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Antibiotic-resistance profile in environmental bacteria isolated from penicillin production wastewater treatment plant and the receiving river

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Summary

The antibiotic-resistance characteristics of bacterial strains in antibiotic production wastewater treatment plants (WWTP) that contain high concentrations of antibiotics are unknown, as are the environmental effects of the discharge of wastewater from such facilities. In this study, 417 strains were individually isolated from the effluent of a WWTP that treated penicillin G production wastewater, as well as from downstream and upstream areas of the receiving river. The minimum inhibition concentrations (MICs) of 18 antibiotics representing seven classes were then determined for each of these strains. Relatively high similarity in the bacterial composition existed between the wastewater and downstream river samples when compared with the upstream sample. High resistance ratios and MIC values were observed for almost all antibiotics in wastewater isolates, followed by strains from downstream river, of which the resistance ratios and levels were still significantly higher than those of upstream strains. The resistance ratios and levels also significantly differed among strains belonged to different species in the penicillin production wastewater effluent and downstream river. In both samples, the resistances to β-lactam antibiotics were more frequent, with much higher levels, than the other class antibiotics. Then five clinically important resistant genes mainly coding for extended-spectrum β-lactamases (ESBLs) were determined for all strains, only *bla*$_{TEM-1}$ which did not belong to ESBL was detected in 17.3% and 11.0% of strains isolated from wastewater and downstream river respectively. Class I integrons were detected in 14% of wastewater isolates and 9.1% of downstream isolates, and primarily contained gene cassettes conferring resistance to aminoglycoside antibiotics. The unexpectedly high levels of multiple antibiotic resistance in strains from wastewater and downstream river were speculated to be mainly due to multidrug efflux systems.

Introduction

There has been considerable concern regarding environments containing antibiotics due to the possibility of antibiotic-resistant strains becoming dominant in the bacterial communities in such ecosystems (Guardabassi *et al*., 1998; Aarestrup *et al*., 2002; Wittwer *et al*., 2005; Jindal *et al*., 2006; Heuer and Smalla, 2007). Until now, most environmental investigations about antibiotic resistance have focused on stock farms, poultry farms, fisheries, surface water, and lakes. The use of biological systems for the treatment of antibiotic production wastewater creates a unique ecosystem that contains much higher concentrations of antibiotics than normal aquatic environments (Li *et al*., 2008), and thus may be an important reservoir of antibiotic-resistant bacteria. Furthermore, the treated wastewater is discharged into the receiving surface water, which might contribute to the occurrence of antibiotic-resistant bacteria in downstream river. However, to our knowledge, no systematic investigations have been performed about bacterial antibiotic resistance in such environments until now. In this study, bacterial resistance characteristics were evaluated in wastewater effluent from a biological system designed to treat penicillin G production wastewater from an antibiotic production facility, as well as in the receiving river. The resistance profiles of bacteria in the upstream river were also determined to evaluate the contribution of wastewater discharge to antibiotic resistance in the downstream river samples.

Meanwhile, most environmental investigations about antibiotic resistance have only included important

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pathogens such as *Escherichia coli* (Hamelin *et al*., 2007), Enterococci (Aarestrup *et al*., 2002), *Aeromonas* spp. (Huddleston *et al*., 2006) and *Campylobacter* (Wittwer *et al*., 2005). It should be noted that the majority of resistant bacteria in environments are indigenous species, which constitute a reservoir that could contribute to the maintenance and spread of antibiotic-resistance genes to human pathogens through horizontal gene transfer facilitated by mobile genetic elements such as plasmids, transposons and integrons (Kruse and Sørum, 1994; Scott *et al*., 1997; Adams *et al*., 1998; Hamelin *et al*., 2007), especially in wastewater treatment plant (WWTP) environments (Szczepanowski *et al*., 2004; Schlüter *et al*., 2007). The elucidation of antibiotic resistance characteristics of the whole bacterial community is in need to assess bacterial resistance comprehensively. Thus, in this study, bacterial isolates were recovered from penicillin production wastewater effluent and river water samples using non-selective culture media, and then tested for susceptibility to 18 kinds of antimicrobial agents after identification.

As penicillin G was first discovered, several new generations of β-lactam antibiotics have been developed, including penicillins, early cephalosporins, second- and third-generation cephalosporins, β-lactam/β-lactamase inhibitor combinations, and carbapenems. All of these antibiotics contain a core β-lactam ring, but have disparate side-chains. β-Lactam antibiotics have become the agents most frequently used for the treatment of bacterial infections by inhibiting bacterial cell wall synthesis, whereas the β-lactam ring is susceptible to hydrolysis by β-lactamase enzymes produced by resistant bacteria (Livermore, 1995). To date, several hundred of β-lactamase have been identified and classified into four groups based on molecular or functional characteristics (Bush *et al*., 1995; Jacoby, 2006). A large number of these enzymes are derivatives of TEM, SHV and oxacillin (OXA) and so on, which are important β-lactamase widely encountered in clinical therapy (Paterson and Bonomo, 2005). On the other hand, class I integrons have been commonly reported to contain antibiotic-resistance gene cassettes and associated with other mobile elements such as plasmids and transposons, which could contribute to the spread of resistance genes (Agersø and Sandvang, 2005). Thus, all bacterial isolates in this study were examined for the occurrence of five clinically important β-lactamase determinants, as well as for the presence of class I integrons to determine the possible horizontal transfer of resistance genes.

It should be noted that bacterial resistance to antibiotics may also be intrinsic except for acquired. For example, enterococci show intrinsic resistance to many β-lactams, and Gram-negative bacteria are intrinsically resistant to macrolides in low concentrations. These should be taken into account when analysing bacterial resistance profiles during environmental investigations.

Results

Characteristics of water samples

Most of penicillin G was eliminated during the wastewater treatment processes. The concentration of penicillin G was 153 $\pm$ 4 μg l^{-1} in raw penicillin production wastewater, and 1.68 $\pm$ 0.48 μg l^{-1} in the wastewater effluent. In the receiving river, the concentration decreased from 0.31 $\pm$ 0.04 μg l^{-1} at the discharging point to under the detection limit (0.03 μg l^{-1}) at the downstream sampling site (approximately 30 km from the discharge point). No penicillin G could be detected in the upstream river samples. More detailed characteristics of the wastewater and surface water such as pH, temperature, chemical oxygen demand (COD) and biochemical oxygen demand for five days (BOD_5) could be found elsewhere (Li *et al*., 2008).

Total cell counts were roughly 5.4×10^7, 3.9×10^7 and 2.3×10^6 cells ml^{-1} in the penicillin production wastewater effluent, downstream river water and upstream river water respectively. It seemed that the discharge of treated wastewater resulted in an increased total count in downstream river when compared with upstream river. Furthermore, 6.8×10^4, 5.3×10^4 and 8.9×10^3 cfu ml^{-1} were individually obtained on Tryptic soy agar (TSA) for treated penicillin production wastewater, downstream river and upstream river. The ratios of cfu versus total cell counts ranged from 0.13% to 0.39%.

Bacterial compositions

A total of 179, 163 and 75 strains were individually isolated from the penicillin production wastewater effluent, the downstream river and the upstream river water samples. Following polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) grouping, 130, 106 and 23 representative PCR products were sequenced, and most of the sequences showed high similarities to known species. All strains were identified to at least the genus level (Table 1), and the majority of them belonged to glucose non-fermenting gram-negative bacilli (Schreckenberger, 1995), with most of isolate species commonly encountered in WWTPs and fresh water. Overall, 28, 30 and 10 bacterial species or genera of unidentified species were isolated from wastewater effluent, downstream river and upstream river samples respectively. Wastewater and downstream river water samples contained 12 common species, while only one species was found in both the upstream and downstream river water, as well as in both the upstream river water and wastewater. When the species abundance was considered, a comparably high similarity in bacterial composition existed between wastewater and downstream river water (Chao's Jaccard Raw

Table 1. Classification of bacterial isolates from penicillin production wastewater effluent, downstream and upstream river.

Genus or species	No. of isolates			Total no.
	Effluent	Downstream	Upstream	
Paracoccus versutus	4			4
Magnetospirillum spp.		1		1
Brevundimonas diminuta	9	28		37
Brevundimonas bullata		1		1
Brevundimonas aurantiaca			1	1
Ochrobactrum intermedium	1			1
Alcaligenes spp.		2		2
Achromobacter xylosoxidans		1		1
Brachymonas denitrificans	38	2		40
Simplicispira psychrophila	1	1		2
Acidovorax defluvii		9		9
Azoarcus spp.		2		2
Thauera aminoaromatica	1	1		2
Thauera phenylacetica		2		2
Thauera aromatica		1		1
Rheinheimera spp.	1			1
Pseudoxanthomonas spp.		1		1
Stenotrophomonas maltophilia	4	4		8
Stenotrophomonas koreensis	1	1		2
Stenotrophomonas acidaminiphila	1			1
Stenotrophomonas spp.	2			2
Pseudomonas fluorescens	12	11	58	81
Pseudomonas libanensis	15			15
Pseudomonas putida	3	10		13
Pseudomonas lundensis	2			2
Pseudomonas mandelii			1	1
Pseudomonas lini			1	1
Pseudomonas spp.	11	2		13
Acinetobacter johnsonii	4			4
Psychrobacter pulmonis	37	1		38
Psychrobacter maritimus	8			8
Psychrobacter spp.	3			3
Shewanella putrefaciens	4			4
Shewanella spp.	1			1
Yersinia kristensenii		5		5
Citrobacter spp.	1			1
Bacillus endophyticus	10	24		34
Bacillus thuringiensis		1		1
Bacillus flexus		1		1
Bacillus firmus		1		1
Bacillus simplex		1		1
Bacillus spp.		3		3
Staphylococcus saprophyticus			3	3
Exiguobacterium sibiricum	1			1
Carnobacterium viridans	1			1
Trichococcus flocculiformis	1	28		29
Trichococcus collinsii		16		16
Microbacterium maritypicum		1		1
Arthrobacter oxydans			5	5
Arthrobacter spp.			2	2
Corynebacterium coyleae			1	1
Corynebacterium spp.			2	2
Rhodococcus erythropolis			1	1
Flavobacterium cucumis		1		1
Flavobacterium spp.	2			2
Total	179	163	75	417

Abundance-based similarity index, 0.543), while the bacterial composition of the upstream river showed much lower similarities with the other two samples (both values of 0.066; Fig. 1).

Antibiotic resistance ratios

The minimum inhibition concentrations (MICs) of 18 antibiotics were determined for all strains in this study, and the

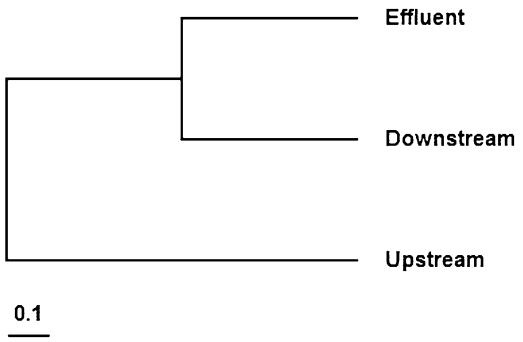

Fig. 1. Dendrograms of dissimilarity (Chao's Jaccard Raw Abundance-based index) between penicillin production wastewater effluent, downstream river and upstream river based on cluster analysis of bacterial composition in water samples, using the UPGMA algorithm. The scale bar indicates linkage distance for the index.

data were interpreted according to Clinical and Laboratory Standards Institute (CLSI) standards with results shown in Table 2. The genera *Thauera*, *Rheinheimera*, *Exiguobacterium*, *Magnetospirillum*, *Azoarcus* and *Microbacterium*, to each of which generally one or two strains belonged in this study, were not included when determining the antibiotic resistance due to a lack of interpretive criteria until now. Overall, among the three water samples of this study, the highest resistance ratios were observed in strains derived from the penicillin production wastewater effluent for all 18 antibiotics, with the exception of three quinolone antibiotics. In addition, the ratios of resistant strains for all antibiotics were significantly higher in wastewater effluent (mean ratio = 63.6 ± 6.4%) than in the downstream and upstream river water (Wilcoxon matched-pair test, both $P < 0.001$), and the ratios were also significantly higher in the downstream (mean ratio = 47.7 ± 5.6%) than in the upstream river (mean ratio = 11.8 ± 3.0%) (Wilcoxon matched-pair test, $P < 0.001$). The resistance ratios individually ranged from 41.6% to 92.2%, 14.7% to 78.5% and 0% to 22.7% in the wastewater effluent, downstream river and upstream river water for all antibiotics except quinolones [ciprofloxacin (CIP), levofloxacin (LEV) and nalidixic acid (NA)]. The highest resistance ratios for the three quinolones antibiotics, which are usually used as veterinary drugs (Ruiz, 2003), were generally found in the upstream river water.

Additionally, the strains from penicillin production wastewater effluent exhibited significantly more frequent resistance to β-lactam antibiotics than the other class antibiotics (Mann–Whitney *U*-test, $P = 0.001$, $n = 8, 10$). The same phenomenon was also observed for the strains from downstream river ($P = 0.002$), but not for the upstream samples ($P = 0.913$). The mean resistance ratios of β-lactam antibiotics were 83.4 ± 3.0%, 65.6 ± 4.9% and 10.7 ± 3.0% in wastewater effluent, downstream river and upstream river respectively. Furthermore, the highest prevalence of resistance among β-lactam antibiotics in wastewater, downstream water and upstream water was always against ampicillin (AMP) (92.2%, 78.5% and 22.7% respectively), followed by OXA (90.5%, 77.9% and 20% respectively), while the lowest resistance were against cefotaxime (CTX) (67.0%, 36.8% and 0% respectively) and ceftazidime (CAZ) (75.4%, 56.4% and 1.3% respectively). These results are reasonable considering that AMP and OXA are penicillins, whereas CTX and CAZ belonged to the third generation of cephalosporins. However, it should be noted that the resistance ratios for the other class antibiotics were generally not low (41.6–83.2% and 14.7–66.3% in wastewater and downstream river respectively), with the exception of CIP and LEV (always less than 6%). The mean ratios for antibiotics except β-lactams, CIP and LEV were up to 59.0 ± 4.9% and 40.4 ± 5.4% in wastewater and downstream river water respectively.

The antibiotic resistance ratios differed significantly for particular bacterial species with a sufficient sample size (five or more strains) in penicillin production wastewater effluent (Kendall's W matched-pair test, $P < 0.001$). Isolates of *Pseudomonas fluorescens*, *Pseudomonas libanensis* and *Brachymonas denitrificans* showed significantly higher resistance ratios than *Bacillus endophyticus*, *Psychrobacter maritimus* and *Psychrobacter pulmonis* (Wilcoxon matched-pair test, all $P < 0.03$). In addition, significant differences also existed in the resistance ratios for particular species with sufficient sample sizes in downstream river water (Kendall's W matched-pair test, $P < 0.001$) and the resistance ratios of *Pseudomonas putida*, *Pseudomonas fluorescens*, *Yersinia kristensenii* and *Brevundimonas diminuta* were significantly higher than those of *Acidovorax defluvii*, *Bacillus endophyticus*, *Trichococcus flocculiformis* and *Trichococcus collinsii* (Wilcoxon matched-pair test, all $P < 0.001$). Over all, strains of *Pseudomonas* sp. generally conferred more frequent antibiotic resistance than most of the other species, although the resistance ratios of *Pseudomonas fluorescens* were not significantly higher than *Arthrobacter oxydans* in the upstream river sample (Wilcoxon matched-pair test, $P = 0.077$). It should be noted that when pooling all bacterial isolates in the three water samples of this study together, eight strains of *Stenotrophomonas maltophilia* were found to have the highest mean antibiotic resistance ratio (81.3 ± 6.5%), which was significantly higher than most of the other species.

The above results were further confirmed by multiple correspondence analysis of water samples, bacterial species and antibiotic resistance (Fig. 2). Dimension 1 explained 43.4% of the observed variation, and dimension 2 explained 12.9% of the variation. Penicillin production wastewater samples were more closely related to most antibiotic resistances when compared with downstream

Table 2. Activities of 18 antimicrobial agents against the isolates from penicillin production wastewater effluent, downstream and upstream river.[a]

	Effluent isolates				Downstream isolates				Upstream isolates			
		MIC (μg ml^{-1})[b]				MIC (μg ml^{-1})[b]				MIC (μg ml^{-1})[b]		
Antimicrobial	Resistance ratio (%)	Range	50%	90%	Resistance ratio (%)	Range	50%	90%	Resistance ratio (%)	Range	50%	90%
Ampicillin	92.2	0.25–> 1024	> 1024	> 1024	78.5	1–> 1024	512	> 1024	22.7	0.25–> 1024	8	256
Carbenicillin	86.6	2–> 1024	> 1024	> 1024	75.4	0.25–> 1024	256	> 1024	18.7	0.25–1024	8	128
Oxacillin	90.5	4–> 1024	> 1024	> 1024	77.9	0.5–> 1024	> 1024	> 1024	20.0	0.25–> 1024	8	256
Piperacillin	88.8	0.5–> 1024	> 1024	> 1024	69.8	0.5–> 1024	256	> 1024	10.7	0.25–1024	8	128
Cephalothin	83.8	2–> 1024	> 1024	> 1024	68.1	0.25–> 1024	128	> 1024	5.3	0.25–512	2	16
Cefoperazone	82.6	1–> 1024	> 1024	> 1024	62.0	0.25–> 1024	128	> 1024	4.0	0.25–1024	1	4
Cefotaxime	67.0	0.25–> 1024	> 1024	> 1024	36.8	0.25–> 1024	32	> 1024	2.7	0.25–512	0.25	2
Ceftazidime	75.4	0.25–> 1024	> 1024	> 1024	56.4	0.25–> 1024	128	> 1024	1.3	0.25–512	0.25	4
Kanamycin	64.8	0.25–> 1024	128	1024	38.7	0.25–> 1024	4	> 1024	5.3	0.25–512	0.5	4
Gentamicin	50.8	0.25–> 1024	16	256	14.7	0.25–> 1024	1	64	0	0.25–4	0.25	2
Erythromycin	62.6	0.25–> 1024	64	512	49.1	0.25–1024	4	512	22.7	0.5–> 1024	2	128
Chloramphenicol	57.0	0.25–1024	32	1024	44.8	0.25–1024	16	64	8	0.5–1024	4	16
Oxytetracycline	43.6	0.25–512	8	512	33.1	0.25–512	2	32	1.3	0.25–32	0.5	4
Tetracycline	41.6	0.25–512	8	512	30.1	0.25–512	1	32	0	0.25–16	0.5	2
Ciprofloxacin	3.9	0.25–8	0.25	2	4.5	0.25–> 1024	1	2	8	0.25–8	0.25	2
Levofloxacin	2.8	0.25–8	0.25	2	5.8	0.25–> 1024	1	2	8	0.25–8	0.25	2
Nalidixic acid	68.2	0.5–> 1024	256	1024	66.3	0.25–1024	256	1024	52	1–> 1024	32	1024
Rifampin	83.2	0.25–> 1024	32	1024	46.0	0.25–> 1024	2	1024	21.3	0.25–> 1024	1	64

a. The *Thauera* spp., *Rheinheimera* spp., *Exiguobacterium* spp., *Magnetospirillum* spp., *Azoarcus* spp. and *Microbacterium* spp. strains for which there were still no interpretive criteria according to CLSI standards had not been taken into account when determining antibiotic resistance ratios, and generally one or two strains were isolated in this study for each genus.
b. 50% and 90% stand for MIC_{50} and MIC_{90} respectively.

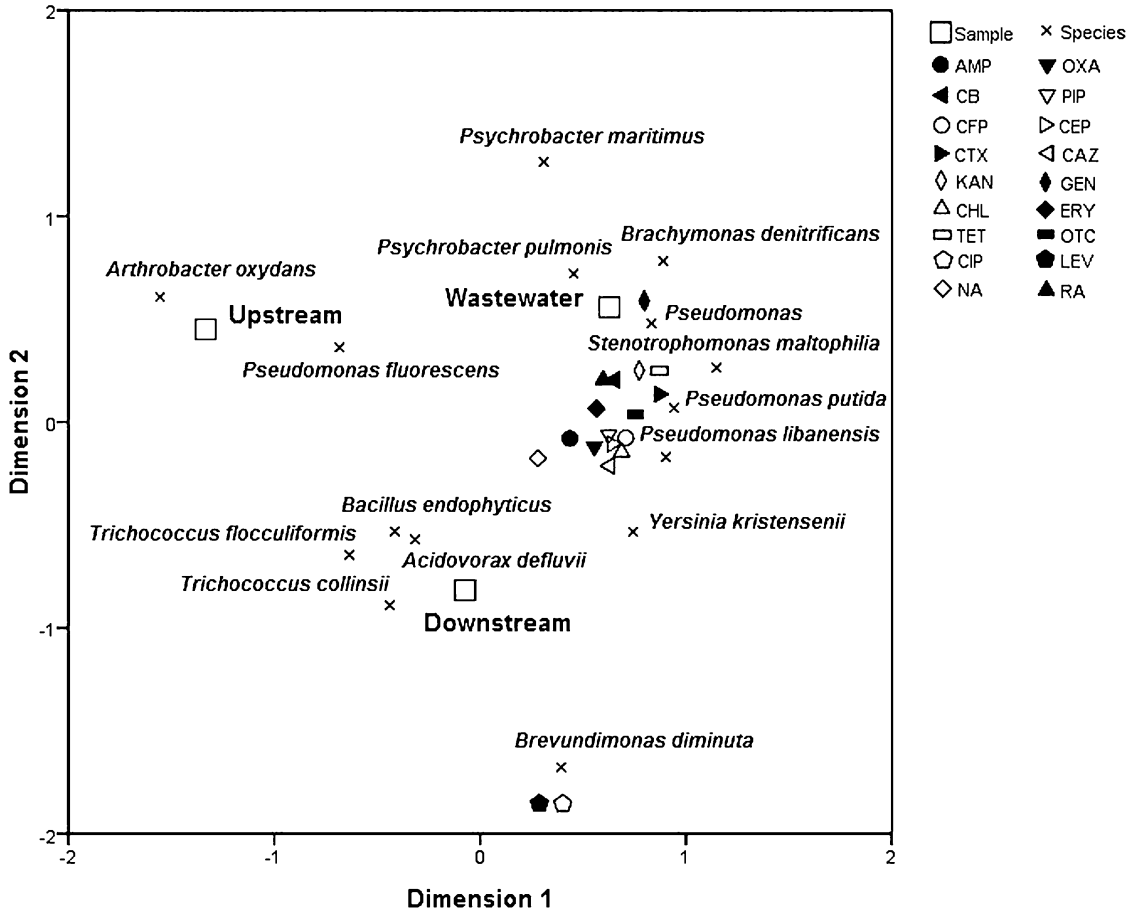

Fig. 2. Multiple correspondence analysis of water samples, bacterial species and antibiotic resistance to 18 antibiotics. Only bacterial species with sufficient sample sizes were shown.

and upstream river samples. In addition, with only the bacterial species containing sufficient sample sizes shown, the strains of *Pseudomonas putida*, *Pseudomonas libanensis*, *Stenotrophomonas maltophilia*, *Pseudomonas* and *Yersinia kristensenii* were comparably more closely related to most antibiotic resistances than the other species. The CIP and LEV resistances were distinctly different from the other 16 antibiotic resistances.

Antibiotic resistance levels

The antibiotic resistance levels for the strains in the three water samples of this study were indicated by the MIC_{50} and MIC_{90} values, as shown in Table 2. The MIC_{50} values of all antibiotics were significantly higher in the penicillin production wastewater effluent than in the downstream and upstream river (Wilcoxon matched-pair test, both $P < 0.001$), and it was also significantly higher in downstream river than in upstream river ($P < 0.001$). For the MIC_{90} of all antibiotics, the values in the wastewater effluent and downstream river were similar (Wilcoxon matched-pair test, $P = 0.625$), and both were significantly higher than those in the upstream river (both $P < 0.001$). Additionally, the values obtained for β-lactams were extremely high in wastewater effluent and downstream river. The MIC_{50} and MIC_{90} values of all β-lactams were greater than 1024 mg l^{-1} in wastewater, with MIC_{50} ranging from 32 to greater than 1024 mg l^{-1} and MIC_{90} all greater than 1024 mg l^{-1} in downstream river water. When compared with β-lactams, the MIC_{50} and MIC_{90} values of the other class antibiotics were significantly lower in these two water samples (Mann–Whitney *U*-test, all $P < 0.002$). The MIC_{50} ranged from 0.25 to 256 mg l^{-1}, and the MIC_{90} were from 2 to greater than 1024 mg l^{-1} in wastewater effluent and downstream river. No significant difference in the MIC_{50} and MIC_{90} values was observed between β-lactams and the other class antibiotics in the upstream water samples (Mann–Whitney *U*-test, $P = 0.255$ and 0.236 respectively).

For particular bacterial species with sufficient sample sizes in the penicillin production wastewater effluent, the MIC_{50} values of all antibiotics differed significantly, as well

as for the species in downstream river (Kendall's W matched-pair test, both $P < 0.001$). Similar to the results of the antibiotic resistance ratios, the MIC_{50} values for the strains of *Pseudomonas fluorescens*, *Pseudomonas libanensis* and *Brachymonas denitrificans* were significantly higher than those for *Bacillus endophyticus* and *Brevundimonas diminuta* in wastewater effluent (Wilcoxon matched-pair test, all $P < 0.03$), and the MIC_{50} values for *Pseudomonas fluorescens*, *Pseudomonas putida* and *Yersinia kristensenii* were significantly higher than those for *Trichococcus collinsii*, *Bacillus endophyticus*, *Trichococcus flocculiformis* and *Acidovorax defluvii* in downstream river water (Wilcoxon matched-pair test, all $P < 0.01$). Moreover, the difference in the MIC_{90} values for particular bacterial species in both wastewater effluent and downstream river water were not as significant as that in the MIC_{50} values (Kendall's W matched-pair test, $P = 0.153$ and 0.014 respectively). When pooling all the bacterial isolates from the three water samples together, the isolates of *Stenotrophomonas maltophilia* (eight strains) showed high resistance to β-lactams, with the MICs generally greater than 1024 mg l^{-1} only except CAZ (64 to >1024 mg l^{-1}). Similar results were also observed for the other five strains of *Stenotrophomonas* spp., including two of *Stenotrophomonas koreensis*, one of *Stenotrophomonas acidaminiphila* and two *Stenotrophomonas* sp. strains.

Multiple antibiotic resistance

Multiple antibiotic resistance (MAR) was dominant for the strains isolated from the penicillin production wastewater effluent, with the ratio up to 97.8%, and nearly 80% of the strains showed co-resistance to 10 or more kinds of antibiotics (Fig. 3). The number of antibiotics to which strains were resistant was significantly lower in downstream river (mean = 8.6 ± 0.4, range = 0–17) than in wastewater effluent (mean = 11.5 ± 0.3, range = 0–17) (Mann–Whitney *U*-test, $P < 0.001$). However, the MAR ratio was still up to 87.7% for the strains in downstream water, with more than 50% of strains being co-resistant to 10 or more kinds of antibiotics. The number of antibiotics to which strains were resistant in downstream river was also significantly higher than in the upstream river (mean = 2.2 ± 0.2, range = 0–10) (Mann–Whitney *U*-test, $P < 0.001$).

Further more, co-resistance to antibiotics from the same class was also frequently observed in this study. Specifi-

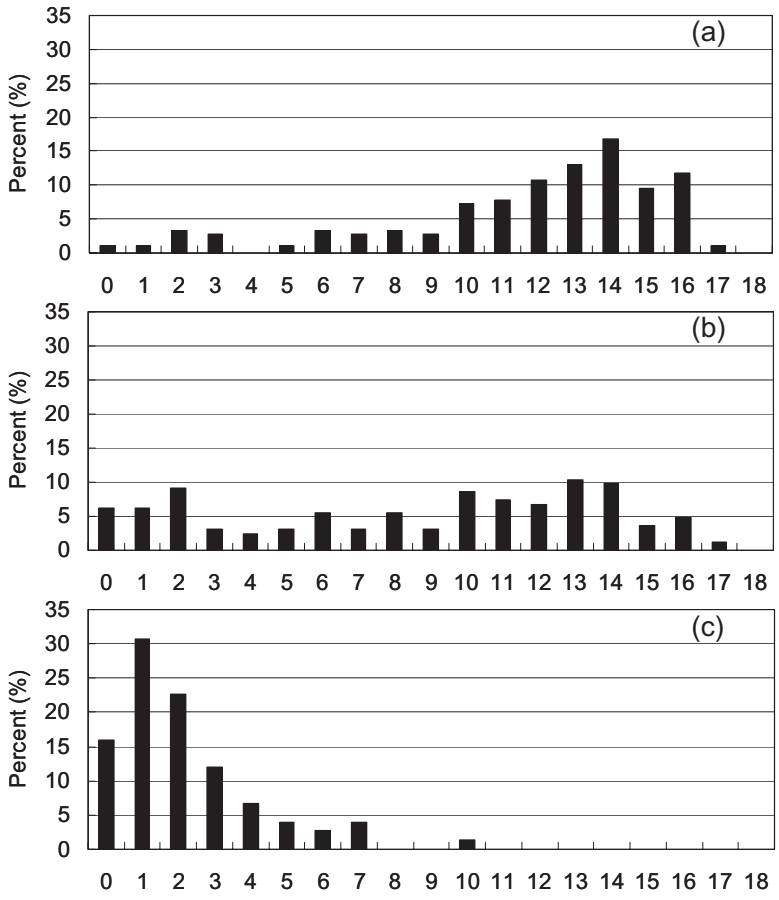

Fig. 3. Multiple antibiotic resistance in penicillin production wastewater effluent (A), downstream (B) and upstream (C) river samples. The *x*-axis represents the number of antibiotics to which an isolate was resistant, and the *y*-axis represents the percentage of that resistance count.

cally, co-resistance was observed when all β-lactam antibiotics (Spearman correlation coefficient, all $r_s > 0.401$, all $P < 0.001$, $n = 406$), aminoglycosides ($r_s = 0.695$, $P < 0.001$, $n = 406$), tetracyclines ($r_s = 0.542$, $P < 0.001$, $n = 406$), and CIP and LEV ($r_s = 0.903$, $P < 0.001$, $n = 406$) were evaluated. Nevertheless, co-resistance to unrelated antibiotics was also common, with resistances significantly correlated ($P < 0.001$), such as CTX and RIF ($r_s = 0.593$, $P < 0.001$, $n = 406$), erythromycin (ERY) and chloramphenicol (CHL) ($r_s = 0.519$, $P < 0.001$, $n = 406$) and so on (Supporting Information, Table S1). Then, among strains from the treated penicillin production wastewater, the most frequent resistance pattern for antibiotics, with the exception of β-lactams, was the combination of kanamycin (KAN), gentamicin (GEN), ERY, CHL, oxytetracycline (OTC), tetracycline (TET), NA and rifampin (RA), with the ratio of 11.2%, followed by that of ERY, CHL, OTC, TET, NA and RA (8.4%), KAN, ERY, CHL, OTC, TET, NA and RA (6.1%), and KAN, GEN and RA (5.6%). Among the downstream strains, the resistance patterns for antibiotics except for β-lactams were rather diverse, with a ratio of each pattern less than 4%.

Significant differences also existed in the number of antibiotics to which particular species were resistant in both penicillin production wastewater effluent and downstream river water (Kruskal–Wallis test, both $P < 0.001$). The strains of *Pseudomonas fluorescens* showed the highest co-resistance number (mean value = 13.9 ± 0.4) in wastewater, followed by *Pseudomonas libanensis* (mean value = 13.3 ± 0.9), while the co-resistance numbers for the strains of *Bacillus endophyticus* (mean value = 7.8 ± 1.9) and *Brevundimonas diminuta* (mean value = 9.4 ± 0.8) were the lowest. Then in downstream river, mean co-resistant numbers were all greater than 11 for *Pseudomonas putida*, *Pseudomonas fluorescens*, *Yersinia kristensenii* and *Brevundimonas diminuta*, which were significantly higher than those for the remaining species, including *Acidovorax defluvii*, *Bacillus endophyticus*, *Trichococcus flocculiformis* and *Trichococcus collinsii* (mean values ranging from 5 to 6). In upstream river, the mean value of co-resistance number for *Pseudomonas fluorescens* was slightly higher (2.5 ± 0.3) than *Arthrobacter oxydans* (1.6 ± 0.4), although the difference had not approached significant.

Resistance genes and class I integrons

All isolates were screened for the β-lactamase coding genes, bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{IMP} and $bla_{CTX\text{-}M}$. Only bla_{TEM} was found in 31 strains (17.3%) from the penicillin production wastewater effluent and 18 strains (11.0%) from the downstream river (Table 3). The positive wastewater isolates mainly included the species *Brevundimonas diminuta* ($n = 6$), *Psychrobacter pulmonis* ($n = 6$) and *Brachymonas denitrificans* ($n = 5$), while the positive downstream strains were mainly identified as *Brevundimonas diminuta* ($n = 5$). To the best of our knowledge, most of the species containing bla_{TEM} in this study have

Table 3. Distribution of bla_{TEM} gene and class I integron gene cassettes among isolates from penicillin production wastewater effluent and downstream river.

	No. of bla_{TEM}-positive strains		Class I integron gene cassette (no.)	
Genus or species	Effluent	Downstream	Effluent	Downstream
Paracoccus versutus			*aadB*, *qacH* (1)	
Brevundimonas diminuta	6	7	*aadA4a* (2); *aadB*, *qacH* (1)	*aadA4a* (1)
Brachymonas denitrificans	5	1	*qac* (1); *aadA4a* (2); *aadB*, *qacH* (3)	*aadB*, *qacH* (2)
Simplicispira psychrophila			*aadA11b* (1)	
Acidovorax defluvii		2		*aadA4a* (1); *aadA5* (3); *aadA1* (1)
Stenotrophomonas acidaminiphila	1		*aadB*, *qacH* (1)	
Stenotrophomonas spp.			*aadA4a* (1)	
Pseudomonas fluorescens	2	1	*aadA4a* (2)	
Pseudomonas libanensis	1			
Pseudomonas putida	1	1		*qac* (1); *orfE* (1)
Pseudomonas lundensis	1			
Pseudomonas spp.	1		*aadA4a* (1)	
Acinetobacter johnsonii	1		*aacA4* (1)	
Psychrobacter pulmonis	6		*aacA4* (1); *aadA4a* (1); *aadB*, *qacH* (2)	
Psychrobacter maritimus	2		*aadA4a* (1); *aadB*, *qacH* (1)	
Shewanella putrefaciens	1		*aadA4a* (1)	
Bacillus endophyticus	2	2	*aadA1* (1)	*aadA1* (1); *aadA11b* (1)
Bacillus simplex		1		
Trichococcus flocculiformis		1		*qac* (1); *aadA11b* (1)
Trichococcus collinsii		2		*aadA4a* (1); *aadA11b* (1)
Flavobacterium spp.	1			
Total	31	18	25	16

not been described before, such as *Brevundimonas diminuta*, *Brachymonas denitrificans*, *Psychrobacter* spp., *Bacillus endophyticus*, *Bacillus simplex*, *Acidovorax defluvii*, *Stenotrophomonas acidaminiphila*, *Acinetobacter johnsonii*, *Pseudomonas libanensis*, *Pseudomonas lundensis*, *Trichococcus* spp. and *Flavobacterium* spp., indicating that bla_{TEM} was widespread in environmental bacteria. Furthermore, the PCR product of bla_{TEM} was sequenced in all bla_{TEM}-positive strains. The deduced amino acid sequence was found to be the same as the TEM-1 reported by Sutcliffe (Sutcliffe, 1978), only with three silent nucleotide substitutions (C18T, C228T and G396T, according to Sutcliffe's numbering system) in several sequences compared with the bla_{TEM-1} sequence (Sutcliffe, 1978), indicating that bla_{TEM-1} is relatively conservative across environments. All bla_{TEM-1}-positive strains showed resistance to AMP at least.

Class I integrons were found in 14% (25 of 179) of the isolates from the penicillin production wastewater effluent and 9.1% (15 of 165) of the isolates from the downstream river (Table 3). The strains from the upstream river didn't contain any integrons. Eighteen and eight integron-positive strains individually isolated from the wastewater effluent and downstream river also contained bla_{TEM-1}. Most of the integrons contained gene cassettes with the following aminoglycoside-resistance genes: *aadA* genes (including *aadA1*, *aadA4a*, *aadA5* and *aadA11b*) encoding aminoglycoside adenyltransferase, which confers streptomycin and spectinomycin resistances; *aadB* encoding aminoglycoside (2′) adenylyltransferase, which confers gentamicin, tobramycin and kanamycin resistances; and *aacA4* encoding aminoglycoside-3′N-acetyltransferase, which confers gentamicin and tobramycin resistances. Furthermore, *qacH* gene which is a multidrug exporter conferring resistance to quaternary ammonium compounds was always detected together with *aadB*, and there were also three strains containing *qac*.

Discussion

The β-lactam antibiotics tested in this study included penicillins, the first and third generations of cephalosporins, whereas most of the strains in the penicillin production wastewater effluent showed resistance to nearly all β-lactam antibiotics evaluated in this study, with resistance levels (mostly > 1024 mg l^{-1}) much higher than those reported in several clinical and environmental studies (generally < 256 mg l^{-1}) (Edelstein *et al*., 2003; Messi *et al*., 2005). The resistance to β-lactams may be due to several mechanisms, and the major one was the presence of β-lactamases, which could hydrolyse the β-lactam ring of β-lactams. Several hundreds of β-lactamase have been found and the most clinically important β-lactamase are extended-spectrum β-lactamases (ESBLs) until now, which are primarily derivates of several existent β-lactamases such as TEM and SHV, with several amino acid substitutions (http://www.lahey.org/studies/webt.htm), and could effectively hydrolyse the third-generation cephalosporins. In this study, most of the strains in the wastewater effluent showed high resistance to the third-generation cephalosporins including cefoperazone (CFP), CTX and CAZ. Resistance genes coding for the majority of ESBLs including TEM, SHV, OXA, IMP and CTX-M were checked, whereas only bla_{TEM-1} was present in less than 20% of the strains, which is even lower than the ratio reported in several environmental studies (Henriques *et al*., 2006). Considering that bla_{TEM-1} didn't belong to ESBL, resistance genes seemed not to play an important role in conferring resistance to the β-lactam antibiotics evaluated in this study.

Furthermore, the isolates from the penicillin production wastewater effluent also showed co-resistance to many other classes of antibiotics, such as aminoglycosides, macrolides, phenicols and ansamycins. Considering the fact that the WWTP was primarily applied to treat penicillin G production wastewater, it is really surprising that the isolates had such wide co-resistance to many unrelated antibiotics, although the resistance levels to the other classes of antibiotics were much lower than those to β-lactams. Similar results have been observed in the intestinal flora of chickens and farm personnel after feeding chickens tetracycline on farms (Levy *et al*., 1976), which had led to the emergence of multiple resistant bacteria in chickens and farm dwellers, especially with lengthened exposure time of chickens to tetracycline. Compared with antibiotic-resistance genes or mutations of antibiotic target sites in bacterial cells that usually lead to resistance to antibiotics from one class, membrane-bound multidrug efflux pumps have been reported able to transport a wide range of antibiotics from different classes with few structural similarities, and have been recognized as a major reason for a number of significant human pathogens resistant to multiple antibiotics, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Stenotrophomonas maltophilia* and the non-pathogen *Pseudomonas putida* (Putman *et al*. 2000). Therefore, the wide distribution of co-resistance to many antibiotics from different classes in wastewater isolates of this study were thus speculated to be caused by multidrug transporters such as *qacH* and *qac* carried by class I integrons in several species of this study. In addition, several *Stenotrophomonas maltophilia* and *Pseudomonas putida* strains have also been isolated in this study and indeed showed high resistance to multiple antibiotics, confirming the existence of multidrug efflux systems to some extent. However, there is still little information available regarding

multidrug efflux systems for most indigenous environmental species until now, and more researches are needed in the future.

Integrons, especially class I integrons, commonly contained antibiotic-resistance gene cassettes, including β-lactamase determinants (Weldhagen, 2004), and have been found to be closely related to MAR of bacteria, as they usually contain several antibiotic-resistance gene cassettes simultaneously (Mazel, 2006). However, the gene cassettes detected in this study mainly conferred resistance to aminoglycoside antibiotics, with no β-lactamase determinants detected and the ratios of class I integrons in treated penicillin production wastewater and downstream river strains were similar to those of previous reports in normal aquatic environments (Schmidt *et al*., 2001; Henriques *et al*., 2006). Thus it seems that the spread of class I integrons have not been significantly promoted in bacterial species isolated from antibiotic-containing environments in this study, and the results furthermore support the speculation that resistance genes like β-lactamase determinants may not be the primary cause of the pervading resistance to antibiotics that was observed in different environmental species in this study.

It should be noted that the strains isolated from the upstream river also showed some resistance to antibiotics. Although penicillin G was undetectable in the upstream water, there may still be some unknown sources of antibiotics or resistance strains such as quinolones, which are usually used as veterinary drugs (Ruiz, 2003). Meanwhile, some researches have indicated that indigenous bacteria in unpolluted environments can also exhibit some antibiotic resistance (D'Costa *et al*., 2006; Dantas *et al*., 2008). Thus, the antibiotic resistance observed in the indigenous species isolated from the upstream river in this study is not surprising. Similar results have also been obtained in previous researches (Kelch and Lee, 1978; Ash *et al*., 2002). Anyway, significant differences have been found in antibiotic resistance between the upstream and downstream samples, which demonstrates that the discharge of treated penicillin production wastewater contributed to the antibiotic resistance in the receiving river.

Experimental procedures

Study site and sampling

The antibiotic production facility located in Hebei Province, China is the largest producer of penicillin G in Asia, with an annual production of approximately 7000 tons. The penicillin G production wastewater from this facility is treated in a biological system consisting successively of an anaerobic digestion, a hydrolysation and two aerobic reactors. Small volumes of several other chemical production wastewaters including avermectins, ivermectin and several kinds of semi-synthetic antibiotics are also treated in this plant. The treated effluent is discharged into the Wangyang River without chlorination. In December 2004, April and August 2005, the wastewater effluent from the WWTP and surface water samples from upstream and downstream of the wastewater discharge point in the Wangyang River were taken in 4 l brown glass bottles and kept at 4°C in darkness for at most 2 days before analysis. The upstream and downstream sampling sites were approximately 5 km and 30 km away from the discharge point respectively. In addition, raw wastewater and effluent samples from each treatment reactor were taken for the analysis of the residual penicillin G. The penicillin G concentrations were then determined using liquid chromatography-electrospray ionization mass spectrometry following a previously described analytical method (Li *et al*., 2008).

Bacterial counts, isolation and identification

Total cell counts were roughly determined by membrane filtration and staining with 4′,6-diamidino-2-phenylindole as described before (Wagner *et al*., 1993). Serial 10-fold dilutions of water samples were prepared in physiological saline, and 0.1 ml aliquots were inoculated onto TSA and R2A non-selective agar media. The plates were incubated aerobically at 30°C for 24 h and up to 10 colonies with different colony morphologies were recovered from each plate. After restreaked for three times and checked for purity microscopically, the isolates were maintained at −80°C in Tryptic soy broth containing 15% glycerol. Colony-forming unit counts were meanwhile determined on TSA.

For the identification of bacterial isolates, the 16S rRNA gene from pure cultures was amplified and sequenced using bacterial universal primers 27F and 1492R (Supporting Information, Table S2). Bacterial DNA used for PCR was prepared by boiling culture cells for 10 min following by centrifugation, and lysozyme (50 mg ml^{-1}) was added and incubated at 37°C for 30 min if needed. The standard 50 μl PCR mixture (Takara, Dalian, China) included 1× PCR buffer containing 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 10 pmol of each primer, 1.25 U of TaKaRa rTaq polymerase, and 1 μl of boiled bacterial culture as template DNA. Polymerase chain reaction conditions were as follows: 95°C for 10 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min 30 s, and a final extension at 72°C for 10 min. After confirmed by electrophoresis in 1.2% (w/v) agarose gel, amplification products were purified with the Qiaquick PCR cleanup kit (Qiagen, Chatsworth, CA) following the manufacturer's instructions, and then digested (3 h, 37°C) with HaeIII (Takara, Dalian, China) and separated by electrophoresis through 2% agarose gels. Sequences were grouped according to their RFLP patterns, and representative PCR products were sequenced with an ABI 3730 automated sequencer (Invitrogen, Shanghai, China). Then isolates were identified by phylogenetic analysis of the 16S rRNA gene sequences with the Ribosomal Database Project II release 9.49 and the GenBank database using the BLASTN program (Altschul *et al*., 1997; Cole *et al*., 2007).

Antibiotic susceptibility testing

The MICs of antibiotics for the bacterial isolates were determined by a standard twofold broth microdilution method using Mueller–Hinton broth according to the CLSI Standards guidelines (Clinical and Laboratory Standards Institute, 2003), with antibiotic concentrations ranging from 0.25 to 1024 mg l^{-1}. The following 18 antibiotics representing seven classes all purchased from Sigma-Aldrich were tested: β-Lactams including AMP, piperacillin, carbenicillin, OXA, cephalothin, CFP, CTX and CAZ; aminoglycosides including KAN and GEN; macrolides including ERY; phenicols including CHL; tetracyclines including OTC and TET; quinolones including CIP, LEV and NA; and ansamycins including RA. *Escherichia coli* ATCC 25922 and ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls.

Resistance genes and class I integrons detection

The presence of five clinically important β-lactam-resistance genes encoding β-lactamase TEM, SHV, OXA, IMP and CTX-M and class I integron in bacterial isolates was determined using PCR method with primers listed in Table S2. Bacterial DNA as template and the standard PCR mixture (50 μl) were the same as above in *Bacterial counts, isolation and identification*. Polymerase chain conditions for the five β-lactam resistance genes were also the same as described above except for various annealing temperatures listed in Table S2. Polymerase chain method described by Lévesque and colleagues (1995) was used for class I integron with primers 5′-CS and 3′-CS. Amplicons were visualized in 2% (w/v) agarose gel stained by ethidium bromide, and purified with the Qiaquick PCR cleanup kit (Qiagen, Chatsworth, CA). Representative bands were sequenced, and the nearest matches were determined in the GenBank database using the BLASTN program.

Statistical analysis

Similarities of bacterial compositions between penicillin production wastewater effluent, downstream and upstream river samples were assessed by Chao's Jaccard Raw Abundance-based similarity index using the software program EstimateS version 8.0 (Colwell, 2005). Then the other statistical analyses were all performed by using the SPSS version 16.0 release.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in this paper could be accessed in the GenBank database under the Accession No. EU434343 to EU434599 for the 16S rRNA genes of the bacterial isolates, EU433996 to EU433997 for the bla_{TEM} genes and EU434600 to EU434618 for the class I integron genes.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Spearman correlation coefficients of co-resistances to antibiotics for all bacterial.

Table S2. Primers and conditions used to amplify five *bla* genes, class I integrons, and 16S rRNA genes by PCR technique.

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