 DOI 10.1099/mic.0.028233-0

Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics

Rafael Szczepanowski,[1] Burkhard Linke,[2] Irene Krahn,[1] Karl-Heinz Gartemann,[3] Tim Gützkow,[1] Wolfgang Eichler,[4] Alfred Pühler[1] and Andreas Schlüter[1]

[1]Institute for Genome Research and Systems Biology, Center for Biotechnology, Universität Bielefeld, Postfach 100131, D-33501 Bielefeld, Germany

[2]Bioinformatics Resource Facility, Center for Biotechnology, Universität Bielefeld, Postfach 100131, D-33501 Bielefeld, Germany

[3]Lehrstuhl für Gentechnologie und Mikrobiologie, Fakultät für Biologie, Universität Bielefeld, Universitätsstraße 25, D-33615 Bielefeld, Germany

[4]Landesamt für Natur, Umwelt und Verbraucherschutz NRW, FB76.2, Auf dem Draap 25, 40221 Düsseldorf, Germany

Correspondence
Andreas Schlüter
Andreas.Schlueter@Genetik.Uni-Bielefeld.de

Received 11 February 2009
Revised 3 April 2009
Accepted 8 April 2009

To detect plasmid-borne antibiotic-resistance genes in wastewater treatment plant (WWTP) bacteria, 192 resistance-gene-specific PCR primer pairs were designed and synthesized. Subsequent PCR analyses on total plasmid DNA preparations obtained from bacteria of activated sludge or the WWTP's final effluents led to the identification of, respectively, 140 and 123 different resistance-gene-specific amplicons. The genes detected included aminoglycoside, β-lactam, chloramphenicol, fluoroquinolone, macrolide, rifampicin, tetracycline, trimethoprim and sulfonamide resistance genes as well as multidrug efflux and small multidrug resistance genes. Some of these genes were only recently described from clinical isolates, demonstrating genetic exchange between clinical and WWTP bacteria. Sequencing of selected resistance-gene-specific amplicons confirmed their identity or revealed that the amplicon nucleotide sequence is very similar to a gene closely related to the reference gene used for primer design. These results demonstrate that WWTP bacteria are a reservoir for various resistance genes. Moreover, detection of about 64 % of the 192 reference resistance genes in bacteria obtained from the WWTP's final effluents indicates that these resistance determinants might be further disseminated in habitats downstream of the sewage plant.

INTRODUCTION

Development and dissemination of antibiotic-resistance genes is a serious problem in the treatment of infectious diseases (Goossens, 2005; Lim & Webb, 2005). An important step in coping with this threat is to elucidate and to understand pathways for resistance gene spread. Many resistance genes are located on mobile genetic elements such as plasmids, transposons and integrons, which function as vectors for these determinants and promote their dissemination (Bennett, 1999; Davies, 1994; Davison, 1999; Hall & Collis, 1995; Mazel & Davies, 1999; Rowe-Magnus & Mazel, 1999; Seveno *et al.*, 2002). Moreover, inappropriate use of antimicrobial drugs favours spread of resistance genes by selection for resistant micro-organisms (Bywater, 2004, 2005; Wassenaar, 2005).

Antibiotic-resistant bacteria of wastewater treatment plants (WWTPs) are the focus of the present study. WWTPs are connected to private households and hospitals where antibiotics are used and resistances in bacteria might arise. Once antibiotic-resistant bacteria reach WWTPs, they potentially can disseminate their resistance freight among members of the endogenous microbial community. Evidence for horizontal transfer of resistance elements in sewage habitats has been obtained for model systems (Geisenberger *et al.*, 1999; Marcinek *et al.*, 1998; Nüßlein *et al.*, 1992). Because of the favourable growth conditions

Abbreviation: WWTP, wastewater treatment plant.

A supplementary table of primers is available with the online version of this paper.

 028233 © 2009 SGM *Printed in Great Britain*

they provide for many micro-organisms, WWTPs have to be considered as hot-spots for horizontal transfer of genetic material, e.g. by means of conjugation (Mach & Grimes, 1982; Mancini *et al.*, 1987). In addition, contamination of sewage with antibiotics might cause a selective advantage for resistant bacteria (Göbel *et al.*, 2005; Golet *et al.*, 2002, 2003; Jarnheimer *et al.*, 2004; Kümmerer, 2003; Kümmerer *et al.*, 2000; Lee *et al.*, 2007; Lindberg *et al.*, 2005, 2006).

Previously, 12 different resistance plasmids, namely pB2/pB3 (Heuer *et al.*, 2004), pB4 (Tauch *et al.*, 2003), pB8 (Schlüter *et al.*, 2005), pB10 (Schlüter *et al.*, 2003), pTB11 (Tennstedt *et al.*, 2005), pRSB101 (Szczepanowski *et al.*, 2004), pRSB105 (Schlüter *et al.*, 2007a), pRSB107 (Szczepanowski *et al.*, 2005), pRSB111 (Szczepanowski *et al.*, 2007), pGNB1 (Schlüter *et al.*, 2007b) and pGNB2 (Bönemann *et al.*, 2006), were isolated from WWTP compartments and analysed at the genomic and functional level. These plasmids confer resistance to different antibiotics such as aminoglycosides, *β*-lactams, chloramphenicol, macrolides, quinolones, fluoroquinolones, tetracycline, trimethoprim and sulphonamides. In addition, some of the plasmids analysed carry heavy metal, quaternary ammonium compound or triphenylmethane dye resistance genes. Moreover, different class 1 integron-specific resistance gene cassettes were identified on plasmids from WWTP bacteria (Tennstedt *et al.*, 2003). A total of 22 different resistance genes and 27 different integron-specific resistance gene cassettes were identified on plasmids harboured by bacteria of activated sludge and the WWTP's final effluents. Other studies investigated the occurrence of resistance genes in different aquatic systems including sewage habitats. Many of these studies focused either on selected antibiotic-resistance genes, e.g. *vanC*, *ampC*, *mecA* (Schwartz *et al.*, 2003; Volkmann *et al.*, 2004), or on genes conferring resistance to a specific class of antimicrobial compounds, e.g. *β*-lastams (Henriques *et al.*, 2006a, b), chloramphenicol (Dang *et al.*, 2008), or tetracyclines (Akinbowale *et al.*, 2007; Chee-Sanford *et al.*, 2001; Guillaume *et al.*, 2000; Smith *et al.*, 2004).

A more comprehensive study investigated the plasmid metagenome of WWTP bacteria with reduced susceptibility to certain antimicrobial drugs by applying the next-generation 454-pyrosequencing technology (Schlüter *et al.*, 2008; Szczepanowski *et al.*, 2008). This approach led to the identification of sequences that are very similar to 81 different antibiotic-resistance genes, three multidrug efflux genes and three quaternary ammonium compound resistance genes. However, detailed analysis of the plasmid metagenome dataset indicated that the corresponding sequencing approach was not carried out to saturation. Thus, it is very likely that low-abundance genes were not detected. Moreover, only one compartment of the wastewater treatment plant was investigated by the cited plasmid metagenome study.

Therefore, the present study was aimed at screening the same WWTP for the occurrence of a large set of known antibiotic-resistance genes by means of a PCR approach which should also allow for detection of low-abundance resistance genes. The identification of resistance genes involved design and testing of 192 resistance-gene-specific PCR primer pairs. The question of whether the set of resistance determinants could also be detected in the WWTP's final effluents was also addressed.

METHODS

Isolation of plasmids from resistant bacteria residing in activated sludge and the final effluents of the WWTP. The WWTP samples were taken in September 2006 from the municipal WWTP Bielefeld-Heepen, Germany. One litre of the final effluent sample was centrifuged (5 min, 8000 ***g***) and the resulting pellet was resuspended in 5 ml Luria Broth. Aliquots (100 μl) of the resuspended final effluent sample and the activated sludge sample were plated in five replicates in serial dilutions onto Luria-Broth agar plates supplemented with one of the following antibiotics: 100 μg ampicillin ml^{-1}, 1 μg cefotaxime ml^{-1}, 15 μg cefuroxime ml^{-1}, 25 μg chloramphenicol ml^{-1}, 1 μg ciprofloxacin ml^{-1}, 200 μg erythromycin ml^{-1}, 15 μg gentamicin ml^{-1}, 50 μg kanamycin ml^{-1}, 1 μg norfloxacin ml^{-1}, 30 μg rifampicin ml^{-1}, 100 μg spectinomycin ml^{-1}, 100 μg streptomycin ml^{-1}, 5 μg tetracycline ml^{-1}. The agar medium was also supplemented with cycloheximide at a final concentration of 75 μg ml^{-1} to avoid growth of fungi. After incubation at 30 °C for 36 h the bacteria were collected separately for each antibiotic used for selection. Total plasmid DNAs from activated sludge or final effluent bacteria were prepared with the NucleoBond kit PC100 on AX 100 columns (Macherey-Nagel) according to the manufacturer's protocol. This method has been shown to be suitable for isolation of plasmids in a size range of 40 to 180 kbp (Stiens *et al.*, 2008; Szczepanowski *et al.*, 2004), with the limitation that larger plasmids cannot be isolated with the same efficiency as smaller plasmids. It should also be mentioned here that the plasmid isolation procedure is biased by the lysis method implemented in the NucleoBond kit PC100 protocol since it cannot be assumed that all kinds of WWTP bacteria are equally well lysed by this method. After DNA isolation, a CsCl high-density gradient centrifugation (Sambrook *et al.*, 1989) using a Vti 65.2 rotor was performed in order to minimize contamination with chromosomal DNA. Plasmid DNA concentrations were determined by using the NanoDrop 1000 instrument (NanoDrop Technologies). For further analyses, 20 μl of each total plasmid DNA preparation (separately held for plasmid DNAs from activated sludge and final effluent bacteria) were mixed, resulting in two master total plasmid DNA samples.

Selection of target reference antibiotic-resistance genes and design of specific PCR primers. For the design of resistance-gene-specific PCR primers, reference resistance gene nucleotide sequences were extracted from different databases: EBI SRS server (http://srs.ebi.ac.uk/), NCBI (http://www.ncbi.nlm.nih.gov/), *β*-lactamase genes (http://www.lahey.org/Studies/) and macrolide and tetracycline resistance genes (http://faculty.washington.edu/marilynr/). In total, about 650 resistance and multidrug efflux permease gene sequences known to confer resistance to different antimicrobial compounds including aminoglycosides, *β*-lactams, chloramphenicol, macrolides, quinolones, fluoroquinolones, rifampicin, tetracyclines, trimethoprim, sulphonamides and quaternary ammonium compounds were selected from these databases. A new database, named ARG-DB (Antibiotic Resistance Gene Database), was set up for the extracted genes and all entries of ARG-DB were compared to each other by applying the BLAST algorithm. Genes with more than 85 % sequence

identity were clustered. Based on CLUSTAL W (Larkin *et al.*, 2007) alignments, a consensus sequence was calculated for each cluster and the gene showing the highest degree of identity to the consensus sequence was defined as representative for the respective cluster. This approach led to the selection of 192 reference resistance genes (see Table 1 and Supplementary Table S1, available with the online version of this paper) each representing a distinct alignment cluster. Specific PCR primers were designed for all reference genes by means of the Primer3 program (Rozen & Skaletsky, 2000) and synthesized. The resulting PCR primer sequences are shown in Table S1. Plasmid incompatibility genes specific for the Inc groups P, Q, W, N (Götz *et al.*, 1996), A/C (Llanes *et al.*, 1996) and F (Eichenlaub *et al.*, 1977) as well as the genes *gfp* (Prasher *et al.*, 1992) and *luc* (accession no. D25416) were chosen as control sequences for primer design.

PCR and amplicon detection. The reaction mix of the PCR was composed of approximately 100 ng total plasmid DNA as template, 2.5 μl reaction buffer (10 ×), 2 mM $MgCl_2$, 0.2 mM of each dNTP,

Table 1. Selected reference antibiotic-resistance genes, and corresponding enzymes

The genes are grouped according to the antimicrobial drug class to which they confer resistance.

Antimicrobial compound class	Encoded enzymes	Gene names
Aminoglycosides	Aminoglycoside acetyltransferases	*aacA*, *aacA1*, *aacA4*, *aacA7*, *aacA29b*, *aacC1*, *aacC2*, *aacC3*, *aacC4*, *aac(3)-Id*, *aac(6′)-Im*
	Aminoglycoside adenylyltransferases	*aadA4*, *aadA7*, *aadA9*, *aadA10*, *aadA12*, *aadD*
	Aminoglycoside phosphotransferases	*aph*, *aphA*, *aphA-3*, *aphA-6*, *aphA-7*, *aph2*, *aph(2′)-Ib*, *strA*, *strB*
β-Lactams	Class A β-lactamases	*ctx-m4*, *ctx-m26*, *ctx-m27*, *ctx-m32*, *ges-3*, *kpc-3*, *per-1*, *per-2*, *shv-34*, bla_{TEM-1}, bla_{TLA-1}, bla_{TLA-2}, *veb-1*
	Class B β-lactamases	*imp-2*, *imp-5*, *imp-9*, *imp-13*, *imp-16*, *imp-16*, *vim-4*, *vim-7*
	Class C β-lactamases	*ampC*, *cmy-9*, *cmy-13*
	Class D β-lactamases	bla_{nps-1}, bla_{nps-2}, *oxa-1*, *oxa-2*, *oxa-5*, *oxa-9*, *oxa-10*, *oxa-12*, *oxa-18*, *oxa-20*, *oxa-22*, *oxa-27*, *oxa-29*, *oxa-40*, *oxa-45*, *oxa-46*, *oxa-48*, *oxa-50*, *oxa- 54*, *oxa-55*, *oxa-58*, *oxa-60*, *oxa-61*, *oxa-75*, *mecA*
Chloramphenicol/florfenicol	Chloramphenicol acetyltransferases	*cat*, *cat*, *cat*, *cat*, *cat2*, *catIII*, *catA*, *catB2*, *catB4*, *catB6*, *catB7*, *catB8*, *catB9*, *catP*, *cat-TC*
	Chloramphenicol/florfenicol transporters	*cmlA1*, *cmxA*, *fexA*, *floR*
	Hydrophobic polypeptide	*cmlB*
Fluoroquinolones	Pentapeptide family proteins	*qnrA3*, *qnrB1*, *qnrB4*, *qnr*
Macrolides	rRNA adenine N^6-methyltransferases	*ermA*, *ermB*, *ermD*, *ermF*, *erm(A)*, *erm(TR)*
	Esterase	*ereA2*, *ereB*
	MFS efflux proteins	*mefA*, *mefE*, *mefE*, *mel*, *msr(A)*
	Macrolide 2′-phosphotransferases	*mph(B)*, *mph(A)*, *mph*, *mphB*, *mph(BM)*
	Hydrolase	*vgh(A)*
	Streptogramin B lactonase	*vgbB*
Rifampicin	ADP-ribosylating transferase	*arr2*
Tetracyclines	Tetracycline transporters	*tet*(A), *tet*(A), *tetA*(C), *tetA*(E), *tetA*(J), *tetBSR*, *tet*(D), *tet*(G), *tet*(H), *tet*(L), *tetA*(Y), *tet*(Z), *effJ*, *tet*(V), *tet*(K), *tet*(30), *tet*(33), *tet*(38), *tetA*(39)
	Tetracycline inactivation proteins	*tet*(37), *tet*(X)
	GTP-binding elongation factor proteins	*tetB*(P), *tet*(M), *tet*(M), *tet*(M), *tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(32)
	Ribosomal protection tetracycline resistance proteins	*tet*(36), *tetQ*, *tet*(T)
	Tetracycline repressor protein	*tet*R(31)
	Tetracycline resistance	*tet*(U)
	Phosphoribosyltransferase	*tet*(34)
Trimethoprim	Dihydrofolate reductases	*dfrII*, *dfrV*, *dfrVI*, *dfrXII*, *dfr13*, *dfr16*, *dfr17*, *dfrA19*, *dfrB2*, *dfrD*, *dhfr*, *dhfR*, *dhfrI*, *dhfrVIII*, *dhfrIX*, *dhfrXV*
Sulfonamides	Dihydropteroate synthetases	*sulI*, *sulII*, *sulIII*
Quaternary ammonium compounds	Small multidrug efflux proteins	*qacB*, *qacD*, *qacE*Δ*1*, *qacF*, *qacF*, *qacG*, *qacG2*, *qacH*
Various antibiotics transported by multidrug efflux genes	Multidrug efflux pumps	*acrB*, *acrD*, *mexB*, *mexD*, *mexD*, *mexF*, *mexI*, *mexY*, *orf11*
Total		192

0.5 μM of each primer, 1 U *Taq* DNA polymerase (BioLine), and filled up to 25 μl with sterile double-distilled water. The initial step of the reaction was denaturation of DNA at 94 °C for 4 min. This step was followed by 35 cycles composed of 1 min denaturation at 94 °C, 1 min annealing at 58 °C and 45 s polymerization at 72 °C. The final polymerization step was performed for 10 min at 72 °C. The amplicons were analysed by gel electrophoresis (in 1 % agarose in Tris/HCl/acetate buffer), stained with ethidium bromide and visualized under UV light.

Sequencing and analysis of selected resistance-gene-specific amplicons. After filter purification by means of MAHVN 4550 (Millipore) and G-50 Fine Sephadex (Sigma-Aldrich) the amplicons were sequenced on an ABI 3730 XL sequencer (Applera, Applied Biosystems) using Big Dye 3.1 chemistry. Assembly of the forward and reverse sequence of each amplicon, and sequence quality control, was carried out by means of the CONSED/AUTOFINISH software tool (Gordon *et al.*, 1998, 2001). Assembled resistance-gene-specific amplicon sequences were compared to the NCBI nucleotide sequence database by means of BLAST (Altschul *et al.*, 1990).

RESULTS AND DISCUSSION

Isolation of antibiotic-resistance plasmids from resistant bacteria obtained from activated sludge and the WWTP's final effluents

To get an overview of the occurrence of resistance determinants in a WWTP habitat, total plasmid DNA preparations isolated from antibiotic-resistant WWTP bacteria were probed for different known resistance genes by means of a PCR approach. Antibiotic-resistant bacteria originating from activated sludge or from the final effluent compartment of the municipal WWTP Bielefeld-Heepen were selected on media supplemented with one of 12 clinically relevant antibiotics (see Methods). Total plasmid DNA was prepared from bacteria able to grow on these selective media and used as template in PCR analyses for the detection of selected resistance determinants. The concentration of the pooled template DNAs was about 80 ng μl^{-1} for each habitat (activated sludge and final effluents). Target reference resistance genes were extracted from different nucleotide sequence databases, and gene-specific PCR primers were designed (see Supplementary Table S1).

Detection of plasmid-encoded resistance genes in resistant bacteria isolated from activated sludge and the WWTP's final effluents

To detect resistance genes and plasmid incompatibility determinants present in bacteria residing in activated sludge and the final effluent compartment of the WWTP, PCR analyses using 200 specific primer pairs were carried out. Total plasmid DNA preparations from antibiotic-resistant WWTP bacteria were used as template DNAs in these PCRs. In total, 145 amplicons (140 specific for resistance genes and five for plasmid incompatibility determinants) were obtained in these PCRs on total plasmid DNA from antibiotic-resistant activated-sludge bacteria (Table 2). The total plasmid DNA preparation originating from bacteria of the final-effluent compartment yielded 129 amplicons (123 specific for resistance genes and six for plasmid-specific genes) (Table 2). PCR results were positive for resistance genes known to confer resistance to different aminoglycoside, *β*-lactam, chloramphenicol, fluoroquinolone, macrolide, rifampicin, tetracycline, trimethoprim and sulfonamide antibiotics as well as to quaternary ammonium compounds (Table 2).

Results of this study were compared to the plasmid metagenome data that were recently obtained for activated sludge bacteria showing reduced susceptibility to selected antimicrobial drugs from the same WWTP. High-throughput 454-pyrosequencing of plasmids from these bacteria revealed that numerous sequences are very similar or even identical to 81 known antibiotic-resistance genes conferring resistance to the major classes of antimicrobial drugs (Schlüter *et al.*, 2008; Szczepanowski *et al.*, 2008). The PCR-based approach led to the detection of 59 additional resistance genes in activated-sludge bacteria that were not apparent in the plasmid metagenome dataset. For instance, 15 additional tetracycline resistance genes appeared in the PCR analysis. In contrast, only sequences for seven different tetracycline-resistance genes, namely *tetA*(A), *tetA*(B), *tetA*(C), *tetA*(D), *tetA*(E), *tetA*(X) and *tet*(39), were identified in the metagenome dataset (Schlüter *et al.*, 2008; Szczepanowski *et al.*, 2008). Moreover, the PCR-based approach led to the detection of 123 different plasmid-encoded resistance-gene-specific amplicons in bacteria isolated from the final effluent of the WWTP analysed here. This compartment was not covered by the cited plasmid metagenome study.

The present study also showed that the resistance gene spectra detected in plasmid DNA preparations originating from activated-sludge and from final-effluent bacteria are quite similar. Only the numbers of detected aminoglycoside, *β*-lactam, macrolide and tetracycline resistance genes differ slightly for the WWTP compartments tested. Interestingly, the same fluoroquinolone, trimethoprim and sulfonamide resistance genes as well as the same genes for multidrug efflux systems could be detected in both plasmid samples (see Table 2). This high congruence of amplicons for the latter resistance genes may be explained by the fact that antibiotics, especially fluoroquinolones, trimethoprim and sulfonamides, are only poorly removed during wastewater treatment processes (Göbel *et al.*, 2005; Golet *et al.*, 2002, 2003; Lindberg *et al.*, 2005, 2006; Nakata *et al.*, 2005) and therefore might exert selective pressure on bacteria within the sewer system or the sewage plant, leading to enrichment of resistant bacteria and their release into the environment with the final effluents.

Detection of resistance genes recently described from clinical isolates in the WWTP compartments analysed

Detection of numerous and various resistance genes in bacteria from activated sludge (140 genes) and the final

Table 2. Resistance genes detected by PCR in total plasmid DNA preparations isolated from bacteria of activated sludge or the WWTP's final effluent

Gene name*	Gene product	Amplicon size (bp)	Resistance to/ function†	Detected in activated sludge	Detected in the final effluents	Accession no.
aacA, *aadB*	Aminoglycoside 6′-*N*-acetyltransferase	197	Km, Tob, Ak	+	+	M86913
aacA1	Aminoglycoside 6′-*N*-acetyltransferase	200	Gm, Km, Tob, Neo	+	−	AB113580
aacA4	Aminoglycoside 6′-acetyltransferase	196	Ak	+	+	AJ744860
aacA7	Aminoglycoside acetyltransferase-6′ type I	175	Gm, Tob, Km	+	−	AF263520
aacA29b	Aminoglycoside 6′-*N*-acetyltransferase	170	Ak, Km	+	+	AY139599
aacC1	Aminoglycoside 3*N*-acetyltransferase	130	Gm	+	+	AY139604
aacC2	Aminoglycoside (3)-*N*-acetyltransferase	148	Gm	+	+	S68058
aacC4	Aminoglycoside (3)-acetyltransferase IV	147	Gm	+	+	X01385
aac(3)-Id‡	3′-*N*-Aminoglycoside acetyltransferase	178	Gm	+	+	AY458224
aac(6′)-Im	6′-Aminoglycoside *N*-acetyltransferase	194	Tob, Ak, Km	+	+	AF337947
aadA4, *aadA5*	Streptomycin 3′-adenylyltransferase	198	Sm, Sp	+	+	AY138986
aadA7	Aminoglycoside (3′)(9)-adenylyltransferase	187	Sm, Sp	+	+	AY463797
aadA9	Streptomycin 3′-adenylyltransferase	184	Sm, Sp	+	−	AJ420072
aadA10, *aadA6/aadA10*‡	Aminoglycoside (3′)(9)-adenylyltransferase	198	Sm, Sp	+	+	U37105
aadA12, *aadA1*, *aadA2*, *aadA8*, *aadA11*, *aadA13*, *aadA23*	Putative streptomycin 3′-adenylyltransferase	186	Putative Sm, Sp	+	+	AY665771
aadD	Kanamycin-nucleotidyltransferase	153	Km	+	+	AB037420
aph	Aminoglycoside 3′-phosphotransferase	173	Km, Neo	+	+	AJ851089
aphA	3′-Aminoglycoside phosphotransferase	198	Km	+	+	AJ744860
aphA-3	3′5′-Aminoglycoside phosphotransferase of type III	139	Km	+	+	V01547
aphA-6	3′-Aminoglycoside phosphotransferase	192	Km, Ak	+	+	X07753
aph2	Aminoglycoside-3′-*O*-phosphotransferase	198	Km, Neo	+	+	U00004
aph(2′)-Ib	Aminoglycoside phosphotransferase	175	Km	+	−	AF337947
strA	Aminoglycoside 3′-phosphotransferase	196	Sm	+	+	NC_004840
strB	Aminoglycoside 6-phosphotransferase	150	Sm	+	+	NC_004840
ctx-m-4	Class A *β*-lactamase	155	Amp, Ctx, Cxm, Atm	+	+	Y14156
ctx-m-27‡	Class A *β*-lactamase	158	Caz, Ctx, Amo, Tic, Prl, Kf, Cxm, Cpo, Atm	+	+	AY156923
ctx-m-32‡	Class A *β*-lactamase	156	Amo, Ctx, Caz, Fep, Prl, Kf, Fox, Cxm	+	+	AJ557142
ges-3‡	Class A extended-spectrum *β*-lactamase	181	Titeracillin, Prl, Caz, Ctx, Atm, Ipm	+	+	AY494717
per-2	Class A extended-spectrum *β*-lactamase	198	Oxyiminocephalosporins, Atm, Cft	+	−	X93314
shv-34	Class A *β*-lactamase	200	Caz, Ctx	+	+	AY036620
$bla_{\mathrm{TEM-1}}$	Class A *β*-lactamase	167	Amp, Pen-G	+	+	AJ851089
$bla_{\mathrm{TLA-2}}$	Class A extended spectrum *β*-lactamase	186	Amo, Tic, Caz, Kf, Cxm, Fox, Ctx, Fep, Atm	+	+	NC_006385
veb-1	Class A extended-spectrum *β*-lactamase	190	Cephalosporins, Atm	+	−	AF010416
vim-4	Metallo-*β*-lactamase	171	*β*-Lactams	−	+	AY509609

Table 2. cont.

Gene name*	Gene product	Amplicon size (bp)	Resistance to/ function†	Detected in activated sludge	Detected in the final effluents	Accession no.
imp-2, *imp-5*	Class B metallo β-lactamase	200	Amp, Ctx, Fep	+	+	AJ243491
imp-9‡, *imp-11*	Class B metallo β-lactamase	178	β-Lactams	+	+	AY033653
imp-13, *imp-2*	Class B metallo β-lactamase	198	Cxm, Caz, Ctx, Cro, Fep, Amp	+	+	AJ550807
ampC	Class C β-lactamase, cephalosporinase	189	Pen, cephalosporins	+	+	J01611
cmy-9, *cmy-10*	Class C β-lactamase	169	β-Lactams	+	+	AB061794
cmy-13‡, *cmy-5*	Class C β-lactamase	150	β-Lactams	+	+	AY339625
bla_{NPS-1}	Class D β-lactamase	188	Amo, azlocillin, Cec, cefazolin, Cfp, Prl	+	−	NC_003430
bla_{NPS-2}	Class D β-lactamase	192	Amp	+	+	NC_006388
oxa-1	Class D β-lactamase	199	β-Lactams	+	+	AY139600
oxa-2, *oxa-21*, *oxa-53*	Class D β-lactamase	177	β-Lactams	+	+	NC_007502
oxa-5	Class D β-lactamase	175	β-Lactams	+	+	X58272
oxa-9	Class D β-lactamase	162	β-Lactams	+	−	M55547
oxa-10, *oxa-56*	Class D β-lactamase	191	β-Lactams	+	+	AY115475
oxa-12	Class D β-lactamase	188	β-Lactams	+	+	U10251
oxa-20	Class D β-lactamase	163	Amo, Tic	−	+	AF024602
oxa-22	Class D β-lactamase	200	Benzylpenicillin, Ob	+	+	AF064820
oxa-27	Class D β-lactamase	180	β-Lactams	+	−	AF201828
oxa-40	Class D β-lactamase	168	Amo, Tic, Caz, Fep, Cpo, Prl, Kf, Cxm, Ipm	+	+	AF509241
oxa-46, *oxa*	Class D β-lactamase	150	Amp, Car, Mez, Kf	+	+	AF317511
oxa-48	Class D β-lactamase	145	Amo, Tic, Fep, Ipm, Cpo, Prl, Ctx	+	+	AY236073
oxa-50	Class D β-lactamase	198	Amp, Tic, Ctx, Prl, Kf, Cxm	+	+	AY306130
oxa-58‡	Class D β-lactamase	152	Amo, Tic, Cpo, Prl, Ipm, Kf	+	+	AY665723
oxa-75	Class D β-lactamase	181	Amp, Prl	+	+	AY859529
cmlA1, *cmlA5*	Chloramphenicol efflux protein	137	Cm	+	+	NC_006388
cmlB	Hydrophobic polypeptide	147	Cm	+	+	AF034958
cmxA	Chloramphenicol export protein	186	Cm	+	+	AF024666
fexA	Florfenicol/chloramphenicol exporter	198	Cm, Ffc	+	−	AJ549214
floR, *cmlA*	Efflux protein	188	Cm, Ffc	+	+	AF118107
cat	Chloramphenicol acetyltransferase	173	Cm	+	+	M11587
cat	Chloramphenicol acetyltransferase	162	Cm	+	+	M35190
cat	Chloramphenicol acetyltransferase	195	Cm	+	+	S48276
cat	Chloramphenicol acetyltransferase	163	Cm	+	+	M58515
cat2, *catII*, *cmlA*	Chloramphenicol acetyltransferase	192	Cm	+	+	AY509004
catIII	Chloramphenicol acetyltransferase	150	Cm	+	+	X07848
catA	Chloramphenicol acetyltransferase	186	Cm	+	+	AJ851089
catB2	Chloramphenicol acetyltransferase	156	Cm	+	+	AY139601
catB4	Chloramphenicol acetyltransferase	188	Cm	+	+	AF322577

Table 2. cont.

Gene name*	Gene product	Amplicon size (bp)	Resistance to/ function†	Detected in activated sludge	Detected in the final effluents	Accession no.
catB6	Chloramphenicol acetyltransferase	144	Cm	−	+	AJ223604
catB7	Chloramphenicol acetyltransferase	152	Cm	+	+	AF036933
catB8	Chloramphenicol acetyltransferase	175	Cm	+	+	AF227506
cat-TC, cat	Chloramphenicol acetyltransferase	194	Cm	+	+	U75299
qnrA3‡, *qnr*	Pentapeptide family, DNA-gyrase and topoisomerase IV protection	168	Nal	+	+	DQ058661
qnrB1‡, *qnrB2*, *qnrB5*	Pentapeptide family, DNA-gyrase and topoisomerase IV protection	191	Cip	+	+	DQ351241
qnrB4	Pentapeptide family	*158*	Quinolones	+	+	DQ303921
qnr, *qnrS2*‡	Quinolone resistance determinant	175	Cip, Nor, Nal	+	+	AB187515
ereA2, *ereA*	Erythromycin esterase type I	177	Em	+	+	AF512546
ereB	Erythromycin esterase type II	158	Em	+	−	X03988
mph(*B*)	Macrolide phosphotransferase	199	Azi, Cla, Em, Rox, Tyl	+	+	AM260957
mph(*A*)	Macrolide 2′-phosphotransferase I	153	Azi, Cla, Em, Rox	+	+	NC_006385
mph	Macrolide 2′-phosphostransferase	200	Em	+	+	DQ839391
mph(B)	Macrolide 2′-phosphotransferase II	200	Macrolides	+	+	D85892
mphBM	Macrolide 2′-phosphotransferase II	200	Macrolides	+	−	AF167161
ermA	rRNA adenine N^6-methyltransferase	185	Em	+	−	X51472
ermB	rRNA adenine N^6-methyltransferase	193	Em	+	+	M11180
ermF	rRNA adenine N^6-methyltransferase	323	MLS	+	+	M14730
mef(A)	Macrolide-efflux protein, MFS permease	179	Em	+	−	AJ715499
mefE, *mefI*	Macrolide-efflux protein, MFS permease	199	Em	+	−	AF274302
mel	Macrolide-efflux protein, macrolide-specific ABC-type efflux carrier	198	Azi, Cla, Em	+	+	DQ839391
msrA	Erythromycin resistance ATP-binding protein MsrA	158	Em	+	−	X52085
arr2	Putative rifampicin ADP-ribosyltransferase	140	Rif	+	+	AF205943
sulI	Dihydropteroate synthetase	185	Sul	+	+	NC_006388
sulII	Dihydropteroate synthetase	147	Sul	+	+	AJ851089
sul3	Dihydropteroate synthetase	199	Sul	+	+	AY316203
dfrII	Dihydrofolate reductase	156	Tp	+	+	AY139601
dfrV	Dihydrofolate reductase	180	Tp	+	+	AY139589
dfr13(dfrXIII)	Dihydrofolate reductase	174	Tp	+	+	Z50802
dfr16	Dihydrofolate reductase	173	Tp	+	+	AY259085
dfr17, *dfrVII*	Dihydrofolate reductase	152	Tp	+	+	AY139588
dfrA19	Dihydrofolate reductase	165	Tp	+	+	AM234698
dfrB2	Dihydrofolate reductase	198	Tp	+	+	AY139592
dfrD	Dihydrofolate reductase	194	Tp	+	+	Z50141
dhfr1	Dihydrofolate reductase	169	Tp	+	+	AJ698325
dhfrVIII	Dihydrofolate reductase	169	Tp	+	+	U10186
dhfrXV	Dihydrofolate reductase	197	Tp	+	+	Z83311

Table 2. cont.

Gene name*	Gene product	Amplicon size (bp)	Resistance to/ function†	Detected in activated sludge	Detected in the final effluents	Accession no.
tetA	MFS tetracycline efflux	200	Tc	+	+	NC_004840
tetA	MFS tetracycline efflux	198	Tc	+	+	NC_006388
tetA	MFS tetracycline efflux	187	Tc	+	+	AJ851089
tetA	MFS tetracycline efflux	176	Tc	+	+	L06940
tetD	MFS tetracycline efflux	155	Tc	+	+	L06798
tetG	MFS tetracycline efflux	140	Tc	+	+	AF133139
tetH	MFS tetracycline efflux	164	Tc	+	+	AJ245947
tetL	MFS tetracycline efflux	176	Tc	+	−	U17153
tet(U)	Replication	198	Low level Tc	+	+	U01917
tetY	MFS tetracycline efflux	146	Tc	+	+	AF070999
tetR(31)	Tetracycline repressor protein	168	Regulates expression of TetA(31)	+	+	AJ250203
effJ (*tet*(35))	Putative tetracycline efflux pump	190	Tc	−	+	AF35362
tet(39)	MFS tetracycline efflux	154	Tc	+	+	AY743590
tetB(P)	GTP-binding elongation factor protein, TetM/TetO family	143	Tc	+	−	L20800
tet(M)	GTP-binding elongation factor protein, TetM/TetO family	197	Tc	+	+	M21136
tet(M)	GTP-binding elongation factor protein, TetM/TetO family	197	Tc	+	+	M85225
tet(M)	GTP-binding elongation factor protein, TetM/TetO family	198	Tc	+	+	X04388
tet(M)	GTP-binding elongation factor protein, TetM/TetO family	198	Tc	+	+	X90939
tet(O)	GTP-binding elongation factor protein, TetM/TetO family	189	Tc	+	−	Y07780
tet(S)	GTP-binding elongation factor protein, TetM/TetO family	172	Tc	+	+	L09756
tet(32)	GTP-binding elongation factor protein, TetM/TetO family	149	Tc	+	−	AJ295238
tet(36)	Ribosomal protection tetracycline resistance protein	192	Tc	−	+	AJ514254
tet(X)	Inactivation of tetracycline	186	Tc	+	+	M37699
qacB	Permease of the MFS family, multidrug efflux protein	164	Multidrug efflux	+	−	AF053771
qacEΔ1	Small multidrug resistance protein, membrane transporter of cations and cationic drugs	198	QAC	+	+	AJ698325
qacF	Small multidrug resistance protein, membrane transporter of cations and cationic drugs	195	QAC	+	−	NC_007502
qacF, *qacH*	Small multidrug resistance protein, membrane transporter of cations and cationic drugs	172	QAC	+	+	AY139598

Table 2. cont.

Gene name*	Gene product	Amplicon size (bp)	Resistance to/ function†	Detected in activated sludge	Detected in the final effluents	Accession no.
qacG2	Small multidrug resistance protein, membrane transporter of cations and cationic drugs	147	QAC	+	+	AJ609296
acrB	RND family, acridine/multidrug efflux pump	160	Multidrug efflux	+	+	M94248
acrD	Cation/multidrug efflux pump	185	Aminoglycosides, Nv	+	+	U12598
mexB	Cation/multidrug efflux pump, RND multidrug efflux transporter	147	Multidrug efflux	+	+	L11616
mexD	RND multidrug efflux transporter	185	Em, Rox	+	+	NC_003430
mexD	Cation/multidrug efflux pump, RND multidrug efflux transporter	182	Multidrug efflux	+	+	U57969
mexF	Cation/multidrug efflux pump, RND multidrug efflux transporter	348	Multidrug efflux	+	+	X99514
mexI	Cation/multidrug efflux pump, RND multidrug efflux transporter	170	Multidrug efflux	+	+	AE004837
mexY	Cation/multidrug efflux pump, RND multidrug efflux transporter	198	Multidrug efflux	+	+	AB015853
orf11	ABC type permease	198	Nal, Nor	+	+	NC_006385
kikA	Killing in *Klebsiella*	198	IncN-specific gene	+	+	AY046276
oriV	Origin of vegetative replication	171	IncW-specific region	−	+	BR000038
oriV	Origin of vegetative replication	192	IncQ-specific region	+	+	NC_001740
rep	Replication initiation protein	163	IncA/C-specific gene	+	+	X73674
repE	Replication initiation protein	192	IncFIA-specific replication gene	+	+	AJ851089
trfA	Replication initiation protein	192	Initiation of replication, IncP-specific gene	+	+	NC_004840

*The PCR product is specific for all genes given in the field.

†Resistance spectra data were extracted from the respective database entry and the literature cited therein. Abbreviations: Ak, amikacin; Amo, amoxicillin; Amp, ampicillin; Atm, aztreonam; Azm, azithromycin; Car, carbenicillin; Caz, ceftazidim; Cec, cefaclor; Cfp, cefoperazon; Cft, ceftibuten; Cip, ciprofloxacin; Clr, clarithromycin; Cm, chloramphenicol; Cpo, cefpirom; Cro, ceftriaxon; Ctx, cefotaxime; Cxm, cefuroxime; Em, erythromycin; Fep, cefepim; Ffc, florfenicol; Fox, cefoxitin; Gm, gentamicin; Ipm, imipenem; Kf, cephalothin; Km, kanamycin; Lev, levofloxacin; Met, meticillin; MLS, macrolide-lincosamide-streptogramin B; Mez, mezlocillin; Nal, nalidixic acid; Neo, neomycin; Nor, norfloxacin; Nv, novobiocin; Ob, cloxacillin; Ofx, ofloxacin; Pen-G, penicillin G; Prl, piperacillin; QAC, quaternary ammonium compounds; Rif, rifampicin; Rox, roxithromycin; Spar, sparfloxacin; Sm, streptomycin; Sp, spectinomycin ; Sul, sulfonamides; Tc, tetracyclines; Tic, ticarcillin; Tob, tobramycin; Tp, trimethoprim; Ty, tylosin.

‡Resistance genes recently described in clinical isolates.

Table 3. Sequencing of randomly selected resistance-gene-specific amplicons obtained from wastewater treatment plant bacteria and annotation results

Gene-specific amplicon	Gene accession no.	Identity*	DNA-sequence identity (%)	Best hit to	Best hit accession no.
ampC	J01611	188 bp/189 bp	99	*ampC*	J01611
bla_{TLA-2}	NC_006385	186 bp/186 bp	100	bla_{TLA-2}	NC_006385
cmy-13	AY339625	149 bp/150 bp	99	*cmy-28*	EF561644
		147 bp/150 bp	97	*cmy-13*	AY339625
ctx-m-4	Y14156	154 bp/155 bp	99	*ctx-m2*	EF592570
		152 bp/155 bp	96	*ctx-m4*	Y14156
ctx-m-32	AJ557142	156 bp/156 bp	100	*ctx-m64*	AB284167
		155 bp/156 bp	99	*ctx-m32*	AJ557142
ctx-m-27	AY156923	158 bp/158 bp	100	*ctx-m27*	AY156923
ges-3	AY494717	181 bp/181 bp	100	*ges-3*	AY494717
imp-9	AY033653	176 bp/178 bp	98	*imp-13*	AJ628135
		157 bp/178 bp	87	*imp-9*	AY033653
imp-13	AJ550807	198 bp/198 bp	100	*imp-13*	AJ550807
oxa-46	AF317511	144 bp/150 bp	96	*oxa-46*	AF317511
oxa-58	AY665723	152 bp/152 bp	100	*oxa-58*	AY665723
shv-34	AY036620	199 bp/200 bp	99	*shv-77*	EF373975
		197 bp/200 bp	98	*shv-34*	AY036620
veb-1	AF010416	190 bp/190 bp	100	*veb-1*	AF010416
cmlB	AF034958	94 bp/101 bp (one read with 101 bp)	93	*cmlB*	AF034958
cmxA	AF024666	186 bp/186 bp	100	*cmxA*	AF024666
floR	AF118107	186 bp/188 bp	98	*floR*	AF118107
qnr	AB187515	175 bp/175 bp	100	*qnr*	AB187515
qnrA3	DQ058661	166 bp/168 bp	98	*qnr*	AY675584
		162 bp/168 bp	96	*qnrA3*	DQ058661
qnrB1	DQ351241	191 bp/191 bp	100	*qnrB2*	AM234698
		190 bp/191 bp	99	*qnrB1*	DQ351241
qnrB4	DQ303921	158 bp/158 bp	100	*qnrB4*	DQ303921
ereA2	AF512546	177 bp/177 bp	100	*ereA2*	AF512546
ermB	M11180	192 bp/193 bp	99	*ermB*	M11180
ermF	M14730	319 bp/323 bp	98	*ermF*	M14730
mef(A)	AJ715499	173 bp/179 bp	96	*mefA*	AJ715499
mefE	AF274302	198 bp/199 bp	99	*mef*	DQ445269
		194 bp/199 bp	96	*mefE*	AF274302
arr2	AF205943	140 bp/140 bp	100	*arr2*	AF205943
tetA(39)	AY743590	99 bp/99 bp (one read with 99 bp)	100	*tetA*(39)	AY743590
tetB(P)	L20800	143 bp /143 bp	100	*tetB*(P)	L20800
tetD	L06798	153 bp/155 bp	98	*tetD*	L06798
tetG	AF133139	138 bp/140 bp	98	*tetG*	AF133140
		132 bp/140 bp	94	*tetG*	AF133139
tetH	AJ245947	163 bp/164 bp	99	*tetH*	AJ245947
tetL	U17153	176 bp/176 bp	100	*tetL*	U17153
tet(M)	X90939	198 bp/198 bp	100	*tet*(M)	EF101931
		194 bp/198 bp	97	*tet*(M)	X90939
tet(M)	M21136	197 bp/197 bp	100	*tetM*	M21136
tet(O)	Y07780	188 bp/189 bp	99	*tet*(O)	Y07780
tet(S)	L09756	172 bp/172 bp	100	*tet*	L09756
tet(X)	M37699	186 bp/186 bp	100	*tetX*	M37699
acrB	M94248	159 bp/160 bp	99	*acrB*	M94248
acrD	U12598	185 bp/185 bp	100	*acrD*	U12598
mexB	L11616	142 bp/147 bp	97	*ttgB*	CT573326
		130 bp/147 bp	88	*mexB*	L11616
mexD	U57969	182 bp/182 bp	100	*mexD*	U57969

Table 3. cont.

Gene-specific amplicon	Gene accession no.	Identity*	DNA-sequence identity (%)	Best hit to	Best hit accession no.
mexD	NC_003430	184 bp/185 bp	99	*mexD*	NC_003430
mexF	X99514	348 bp/348 bp	100	*mexF*	AE004091
		345 bp/348 bp	99	*mexF*	X99514
mexI	AE004837	170 bp/170 bp	100	*mexI*	AE004837 (new accession no. AE004091)
mexY	AB015853	198 bp/198 bp	100	*mexY*	AB015853

*In some cases the two best hits are given: (i) best hit to a related gene; (ii) hit to the reference gene.

effluents of the WWTP (123 genes) raises the question whether resistance genes recently described for clinical isolates are also present in and are released from the municipal sewage plant under study. It appeared that the aminoglycoside resistance genes *aadA6/aadA10* (Fiett *et al.*, 2006) and *aac(3)-Id* (Doublet *et al.*, 2004), the *β*-lactam resistance genes *ctx-m-27* (Bonnet *et al.*, 2003), *ctx-m-32* (Cartelle *et al.*, 2004), *ges-3* (Vourli *et al.*, 2004), *imp-9* (Xiong *et al.*, 2006), *imp-13* (Toleman *et al.*, 2003) and *oxa-58* (Poirel *et al.*, 2005), and the fluoroquinolone resistance genes *qnrA3* (Heritier *et al.*, 2004), *qnrB1* (Jacoby *et al.*, 2006) and *qnrS* (Hata *et al.*, 2005), which were recently described as new genes or novel variants of known genes in clinical isolates, could be identified in the WWTP analysed (Table 2).

Sequencing of selected resistance-gene-specific amplicons to verify their identity

To verify the identity of the PCR products obtained in the analyses described above, 45 amplicons were randomly selected and sequenced. Sequencing and annotation results are summarized in Table 3. The nucleotide sequences of 20 amplicons (*bla*$_{\text{TLA-2}}$, *ctx-m27*, *ges-3*, *imp-13*, *oxa-58*, *veb-1*, *cmxA*, *qnr*, *qnrB4*, *ereA2*, *arr2*, *tetB*(P), *tetL*, *tet*(M), *tet*(S), *tet*(X), *acrD*, *mexD*, *mexI*, *mexY*) are identical to the corresponding reference sequences, and eight amplicons (*ampC*, *ctx-m-32*, *qnrB1*, *ermB*, *tetH*, *tet*(O), *acrB*, *mexD*) display only one nucleotide exchange compared to the reference sequence. Moreover, the nucleotide sequences of 15 further amplicons are 87 % to 98 % identical to the corresponding reference gene. In the case of two amplicons, namely those for the genes *tetA*(39) and *cmlB*, only the sequence from one sequencing direction could be obtained. The resulting short sequence reads show, respectively, 100 % identity (over a length of 99 bases) to *tetA*(39) and 93 % identity (over a length of 101 bases) to *cmlB*. Although some amplicon sequences do not show the highest degree of identity to the corresponding reference resistance gene, they are very similar or even identical to a closely related resistance determinant. For example, the amplicon sequence obtained with primers designed on the resistance gene *ctx-m-32* is identical to the sequence of *ctx-m-64* and has only one mismatch compared to the reference gene *ctx-m-32*. These results show that the sequenced amplicons really contain resistance-gene-specific nucleotide sequences.

Conclusions

This comprehensive study provides evidence that bacteria residing in different compartments of the WWTP analysed harbour various plasmid-borne resistance determinants representing all common classes. To our best knowledge, this is the first study that describes detection of resistance genes known to confer resistance to all common classes of antibiotics in two different compartments of the same WWTP. The mobile pool of resistance genes shared by bacteria of the WWTP analysed even includes resistance genes that have only recently been described for clinical isolates, indicating genetic exchange between clinical and WWTP bacteria. Moreover, detection of these newer resistance genes on plasmids isolated from bacteria of the WWTP's final effluents confirms that these determinants are released into the environment, which might facilitate further dissemination among environmental bacteria. Moreover, it appeared that wastewater purification processes operating within the WWTP analysed are not appropriate to significantly reduce the spectrum of resistance genes that are detectable in the final effluents.

The composition of the plasmid pool analysed was biased, since plasmids were isolated from bacteria showing reduced susceptibility to different antibiotics. Accordingly, future projects will aim at the detection of antibiotic-resistance determinants in whole-community plasmid DNA preparations. In this context the microarray technology seems to be very well suited for simultaneous detection of hundreds of resistance determinants in samples derived from different WWTPs. Likewise, it would be informative to compare plasmid samples obtained from WWTPs that receive effluents from hospitals with those that are not connected to any medical facilities.

ACKNOWLEDGEMENTS

The authors thank the Bioinformatics Resource Facility (BRF) at the CeBiTec of Bielefeld University for support regarding bioinformatics issues. The work was supported by the Landesamt für Natur, Umwelt und Verbraucherschuts NRW (Germany).

REFERENCES

Akinbowale, O. L., Peng, H. & Barton, M. D. (2007). Diversity of tetracycline resistance genes in bacteria from aquaculture sources in Australia. *J Appl Microbiol* **103**, 2016–2025.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Bennett, P. M. (1999). Integrons and gene cassettes: a genetic construction kit for bacteria. *J Antimicrob Chemother* **43**, 1–4.

Bönemann, G., Stiens, M., Pühler, A. & Schlüter, A. (2006). Mobilizable IncQ-related plasmid carrying a new quinolone resistance gene, *qnrS2*, isolated from the bacterial community of a wastewater treatment plant. *Antimicrob Agents Chemother* **50**, 3075–3080.

Bonnet, R., Recule, C., Baraduc, R., Chanal, C., Sirot, D., De Champs, C. & Sirot, J. (2003). Effect of D240G substitution in a novel ESBL CTX-M-27. *J Antimicrob Chemother* **52**, 29–35.

Bywater, R. J. (2004). Veterinary use of antimicrobials and emergence of resistance in zoonotic and sentinel bacteria in the EU. *J Vet Med B Infect Dis Vet Public Health* **51**, 361–363.

Bywater, R. J. (2005). Identification and surveillance of antimicrobial resistance dissemination in animal production. *Poult Sci* **84**, 644–648.

Cartelle, M., del Mar Tomas, M., Molina, F., Moure, R., Villanueva, R. & Bou, G. (2004). High-level resistance to ceftazidime conferred by a novel enzyme, CTX-M-32, derived from CTX-M-1 through a single Asp240-Gly substitution. *Antimicrob Agents Chemother* **48**, 2308–2313.

Chee-Sanford, J. C., Aminov, R. I., Krapac, I. J., Garrigues-Jeanjean, N. & Mackie, R. I. (2001). Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl Environ Microbiol* **67**, 1494–1502.

Dang, H., Ren, J., Song, L., Sun, S. & An, L. (2008). Diverse tetracycline resistant bacteria and resistance genes from coastal waters of Jiaozhou Bay. *Microb Ecol* **55**, 237–246.

Davies, J. (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science* **264**, 375–382.

Davison, J. (1999). Genetic exchange between bacteria in the environment. *Plasmid* **42**, 73–91.

Doublet, B., Weill, F. X., Fabre, L., Chaslus-Dancla, E. & Cloeckaert, A. (2004). Variant *Salmonella* genomic island 1 antibiotic resistance gene cluster containing a novel 3′-*N*-aminoglycoside acetyltransferase gene cassette, *aac(3)-Id*, in *Salmonella enterica* serovar *newport*. *Antimicrob Agents Chemother* **48**, 3806–3812.

Eichenlaub, R., Figurski, D. & Helinski, D. R. (1977). Bidirection replication from a unique origin in a mini-F plasmid. *Proc Natl Acad Sci U S A* **74**, 1138–1141.

Fiett, J., Baraniak, A., Mrowka, A., Fleischer, M., Drulis-Kawa, Z., Naumiuk, L., Samet, A., Hryniewicz, W. & Gniadkowski, M. (2006). Molecular epidemiology of acquired-metallo-beta-lactamase-producing bacteria in Poland. *Antimicrob Agents Chemother* **50**, 880–886.

Geisenberger, O., Ammendola, A., Christensen, B. B., Molin, S., Schleifer, K. H. & Eberl, L. (1999). Monitoring the conjugal transfer of plasmid RP4 in activated sludge and in situ identification of the transconjugants. *FEMS Microbiol Lett* **174**, 9–17.

Göbel, A., Thomsen, A., McArdell, C. S., Alder, A. C., Giger, W., Theiss, N., Löffler, D. & Ternes, T. A. (2005). Extraction and determination of sulfonamides, macrolides, and trimethoprim in sewage sludge. *J Chromatogr A* **1085**, 179–189.

Golet, E. M., Strehler, A., Alder, A. C. & Giger, W. (2002). Determination of fluoroquinolone antibacterial agents in sewage sludge and sludge-treated soil using accelerated solvent extraction followed by solid-phase extraction. *Anal Chem* **74**, 5455–5462.

Golet, E. M., Xifra, I., Siegrist, H., Alder, A. C. & Giger, W. (2003). Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. *Environ Sci Technol* **37**, 3243–3249.

Goossens, H. (2005). European status of resistance in nosocomial infections. *Chemotherapy* **51**, 177–181.

Gordon, D., Abajian, C. & Green, P. (1998). Consed: a graphical tool for sequence finishing. *Genome Res* **8**, 195–202.

Gordon, D., Desmarais, C. & Green, P. (2001). Automated finishing with autofinish. *Genome Res* **11**, 614–625.

Götz, A., Pukall, R., Smit, E., Tietze, E., Prager, R., Tschäpe, H., van Elsas, J. D. & Smalla, K. (1996). Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. *Appl Environ Microbiol* **62**, 2621–2628.

Guillaume, G., Verbrugge, D., Chasseur-Libotte, M., Moens, W. & Collard, J. (2000). PCR typing of tetracycline resistance determinants (Tet A–E) in *Salmonella enterica* serotype Hadar and in the microbial community of activated sludges from hospital and urban wastewater treatment facilities in Belgium. *FEMS Microbiol Ecol* **32**, 77–85.

Hall, R. M. & Collis, C. M. (1995). Mobile gene cassettes and integrons – capture and spread of genes by site-specific recombination. *Mol Microbiol* **15**, 593–600.

Hata, M., Suzuki, M., Matsumoto, M., Takahashi, M., Sato, K., Ibe, S. & Sakae, K. (2005). Cloning of a novel gene for quinolone resistance from a transferable plasmid in *Shigella flexneri* 2b. *Antimicrob Agents Chemother* **49**, 801–803.

Henriques, I., Moura, A., Alves, A., Saavedra, M. J. & Correia, A. (2006a). Analysing diversity among β-lactamase encoding genes in aquatic environments. *FEMS Microbiol Ecol* **56**, 418–429.

Henriques, I. S., Fonseca, F., Alves, A., Saavedra, M. J. & Correia, A. (2006b). Occurrence and diversity of integrons and β-lactamase genes among ampicillin-resistant isolates from estuarine waters. *Res Microbiol* **157**, 938–947.

Heritier, C., Poirel, L. & Nordmann, P. (2004). Genetic and biochemical characterization of a chromosome-encoded carbapenem-hydrolyzing ambler class D beta-lactamase from *Shewanella algae*. *Antimicrob Agents Chemother* **48**, 1670–1675.

Heuer, H., Szczepanowski, R., Schneiker, S., Pühler, A., Top, E. M. & Schlüter, A. (2004). The complete sequences of plasmids pB2 and pB3 provide evidence for a recent ancestor of the IncP-1β group without any accessory genes. *Microbiology* **150**, 3591–3599.

Jacoby, G. A., Walsh, K. E., Mills, D. M., Walker, V. J., Oh, H., Robicsek, A. & Hooper, D. C. (2006). *qnrB*, another plasmid-mediated gene for quinolone resistance. *Antimicrob Agents Chemother* **50**, 1178–1182.

Jarnheimer, P. A., Ottoson, J., Lindberg, R., Stenstrom, T. A., Johansson, M., Tysklind, M., Winner, M. M. & Olsen, B. (2004). Fluoroquinolone antibiotics in a hospital sewage line; occurrence, distribution and impact on bacterial resistance. *Scand J Infect Dis* **36**, 752–755.

Kümmerer, K. (2003). Significance of antibiotics in the environment. *J Antimicrob Chemother* **52**, 5–7.

Kümmerer, K., Al-Ahmad, A. & Mersch-Sundermann, V. (2000). Biodegradability of some antibiotics, elimination of the genotoxicity

and affection of wastewater bacteria in a simple test. *Chemosphere* **40**, 701–710.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007). CLUSTAL W and CLUSTAL_X version 2.0. *Bioinformatics* **23**, 2947–2948.

Lee, H. B., Peart, T. E. & Svoboda, M. L. (2007). Determination of ofloxacin, norfloxacin, and ciprofloxacin in sewage by selective solid-phase extraction, liquid chromatography with fluorescence detection, and liquid chromatography-tandem mass spectrometry. *J Chromatogr A* **1139**, 45–52.

Lim, S. M. & Webb, S. A. (2005). Nosocomial bacterial infections in intensive care units. I. Organisms and mechanisms of antibiotic resistance. *Anaesthesia* **60**, 887–902.

Lindberg, R. H., Wennberg, P., Johansson, M. I., Tysklind, M. & Andersson, B. A. (2005). Screening of human antibiotic substances and determination of weekly mass flows in five sewage treatment plants in Sweden. *Environ Sci Technol* **39**, 3421–3429.

Lindberg, R. H., Olofsson, U., Rendahl, P., Johansson, M. I., Tysklind, M. & Andersson, B. A. (2006). Behavior of fluoroquinolones and trimethoprim during mechanical, chemical, and active sludge treatment of sewage water and digestion of sludge. *Environ Sci Technol* **40**, 1042–1048.

Llanes, C., Gabant, P., Couturier, M., Bayer, L. & Plesiat, P. (1996). Molecular analysis of the replication elements of the broad-host-range RepA/C replicon. *Plasmid* **36**, 26–35.

Mach, P. A. & Grimes, D. J. (1982). R-plasmid transfer in a wastewater treatment plant. *Appl Environ Microbiol* **44**, 1395–1403.

Mancini, P., Fertels, S., Nave, D. & Gealt, M. A. (1987). Mobilization of plasmid pHSV106 from *Escherichia coli* HB101 in a laboratory-scale waste treatment facility. *Appl Environ Microbiol* **53**, 665–671.

Marcinek, H., Wirth, R., Muscholl-Silberhorn, A. & Gauer, M. (1998). *Enterococcus faecalis* gene transfer under natural conditions in municipal sewage water treatment plants. *Appl Environ Microbiol* **64**, 626–632.

Mazel, D. & Davies, J. (1999). Antibiotic resistance in microbes. *Cell Mol Life Sci* **56**, 742–754.

Nakata, H., Kannan, K., Jones, P. D. & Giesy, J. P. (2005). Determination of fluoroquinolone antibiotics in wastewater effluents by liquid chromatography-mass spectrometry and fluorescence detection. *Chemosphere* **58**, 759–766.

Nüßlein, K., Maris, D., Timmis, K. & Dwyer, D. F. (1992). Expression and transfer of engineered catabolic pathways harbored by *Pseudomonas* spp. introduced into activated sludge microcosms. *Appl Environ Microbiol* **58**, 3380–3386.

Poirel, L., Marqué, S., Héritier, C., Segonds, C., Chabanon, G. & Nordmann, P. (2005). OXA-58, a novel class D β-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **49**, 202–208.

Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G. & Cormier, M. J. (1992). Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* **111**, 229–233.

Rowe-Magnus, D. A. & Mazel, D. (1999). Resistance gene capture. *Curr Opin Microbiol* **2**, 483–488.

Rozen, S. & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**, 365–386.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schlüter, A., Heuer, H., Szczepanowski, R., Forney, L. J., Thomas, C. M., Pühler, A. & Top, E. M. (2003). The 64 508 bp IncP-1β antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1β group. *Microbiology* **149**, 3139–3153.

Schlüter, A., Heuer, H., Szczepanowski, R., Poler, S. M., Schneiker, S., Pühler, A. & Top, E. M. (2005). Plasmid pB8 is closely related to the prototype IncP-1β plasmid R751 but transfers poorly to *Escherichia coli* and carries a new transposon encoding a small multidrug resistance efflux protein. *Plasmid* **54**, 135–148.

Schlüter, A., Szczepanowski, R., Kurz, N., Schneiker, S., Krahn, I. & Pühler, A. (2007a). Erythromycin resistance-conferring plasmid pRSB105, isolated from a sewage treatment plant, harbors a new macrolide resistance determinant, an integron-containing Tn*402*-like element, and a large region of unknown function. *Appl Environ Microbiol* **73**, 1952–1960.

Schlüter, A., Krahn, I., Kollin, F., Bönemann, G., Stiens, M., Szczepanowski, R., Schneiker, S. & Pühler, A. (2007b). IncP-1β plasmid pGNB1 isolated from a bacterial community from a wastewater treatment plant mediates decolorization of triphenyl-methane dyes. *Appl Environ Microbiol* **73**, 6345–6350.

Schlüter, A., Krause, L., Szczepanowski, R., Goesmann, A. & Pühler, A. (2008). Genetic diversity and composition of a plasmid metagenome from a wastewater treatment plant. *J Biotechnol* **136**, 65–76.

Schwartz, T., Kohnen, W., Jansen, B. & Obst, O. (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol Ecol* **43**, 325–335.

Seveno, N. A., Kallifidas, D., Smalla, K., van Elsas, J. D., Collard, J. M., Karagouni, A. D. & Wellington, E. M. H. (2002). Occurrence and reservoirs of antibiotic resistance genes in the environment. *Rev Med Microbiol* **13**, 15–27.

Smith, M. S., Yang, R. K., Knapp, C. W., Niu, Y., Peak, N., Hanfelt, M. M., Galland, J. C. & Graham, D. W. (2004). Quantification of tetracycline resistance genes in feedlot lagoons by real-time PCR. *Appl Environ Microbiol* **70**, 7372–7377.

Stiens, M., Becker, A., Bekel, T., Gödde, V., Goesmann, A., Niehaus, K., Schneiker-Bekel, S., Selbitschka, W., Weidner, S. & other authors (2008). Comparative genomic hybridisation and ultrafast pyrosequencing revealed remarkable differences between the *Sinorhizobium meliloti* genomes of the model strain Rm1021 and the field isolate SM11. *J Biotechnol* **136**, 31–37.

Szczepanowski, R., Krahn, I., Linke, B., Goesmann, A., Pühler, A. & Schlüter, A. (2004). Antibiotic multiresistance plasmid pRSB101 isolated from a wastewater treatment plant is related to plasmids residing in phytopathogenic bacteria and carries eight different resistance determinants including a multidrug transport system. *Microbiology* **150**, 3613–3630.

Szczepanowski, R., Braun, S., Riedel, V., Schneiker, S., Krahn, I., Pühler, A. & Schlüter, A. (2005). The 120 592 bp IncF plasmid pRSB107 isolated from a sewage-treatment plant encodes nine different antibiotic-resistance determinants, two iron-acquisition systems and other putative virulence-associated functions. *Microbiology* **151**, 1095–1111.

Szczepanowski, R., Krahn, I., Bohn, N., Pühler, A. & Schlüter, A. (2007). Novel macrolide resistance module carried by the IncP-1β resistance plasmid pRSB111, isolated from a wastewater treatment plant. *Antimicrob Agents Chemother* **51**, 673–678.

Szczepanowski, R., Bekel, T., Goesmann, A., Krause, L., Krömeke, H., Kaiser, O., Eichler, W., Pühler, A. & Schlüter, A. (2008). Insight into the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to antimicrobial drugs analysed by the 454-pyrosequencing technology. *J Biotechnol* **136**, 54–64.

Tauch, A., Schlüter, A., Bischoff, N., Goesmann, A., Meyer, F. & Pühler, A. (2003). The 79,370 bp conjugative plasmid pB4 consists of an IncP-1β backbone loaded with a chromate resistance transposon, the *strA-strB* streptomycin resistance gene pair, the oxacillinase gene $bla_{\text{NPS-1}}$, and a tripartite antibiotic efflux system of the resistance-nodulation-division family. *Mol Genet Genomics* **268**, 570–584.

Tennstedt, T., Szczepanowski, R., Braun, S., Pühler, A. & Schlüter, A. (2003). Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant. *FEMS Microbiol Ecol* **45**, 239–252.

Tennstedt, T., Szczepanowski, R., Krahn, I., Pühler, A. & Schlüter, A. (2005). Sequence of the 68,869 bp IncP-1α plasmid pTB11 from a waste-water treatment plant reveals a highly conserved backbone, a Tn*402*-like integron and other transposable elements. *Plasmid* **53**, 218–238.

Toleman, M. A., Biedenbach, D., Bennett, D., Jones, R. N. & Walsh, T. R. (2003). Genetic characterization of a novel metallo-beta-lactamase gene, $bla_{\text{IMP-13}}$, harboured by a novel Tn*5051*-type transposon disseminating carbapenemase genes in Europe: report from the SENTRY worldwide antimicrobial surveillance programme. *J Antimicrob Chemother* **52**, 583–590.

Volkmann, H., Schwartz, T., Bischoff, P., Kirchen, S. & Obst, U. (2004). Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *J Microbiol Methods* **56**, 277–286.

Vourli, S., Giakkoupi, P., Miriagou, V., Tzelepi, E., Vatopoulos, A. C. & Tzouvelekis, L. S. (2004). Novel GES/IBC extended-spectrum β-lactamase variants with carbapenemase activity in clinical enterobacteria. *FEMS Microbiol Lett* **234**, 209–213.

Wassenaar, T. M. (2005). Use of antimicrobial agents in veterinary medicine and implications for human health. *Crit Rev Microbiol* **31**, 155–169.

Xiong, J., Hynes, M. F., Ye, H., Chen, H., Yang, Y., M'Zali, F. & Hawkey, P. M. (2006). $bla_{\text{IMP-9}}$ and its association with large plasmids carried by *Pseudomonas aeruginosa* isolates from the People's Republic of China. *Antimicrob Agents Chemother* **50**, 355–358.

Edited by: L. Heide