probes targeting different families of genes encoding Gm-modifying enzymes in non-producing strains were developed and applied for a molecular analysis of Gm-resistant isolates, exogenously isolated Gm-resistant transconjugants and directly extracted community DNA. In addition PCR-based screening of community DNA from 40 environmental samples was performed to provide data on the occurrence of broad host range plasmids.

2. Materials and methods

2.1. Strains and plasmids

The following reference strains carrying Gm resistance (Gm^r) genes were used for testing the primer systems and generation of probes: *Escherichia coli* S17-1 pAB2002 (*aac*(*3*)-Ia), *Pseudomonas aeruginosa* 88.341F (*aac*(*3*)-Ib), *E. coli* DH5α pWP866 (*aac*(*3*)-IIa), *Enterobacter aerogenes* 17798 VDK (*aac*(*3*)-IIa), *E. coli* DH5α pSCH4203 (*aac*(*3*)-IIb), *E. coli* DH5α pSCH4101 (*aac*(*3*)-VIa), *P. aeruginosa* PST-1 (*aac*(*3*)-IIIa), *E. coli* DH5α pHLW1 (*aac*(*3*)-IV), *Acinetobacter baumannii* LBL.3 (*aac*(*6′*)-Ib), *P. aeruginosa* F-03 (*aac*(*6′*)-IIa), *E. coli* DH5α pSCH5102 (*aac*(*6′*)-IIb), *E. coli* CV600 pIE723 (*ant*(*2″*)-I), *Staphylococcus aureus* pSK1 (*aph*(*2″*)-Ia), *E. coli* DH5α pAM6306 (*aph*(*2″*)-Ic), and *E. coli* NC95 (*aph*(*2″*)-Id).

The following rifampicin-resistant mutants were used as recipients in exogenous matings: *Pseudomonas putida* UWC1 (γ-subclass of Proteobacteria), *Ralstonia eutropha* JMP228 (β-subclass of Proteobacteria), *Agrobacterium tumefaciens* UBAPF2 (α-subclass of Proteobacteria) and *E. coli* CV601 (γ-subclass of Proteobacteria) [18]. All strains were *gfp* marked by introducing the mini-transposon vector pAG508 which was kindly provided by M. Straetz, GBF as described by Suarez et al. [21]. The resulting strains were kanamycin and rifampicin resistant and showed the typical green fluorescence allowing their unequivocal detection.

2.2. Sampling and sample processing

Table 1 summarises the type of samples, date of sampling and the geographic origin from which they were taken. All samples were composed of at least three single samples and mixed whilst fresh. Plating and exogenous isolation of transferable Gm resistance was done shortly after sampling using the recovered bacterial fraction. Pellets of the bacterial fraction or membrane filters with concentrated cells were kept frozen until DNA extraction.

2.3. Recovery of the bacterial fraction

The bacterial fraction was recovered from 5 g of animal faeces, resuspended in 15 ml sterile saline (0.85% NaCl) by Stomacher treatment for 1 min at high speed followed by low speed centrifugation to remove big particles. The pellet was re-extracted twice. The same protocol was used for soil except that a Griffin shaker was used since small stones in soil samples caused Stomacher bags to burst. From rhizosphere the bacterial fraction was obtained by shaking 5 g sample material in sodium pyrophosphate and 10 g gravel for 10 min at 200 rpm. Thirty litres of seawater were filtered through a 47-mm-diameter glass fibre filter (Gelman type A/E) to remove larger particles and eukaryotes. Microorganisms were then collected by filtration on a

0.22-μm (pore size) membrane filter (GV Durapore/Millipore). Bacteria were removed from the filter by vortex mixing for 5 min in 10 ml Ringer solution. Thoroughly mixed sewage samples composed of three single samples were used immediately, without treatment.

2.4. Cultivation conditions

Serial dilutions of the bacterial fraction were spread onto (i) R2A (Difco), supplemented with cycloheximide (200 mg l^{-1}, Sigma), benlate (30 mg l^{-1}, DuPont, contains 50% benomyl), nystatin (50 mg l^{-1}, Sigma) and with or without Gm (10 mg l^{-1}, Sigma). Plate counts were determined after 3–6 days incubation at 28°C. For each of the samples, 24 colonies with different morphology were picked from the Gm-selective medium.

2.5. FAME analysis of Gm-resistant isolates

For each sample of the first sampling event 12 morphologically different colonies (if possible) were selected and streaked on Trypticase soy broth (Becton Dickinson, Cockeysville, MD, USA) supplemented with Bacto-agar (15 g l^{-1}, Difco Laboratories). The strains were identified or classified by fatty acid analysis using the Microbial Identification System (MIS, MIDI Inc., Newark, DE, USA).

2.6. Exogenous isolation of mobile genetic elements carrying Gm^r genes

Exogenous plasmid isolations were essentially performed as described by Bale et al. [22] and Hill et al. [23]. Recipient strains were grown in 5 ml Luria broth (LB) supplemented with rifampicin (50 mg l^{-1}) at 28°C for approximately 20 h. Cells were harvested by centrifugation for 5 min, 4000 × *g* at room temperature. The pellet was resuspended in 10 ml LB and incubated for another 1–2 h at room temperature during the preparation of donor cells. Before the mating 200 μl culture of the recipient strain and approximately 1 ml donor cells (bacterial fraction recovered from the environmental sample) were mixed (donor to recipient ratio of 1:1; approximately 10^8 cells ml^{-1}) and pelleted by centrifugation at 10 000 × *g* for 5 min. The cell pellet was resuspended in 100 μl LB and transferred to sterile Millipore filters (0.22 μm, cat. No. GVWP02500) placed on yeast agar plates (15 g yeast extract Difco, 10 g Bacto-peptone Difco, 6 g NaCl, 12.5 g Bacto-agar Difco, 1 l deionised water, pH 7.5, supplemented with 200 mg cycloheximide, 30 mg benlate, and 50 mg nystatin). The latter two antifungal compounds were used only for samples from cattle faeces. After 20–24 h incubation at 28°C the cells from the filters were resuspended in 10 ml sterile 0.85% NaCl. To select for Gm^r transconjugants serial dilutions were plated on Plate Count Agar (PCA, Merck, Darmstadt, Germany) supplemented with cycloheximide (200 mg l^{-1}), nystatin (50 mg l^{-1}), rifampicin (50 mg l^{-1}), and Gm (10 mg l^{-1}). Recipient numbers were determined by applying three replicate 20-μl drops per serial dilution on PCA with rifampicin (50 mg l^{-1}). As controls either 200 μl sterile water and 1 ml donor cells or 200 μl recipient and 1 ml sterile water were mixed, pelleted and resuspended in 100 μl LB before transferring the cell suspension to a filter as described above. Undiluted suspensions of the control filters were plated onto the selective medium for Gm^r transconjugants to check for background growth of the donor and for spontaneous mutants of the recipient strains. After 2 days' incubation at 28°C the number of transconjugants and recipients was determined. Transconjugants were picked and streaked onto PCA supplemented with rifampicin (50 mg l^{-1}) and Gm (either 10 mg l^{-1} or 50 mg l^{-1}). Recipient strain identity was confirmed by GFP fluorescence.

For the second sampling the culturable bacterial fraction grown on R2A with Gm was resuspended from plates and used as donors in matings with *gfp*-marked recipient strains.

2.7. Total community DNA extraction

Total community DNA was extracted directly or from the bacterial fraction after a harsh lysis step. DNA from manure and faeces samples was extracted and purified essentially as described by van Elsas and Smalla [24]. Briefly, the DNA was extracted from manure slurries, soil and rhizosphere samples by lysozyme, bead beating, and alkaline sodium dodecyl sulfate treatments followed by phenol, phenol–chloroform and chloroform extraction. The crude DNA was purified by two salt precipitation steps (cesium chloride and potassium acetate precipitation, Wizard DNA cleanup (Promega, Madison, WI, USA) or Ultra clean (MOBIO, Solana Beach, CA, USA)). The DNA extraction protocols used for other environmental samples are summarised in Table 1.

2.8. Plasmid and genomic DNA isolations

Plasmid DNA was extracted according to the protocol described by Smalla et al. [18]. Genomic DNA was isolated from a loop of cells freshly grown on PCA (Gm 20 mg l^{-1}). Crude cell lysates were obtained using the protocol provided with the Qiagen genomic DNA extraction kit (Qiagen, Hilden, Germany). DNA extraction from the lysates was done with Ultra clean (MOBIO).

2.9. Dot-blot and Southern blot analysis

Genomic DNA extracted from Gm-resistant isolates or transconjugants was analysed by dot-blot hybridisation for the presence of the known Gm^r genes. Dot and Southern blotting was performed by standard protocols [25].

Hybridisations of Southern- or dot-blotted plasmid DNA or PCR products with digoxigenin-labelled probes were performed according to the manufacturer's instructions (Roche Diagnostic, Mannheim, Germany).

2.10. PCR-based detection of Gm^r genes and broad host range plasmids

Six PCR primer sets were designed to amplify all known genes coding for Gm-modifying enzymes and similar but not yet discovered sequences, except *aac*(*2′*)-I, which has its main function in peptidoglycan acetylation in *Providencia stuartii* and may not play a major role in Gm resistance of environmental bacteria. Sequences from GenBank with the following accession numbers were used for pairwise comparisons and multiple alignments to identify common conserved primer annealing sites: *aac*(*3*)-I: U72638, AJ006518, U12338, X15852, U25061, S68049, AJ009820, U90945, L06157; *aac*(*3*)-II/VI: M88012, M97172 (originally named *aac*(3)-Vb), X13541, S68058, X51534, X13542, M62833, L22613, X54723, X13543; *aac*(*3*)-III/IV: L06161, L06160, X55652, X01385; *aac*(*6′*)-II/Ib (Ib does only confer resistance to Gm after mutation in one base (Ib′) but was included due to its high similarity to II): M29659, L06163, AF012280, M23634, M55547, M21682, AF012279, AJ009820, L25666, L25617, X60321, U59183; *ant*(*2″*)-I: X04555, X64369, X74412, and other sequences identical to the latter; *aph*(*2″*)-I: U51479, AF016483, U50978, M18086, M13771, AF051917 (the latter four are part of the bifunctional *aac*(*6′*)-*aph*(*2″*) gene). The primer design was based on codons of conserved amino acid motifs which might be of functional importance. Degeneracy in the third codon bases at the 3′ terminus of the primers was obligate, a moderate number of mismatches at the 5′ termini was tolerated. PCR was performed with either total community DNA, heat-denatured cells (1 μl of a 1/10 diluted overnight broth), genomic or plasmid DNA as template. PCR mixtures contained AmpliTaq Gold (Applied Biosystems), 1×PCR buffer, 0.2 mM deoxynucleoside triphosphates, 3 mM $MgCl_2$, 100 μg ml^{-1} bovine serum albumin and 0.2 μM of each primer for Gm^r gene amplification. After a 10-min denaturation step at 94°C, 35 cycles consisting of 1 min at 94°C, 1 min primer annealing and 1 min at 72°C were performed, followed by a final 10-min extension step at 72°C. PCR products (10 μl) were analysed on 1% agarose gels with Tris–borate EDTA buffer, Southern blotted [25] and hybridised using digoxigenin-labelled probes. Probes were generated by random prime labelling of PCR products (ca. 300 ng) with digoxigenin dUTP according to the instructions of the manufacturer (Roche, Mannheim, Germany). PCR products were excised after preparative agarose gel electrophoresis and recovered from the agarose using the QIAEX II gel extraction kit. For each of the Gm^r gene families a mixed DNA probe consisting of PCR products obtained with the different reference strains was used (see Table 2). PCR-based detection of BHR plasmids and generation of probes was done as described by Götz et al. [5]. The amplification of 16S rDNA fragments from isolates or from community DNA was done as described previously [26].

2.11. Cloning and sequencing

To enhance the cloning efficiency, PCR conditions were slightly modified, with an additional step of 2 h at 72°C after adding fresh dATP (2 mM) and 2 U AmpliTaq DNA polymerase (Stoffel fragment, Applied Biosystems, Perkin-Elmer) to support the formation of 3′-A overhang [27]. After checking the PCR product quality by agarose gel electrophoresis, the PCR product was ligated into the pGEM-T vector (Promega, Madison, WI, USA) and transformed into competent cells (*E. coli* JM 109, Promega) as described by the manufacturer. Plasmids with the correct insert, as revealed by PCR screening, were selected and inserts were sequenced with vector-specific primers (IIT GmbH, Bielefeld, Germany). Data analysis was done for 16S rDNA sequences with the ARB software [28]. The accession numbers of the Gm^r genes sequenced are AJ493429–AJ493438 and those of the *trfA* sequences determined for nine IncP plasmids exogenously isolated from sewage are AJ493452–AJ493460.

3. Results

3.1. Primer design and testing

Six clusters of Gm^r genes were identified based on conserved motifs in published sequences: *aac*(*3*)-I, *aac*(*3*)-II/VI, *aac*(*3*)-III/IV, *aac*(*6′*)-II/Ib, *ant*(*2″*)-I, and *aph*(*2″*)-I. Primers were designed which anneal to these conserved sites and which are degenerated at the 3′ termini (Table 2) to allow amplification not only of the known genes but also of similar but not yet discovered sequences. Most Gm^r genes originate from *Pseudomonas* or Enterobacteriaceae except the *aph*(*2″*)-I genes for which the original hosts were *Staphylococcus*, *Streptococcus* and *Enterococcus*. The primer systems allowed the specific amplification of all Gm^r genes from the reference strains (see Section 2). The specificity of probes generated by random labelling of the PCR products was verified.

3.2. Prevalence of resistance genes in culturable, resistant non-producing strains, genetic localisation and host background

In all environmental samples a considerable proportion of the culturable bacterial fraction was resistant to gentamicin. For each of the 20 samples of the first sampling event, 12 Gm-resistant bacterial isolates with different colony morphology were selected to investigate the preva-

Table 1
Survey of sampling

Type of sample and designation (in parentheses)	Site, country	Sampling date	DNA extraction
Faeces of broiler fed chickens with (F1) or without (F2) flavomycin	Celle, Germany	May 1999	[24]
Faeces of laying hens fed with (F3) or without (F4) Zn-bacitracin	Celle, Germany	May 1999	[24]
Manure from piglets treated with neomycin (M1) or non-treated (M2)	Hannover, Germany	March 2000	[24]
Faeces of cattle fed with (F5) or without (F6) monensin	Braunschweig, Germany	March 2000	[24]
Sewage from a hospital wastewater treatment plant; samples taken at three different locations in the aeration tank (SD2)	Brussels, Belgium	I: May 1999 II: February 2000	[43]
Sewage from a hospital wastewater treatment plant; samples taken at three different locations in the aeration tank (SD4)	Ghent, Belgium	I: May 1999 II: February 2000	[43]
Sewage from the community wastewater treatment plant; samples taken at three different locations in the aeration tank (SD1)	Wavre, Belgium	I: May 1999 II: February 2000	[43]
Sewage from the community wastewater treatment plant; samples taken at three different locations in the aeration tank (SD3)	Rosière, Belgium	I: May 1999 II: February 2000	[43]
Seawater from a fish farm (from surface to 3 m depth): polluted site (SW1)	Epidauros, Greece	I: June 1999 II: September 1999	[44]
Seawater close to the wastewater outflow in Saronicos Bay (from surface to 3 m depth): polluted site (SW2)	Athens, Greece	I: June 1999 II: September 1999	[44]
Seawater close to Fleves Island (from surface to 3 m depth): non-polluted site (SW3)	Fleves Island, Greece	I: June 1999 II: Sepember 1999	[44]
Seawater near shore Eretria (from surface to 3 m depth): non-polluted site (SW4)	Euboea Island, Greece	I: June 1999 II: September 1999	[44]
Grass rhizosphere from an apple orchard treated with plantomycin (active substance streptomycin; 1997, 1998) (R1); grass rhizosphere from non-treated soil from the same site (R2)	Dossenheim, Germany	I: June 1999 II: November 1999	[45]
Rhizosphere of white radish, fields treated with $CuSO_4$ (R3) or non-treated (R4), manured	Ens, Netherlands	June 1999	[45]
Cauliflower rhizosphere from soil treated with $CuSO_4$ (R5) or non-treated (R6)	Ens, Netherlands	November 1999	[45]
Soil from an apple orchard treated with plantomycin (1997, 1998) (S1) or non-treated (S2)	Dossenheim, Germany	I: June 1999 II: November 1999	[46]
Soil S3 (brown clay soil) from pasture treated with sewage sludge (polluted): sampling I 2 weeks, sampling II 7 months after sludge application	Droitwich, UK	I: November 1999 II: June 2000	[46]
Soil S4: chalky loam (unpolluted); sampling I: field covered with winter wheat; sampling II: soil covered with grass	Cotswolds, UK	I: November 1999 II: June 2000	[46]

Table 2
Detection by PCR of genes coding for Gm-modifying enzymes

Gene cluster	Genes (alternative names) [accession No.]	Consensus sequences at primer annealing sites (5′-3′)	Primer name	Primer sequences (5′-3′)	Size (bp)	T_a (°C)
aac(3)-I	Ia (*aacC1*) [X15852]	TTC ATC GCG CTY GCT GCY TTY GA	Faac3-1	TTC ATC GCG CTT GCT GCY TTY GA	239	58
	Ib [L06157]	GC SAC TGC SGG ATC GTC ACC GTA	Raac3-1	GC CAC TGC GGG ATC GTC RCC RTA		
aac(3)-II/VI	IIa [X13543, X13543]	GCS CAY CCC GAY GCM TCS ATG G	Faac3-2	GCG CAC CCC GAT GCM TCS ATG G	189	58
	IIb (Vb) [M97172]		Faac3-6	GCC CAT CCC GAC GCA TCS ATG G		
	IIc [X54723]	SGC VAY SGC YTC GGC RTA RTG SA	Raac3-2	GGC AAC GGC CTC GGC GTA RTG SA		
	VIa [M88012]		Raac3-6	CGC CAC CGC TTC GGC ATA RTG SA		
aac(3)-III/IV	IIIa (*aacC3*) [X55652]	III: GAM AAY RGC GTG CTD SCG GAA T	Faac3-3	GAC AAT GGC GTG CTA SCS GAR T	241	58
	IIIb [L06160]	IV: GAC GAC GAG CCG TTC GAT CC	Faac3-4	GAC GAC GAG CCG TTC GAY CC	280	58
	IIIc [L06161]	C CAK MWR YTC SGC RWG RTG CAG	Raac3-3a	C CAG ATA TTC CGC GTG RTG SAG		
	IVa (*aacC4*) [X01385]		Raac3-3	C CAG ATG CTC GGC ATG RTG SAG		
			Raac3-4	C CAT CAA CTC GGC AAG ATG SAG		
aac(6′)-II/Ib	Ib (*aacA4*) [M55547]	CH CAG TCG TAC GTY GCK CTY GG	Faac6	CA CAG TCG TAC GTT GCK CTB GG	235	58
	IIa [M29695]	CC YGC YTT CTC RTA GCA KCG KAT	Raac6	CC TGC CTT CTC GTA GCA KCG DAT		
	IIb [L06163]					
ant(2″)-I	Ia (*aadB*) [U14415]	TGG GCG ATC GAT GCA CGG CTA G	Fant	TGG GCG ATC GAT GCA CGG CTR G	428	58
		AA AGC GGC ACG CAA GAC CTC AAC	Rant	AA AGC GGC ACG CAA GAC CTC MAC		
aph(2″)-I	Ia (*aphD*) [M18086]	GCC ACA AAT GTT AAG GCA ATG A	Fapha	GCC ACA AAT GTT AAG GCA ATG A	644	50
	Ic [U51479]	CCC AAG AGT CAA CAA GGT GCA GA	Faphc	CCC AAG AGT CAA CAA GGT GCA GA	527	55
	Id [AF016483]	GGC AAT GAC TGT ATT GCA TAT GA	Faphd	GGC AAT GAC TGT ATT GCA TAT GA	572	50
		GM ATC WCC AAA ATC RAT WAT KCC	Raph	GA ATC TCC AAA ATC RAT WAT KCC		

lence, genetic localisation and host background of Gm^r genes in cultured strains. The taxonomic composition of Gm-resistant bacteria was found to be different for the various environments analysed, with a surprisingly high proportion of Gm-resistant bacteria belonging to the CFB group particularly in manure (56%), rhizosphere (30%), soil (43%), seawater (18%), and sewage (16%). For rhizosphere and bulk soil samples about 62% and 43% of the Gm-resistant isolates, respectively, were Gram-positive bacteria. Only amongst the Gm-resistant isolates from sewage and seawater a high proportion of γ-Proteobacteria was found (40% and 48%, respectively). Proteobacterial isolates of the α-subdivision were frequent in seawater (15%), and of the β-subdivision in sewage (32%) and faeces (10%). Genomic DNA from 34 of 207 isolates analysed hybridised with one or more of the probes derived from the known Gm^r gene clusters (Table 3). These strains originated from faeces of layers (laying hens), sewage, or seawater taken close to a wastewater outflow, except for two strains from a soil sample (sample S2) and one strain from unpolluted coastal water (sample SW3). Three strains from seawater samples taken close to a wastewater outflow contained multiple Gm^r genes. When genomic DNA extracted from isolates was analysed by agarose gel electrophoresis, plasmid DNA could be detected for several Gm-resistant strains, and for 18 isolates the hybridisation signal indicated that the Gm^r gene was localised on a plasmid. Gm^r genes were detected in organisms in which the genes had been detected previously (Enterobacteriaceae, or the genera *Pseudomonas* and *Acinetobacter*) and also in genera where they had not been detected before (Table 3). Isolates hybridising with the probe for the *aac*(*3*)-II/VI cluster (17) belonged to a diverse range of genera and originated from faeces of layers, from polluted and unpolluted seawater, sewage and bulk soil. Several strains among them were affiliated to *Sphingobacterium* and other CFB bacteria. An *Acinetobacter johnsonii* strain isolated from polluted seawater was additionally *aac*(*3*)-I probe positive. Eleven *ant*(*2″*)-I probe-positive strains were isolated from faeces, sewage and seawater. Seven isolates from sewage and wastewater-polluted seawater were *aac*(*6′*)-II/Ib probe positive. One *Pseudomonas stutzeri* strain originating from faeces had *aac*(*3*)-IV. PCR products amplified from seven isolates, representing four of the five Gm families detected in isolates, were successfully cloned and sequenced. Interestingly, most sequences determined showed 100% identity with the sequenced Gm^r gene although some of them were detected in hosts which were phylogenetically distant to the hosts of the originally sequenced genes (Table 3).

3.3. Exogenous isolation of Gm^r genes located on mobile genetic elements

As a consequence of the problems encountered with the apparent background of rifampicin- and Gm-resistant

Table 3
Bacterial hosts of gentamicin^r genes

Gene cluster	Gentamicin-resistant isolates[a]
aac(*3*)-I	γ-Subdivision of Proteobacteria: *Acinetobacter johnsonii* (MIS 0.54; 99.5% sequence identity to *aac*(*3*)-Ia, accession No. AF318077)
aac(*3*)-II/VI	CFB group: *Sphingobacterium multivorum* (MIS 0.52; 93.8% 16S rRNA identity with *S. multivorum* OM-A8), *Empedobacter* (MIS 0.18), *Pedobacter heparinus* (MIS 0.42; 95.3% 16S rRNA identity with *P. heparinus* DSM2366^T) α-Subdivision of Proteobacteria: '*Zoogloea ramigera*' (99.8% 16S rRNA identity with misnamed strain ATCC 19623; 98.6% sequence identity to *aac*(*3*)-II, accession No. X13542) γ-Subdivision of Proteobacteria: *Pseudomonas pseudoalcaligenes* (MIS 0.75), *E. coli* (MIS 0.85), *Citrobacter freundii* (MIS 0.79), *A. johnsonii* (MIS 0.54; 100% sequence identity to *aac(3)*-II, accession No. X13542), *Aeromonas caviae* (MIS 0.83), *Stenotrophomonas maltophilia* (MIS 0.80), *Xanthomonas campestris* (96.4% 16S rRNA identity with *X. campestris* LMG726)
aac(*3*)-III/IV	γ-Subdivision of Proteobacteria: *Pseudomonas stutzeri* (MIS 0.65; 100% sequence identity to *aac*(*3*)-IV, accession No. X01385)
aac(*6′*)-II/Ib	α-Subdivision of Proteobacteria: *Agrobacterium radiobacter* (MIS 0.23; 100% sequence identity to *aac*(*6′*)-Ib, accession No. AF371964) β-Subdivision of Proteobacteria: *Neisseria mucosa* (MIS 0.56; 100% sequence identity to *aac*(*6′*)-Ib, accession No. AF371964) γ-Subdivision of Proteobacteria: *Pseudomonas putida* (MIS 0.87)
ant(*2″*)-I	β-Subdivision of Proteobacteria: *Neisseria sicca* (MIS 0.64; 100% sequence identity to *ant*(*2″*)-I, accession No. AF205943), *N. mucosa* (MIS 0.56), *Achromobacter xylosoxidans* (MIS 0.76; 100% sequence identity to *ant*(*2″*)-I, accession No. AF205943) γ-Subdivision of Proteobacteria: *Proteus vulgaris* (99.0% 16S rRNA identity with *P. vulgaris* IFAM1731), *P. stutzeri* (MIS 0.88), *P. pseudoalcaligenes* (MIS 0.75)
None of the six gene clusters found	Most frequent Gram-negative gentamicin-resistant isolates: CFB group: *Sphingobacterium spiritivorum* (MIS 0.84), *S. multivorum* (MIS 0.77), *Empedobacter brevis* (MIS 0.44), *Myroides odoratus* (MIS 0.83) α-Subdivision of Proteobacteria: *A. radiobacter* (MIS 0.68) β-Subdivision of Proteobacteria: *A. xylosoxydans* (MIS 0.84) γ-Subdivision of Proteobacteria: *P. putida* (MIS 0.74), *S. maltophilia* (MIS 0.90), *P. aeruginosa* (MIS 0.98), *Yersinia pseudotuberculosis* (MIS 0.57)

[a]MIS, similarity of the fatty acid methyl ester profile to TSBA database entry of the Microbial Identification System (MIDI Inc.), highest similarity in case of several isolates of a species.

bacteria in most of the environmental samples from the first sampling event, the recipient strains were additionally marked with a kanamycin resistance gene, for improved selection, and *gfp*, to allow identification after re-isolation from the mating mixture. Potential transconjugants which captured Gm resistance could be easily confirmed by their resistance to kanamycin, rifampicin and by green fluorescence. In the second sampling Gm resistance was captured in an exogenous mating in *gfp*-tagged recipients from sewage, seawater, rhizosphere, piggery manure and cattle faeces, but not from bulk soil. A selection of transconjugants from piggery manure, cattle faeces and sewage was first tested by dot-blot hybridisation using probes for *aac*(*3*)-I, *aac*(*3*)-II/VI, *aac*(*3*)-III/IV, *aac*(*6′*)-II/Ib, *ant*(*2″*)-I. Dot-blot-positive transconjugants were confirmed by PCR. Transconjugants from rhizosphere, soil (only from the culturable fraction) and seawater were screened for the presence of known Gm^r genes by PCR.

3.3.1. *Manure from pigs treated with neomycin, or untreated*

Gm resistance was acquired in all four recipients from manure of non-treated piglets at frequencies between 4×10^{-7} and 3×10^{-5} (cfu transconjugants per cfu recipient). A total of 28 transconjugants with exogenously captured Gm resistance and nine transconjugants obtained from the Gm-resistant culturable population were screened by dot-blot hybridisation. Eleven transconjugants captured a gene of the *ant*(*2″*)-I cluster in *P. putida* and *R. eutropha*. All *ant*(*2″*)-I-positive transconjugants carried IncP-1 plasmids. Seven transconjugants obtained a gene of the *aac*(3)-III/IV cluster in *P. putida* and *E. coli* exogenously from the microbial manure fraction. The *aac*(*3*)-III/IV was also captured from the culturable fraction in *P. putida* and *E. coli*. Sequencing of the PCR-amplified 280-bp *aac*(*3*)-III/IV fragment showed that the resistance genes acquired by *E. coli* directly from the bacterial cell pellet and from the culturable fraction were 100% identical to the reference sequence of *aac*(*3*)-IV (X01385). Surprisingly, no Gm^r transconjugants were obtained from the manure of pigs treated with neomycin. Obviously there was still an inhibiting effect of neomycin which affected the mating partners.

3.3.2. *Faeces of cattle fed with monensin as a feed additive, and without monensin*

Gm resistance was captured after an exogenous isolation from both kinds of faeces only in *E. coli* but not in *P. putida*, *A. tumefaciens* or *R. eutropha*. Transfer frequencies were more than one order of magnitude higher in the mating with the donor bacteria originating from faeces of treated cattle (4.9×10^{-7} compared to 5.7×10^{-8}). A total of 11 *E. coli* transconjugants from exogenous isolations with faeces of treated and untreated cattle were screened by dot-blot hybridisation and PCR confirmation of dot-blot positives. Genes of the *aac*(*3*)-II/VI cluster

Table 4
Prevalence of gentamicin[r] gene clusters in environmental DNA

Habitat	Samples and treatments[a]		Hybridisation signal with probes for different gene clusters[b]											
	Sampling I	Sampling II	*aac*(*3*)-I		*aac*(*3*)-II/VI		*aac*(*3*)-III/IV		*aac*(*6′*)-II/Ib		*ant*(*2″*)-I		*aph*(*2″*)-I	
			I	II	I	II	I	II	I	II	I	II	I	II
Manure	F1: Broiler+Fm	M1: Pig+Nm		++		++	+	++		++	++	++	+	++
	F2: Broiler, control	M2: Pig, control		+		++	+	++		++		++	+	++
	F3: Layers+Bc	F5: Cattle+Mn	++	++				++			+	+	+	+
	F4: Layer, control	F6: Cattle, control				++		++		(+)	++	+		+
Rhizosphere	R1: Grass+Sm (Dossenheim)		+	+					+	+				
	R2: Grass, control (Dossenheim)			+					(+)	+				
	R3: White radish+Cu	R5: Cauliflower+Cu	+						+	+			+	+
	R4: White radish	R6: Cauliflower							(+)	+				
Coastal water	SW1: Fish farm at Epidauros													
	SW2: Close to wastewater outflow		++	++	+	+		+	+	++	++	+		
	SW3: Close to Fleves Island, pristine			++								+		
	SW4: Near shore of Eretria, pristine								(+)					
Soil	S1: Apple orchard near Dossenheim+Sm		+	+					(+)	(+)				
	S2: Apple orchard near Dossenheim, control					+			(+)			+		
	S3: Droitwich pasture+sewage sludge					+			(+)	(+)				
	S4: Field near Cotswold, pristine		+						+	(+)				
Sewage	SD1: Wavre, community wastewater		+	+	++				++					
	SD2: Erasmus, hospital wastewater		++	++	++	++			++	++	++	+		
	SD3: Rosière, community wastewater		++	+	++	+			++	+	++			
	SD4: Ghent, hospital wastewater		++	+	++	+			++	+	++			

[a]See Table 1 for sample descriptions. Treatments: flavomycin (Fm), neomycin (Nm), Zn-bacitracin (Bc), monensin (Mn), streptomycin (Sm), copper (Cu).
[b]Signal intensity at medium stringency (70–100% similarity of probe to target) very strong: ++, strong: +, weak: (+).

were detected in seven of the 11 tested transconjugants. The sequence of the PCR product amplified from *aac*(*3*)-II/VI-positive transconjugant was identical to the reference sequence of *aac*(*3*)-II of pWP14. None of the other Gm[r] genes was detected. In a mating with resuspended cell lawns of Gm-resistant isolates the phenotype could be transferred to *R. eutropha*, *P. putida* and *E. coli*. All 14 Gm-resistant transconjugants of 28 tested hybridised with the *aac*(*3*)-II/VI probe.

3.3.3. Sewage from four sewage treatment plants

Transconjugants which acquired Gm resistance were obtained from all sewage samples (transfer frequencies ranging from 10^{-5} to 3×10^{-8}) independent of whether the sewage originated from a sewage treatment plant with community or hospital sewage. Forty-six of the 54 transconjugants screened by PCR for the presence of BHR plasmids carried IncP-1 plasmids. From the Wavre sewage treatment plant (SD1) Gm resistance was only acquired in *R. eutropha*. All seven transconjugants tested carried the *aac*(*6′*)-II/Ib gene located on an IncPβ plasmid as evidenced by hybridisation with the IncP *trfA2* probe. A total of six Gm[r] transconjugants were received after a mating with sewage from the Rosière sewage treatment plant (SD3) in *R. eutropha* (3), in *P. putida* (1) and in *E. coli* (2). Three of the transconjugants carried an IncQ plasmid. While the *P. putida* transconjugant hybridised with the *aac*(*6′*)-II/Ib gene and the *aac*(*3*)-II/VI probe, the two *E. coli* and three *R. eutropha* transconjugants did not hybridise with any of the Gm[r] probes.

A total of 17 transconjugants (*A. tumefaciens*, *P. putida*, and *E. coli*) obtained after a mating with sewage from the Erasmus Hospital sewage treatment plant (SD2) was screened by dot-blot hybridisation. The *aac*(*6′*)-II/Ib localised on IncP-1 plasmids was detected in almost all transconjugants. Gm resistance was captured in all four recipients after a mating with sewage bacteria from the Ghent hospital sewage treatment plant (SD4). Again the *aac*(*6′*)-II/Ib gene localised on an IncP-1 plasmid was captured in almost all *A. tumefaciens*, *R. eutropha*, *P. putida*, and *E. coli* transconjugants. In addition, in all *E. coli* transconjugants the *ant*(*2″*)-I was detected. Since IncPβ plasmids carrying the Gm[r] gene *aac*(*6′*)-II/Ib were isolated from three of four sewage treatment plants, a more thorough analysis of these plasmids was performed. A 543-bp fragment of the *trfA* gene (encoding the replication-initiating protein) was amplified from nine IncP plasmids. Based on the *trfA* sequences a high similarity of these plasmids with the IncPβ plasmid R751 can be assumed. However, the *Sph*I digestion patterns of IncP plasmid DNA indicated that different IncPβ plasmids were isolated (data not shown).

Gm resistance could be captured in *A. tumefaciens* (SD1, SD2, SD4), *P. putida* (SD1, SD2), and *R. eutropha*

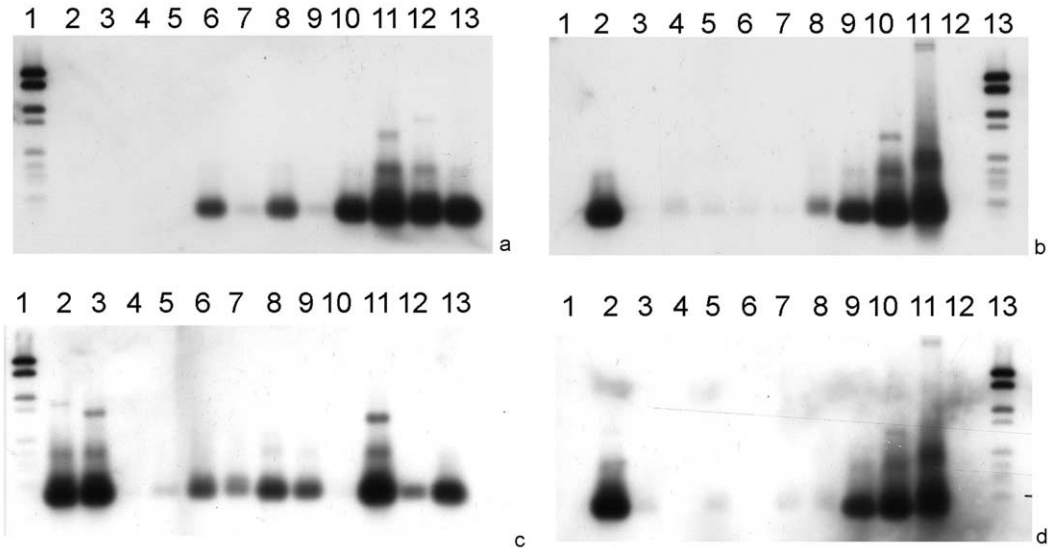

Fig. 1. Hybridisation of Southern-blotted PCR products obtained with the *aac*(6′)-II/Ib primer system from total community DNA. First sampling: (a) lanes: 1, dig ladder; 2–5, faeces F1, F2, F3, F4; 6–9, rhizosphere R1, R2, R3, R4; 10–13, sewage SD1, SD2, SD3, SD4; (b) lanes: 1–4, seawater SW1, SW2, SW3, SW4; 5–8, soil S1, S2, S3, S4; 9–11, positive controls; 12, negative control; 13, dig ladder. Second sampling: (c) lanes: 1, dig ladder; 2, 3, manure M1, M2; 4, 5, faeces F5, F6; 6–9, rhizosphere R1, R2, R5, R6; 10–13, sewage SD1, SD2, SD3, SD4; (d) lanes: 1–4, seawater SW1, SW2, SW3, SW4; 5–8, soil S1, S2, S3, S4; 9–11, positive controls; 12, negative control; 13, dig ladder.

(SD1) after mating with the culturable fraction of Gm-resistant bacteria (from cell lawns). Interestingly, only two Gm transconjugants from SD2 hybridised with a Gm probe (*aac*(6′)-II/Ib). However, none of the transconjugants captured the resistance via an IncPβ plasmid.

3.3.4. *Seawater taken from differently polluted sites in Greece*

Gm^r transconjugants were obtained from seawater bacteria from three of the four sites in all four recipients with transfer frequencies ranging from 5×10^{-6} to 2×10^{-8}. Interestingly, no transconjugants were recovered after mating the bacterial fraction of fish farm sediments with the recipients. Fifteen Gm-resistant transconjugants (*P. putida*, *A. tumefaciens*) were screened by PCR for the presence of Gm^r genes. Except for one transconjugant all others gave PCR products with the *aac*(*3*)-II/VI primers (11/15) and/or the *aac*(6′)-II/Ib primers which hybridised with the respective probes (9/15). In six transconjugants both the *aac*(*3*)-II/VI and the *aac*(6′)-II/Ib Gm^r genes were detected. However, BHR plasmids could not be detected in any of the transconjugants from seawater.

3.3.5. *Rhizosphere of grass growing below apple trees treated with streptomycin, or untreated*

Ten transconjugants (*A. tumefaciens*, *P. putida*, and *E. coli*) which acquired Gm resistance after mating with bacteria from the rhizosphere of grass from areas treated with streptomycin, or untreated, were screened by PCR for the presence of known Gm^r genes. Strong PCR products of the expected size were observed with primers targeting the *aac*(*3*)-III/IV and the *ant*(*2″*)-I clusters for the four *A. tumefaciens* transconjugants tested. Interestingly, none of the PCR products hybridised indicating sequences divergent from those of the sequenced genes. Therefore, the PCR products obtained from two transconjugants with the *ant*(*2″*)-I-specific primers were cloned and sequenced. The sequence showed no homology to any sequence in the database. Only for one Gm-resistant *A. tumefaciens* transconjugant a weak PCR product hybridised with the *aac*(*3*)-II/VI probe.

3.3.6. *Rhizosphere from soils treated with copper, or untreated*

A total of nine Gm-resistant transconjugants (six *P. putida*, three *A. tumefaciens*) obtained in matings with rhizosphere bacterial communities from soils treated with copper or untreated were screened by PCR. Strong PCR products of the expected size were obtained with the *aac*(*3*)-III/IV and the *ant*(*2″*)-I primer system from all *A. tumefaciens* transconjugants. However, the absence of hybridisation indicated sequence divergence. Again the sequence of cloned *ant*(*2″*)-I PCR products showed no homology to any sequence in the database.

3.3.7. *Bulk soil*

No Gm resistance was captured by exogenous isolation from bulk soils. Three Gm-resistant *P. putida* transconjugants originating from a mating of the culturable fraction with each of the four recipients were screened by PCR. Only one transconjugant gave hybridising PCR products with the *aac*(6′)-II/Ib gene system.

Table 5
Prevalence of broad host range plasmids in environmental DNA

Habitat	Samples and treatments[a]		Hybridisation signal[b]									
	Sampling I	Sampling II	IncPα		IncPβ		IncQ		IncN		IncW	
			I	II	I	II	I	II	I	II	I	II
Manure	F1: Broiler+Fm	M1: Pig+Nm		+		+	+	+		+		+
	F2: Broiler, control	M2: Pig, control		+		+	+	+		+	+	+
	F3: Layers+Bc	F5: Cattle+Mn	+					+	+			+
	F4: Layer, control	F6: Cattle, control										
Rhizosphere	R1: Grass+Sm (Dossenheim)			+		+						
	R2: Grass, control (Dossenheim)											
	R3: White radish+Cu	R5: Cauliflower+Cu				+	+					
	R4: White radish	R6: Cauliflower	+	+		+	+					
Coastal water	SW1: Fish farm at Epidauros		+		+				+			
	SW2: Close to wastewater outflow		+	+	+	+	+	+	+	+		
	SW3: Close to Fleves Island, pristine			+	+	+			+			
	SW4: Near shore of Eretria, pristine			+	+				+			
Soil	S1: Apple orchard near Dossenheim+Sm				+	+						
	S2: Apple orchard near Dossenheim, control				+	+						
	S3: Droitwich pasture+sewage sludge		+		+	+						
	S4: Field near Cotswold, pristine				+		+	+				
Sewage	SD1: Wavre, community wastewater			+		+						
	SD2: Erasmus, hospital wastewater		+	+	+	+	+	+	+			
	SD3: Rosière, community wastewater		+	+	+		+		+			
	SD4: Ghent, hospital wastewater		+	+	+	+						

[a]See Table 1 for sample descriptions. Treatments: flavomycin (Fm), neomycin (Nm), Zn-bacitracin (Bc), monensin (Mn), streptomycin (Sm), copper (Cu).
[b]Probes targeted the genes *trfA2* (IncP), *oriV* (IncQ, IncW), or *rep* (IncN).

3.4. PCR-based detection of Gm^r genes and mobile genetic elements in community DNA

The primer systems were applied to the environmental DNA of the first and second sampling. Due to the degeneracy of the primers in third codon positions, specific and some unspecific amplicons were derived from environmental DNA. Southern blots of the PCR products were always probed with the PCR-derived probes for the respective gene cluster to increase the specificity and sensitivity. Hybridisation signals of the expected size were detected in many environmental DNA samples (Table 4). Some of the genes showed habitat-specific prevalence. The gene clusters *aac*(*3*)-II/VI and *ant*(*2″*)-I were mainly detected in manure, sewage or seawater (wastewater outflow). The *aac*(*3*)-III/IV cluster was detected only in faeces of broiler chickens and cattle, piggery manure, and in one sample of polluted seawater. The *aph*(*2″*)-I gene, which is typically found in Gram-positive cocci, was prevalent in all types of manure. At medium stringency the *aph*(*2″*)-I gene was also detected in the rhizosphere of white radish grown in Cu-treated soil. Streptomycin- and Cu-treated sites (D+, Cu+, W1) showed enrichment of *aac*(*3*)-I compared to the control sites but only for samples taken in spring. The *aac*(*6′*)-II/Ib fragment was detected in piggery manure, municipal and hospital sewage, rhizosphere of white radish and cauliflower, and seawater (Fig. 1). The intensity of the hybridisation signals indicated that an enrichment of genes belonging to the *aac*(*6′*)-II/Ib cluster occurred in rhizosphere samples of the copper- or streptomycin-treated samples taken at the first sampling time. However, this gene cluster also includes genes which code for an aminoglycoside-modifying enzyme not targeting Gm. There was no clear evidence for an effect of antibiotic feeding on the abundance of Gm^r genes in total community DNA. Gm^r genes were most frequently found in sewage, wastewater, animal faeces and manure. In seven of the 40 environmental samples from both sampling times (fish farm water, unpolluted seawater, untreated rhizosphere) none of the known Gm^r genes was detected. Most remarkable is the finding that all known genes encoding Gm resistance could be detected in DNA extracted directly from manure of pigs treated with neomycin, or untreated.

The set of community DNA was screened by PCR for the presence of the BHR plasmids IncQ, IncP, IncN, IncW. Southern blot hybridisations of PCR products amplified with replicon-specific primers [5] indicated a habitat-specific dissemination of BHR plasmids (see Table 5). Piggery manure, sewage and seawater are environments with high abundance of BHR plasmids. Again, in piggery manure all known BHR plasmids could be detected. IncPβ plasmids seem to be also present in soils and rhizospheres. In particular, with respect to the spread of antibiotic resistance genes amongst a wide range of Gram-negative bacteria the PCR-based detection of IncP plasmid-specific sequences in 27 of 40 samples is remarkable (Fig. 2a,b). IncQ plasmid-specific sequences were detected in 13 of 40 samples with a high abundance in pig manure, faeces from

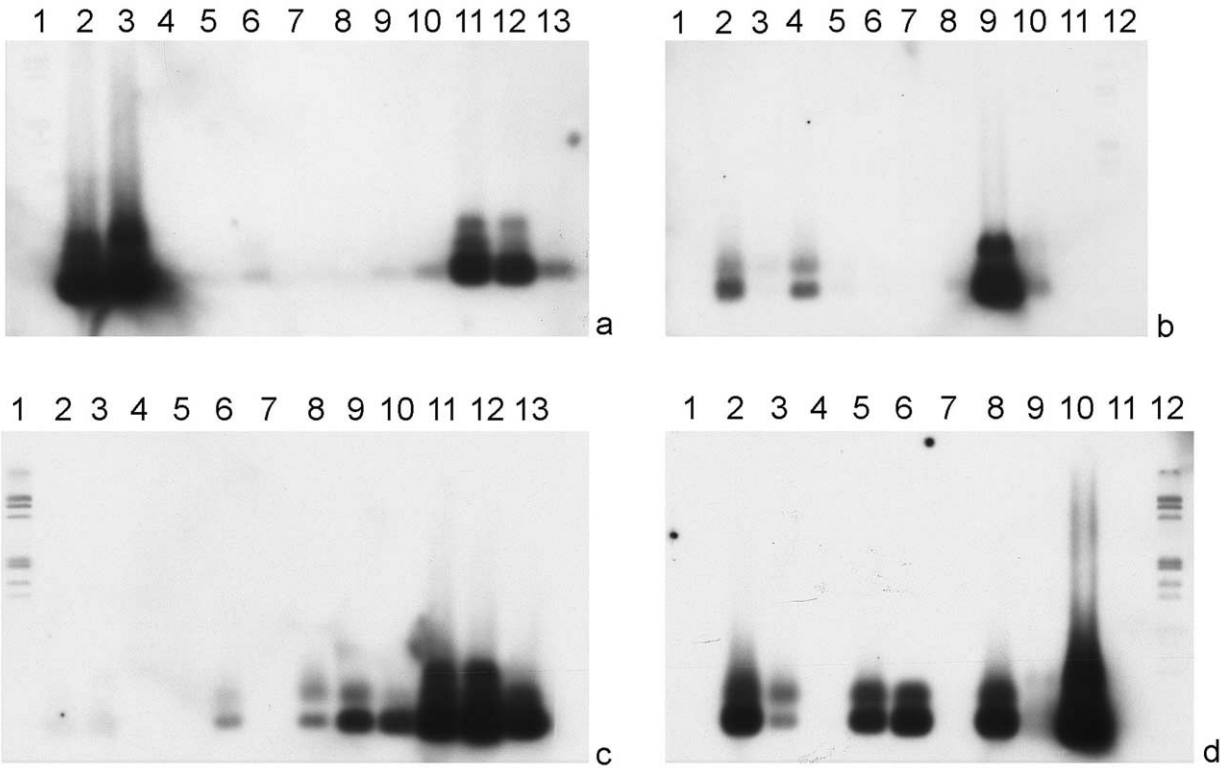

Fig. 2. Hybridisation of Southern-blotted PCR products obtained with the *trfA2* primer system [5] from total community DNA (second sampling). a,c: Lanes 1, 2, manure M1, M2; 3, 4, faeces F5, F6; 5–8, rhizosphere R1, R2, R5, R6; 9–12, sewage SD1, SD2, SD3, SD4; b,d: lanes: 1–4, seawater SW1, SW2, SW3, SW4; 5–8, soil S1, S2, S3, S4; 9, RP4; 10, R751; 11, negative control; 12, dig ladder. a,c: Hybridised with a *trfA* probe derived from the IncPα plasmid RP4. b,d: Hybridised with a *trfA* probe derived from the IncPβ plasmid R751.

broiler (flavomycin-treated) and cattle (monensin-treated) faeces, sewage (3/8) and copper-treated rhizosphere (1/2). IncW plasmid-specific sequences were only found in the faeces of broiler chickens fed without flavomycin and in pig manure but not in all other environments. IncN plasmid-specific sequences were detected in 10 of the 40 samples: seawater samples (5/8), pig manure (2/2), sewage (2/8) and faeces of Zn-bacitracin-treated layers. There is some evidence that BHR plasmids were more abundant in samples originating from antibiotic- or copper-treated habitats: IncQ plasmids seem to be enriched in copper-treated rhizosphere of white radish, in the faeces of cattle treated with monensin, and in the faeces of broiler chickens fed with flavomycin, compared to the non-treated control group. IncPα- and IncN-specific sequences were detected in faeces of layers treated with Zn-bacitracin but not in faeces of non-treated layers.

4. Discussion

The reservoirs of Gm^r genes were monitored using a multiphasic approach consisting of selective plating, exogenous isolation of transferable elements carrying Gm^r genes as well as the PCR-based detection of Gm^r genes. Each of the approaches used has its advantages and limitations [6] but when applied simultaneously a broader picture of reservoirs of Gm^r genes and their mobility should become possible. Traditional selective cultivation allowed us to provide data on the proportion of Gm-resistant bacteria accessible to cultivation and their taxonomic composition. The taxonomic composition was different for the various environments analysed and only a rather small proportion of the isolates mainly originating from sewage, seawater and manure hybridised with Gm probes. Gm^r genes were also detected in genera which were phylogenetically distant or at least different from the bacterial species from which the sequence was originally described. Interestingly, the sequence of PCR products amplified from such a host was 100% identical to the reference sequence, providing further evidence of horizontal spread of antibiotic resistance genes. Indeed plasmid localisation of the Gm^r gene was often observed by Southern hybridisation of genomic DNA (data not shown). Several Gm^r genes were also described to occur as gene cassettes located in integrons or secondary integration sites [29,30].

In contrast to the selective plating method, the exogenous isolation method allowed the isolation of antibiotic resistance independent from the culturability of the original host and more importantly it provided information on the occurrence of this trait on self-transferable mobile genetic elements. MGE conferring Gm resistance could be captured directly from all environments analysed except

for soil. Since it was possible to obtain a few transconjugants from the culturable fraction of one soil sample, the failure to recover Gm^r genes directly from the soil bacterial fraction might be due to low metabolic activity of the soil bacteria used as donors. Since only Gram-negative bacteria were used as recipients in biparental matings it is not too surprising that the *aph*(*2″*) cluster was not captured on an MGE. Considering transfer frequencies sewage and pig manure could be clearly identified as environments with high mobility of Gm^r genes. The proportion of exogenously isolated Gm^r transconjugants which hybridised with the Gm probes used was considerably higher than for Gm^r isolates. This finding supports the assumption that a considerable proportion of the cultured bacterial fraction might resist antibiotics due to different mechanisms such as cell wall impermeability. The Gm-resistant bacterial strains and mobile plasmids conferring Gm resistance, where the genes for Gm-modifying enzymes known from clinical isolates [31,32] could not be detected, might also indicate unknown resistance genes which are not yet abundant in the clinical environment. For isolates as well as exogenous transconjugants the majority of the Gm probe positives originated from sewage, manure or seawater samples taken close to a wastewater outflow. This result seems to reflect the anthropogenic use of gentamicin in human and animal therapy. Most striking was the high proportion of transconjugants which captured Gm^r genes located on IncP-1 plasmids in mating experiments with the bacterial fraction of samples from four different sewage treatment plants in Belgium and from piggery manure. IncPβ plasmids conferring Gm resistance were captured exogenously in all four recipients used for mating, underlining their broad host range [33,34]. Also other authors have repeatedly obtained IncP-1 plasmids when exogenous plasmid isolation was used [13,18,19]. Thus there is increasing evidence that two of the most extensively studied plasmids from the clinical environment, the IncP-1α [35] and the IncP-1β plasmid [36], occur in various environments. This observation is also supported by the detection of IncP-1α- and IncP-1β-specific sequences in community DNA from different environments, in this and other studies [5,7,37].

The detection of the Gm^r genes in bacterial isolates and transconjugants confirmed the molecular detection in the respective environmental DNA. As expected, isolates could not be recovered from all the samples, where specific Gm^r genes were detected in community DNA by PCR and Southern blot hybridisation. This can be explained by the higher sensitivity of the PCR-based detection in community DNA (in contrast only numerically dominant Gm-resistant isolates were analysed), the limited number of isolates analysed and potentially non-culturable Gm-resistant environmental bacteria, although in one isolate from faeces of chickens *aac*(*3*)-III/IV was detected while it was not detectable in the total community DNA.

Antibiotic-selective pressure is not necessarily required for the selection of antibiotic resistance genes carried by replicons that contain not only one antibiotic resistance gene but also other selectable markers [38]. Therefore, the different kinds of selective pressure studied – although not including gentamicin and for some of the samples not well defined – could have resulted in an increased abundance of Gm^r genes, but such an effect was not consistently supported by the data. However, PCR-based detection of BHR plasmids in community DNA as well as the increased transfer frequencies of capturing Gm^r from faeces of monensin-treated cattle indicated a correlation of MGE abundance and selective pressure.

Gentamicin is still an important clinical antibiotic. Considering that genes conferring resistance to gentamicin were detected in all environments analysed, often localised on BHR plasmids and in a wide range of hosts, the successful medical use of this antibiotic might be increasingly jeopardized.

Increasing problems linked with the medical use of antibiotics can be predicted. Our data suggest that the traditional perception that in some way the hospital environment is closed and antimicrobial resistance arises in patients in the hospital needs to be corrected. There is substantial mobility of antibiotic resistance genes between bacteria of the same species but also between phylogenetically distant bacteria, and there is substantial movement of bacteria of different environmental habitats somehow related to the food chain and human activity [39–42].

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