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

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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.

Received 11 February 2009

Revised 3 April 2009

Accepted 8 April 2009

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.

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 (Szczezanowski *et al.*, 2004), pRSB105 (Schlüter *et al.*, 2007a), pRSB107 (Szczezanowski *et al.*, 2005), pRSB111 (Szczezanowski *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 -lactams (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; Szczezanowski *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 μ l) 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 μ g ampicillin ml⁻¹, 1 μ g cefotaxime ml⁻¹, 15 μ g cefuroxime ml⁻¹, 25 μ g chloramphenicol ml⁻¹, 1 μ g ciprofloxacin ml⁻¹, 200 μ g erythromycin ml⁻¹, 15 μ g gentamicin ml⁻¹, 50 μ g kanamycin ml⁻¹, 1 μ g norfloxacin ml⁻¹, 30 μ g rifampicin ml⁻¹, 100 μ g spectinomycin ml⁻¹, 100 μ g streptomycin ml⁻¹, 5 μ g tetracycline ml⁻¹. The agar medium was also supplemented with cycloheximide at a final concentration of 75 μ g 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; Szczezanowski *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 μ l 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/marilyn/>). 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 MgCl₂, 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	<i>aacA</i> , <i>aacA1</i> , <i>aacA4</i> , <i>aacA7</i> , <i>aacA29b</i> , <i>aacC1</i> , <i>aacC2</i> , <i>aacC3</i> , <i>aacC4</i> , <i>aac(3)-Id</i> , <i>aac(6')-Im</i>
	Aminoglycoside adenyltransferases	<i>aadA4</i> , <i>aadA7</i> , <i>aadA9</i> , <i>aadA10</i> , <i>aadA12</i> , <i>aadD</i>
	Aminoglycoside phosphotransferases	<i>aph</i> , <i>aphA</i> , <i>aphA-3</i> , <i>aphA-6</i> , <i>aphA-7</i> , <i>aph2</i> , <i>aph(2')-Ib</i> , <i>strA</i> , <i>strB</i>
β-Lactams	Class A β-lactamases	<i>ctx-m4</i> , <i>ctx-m26</i> , <i>ctx-m27</i> , <i>ctx-m32</i> , <i>ges-3</i> , <i>kpc-3</i> , <i>per-1</i> , <i>per-2</i> , <i>shv-34</i> , <i>bla_{TEM-1}</i> , <i>bla_{TLA-1}</i> , <i>bla_{TLA-2}</i> , <i>veb-1</i>
	Class B β-lactamases	<i>imp-2</i> , <i>imp-5</i> , <i>imp-9</i> , <i>imp-13</i> , <i>imp-16</i> , <i>imp-16</i> , <i>vim-4</i> , <i>vim-7</i>
	Class C β-lactamases	<i>ampC</i> , <i>cmy-9</i> , <i>cmy-13</i>
	Class D β-lactamases	<i>bla_{nps-1}</i> , <i>bla_{nps-2}</i> , <i>oxa-1</i> , <i>oxa-2</i> , <i>oxa-5</i> , <i>oxa-9</i> , <i>oxa-10</i> , <i>oxa-12</i> , <i>oxa-18</i> , <i>oxa-20</i> , <i>oxa-22</i> , <i>oxa-27</i> , <i>oxa-29</i> , <i>oxa-40</i> , <i>oxa-45</i> , <i>oxa-46</i> , <i>oxa-48</i> , <i>oxa-50</i> , <i>oxa-54</i> , <i>oxa-55</i> , <i>oxa-58</i> , <i>oxa-60</i> , <i>oxa-61</i> , <i>oxa-75</i> , <i>mecA</i>
Chloramphenicol/florfenicol	Chloramphenicol acetyltransferases	<i>cat</i> , <i>cat</i> , <i>cat</i> , <i>cat2</i> , <i>catIII</i> , <i>catA</i> , <i>catB2</i> , <i>catB4</i> , <i>catB6</i> , <i>catB7</i> , <i>catB8</i> , <i>catB9</i> , <i>catP</i> , <i>cat-TC</i>
	Chloramphenicol/florfenicol transporters	<i>cmlA1</i> , <i>cmxA</i> , <i>fexA</i> , <i>florR</i>
	Hydrophobic polypeptide	<i>cmlB</i>
Fluoroquinolones	Pentapeptide family proteins	<i>qnrA3</i> , <i>qnrB1</i> , <i>qnrB4</i> , <i>qnr</i>
Macrolides	rRNA adenine N ⁶ -methyltransferases	<i>ermA</i> , <i>ermB</i> , <i>ermD</i> , <i>ermF</i> , <i>erm(A)</i> , <i>erm(TR)</i>
	Esterase	<i>ereA2</i> , <i>ereB</i>
	MFS efflux proteins	<i>mefA</i> , <i>mefE</i> , <i>mefE</i> , <i>mel</i> , <i>msr(A)</i>
	Macrolide 2'-phosphotransferases	<i>mph(B)</i> , <i>mph(A)</i> , <i>mph</i> , <i>mphB</i> , <i>mph(BM)</i>
	Hydrolase	<i>vgh(A)</i>
	Streptogramin B lactonase	<i>vgbB</i>
Rifampicin	ADP-ribosylating transferase	<i>arr2</i>
Tetracyclines	Tetracycline transporters	<i>tet(A)</i> , <i>tet(A)</i> , <i>tetA(C)</i> , <i>tetA(E)</i> , <i>tetA(I)</i> , <i>tetBSR</i> , <i>tet(D)</i> , <i>tet(G)</i> , <i>tet(H)</i> , <i>tet(L)</i> , <i>tetA(Y)</i> , <i>tet(Z)</i> , <i>effJ</i> , <i>tet(V)</i> , <i>tet(K)</i> , <i>tet(30)</i> , <i>tet(33)</i> , <i>tet(38)</i> , <i>tetA(39)</i>
	Tetracycline inactivation proteins	<i>tet(37)</i> , <i>tet(X)</i>
	GTP-binding elongation factor proteins	<i>tetB(P)</i> , <i>tet(M)</i> , <i>tet(M)</i> , <i>tet(M)</i> , <i>tet(M)</i> , <i>tet(O)</i> , <i>tet(S)</i> , <i>tet(W)</i> , <i>tet(32)</i>
	Ribosomal protection tetracycline resistance proteins	<i>tet(36)</i> , <i>tetQ</i> , <i>tet(T)</i>
	Tetracycline repressor protein	<i>tetR(31)</i>
	Tetracycline resistance	<i>tet(U)</i>
	Phosphoribosyltransferase	<i>tet(34)</i>
Trimethoprim	Dihydrofolate reductases	<i>dfrII</i> , <i>dfrV</i> , <i>dfrVI</i> , <i>dfrXII</i> , <i>dfr13</i> , <i>dfr16</i> , <i>dfr17</i> , <i>dfrA19</i> , <i>dfrB2</i> , <i>dfrD</i> , <i>dhfr</i> , <i>dhfr</i> , <i>dhfrI</i> , <i>dhfrVIII</i> , <i>dhfrIX</i> , <i>dhfrXV</i>
		<i>sull</i> , <i>sullI</i> , <i>sullII</i>
Sulfonamides	Dihydropteroate synthetases	<i>qacB</i> , <i>qacD</i> , <i>qacEΔ1</i> , <i>qacF</i> , <i>qacF</i> , <i>qacG</i> , <i>qacG2</i> , <i>qacH</i>
Quaternary ammonium compounds	Small multidrug efflux proteins	
Various antibiotics transported by multidrug efflux genes	Multidrug efflux pumps	<i>acrB</i> , <i>acrD</i> , <i>mexB</i> , <i>mexD</i> , <i>mexD</i> , <i>mexF</i> , <i>mexI</i> , <i>mexY</i> , <i>orf11</i>
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 (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.
<i>aacA, aadB</i>	Aminoglycoside 6'-N-acetyltransferase	197	Km, Tob, Ak	+	+	M86913
<i>aacA1</i>	Aminoglycoside 6'-N-acetyltransferase	200	Gm, Km, Tob, Neo	+	—	AB113580
<i>aacA4</i>	Aminoglycoside 6'-acetyltransferase	196	Ak	+	+	AJ744860
<i>aacA7</i>	Aminoglycoside acetyltransferase-6' type I	175	Gm, Tob, Km	+	—	AF263520
<i>aacA29b</i>	Aminoglycoside 6'-N-acetyltransferase	170	Ak, Km	+	+	AY139599
<i>aacC1</i>	Aminoglycoside 3N-acetyltransferase	130	Gm	+	+	AY139604
<i>aacC2</i>	Aminoglycoside (3)-N-acetyltransferase	148	Gm	+	+	S68058
<i>aacC4</i>	Aminoglycoside (3)-acetyltransferase IV	147	Gm	+	+	X01385
<i>aac(3)-Id‡</i>	3'-N-Aminoglycoside acetyltransferase	178	Gm	+	+	AY458224
<i>aac(6')-Im</i>	6'-Aminoglycoside N-acetyltransferase	194	Tob, Ak, Km	+	+	AF337947
<i>aadA4, aadA5</i>	Streptomycin 3'-adenylyltransferase	198	Sm, Sp	+	+	AY138986
<i>aadA7</i>	Aminoglycoside (3')(9)-adenylyltransferase	187	Sm, Sp	+	+	AY463797
<i>aadA9</i>	Streptomycin 3'-adenylyltransferase	184	Sm, Sp	+	—	AJ420072
<i>aadA10, aadA6/aadA10‡</i>	Aminoglycoside (3')(9)-adenylyltransferase	198	Sm, Sp	+	+	U37105
<i>aadA12, aadA1, aadA2, aadA8, aadA11, aadA13, aadA23</i>	Putative streptomycin 3'-adenylyltransferase	186	Putative Sm, Sp	+	+	AY665771
<i>aadD</i>	Kanamycin-nucleotidyltransferase	153	Km	+	+	AB037420
<i>aph</i>	Aminoglycoside 3'-phosphotransferase	173	Km, Neo	+	+	AJ851089
<i>aphA</i>	3'-Aminoglycoside phosphotransferase	198	Km	+	+	AJ744860
<i>aphA-3</i>	3'5'-Aminoglycoside phosphotransferase of type III	139	Km	+	+	V01547
<i>aphA-6</i>	3'-Aminoglycoside phosphotransferase	192	Km, Ak	+	+	X07753
<i>aph2</i>	Aminoglycoside-3'-O-phosphotransferase	198	Km, Neo	+	+	U00004
<i>aph(2')-Ib</i>	Aminoglycoside phosphotransferase	175	Km	+	—	AF337947
<i>strA</i>	Aminoglycoside 3'-phosphotransferase	196	Sm	+	+	NC_004840
<i>strB</i>	Aminoglycoside 6-phosphotransferase	150	Sm	+	+	NC_004840
<i>ctx-m-4</i>	Class A β -lactamase	155	Amp, Ctx, Cxm, Atm	+	+	Y14156
<i>ctx-m-27‡</i>	Class A β -lactamase	158	Caz, Ctx, Amo, Tic, Prl, Kf, Cxm, Cpo, Atm	+	+	AY156923
<i>ctx-m-32‡</i>	Class A β -lactamase	156	Amo, Ctx, Caz, Fep, Prl, Kf, Fox, Cxm	+	+	AJ557142
<i>ges-3‡</i>	Class A extended-spectrum β -lactamase	181	Tetracycline, Prl, Caz, Ctx, Atm, Ipm	+	+	AY494717
<i>per-2</i>	Class A extended-spectrum β -lactamase	198	Oxyiminocephalosporins, Atm, Cft	+	—	X93314
<i>shv-34</i>	Class A β -lactamase	200	Caz, Ctx	+	+	AY036620
<i>bla_{TEM-1}</i>	Class A β -lactamase	167	Amp, Pen-G	+	+	AJ851089
<i>bla_{TLA-2}</i>	Class A extended spectrum β -lactamase	186	Amo, Tic, Caz, Kf, Cxm, Fox, Ctx, Fep, Atm	+	+	NC_006385
<i>veb-1</i>	Class A extended-spectrum β -lactamase	190	Cephalosporins, Atm	+	—	AF010416
<i>vim-4</i>	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.
<i>imp-2, imp-5</i>	Class B metallo β -lactamase	200	Amp, Ctx, Fep	+	+	AJ243491
<i>imp-9‡, imp-11</i>	Class B metallo β -lactamase	178	β -Lactams	+	+	AY033653
<i>imp-13, imp-2</i>	Class B metallo β -lactamase	198	Cxm, Caz, Ctx, Cro, Fep, Amp	+	+	AJ550807
<i>ampC</i>	Class C β -lactamase, cephalosporinase	189	Pen, cephalosporins	+	+	J01611
<i>cmy-9, cmy-10</i>	Class C β -lactamase	169	β -Lactams	+	+	AB061794
<i>cmy-13‡, cmy-5</i>	Class C β -lactamase	150	β -Lactams	+	+	AY339625
<i>bla_{NPS-1}</i>	Class D β -lactamase	188	Amo, azlocillin, Cec, cefazolin, Cfp, Prl	+	—	NC_003430
<i>bla_{NPS-2}</i>	Class D β -lactamase	192	Amp	+	+	NC_006388
<i>oxa-1</i>	Class D β -lactamase	199	β -Lactams	+	+	AY139600
<i>oxa-2, oxa-21, oxa-53</i>	Class D β -lactamase	177	β -Lactams	+	+	NC_007502
<i>oxa-5</i>	Class D β -lactamase	175	β -Lactams	+	+	X58272
<i>oxa-9</i>	Class D β -lactamase	162	β -Lactams	+	—	M55547
<i>oxa-10, oxa-56</i>	Class D β -lactamase	191	β -Lactams	+	+	AY115475
<i>oxa-12</i>	Class D β -lactamase	188	β -Lactams	+	+	U10251
<i>oxa-20</i>	Class D β -lactamase	163	Amo, Tic	—	+	AF024602
<i>oxa-22</i>	Class D β -lactamase	200	Benzylpenicillin, Ob	+	+	AF064820
<i>oxa-27</i>	Class D β -lactamase	180	β -Lactams	+	—	AF201828
<i>oxa-40</i>	Class D β -lactamase	168	Amo, Tic, Caz, Fep, Cpo, Prl, Kf, Cxm, Ipm	+	+	AF509241
<i>oxa-46, oxa</i>	Class D β -lactamase	150	Amp, Car, Mez, Kf	+	+	AF317511
<i>oxa-48</i>	Class D β -lactamase	145	Amo, Tic, Fep, Ipm, Cpo, Prl, Ctx	+	+	AY236073
<i>oxa-50</i>	Class D β -lactamase	198	Amp, Tic, Ctx, Prl, Kf, Cxm	+	+	AY306130
<i>oxa-58‡</i>	Class D β -lactamase	152	Amo, Tic, Cpo, Prl, Ipm, Kf	+	+	AY665723
<i>oxa-75</i>	Class D β -lactamase	181	Amp, Prl	+	+	AY859529
<i>cmlA1, cmlA5</i>	Chloramphenicol efflux protein	137	Cm	+	+	NC_006388
<i>cmlB</i>	Hydrophobic polypeptide	147	Cm	+	+	AF034958
<i>cmxA</i>	Chloramphenicol export protein	186	Cm	+	+	AF024666
<i>fexA</i>	Florfenicol/chloramphenicol exporter	198	Cm, Ffc	+	—	AJ549214
<i>floR, cmlA</i>	Efflux protein	188	Cm, Ffc	+	+	AF118107
<i>cat</i>	Chloramphenicol acetyltransferase	173	Cm	+	+	M11587
<i>cat</i>	Chloramphenicol acetyltransferase	162	Cm	+	+	M35190
<i>cat</i>	Chloramphenicol acetyltransferase	195	Cm	+	+	S48276
<i>cat</i>	Chloramphenicol acetyltransferase	163	Cm	+	+	M58515
<i>cat2, catII, cmlA</i>	Chloramphenicol acetyltransferase	192	Cm	+	+	AY509004
<i>catIII</i>	Chloramphenicol acetyltransferase	150	Cm	+	+	X07848
<i>catA</i>	Chloramphenicol acetyltransferase	186	Cm	+	+	AJ851089
<i>catB2</i>	Chloramphenicol acetyltransferase	156	Cm	+	+	AY139601
<i>catB4</i>	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.
<i>catB6</i>	Chloramphenicol acetyltransferase	144	Cm		–	+	AJ223604
<i>catB7</i>	Chloramphenicol acetyltransferase	152	Cm		+	+	AF036933
<i>catB8</i>	Chloramphenicol acetyltransferase	175	Cm		+	+	AF227506
<i>cat-TC, cat</i>	Chloramphenicol acetyltransferase	194	Cm		+	+	U75299
<i>qnrA3‡, qnr</i>	Pentapeptide family, DNA-gyrase and topoisomerase IV protection	168	Nal		+	+	DQ058661
<i>qnrB1‡, qnrB2, qnrB5</i>	Pentapeptide family, DNA-gyrase and topoisomerase IV protection	191	Cip		+	+	DQ351241
<i>qnrB4</i>	Pentapeptide family	158	Quinolones		+	+	DQ303921
<i>qnr, qnrS2‡</i>	Quinolone resistance determinant	175	Cip, Nor, Nal		+	+	AB187515
<i>ereA2, ereA</i>	Erythromycin esterase type I	177	Em		+	+	AF512546
<i>ereB</i>	Erythromycin esterase type II	158	Em		+	–	X03988
<i>mph(B)</i>	Macrolide phosphotransferase	199	Azi, Cla, Em, Rox, Tyl		+	+	AM260957
<i>mph(A)</i>	Macrolide 2'-phosphotransferase I	153	Azi, Cla, Em, Rox		+	+	NC_006385
<i>mph</i>	Macrolide 2'-phosphotransferase	200	Em		+	+	DQ839391
<i>mph(B)</i>	Macrolide 2'-phosphotransferase II	200	Macrolides		+	+	D85892
<i>mphBM</i>	Macrolide 2'-phosphotransferase II	200	Macrolides		+	–	AF167161
<i>ermA</i>	rRNA adenine N ⁶ -methyltransferase	185	Em		+	–	X51472
<i>ermB</i>	rRNA adenine N ⁶ -methyltransferase	193	Em		+	+	M11180
<i>ermF</i>	rRNA adenine N ⁶ -methyltransferase	323	MLS		+	+	M14730
<i>mef(A)</i>	Macrolide-efflux protein, MFS permease	179	Em		+	–	AJ715499
<i>mefE, mefI</i>	Macrolide-efflux protein, MFS permease	199	Em		+	–	AF274302
<i>mel</i>	Macrolide-efflux protein, macrolide-specific ABC-type efflux carrier	198	Azi, Cla, Em		+	+	DQ839391
<i>msrA</i>	Erythromycin resistance ATP-binding protein MsrA	158	Em		+	–	X52085
<i>arr2</i>	Putative rifampicin ADP-ribosyltransferase	140	Rif		+	+	AF205943
<i>sulI</i>	Dihydropteroate synthetase	185	Sul		+	+	NC_006388
<i>sulII</i>	Dihydropteroate synthetase	147	Sul		+	+	AJ851089
<i>sul3</i>	Dihydropteroate synthetase	199	Sul		+	+	AY316203
<i>dfrII</i>	Dihydrofolate reductase	156	Tp		+	+	AY139601
<i>dfrV</i>	Dihydrofolate reductase	180	Tp		+	+	AY139589
<i>dfr13(dfrXIII)</i>	Dihydrofolate reductase	174	Tp		+	+	Z50802
<i>dfr16</i>	Dihydrofolate reductase	173	Tp		+	+	AY259085
<i>dfr17, dfrVII</i>	Dihydrofolate reductase	152	Tp		+	+	AY139588
<i>dfrA19</i>	Dihydrofolate reductase	165	Tp		+	+	AM234698
<i>dfrB2</i>	Dihydrofolate reductase	198	Tp		+	+	AY139592
<i>dfrD</i>	Dihydrofolate reductase	194	Tp		+	+	Z50141
<i>dhfr1</i>	Dihydrofolate reductase	169	Tp		+	+	AJ698325
<i>dhfrVIII</i>	Dihydrofolate reductase	169	Tp		+	+	U10186
<i>dhfrXV</i>	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.
<i>tetA</i>	MFS tetracycline efflux	200	Tc		+	+	NC_004840
<i>tetA</i>	MFS tetracycline efflux	198	Tc		+	+	NC_006388
<i>tetA</i>	MFS tetracycline efflux	187	Tc		+	+	AJ851089
<i>tetA</i>	MFS tetracycline efflux	176	Tc		+	+	L06940
<i>tetD</i>	MFS tetracycline efflux	155	Tc		+	+	L06798
<i>tetG</i>	MFS tetracycline efflux	140	Tc		+	+	AF133139
<i>tetH</i>	MFS tetracycline efflux	164	Tc		+	+	AJ245947
<i>tetL</i>	MFS tetracycline efflux	176	Tc		+	—	U17153
<i>tet(U)</i>	Replication	198	Low level Tc		+	+	U01917
<i>tetY</i>	MFS tetracycline efflux	146	Tc		+	+	AF070999
<i>tetR(31)</i>	Tetracycline repressor protein	168	Regulates expression of TetA(31)		+	+	AJ250203
<i>effJ (tet(35))</i>	Putative tetracycline efflux pump	190	Tc		—	+	AF35362
<i>tet(39)</i>	MFS tetracycline efflux	154	Tc		+	+	AY743590
<i>tetB(P)</i>	GTP-binding elongation factor protein, TetM/TetO family	143	Tc		+	—	L20800
<i>tet(M)</i>	GTP-binding elongation factor protein, TetM/TetO family	197	Tc		+	+	M21136
<i>tet(M)</i>	GTP-binding elongation factor protein, TetM/TetO family	197	Tc		+	+	M85225
<i>tet(M)</i>	GTP-binding elongation factor protein, TetM/TetO family	198	Tc		+	+	X04388
<i>tet(M)</i>	GTP-binding elongation factor protein, TetM/TetO family	198	Tc		+	+	X90939
<i>tet(O)</i>	GTP-binding elongation factor protein, TetM/TetO family	189	Tc		+	—	Y07780
<i>tet(S)</i>	GTP-binding elongation factor protein, TetM/TetO family	172	Tc		+	+	L09756
<i>tet(32)</i>	GTP-binding elongation factor protein, TetM/TetO family	149	Tc		+	—	AJ295238
<i>tet(36)</i>	Ribosomal protection tetracycline resistance protein	192	Tc		—	+	AJ514254
<i>tet(X)</i>	Inactivation of tetracycline	186	Tc		+	+	M37699
<i>qacB</i>	Permease of the MFS family, multidrug efflux protein	164	Multidrug efflux		+	—	AF053771
<i>qacEΔ1</i>	Small multidrug resistance protein, membrane transporter of cations and cationic drugs	198	QAC		+	+	AJ698325
<i>qacF</i>	Small multidrug resistance protein, membrane transporter of cations and cationic drugs	195	QAC		+	—	NC_007502
<i>qacF, qacH</i>	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.
<i>qacG2</i>	Small multidrug resistance protein, membrane transporter of cations and cationic drugs	147	QAC	+	+	AJ609296
<i>acrB</i>	RND family, acridine/multidrug efflux pump	160	Multidrug efflux	+	+	M94248
<i>acrD</i>	Cation/multidrug efflux pump	185	Aminoglycosides, Nv	+	+	U12598
<i>mexB</i>	Cation/multidrug efflux pump, RND multidrug efflux transporter	147	Multidrug efflux	+	+	L11616
<i>mexD</i>	RND multidrug efflux transporter	185	Em, Rox	+	+	NC_003430
<i>mexD</i>	Cation/multidrug efflux pump, RND multidrug efflux transporter	182	Multidrug efflux	+	+	U57969
<i>mexF</i>	Cation/multidrug efflux pump, RND multidrug efflux transporter	348	Multidrug efflux	+	+	X99514
<i>mexI</i>	Cation/multidrug efflux pump, RND multidrug efflux transporter	170	Multidrug efflux	+	+	AE004837
<i>mexY</i>	Cation/multidrug efflux pump, RND multidrug efflux transporter	198	Multidrug efflux	+	+	AB015853
<i>orf11</i>	ABC type permease	198	Nal, Nor	+	+	NC_006385
<i>kikA</i>	Killing in <i>Klebsiella</i>	198	IncN-specific gene	+	+	AY046276
<i>oriV</i>	Origin of vegetative replication	171	IncW-specific region	–	+	BR000038
<i>oriV</i>	Origin of vegetative replication	192	IncQ-specific region	+	+	NC_001740
<i>rep</i>	Replication initiation protein	163	IncA/C-specific gene	+	+	X73674
<i>repE</i>	Replication initiation protein	192	IncFIA-specific replication gene	+	+	AJ851089
<i>trfA</i>	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, ceftiprom; Cro, ceftriaxon; Ctx, cefotaxime; Cxm, cefuroxime; Em, erythromycin; Fep, cefepim; Ffc, florfenicol; Fox, ceftoxitin; Gm, gentamicin; Ipm, imipenem; Kf, cephalothin; Km, kanamycin; Lev, levofloxacin; Met, metacillin; 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, sparflaxacin; 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.
<i>ampC</i>	J01611	188 bp/189 bp	99	<i>ampC</i>	J01611
<i>bla_{TLA-2}</i>	NC_006385	186 bp/186 bp	100	<i>bla_{TLA-2}</i>	NC_006385
<i>cmv-13</i>	AY339625	149 bp/150 bp	99	<i>cmv-28</i>	EF561644
		147 bp/150 bp	97	<i>cmv-13</i>	AY339625
<i>ctx-m-4</i>	Y14156	154 bp/155 bp	99	<i>ctx-m2</i>	EF592570
		152 bp/155 bp	96	<i>ctx-m4</i>	Y14156
<i>ctx-m-32</i>	AJ557142	156 bp/156 bp	100	<i>ctx-m64</i>	AB284167
		155 bp/156 bp	99	<i>ctx-m32</i>	AJ557142
<i>ctx-m-27</i>	AY156923	158 bp/158 bp	100	<i>ctx-m27</i>	AY156923
<i>ges-3</i>	AY494717	181 bp/181 bp	100	<i>ges-3</i>	AY494717
<i>imp-9</i>	AY033653	176 bp/178 bp	98	<i>imp-13</i>	AJ628135
		157 bp/178 bp	87	<i>imp-9</i>	AY033653
<i>imp-13</i>	AJ550807	198 bp/198 bp	100	<i>imp-13</i>	AJ550807
<i>oxa-46</i>	AF317511	144 bp/150 bp	96	<i>oxa-46</i>	AF317511
<i>oxa-58</i>	AY665723	152 bp/152 bp	100	<i>oxa-58</i>	AY665723
<i>shv-34</i>	AY036620	199 bp/200 bp	99	<i>shv-77</i>	EF373975
		197 bp/200 bp	98	<i>shv-34</i>	AY036620
<i>veb-1</i>	AF010416	190 bp/190 bp	100	<i>veb-1</i>	AF010416
<i>cmlB</i>	AF034958	94 bp/101 bp (one read with 101 bp)	93	<i>cmlB</i>	AF034958
<i>cmxA</i>	AF024666	186 bp/186 bp	100	<i>cmxA</i>	AF024666
<i>floR</i>	AF118107	186 bp/188 bp	98	<i>floR</i>	AF118107
<i>qnr</i>	AB187515	175 bp/175 bp	100	<i>qnr</i>	AB187515
<i>qnrA3</i>	DQ058661	166 bp/168 bp	98	<i>qnr</i>	AY675584
		162 bp/168 bp	96	<i>qnrA3</i>	DQ058661
<i>qnrB1</i>	DQ351241	191 bp/191 bp	100	<i>qnrB2</i>	AM234698
		190 bp/191 bp	99	<i>qnrB1</i>	DQ351241
<i>qnrB4</i>	DQ303921	158 bp/158 bp	100	<i>qnrB4</i>	DQ303921
<i>ereA2</i>	AF512546	177 bp/177 bp	100	<i>ereA2</i>	AF512546
<i>ermB</i>	M11180	192 bp/193 bp	99	<i>ermB</i>	M11180
<i>ermF</i>	M14730	319 bp/323 bp	98	<i>ermF</i>	M14730
<i>mef(A)</i>	AJ715499	173 bp/179 bp	96	<i>mefA</i>	AJ715499
<i>mefE</i>	AF274302	198 bp/199 bp	99	<i>mef</i>	DQ445269
		194 bp/199 bp	96	<i>mefE</i>	AF274302
<i>arr2</i>	AF205943	140 bp/140 bp	100	<i>arr2</i>	AF205943
<i>tetA(39)</i>	AY743590	99 bp/99 bp (one read with 99 bp)	100	<i>tetA(39)</i>	AY743590
<i>tetB(P)</i>	L20800	143 bp /143 bp	100	<i>tetB(P)</i>	L20800
<i>tetD</i>	L06798	153 bp/155 bp	98	<i>tetD</i>	L06798
<i>tetG</i>	AF133139	138 bp/140 bp	98	<i>tetG</i>	AF133140
		132 bp/140 bp	94	<i>tetG</i>	AF133139
<i>tetH</i>	AJ245947	163 bp/164 bp	99	<i>tetH</i>	AJ245947
<i>tetL</i>	U17153	176 bp/176 bp	100	<i>tetL</i>	U17153
<i>tet(M)</i>	X90939	198 bp/198 bp	100	<i>tet(M)</i>	EF101931
		194 bp/198 bp	97	<i>tet(M)</i>	X90939
<i>tet(M)</i>	M21136	197 bp/197 bp	100	<i>tetM</i>	M21136
<i>tet(O)</i>	Y07780	188 bp/189 bp	99	<i>tet(O)</i>	Y07780
<i>tet(S)</i>	L09756	172 bp/172 bp	100	<i>tet</i>	L09756
<i>tet(X)</i>	M37699	186 bp/186 bp	100	<i>tetX</i>	M37699
<i>acrB</i>	M94248	159 bp/160 bp	99	<i>acrB</i>	M94248
<i>acrD</i>	U12598	185 bp/185 bp	100	<i>acrD</i>	U12598
<i>mexB</i>	L11616	142 bp/147 bp	97	<i>ttgB</i>	CT573326
		130 bp/147 bp	88	<i>mexB</i>	L11616
<i>mexD</i>	U57969	182 bp/182 bp	100	<i>mexD</i>	U57969

Table 3. cont.

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

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Edited by: L. Heide