Antibiotic resistance among aquatic bacteria in natural freshwater environments of Korea

Tae Woon Kim, Yochan Joung, Ji-Hye Han, Wonwha Jung and Seung Bum Kim

ABSTRACT

The taxonomic diversity and antibiotic resistance among freshwater bacterial communities in the major water bodies of Korea was examined using 437 penicillin-resistant, and 110 tetracycline-resistant bacterial isolates. Based on 16S rRNA gene sequence analysis, most isolates were assigned to Proteobacteria, which was then followed by Bacteroidetes. Strains of *Aeromonas* were found as the most abundant penicillin-resistant populations, whereas those affiliated to diverse species including enteric groups were found as the most abundant tetracycline-resistant populations. Most strains exhibited multiple antibiotic resistance, and all tested strains were resistant to penicillin and hygromycin. High levels of resistance were observed for antibiotics acting on cell wall synthesis, whereas low levels were for those acting on DNA replication or transcription in general. It is apparent from this study that penicillin resistance is widespread among environmental bacteria, although the antibiotic has been generally non-detectable in the environment. It is also likely from the taxonomic composition of the resistant communities that various sources including terrestrial animals and humans may contribute to antibiotic resistance in the freshwater environment.

Key words | *Aeromonas*, penicillin, Proteobacteria, resistome, tetracycline

Tae Woon Kim
Yochan Joung
Ji-Hye Han
Seung Bum Kim (corresponding author)
Department of Microbiology and Molecular Biology, College of Bioscience and Biotechnology,
Chungnam National University,
99 Daehak-Ro, Yuseong,
Daejeon 305-764,
Republic of Korea
E-mail: *sbk01@cnu.ac.kr*

Wonwha Jung
National Institute of Environmental Research,
Incheon,
Korea

INTRODUCTION

Since the first use of antibiotics in the 1940s, a huge number of antibiotics have been discovered and many of them have been mass produced for pharmaceutical and agricultural purposes. Recent reports indicate the continuous increase of antibiotic production and use worldwide (Sarmah *et al.* 2006; Hamad 2010). Antibiotics are naturally occurring substances, and thus antibiotic resistance would also be present within the natural microbial community. However, the widespread and extensive use of antibiotics in hospitals, agriculture and aquaculture exerts more selective pressures on environmental microbes, and may accelerate evolution and dissemination of antibiotic resistance. The spread of antibiotic resistance among pathogenic microbes is an obvious example of microbial evolution in action, which can pose a significant problem to humans and livestock. The evolution and dissemination of antibiotic resistance among bacteria in environment has been well reviewed (Baquero & Blazquez 1997; Gomez-Lus 1998; Alonso *et al.* 2001; Normark & Normark 2002; Baquero *et al.* 2008; Davies & Davies 2010; Young *et al.* 2013).

The mechanisms of antibiotic resistance would be diverse, such as alterations of target sites, efflux of antibiotics, or their degradation or modifications, and the target sites for antibiotics include large or small subunit ribosomes, cell membranes, enzymes for nucleic acid synthesis such as DNA or RNA polymerases, components involved in cell wall biosynthesis, or components of metabolic pathways such as that of folate metabolism (D'Costa *et al.* 2006; Davies & Davies 2010). Mutations or transmission of resistance genes by horizontal gene transfer involving plasmid- or phage-mediated processes are considered the main genetic basis for the evolution and dissemination of resistance genes (Davies & Davies 2010).

The term resistome has been used to describe the total complements of antibiotic resistance in the environment,

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which is known to span all known classes of natural and synthetic antibiotics (D'Costa *et al.* 2006; Wright 2007). Antibiotic resistance in various environments can be intrinsic, or due to anthropogenic activities (Davies & Davies 2010; Martinez 2012; Vaz-Moreira *et al.* 2014). Antibiotic resistance genes from pathogens may comprise a small fraction of the resistome, and resistant genes from non-pathogenic bacteria include those from antibiotic producers and 'cryptic resistance genes' (Wright 2007). Studies indicate that many of the environmental bacteria are likely resistant to multiple antibiotics (D'Costa *et al.* 2007; Davies & Davies 2010; Diene & Rolain 2013). Thus, natural antibiotic resistance would no doubt be present in a considerable amount within environmental bacterial communities, although more studies are necessary to understand the nature of such resistance.

There have been some studies on antibiotic resistant bacteria in the natural or artificial freshwater environment (Ash *et al.* 2002; Lobova *et al.* 2002; Schwartz *et al.* 2003; Papadopoulou *et al.* 2008; Moore *et al.* 2010a, b; Falcone-Dias *et al.* 2012; Ozaktas *et al.* 2012; Marti *et al.* 2013), and there are a few reviews on the antibiotic resistance in the freshwater environment (Baquero *et al.* 2008; Kummerer 2009; Vaz-Moreira *et al.* 2014). However, not much is known on the taxonomic distribution of antibiotic resistant bacteria and their resistance to multiple antibiotics in natural freshwater bodies.

This study primarily focuses on the concentration and taxonomic diversity of antibiotic resistant bacteria using a culture-based approach and also the resistance potentials to various antibiotics in the natural freshwater environment of Korea. Two antibiotics, penicillin and tetracycline, were employed for the detection and isolation of resistant bacteria. For the representative isolates, resistance to multiple antibiotics were tested and compared to examine natural antibiotic resistance among the aquatic freshwater community.

MATERIALS AND METHODS

Samplings

Water samples were collected at 2 month intervals from April to October in 2012. Samples were taken from the surface water at two sites from each of the major inland water bodies of Korea, namely the Keum River (GPS N36.4706/E127.4690, N36.4586/E127.4018), Nakdong River (N36.1126/E128.3982, N36.0502/E128.2341), Lake Soyang and Juam Reservoir (N34.5856/E127.1308, N34.5950/E127.0806), respectively. The water bodies are the major sources of drinking water for each region, and the sampling sites were located in the upstream regions of each water body so as to minimize effects of anthropogenic activities. The water samples were kept at 4 °C and transported to the laboratory for immediate analysis.

Determination of viable counts and isolation of bacteria

A volume of 100 μL from each sample was inoculated onto the Mueller Hinton agar plate (17.5 g casein acid hydrolysate, 2 g beef extract, 1.5 g starch and 17 g agar/L D.W.) supplemented with either penicillin G or tetracycline at 0.1 mg/mL concentration, and incubated at 37 °C for 2 days for viable counts. The counts for each sample were calculated from duplicate plates at two different dilution rates. Variations in viable counts were statistically evaluated using the Student *t*-test. Significance level was set at P values of <0.05.

Taxonomic identification of isolated bacteria

For isolation of bacteria, single colonies were picked and streaked on fresh Mueller Hinton agar plates. Isolates were subcultured twice to check the purity. Bacterial colonies were suspended in 1 mL 80% extraction buffer and were subjected to boiling for polymerase chain reaction (PCR) amplification of 16S rRNA genes. The 16S rRNA gene of the cells was PCR amplified and purified as described previously (Park *et al.* 2005). The obtained 16S rRNA gene sequences were identified using the EzBioCloud server (http://www.ezbiocloud.net/eztaxon/) (Kim *et al.* 2012).

Antibiotic resistance profiling of representative isolates

Selected strains of penicillin- and tetracycline-resistant isolates were tested against tetracycline and penicillin as well as 11 additional antibiotics, namely amikacin, ampicillin, chloramphenicol, ciprofloxacin, erythromycin, hygromycin, gentamycin, kanamycin, novobiocin, rifampicin and

trimethoprim (Sigma-Aldrich, USA) at a fixed concentration of 50 μg/mL. Antibiotic containing Mueller Hinton agar plates were prepared, and 10 μL aqueous suspension of strains ($A_{600} = 0.3$) were spotted onto the plates. Plates were incubated overnight at 37 °C. Any growth was recorded after the incubation for 18–24 hours.

RESULTS

Viable counts of antibiotic resistance bacteria

The viable counts of penicillin-resistant bacteria in four water bodies over four sampling times were in the range of 27 ± 11 and $3.3 \pm 2.8 \times 10^4$ CFU/mL (average $= 2.7 \pm 2.1 \times 10^3$), and those of tetracycline-resistant bacteria were between 0 and 99 ± 46 CFU/mL (average $= 23 \pm 7.4$) CFU/mL. The distribution of antibiotic resistant populations differed among water bodies, as the highest average viable count of $9.0 \pm 7.0 \times 10^3$ CFU/mL was recorded in Keum River for penicillin-resistant bacteria, while that of 38 ± 18 CFU/mL was recorded in Lake Soyang for tetracycline-resistant bacteria (Figure 1). The Juam Reservoir recorded lowest average counts in both cases, as the averages were $3.6 \pm 0.76 \times 10^2$ CFU/mL for penicillin-resistant bacteria and 12 ± 8.5 CFU/mL for tetracycline-resistant bacteria. The average count of penicillin-resistant bacteria in the lotic waters was $5.1 \pm 3.8 \times 10^3$ CFU/mL, which was comparable to that in the lentic waters ($3.7 \pm 0.58 \times 10^2$ CFU/mL). There was no statistically significant difference in the average counts of tetracycline-resistant bacteria between the lotic (20 ± 9.2 CFU/mL) and lentic (25 ± 11 CFU/mL) waters. There was no apparent correlation between the counts of penicillin-resistant and tetracycline-resistant bacteria as the correlation coefficient between them was 0.262. Seasonal variations were observed for penicillin-resistant bacteria in the two rivers, as the counts were lowest in April and highest in August, but no clear trend could be observed in lentic waters (Figure 1).

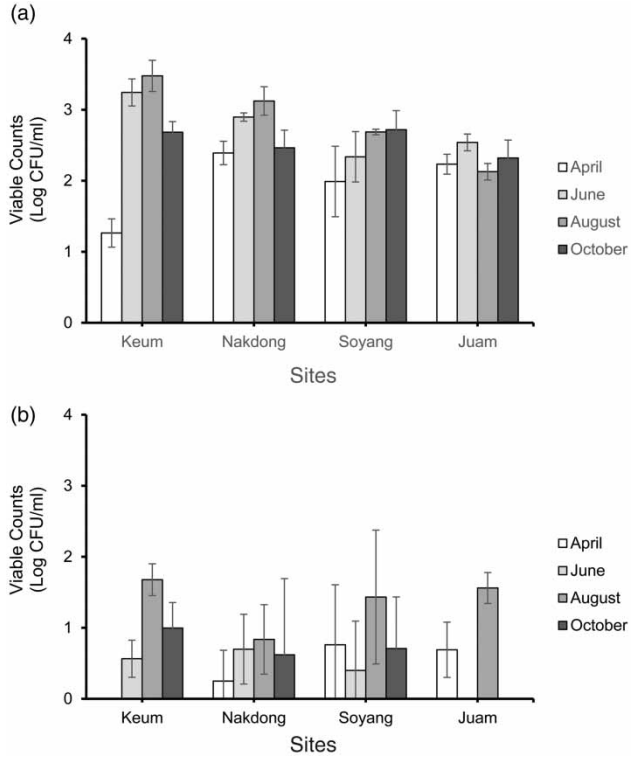

Figure 1 | Viable counts of (a) penicillin-resistant and (b) tetracycline-resistant bacteria in the major water bodies.

Taxonomic composition of antibiotic resistant bacteria

Based on 16S rRNA gene sequence analysis, the penicillin-resistant isolates were assigned to Proteobacteria (94.1% of the total isolates), Bacteroidetes (4.3%), Firmicutes (0.9%) and Actinobacteria (0.7%) (Figure 2(a)). Gammaproteobacteria were the majority, comprising 82.2% of the total isolates, which was then followed by Betaproteobacteria (8.2%) and Alphaproteobacteria (3.7%). The tetracycline-resistant isolates were assigned to two phyla, Proteobacteria (80.0%) and Bacteroidetes (20.0%) (Figure 2(b)). Gammaproteobacteria were again the majority, comprising 68.2% of the total isolates, which was followed by Betaproteobacteria (10.9%) and Alphaproteobacteria (0.9%).

Aeromonas (47.1%) and *Pseudomonas* (19.0%) were found as the major penicillin-resistant genera (Figure 2(c)), and 42 other genera including *Acinetobacter* (5.7%), *Enterobacter* (3.0%), *Chryseobacterium* (2.1%) and *Flavobacterium* (2.1%) were also found (Table 1). In contrast, 25 genera including *Acinetobacter* (11.8%), *Chryseobacterium* (11.8%), *Pseudomonas* (10.9%), *Klebsiella* (10.0%), *Serratia* (8.2%), *Elizebethkingia* (7.3%), *Escherichia* (5.5%) and *Providencia* (5.5%) were found as the main tetracycline-resistant genera (Figure 2(d)). *Acinetobacter*, *Aeromonas*,

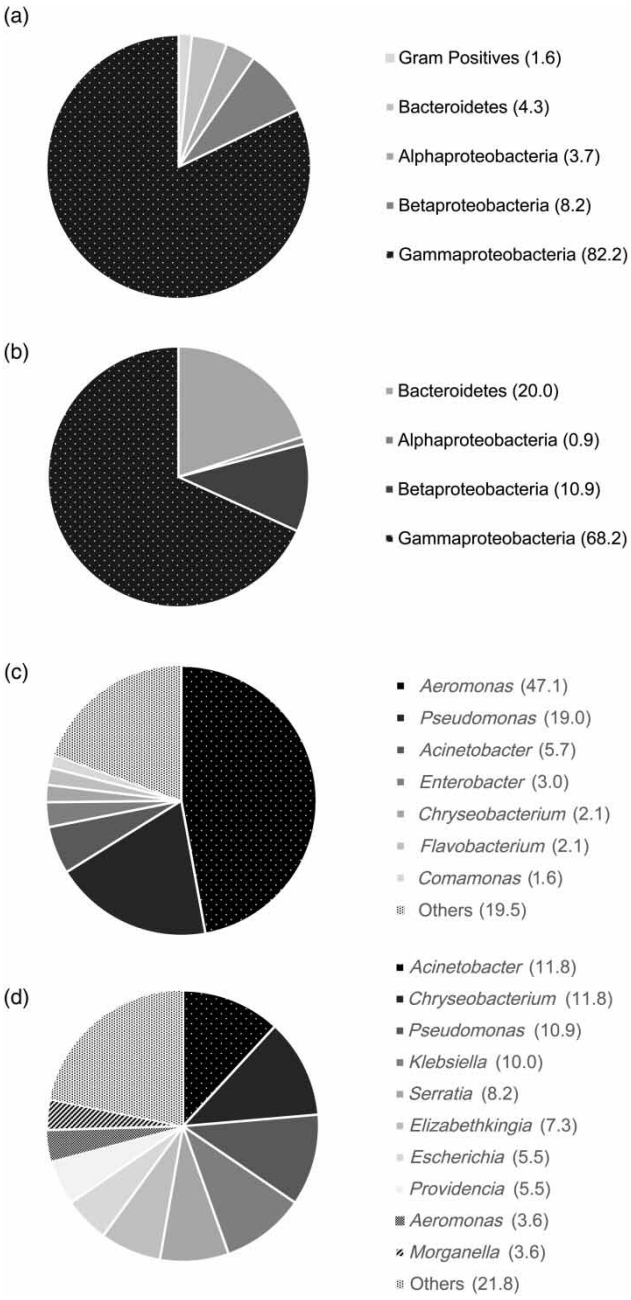

Figure 2 | Taxonomic composition of penicillin-resistant (a) and tetracycline-resistant bacteria (b) in the major water bodies.

Chryseobacterium, *Enterobacter*, *Klebsiella*, *Pseudomonas* and *Serratia* were commonly occurring genera for both penicillin and tetracycline-resistant isolates.

At the species level, the closest matches, not species identity, were searched and recorded since species assignment was not possible using 16S rRNA gene analysis alone. The strains affiliated to *Aeromonas ichthiosmia* (10.1%), *Aeromonas popoffii* (9.6%) and *Aeromonas veronii* (8.2%) were found as the most abundant penicillin-resistant groups. The strains affiliated to *Aeromonas hydrophila* (4.3%), *Pseudomonas koreensis* (4.3%), *Aeromonas jandaei* (3.2%), *Aeromonas media* (2.7%) and *Aeromonas punctata* subsp. *caviae* (2.7%) were also found as the common penicillin-resistant groups. In contrast, the strains affiliated to *Elizabethkingia anopheles* (7.3%), *Acinetobacter bouvetii* (4.5%), *Chryseobacterium indologenes* (4.5%), *Escherichia coli* (4.5%), *Klebsiella pneumonia* subsp. *ozaenae* (4.5%), *Serratia marcescens* (4.5%), *Chryseobacterium joostei* (3.6%) and *Serratia nematodiphila* (3.6%) were found as the most abundant tetracycline-resistant groups.

Antibiotic resistance profile of representative isolates

Selected penicillin-resistant and tetracycline-resistant strains were tested for antibiotic resistance against 12 other antibiotics (Tables 2 and 3). Strains within the same genera generally exhibited similar resistance profiles. The penicillin-resistant strains were least resistant to ciprofloxacin and rifampicin as only 4.7% of the tested strains were resistant to each of these antibiotics. The tetracycline-resistant strains were least resistant to ciprofloxacin (6.7%) and gentamycin (13.3%). Both the penicillin- and tetracycline-resistant strains were highly resistant to penicillin, hygromycin and ampicillin and erythromycin. Notably, all tested strains were resistant to penicillin and hygromycin. The penicillin-resistant strains were also resistant to an average of 4.2 additional antibiotics among 12 tested ones, and the tetracycline-resistant strains were also resistant to an average of 5.9 additional antibiotics.

The strains of *Alcaligenes*, *Chryseobacterium* and *Shigella* generally exhibited the broadest multiple antibiotic resistance, which was then followed by *Escherichia*, *Serratia* and *Pseudomonas* for both antibiotic resistant populations. As for individual strains, however, strain AUNP09, a penicillin-resistant strain affiliated to *Pseudomonas geniculata*, and strain JUD01, a penicillin-resistant strain affiliated to *Raoultella ornithinolytica*, were found as the two broadest multiple antibiotic resistant bacteria, resistant to 10 and nine additional antibiotics, respectively (Table 2). In addition, those affiliated to *E. coli*, *Chryseobacterium arthrosphaerae* and *Alcaligenes faecalis* subsp. *faecalis* were found to

Table 1 | Generic composition of antibiotic resistant bacteria (%)

	Penicillin-resistant				Tetracycline-resistant			
Site	**Keum**	**Nakdong**	**Soyang**	**Juam**	**Keum**	**Nakdong**	**Soyang**	**Juam**
No. of isolates	146	115	100	76	64	35	9	2
Acetobacter	0.7							
Acidovorax	2.7	0.9						
Acinetobacter	11.0	7.8			15.6	8.6		
Aeromonas	35.6	29.6	72.0	63.2	3.1	5.7		
Alcaligenes	0.7				4.7			
Aquitalea	0.7		1.0	1.3				
Asticcacaulis				1.3				
Azospirillum		0.9		2.6				
Bacillus	0.7			3.9				
Burkholderia					3.1			
Caulobacter	1.4	0.9		1.3				
Cedecea				1.3				
Chromobacterium	2.7			2.6				
Chryseobacterium	1.4	5.2		1.3	12.5	14.3		
Citrobacter				2.6				
Comamonas	2.7	2.6				2.9		
Cupriavidus		0.9						
Curtobacterium				1.3				
Dickeya	0.7							
Elizabethkingia	0.7				3.1	11.4		100
Enterobacter	2.1	6.1	1.0	2.6		2.9	11.1	
Escherichia	0.7	0.9			4.7	8.6		
Ewingella							11.1	
Flavimonas								
Flavobacterium	2.1	5.2						
Gluconobacter					1.6			
Haemophilus	2.7		1.0					
Hafnia								
Hydrogenophaga		4.3						
Iodobacter	1.4							
Kinneretia	1.4							
Klebsiella	1.4	2.6			1.6	28.6		
Kluyvera					1.6			
Laribacter					3.1			
Leclercia	1.4							
Massilia	0.7							
Microvirgula				1.3	1.6			

(*continued*)

Table 1 | continued

	Penicillin-resistant				Tetracycline-resistant			
Site	**Keum**	**Nakdong**	**Soyang**	**Juam**	**Keum**	**Nakdong**	**Soyang**	**Juam**
Moraxella							11.1	
Morganella					6.3			
Nocardia				1.3				
Novosphingobium		1.7						
Ochrobactrum		0.9						
Providencia	1.4				9.4			
Pseudomonas	17.1	26.1	22.0	7.9	14.1	8.6		
Ralstonia								
Raoultella	1.4							
Rheinheimera		0.9						
Rhizobium		1.7						
Roseomonas	0.7							
Serratia	0.7	0.9	3.0	1.3	4.7	5.7	44.4	
Shigella					3.1	2.9		
Simplicispira					1.6			
Sphingomonas	0.7							
Staphylococcus								
Streptomyces				1.3				
Variovorax				1.3				
Vogesella	0.7				3.1			
Wautersiella					1.6			
Yersinia	1.4							
Yokenella	0.7						22.2	

exhibit the broadest multiple antibiotic resistance among penicillin-resistant isolates. Among the tetracycline-resistant strains, strain AUDT16 affiliated to *Alcaligenes faecalis*, OCDT03 affiliated to *Chryseobacterium arthrosphaerae*, AUDT13 affiliated to *Morganella morganii* subsp. *sibonii*, AUNT12 affiliated to *Pseudomonas geniculate*, and AUDT20 affiliated to *Shigella flexneri* exhibited the broadest multiple antibiotic resistance (resistant to eight additional antibiotics) among tetracycline-resistant isolates (Table 3).

DISCUSSION

The low viable counts of tetracycline-resistant bacteria compared to the penicillin-resistant bacteria indicate that the tetracycline-resistant populations, exhibiting higher degree of multiple antibiotic resistance, constitute only small proportions within the total communities. This is notable as tetracyclines are used as the major veterinary pharmaceuticals and its presence in detectable concentration in freshwater environment has been reported, while penicillin has been virtually non-detectable in nationwide surveys (Kim *et al.* 2008; Son & Jang 2011). Tetracycline is known as a relatively resilient antibiotic to biodegradation, whereas penicillin is known to be subject to biodegradation more readily than other antibiotics (Gartiser *et al.* 2007; Son & Jang 2011). Another notable finding is that the antibiotic resistant populations in river waters are more diverse in taxonomic compositions than those in lake waters. Moreover, some taxa, namely *Acinetobacter*, *Chryseobacterium*,

Table 2 | Antibiotic resistance profiles of penicillin-resistant isolates

		Antibiotics[a]											
Strain	**Identification**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
AUNP27	*Acinetobacter beijerinckii*	−	+	−	−	+	−	−	−	−	−	−	+
AUDP59	*Acinetobacter calcoaceticus*	−	+	−	−	+	−	−	−	(+)	−	−	−
AUDP15	*Acinetobacter johnsonii*	−	+	−	−	−	−	−	−	ND	−	−	−
AUDP13	*Acinetobacter junii*	−	+	−	−	−	−	−	−	−	−	−	−
AUDP20	*Acinetobacter junii*	−	+	−	−	−	−	−	−	−	−	−	−
AUDP42	*Acinetobacter junii*	−	+	−	−	−	−	−	−	−	−	−	−
AUNP06	*Acinetobacter junii*	−	+	−	+	−	−	−	−	−	−	−	+
AUNP25	*Acinetobacter junii*	−	+	−	+	+	−	−	−	−	−	−	+
AUNP26	*Acinetobacter junii*	−	+	−	−	−	−	−	−	−	−	−	−
AUDP40	*Acinetobacter nosocomialis*	−	+	−	−	+	−	−	−	(+)	−	+	−
AUDP12	*Acinetobacter parvus*	−	+	−	−	−	+	−	−	−	−	+	−
AUNP18	*Acinetobacter parvus*	−	+	−	−	−	+	−	−	−	−	−	−
AUDP02	*Acinetobacter tandoii*	−	+	−	−	−	−	−	−	ND	−	−	+
OCDP14	*Aeromonas jandaei*	(+)	+	−	−	+	−	−	−	+	−	−	−
JUN02	*Aeromonas media*	−	+	−	−	+	−	−	−	+	−	−	+
OCDP12	*Aeromonas popoffii*	−	+	−	−	−	−	−	−	−	−	−	−
OCDP13	*Aeromonas punctata* subsp. *caviae*	+	+	(+)	+	+	+	−	−	+	−		−
OCNP03	*Aeromonas veronii*	−	+	−	+	+	−	−	−	(+)	−	−	−
AUDP25	*Alcaligenes faecalis* subsp. *faecalis*	(+)	+	−	+	+	+	−	+	+	−	−	+
AUDP30	*Chryseobacterium aestuarii*	+	+	+	+	+	−	−	−	+	−	+	−
JUN01	*Chryseobacterium arthrosphaerae*	+	+	+	+	+	+	−	−	+	−	+	−
OCDP15	*Chryseobacterium vietnamense*	+	+	(+)	−	+	+	−	−	+	−	−	−
AUNP23	*Comamonas aquatica*	−	+	−	−	+	−	−	−	(+)	−	−	−
AUNP16	*Comamonas thiooxydans*	−	+	−	−	+	−	−	−	(+)	−	−	−
AUDP61	*Enterobacter asburiae*	−	+	−	−	+	−	−	−	+	−	−	−
OCNP21	*Enterobacter asburiae*	−	+	−	−	+	−	−	−	+	(+)	−	−
AUDP46	*Enterobacter ludwigii*	−	+	−	−	+	−	−	−	+	(+)	−	−
OCDP16	*Enterobacter ludwigii*	−	+	−	−	+	−	−	−	+	(+)	−	−
AUDP57	*Enterobacter mori*	−	+	−	−	+	−	−	−	+	+	−	−
OCNP07	*Escherichia coli*	−	+	(+)	+	+	+	+	−	+	−	−	+
AUDP54	*Klebsiella pneumoniae* subsp. *ozaenae*	−	+	−	−	+	−	−	−	+	−	−	−
AUDP36	*Klebsiella variicola*	−	+	−	−	+	−	−	−	+	−	(+)	−
AUNP32	*Klebsiella variicola*	−	+	−	−	+	−	−	−	+	−	−	−
OCNP02	*Pseudomonas alcaligenes*	−	+	−	+	−	+	−	−	+	−	−	−
OCNP09	*Pseudomonas alcaligenes*	−	+	−	−	+	−		−	−	−	−	−
APN27	*Pseudomonas chlororaphis* subsp. *piscium*	−	+	−	−	+	−	−	−	+	+	+	+
AUNP09	*Pseudomonas geniculata*	+	+	+	+	+	+	(+)	−	+	+	−	+
APN06	*Pseudomonas koreensis*	−	+	−	−	+	−	−	−	−	+	−	+

(*continued*)

Table 2 | continued

Strain	Identification	Antibiotics[a] 1	2	3	4	5	6	7	8	9	10	11	12
OCDP01	*Pseudomonas parafulva*	−	+	−	−	+	−	−	−	−	−	−	+
OCDP02	*Pseudomonas taiwanensis*	−	+	−	+	+	−	−	−	+	−	+	+
JUD01	*Raoultella ornithinolytica*	−	+	−	+	+	+	+	−	+	+	+	+
OCDP32	*Roseomonas cervicalis*	−	+	−	−	+	−	−	−	−	−	−	+
AUNP20	*Serratia marcescens* subsp. *sakuensis*	−	+	−	−	+	+	−	+	+	+	−	−
OCDP08	*Serratia nematodiphila*	(+)	+	−	−	+	+	−	−	+	−	−	−
AUDP11	*Yokenella regensburgei*	−	+	−	−	+	−	−	−	+	(+)	−	−
AUNP08	*Yokenella regensburgei*	−	+	−	−	−	−	−	ND	+	−	−	−
	Overall resistance to each antibiotic (%)	18.6	100	14.0	27.9	76.7	27.9	4.7	4.8	73.1	20.9	18.6	32.6

[a]1, amikacin; 2, hygromycin; 3, gentamycin; 4, kanamycin; 5, ampicillin; 6, tetracycline; 7, ciprofloxacin; 8, rifampicin; 9, erythromycin; 10, novobiocin; 11, chloramphenicol; 12, trimethoprim. +, positive; −, negative; (+), weak; ND, not detected.

Comamonas, *Klebsiella*, *Escherichia* and *Flavobacterium* were found only in river waters in this study (Table 1). These observations altogether imply that penicillin resistance is widespread in the river environment.

Members of Proteobacteria, in particular Gammaproteobacteria, are apparently the main source for both penicillin and tetracycline-resistance. The prevalence of Proteobacteria is in line with previous observations in aquatic environment (Ash *et al.* 2002; Falcone-Dias *et al.* 2012; Sigala & Unc 2013; Young *et al.* 2013). Although the composition at genus level was different between the penicillin- and tetracycline-resistant populations, the presence of the genus *Pseudomonas* as the main constituent was common for both. In addition to *Pseudomonas*, *Acinetobacter*, *Aeromonas*, *Chryseobacterium*, *Enterobacter*, *Escherichia*, *Klebsiella* and *Serratia* constituted the main antibiotic resistant community, each of which has also been reported as a main antibiotic resistant population in the bacterial community of natural or artificial aquatic environments, for example rivers (*Acinetobacter*, *Alcaligenes*, *Citrobacter*, *Enterobacter*, *Pseudomonas* and *Serratia*), swimming pools (*Pseudomonas*, *Leuconostoc*, *Staphylococcus*, *Chryseobacterium*, *Aeromonas*, *Enterobacter*, *Klebsiella* and *Ochrobactrum*), wastewater systems (*Pseudomonas*, *Shewanella*, *Escherichia*, *Acinetobacter*, *Arcobacter* and *Yersinia*), and bottled mineral water (*Arthrobacter*, *Acidovorax*, *Ralstonia*, *Curvibacter*, *Acidovorax* and *Hydrogenophaga*) (Ash *et al.* 2002; Papadopoulou *et al.* 2008; Falcone-Dias *et al.* 2012; Sigala & Unc 2013; Young *et al.* 2013). Based on those previous studies and this study, the eight main genera can be considered to comprise the 'core resistome' in the natural freshwater environment. *Alcaligenes*, *Comamonas*, *Elizabethkingia*, *Microvirgula*, *Providencia* and *Yokenella* were also common constituents but in minor proportions. The isolates belonging to *Enterobacteriaceae* comprised 40.1% of the total tetracycline-resistant bacteria, but only 8.9% of the total penicillin-resistant bacteria. A small overlap was found between the two antibiotic resistant populations, as 25 species out of 161 species, i.e. species of *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Chryseobacterium*, *Comamonas*, *Elizabethkingia*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Microvirgula*, *Providencia*, *Pseudomonas*, *Serratia* and *Yokenella*, were recovered in both antibiotic resistant populations.

Among the species identified in this study, those classified as risk group category 2 pathogens defined by the Korea Center for Disease Control and Prevention (www.cdc.go.kr) include *Acinetobacter baumanii* (tetracycline-resistant), *Aeromonas hydrophila* (penicillin-resistant), *Aeromonas punctata* subsp. *punctata* (penicillin-resistant), species of *Klebsiella* (penicillin and/or tetracycline-resistant), *Moraxella osloensis* (tetracycline-resistant), *Pseudomonas aeruginosa* (tetracycline-resistant) and *Shigella flexneri* (tetracycline-resistant). Apart from *A. hydrophila*, all other species were detected at low numbers in single, or only a few, occasions. No species classified as risk group 3 was detected. Among the ESKAPE pathogens (Rice 2008),

Table 3 | Antibiotic resistance profiles of tetracycline-resistant isolates

		Antibiotics[a]											
Strain	**Identification**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
AUNT08	*Acinetobacter guillouiae*	−	+	−	−	−	+	−	−	−	−	−	+
AUNT04	*Acinetobacter tandoii*	−	+	−	−	−	+	−	−	−	−	−	+
AUNT05	*Acinetobacter tandoii*	−	+	−	−	−	+	−	−	−	−	−	+
AUDT09	*Alcaligenes faecalis* subsp. *Faecalis*	(+)	+	−	+	+	+	−	−	+	−	−	+
AUDT16	*Alcaligenes faecalis* subsp. *Faecalis*	(+)	+	−	+	+	+	−	+	+	−	−	+
AUDT10	*Alcaligenes faecalis* subsp. *parafaecalis*	(+)	+	−	+	+	+	−	−	+	−	−	+
OCDT03	*Chryseobacterium arthrosphaerae*	+	+	+	+	+	+	−	−	+	−	+	−
AUNT11	*Chryseobacterium indologenes*	+	+	+	−	+	+	−	−	+	−	+	−
AUNT15	*Comamonas testosteroni*	+	+	+	−	+	+	−	−	+	−	−	+
OCNT01	*Enterobacter ludwigii*	−	+	−	−	−	+	−	−	+	−	−	−
AUDT19	*Escherichia coli*	−	+	−	−	−	+	−	−	+	(+)	−	+
AUNT21	*Escherichia coli*	−	+	−	−	+	+	−	−	+	−	+	+
AUNT20	*Escherichia coli*	−	+	−	−	+	+	−	−	+	−	+	−
AUNT06	*Escherichia fergusonii*	−	+	−	−	+	+	(+)	−	+	−	−	+
AUNT01	*Klebsiella oxytoca*	−	+	−	+	+	+	−	−	+	−	−	+
AUNT14	*Klebsiella pneumoniae* subsp. *ozaenae*	−	+	−	−	+	+	−	+	+	−	−	−
AUNT18	*Klebsiella pneumoniae* subsp. *ozaenae*	−	+	−	−	+	+	−	(+)	+	−	(+)	−
AUNT19	*Klebsiella pneumoniae* subsp. *ozaenae*	−	+	−	−	+	+	−	−	+	−	(+)	−
AUNT22	*Klebsiella pneumoniae* subsp. *ozaenae*	−	+	−	−	+	+	−	−	+	−	(+)	−
OCNT03	*Klebsiella pneumoniae* subsp. *ozaenae*	−	+	−	−	+	+	−	−	+	−	−	+
AUNT02	*Klebsiella variicola*	−	+	−	−	+	+	−	(+)	+	−	−	−
OCNT02	*Klebsiella variicola*	−	+	−	−	+	+	−	−	+	−	−	+
AUDT33	*Morganella morganii* subsp. *Morganii*	−	+	−	−	+	+	−	−	+	+	+	−
AUDT11	*Morganella morganii* subsp. *Sibonii*	−	+	−	−	+	+	−	−	+	+	+	−
AUDT13	*Morganella morganii* subsp. *Sibonii*	(+)	+	−	−	+	+	−	−	+	+	+	+
AUDT05	*Providencia alcalifaciens*	−	+	−	−	+	+	−	−	+	+	−	−
OCDT02	*Providencia alcalifaciens*	−	+	−	−	+	+	−	−	+	+	−	−
AUDT14	*Providencia stuartii*	−	+	−	−	+	+	−	−	+	+	+	−
AUNT12	*Pseudomonas geniculata*	(+)	+	−	+	+	+	−	−	+	+	−	+
AUNT03	*Serratia marcescens* subsp. *marcescens*	−	+	−	−	+	+	−	+	+	+	−	−
AUNT17	*Serratia nematodiphila*	(+)	+	−	−	+	+	−	(+)	+	+	−	−
AUDT20	*Shigella flexneri*	−	+	+	−	+	+	+	−	+	+	+	−
AUDT21	*Shigella flexneri*	−	+	−	+	+	+	−	−	+	+	−	−
	Overall resistance to each antibiotic (%)	30.0	100	13.3	16.7	86.7	100	6.7	20.0	93.3	36.7	36.7	46.7

[a]1, amikacin; 2, hygromycin; 3, gentamycin; 4, kanamycin; 5, ampicillin; 6, penicillin; 7, ciprofloxacin; 8, rifampicin; 9, erythromycin; 10, novobiocin; 11, chloramphenicol; 12, trimethoprim. +, positive −, negative; (+), weak.

A. baumanii, *P. aeruginosa*, *Klebsiella pneumoniae*, and species of *Enterobacter*, but not *Enterococcus faecium* and *Staphylococcus aureus*, were detected.

The intrinsic resistance of pathogenic microbes to antibiotics has been previously studied (Fajardo *et al.* 2008; Alvarez-Ortega *et al.* 2011), but not much is known on the

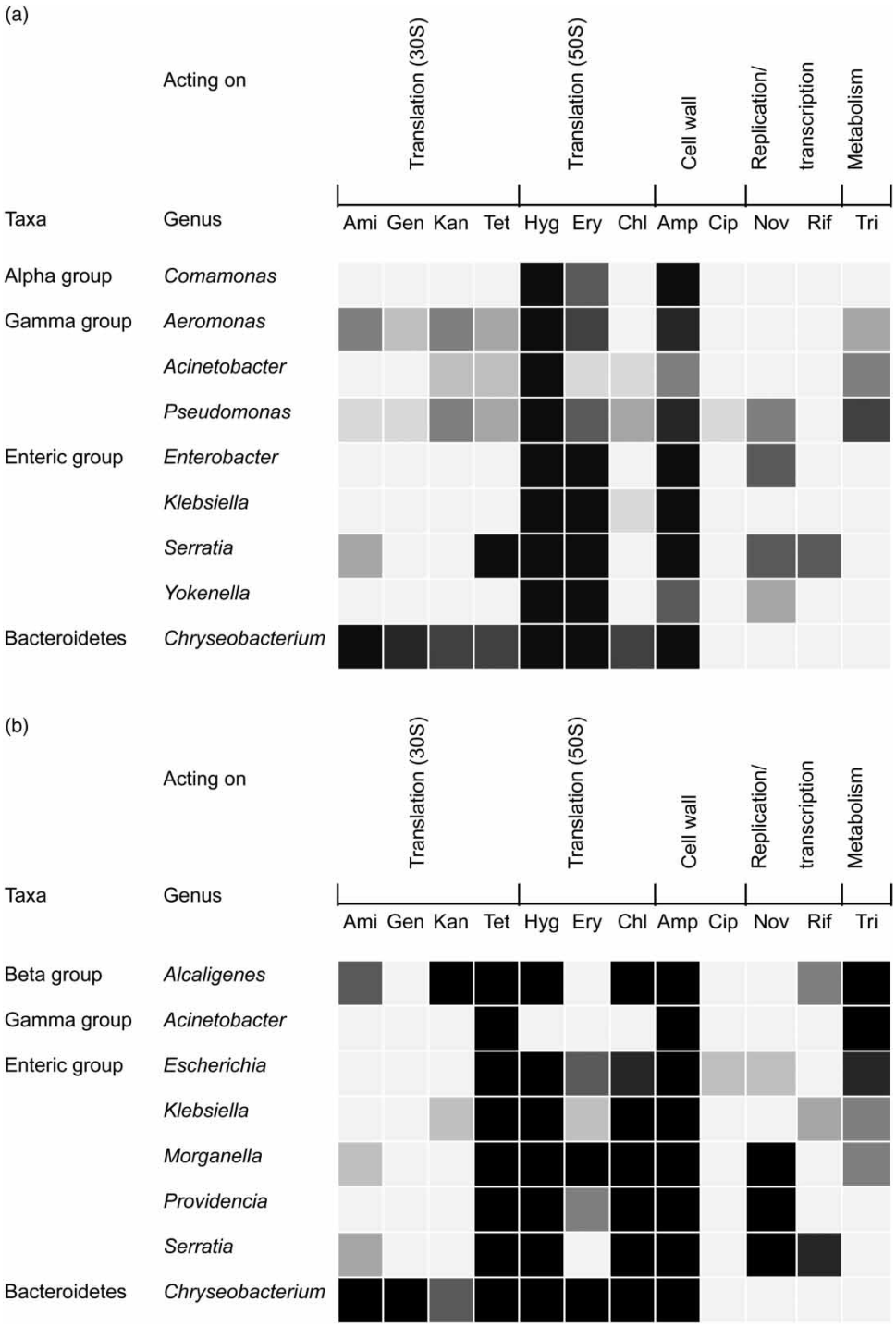

Figure 3 | Antibiotic resistance profile of main bacterial genera according to taxonomic classification and mechanisms of action. (a) Penicillin-resistant bacteria; (b) tetracycline-resistant bacteria. Darkness indicates degrees of resistance. Alpha, Beta and Gamma groups indicate Alpha-, Beta- and Gammaproteobacteria, respectively. Ami, amikacin; Gen, gentamycin; Kan, kanamycin; Tet, tetracycline; Hyg, hygromycin; Ery, erythromycin; Chl, chloramphenicol; Amp, ampicillin; Cip, ciprofloxacin; Nov, novobiocin; Rif, rifampicin; Tri, trimethoprim.

resistance of environmental strains. Through the genome analysis, the pathogenic members of *Pseudomonas*, *Acinetobacter* and *Aeromonas* are known to contain the genetic elements that may render intrinsic resistance to antibiotics (Fournier *et al.* 2006; Diene & Rolain 2013), although it is not clear to what extent such genetic elements are distributed among environmental bacteria. Of the 55 genera confirmed in this study, *Aquitalea*, *Asticcacaulis*, *Curtobacterium*, *Iodobacter*, *Kinneretia*, *Microvirgula*, *Simplicispira* and *Vogesella* are generally known as environmental organisms, and antibiotic resistance for these taxa has not been reported before. However, most of the main taxa identified in this study have been reported as isolates from human or animal sources, for example, species of *Aeromonas* and other members of Gammaproteobacteria (Brenner & Farmer III 2005), and also species belonging to the family *Enterobacteriaceae* (Martin-Carnahan & Joseph 2005).

The strains affiliated to *Alcaligenes faecalis*, *Chryseobacterium arthrosphaerae*, and *Pseudomonas geniculata* were among the top broad-spectrum multiple antibiotic resistant bacteria in both resistant populations. Enterobacterial strains together with *Alcaligenes*, *Chryseobacterium* and *Pseudomonas* exhibited high levels of multiple antibiotic resistance in general. This observation is clearly comparable to the multiple antibiotic resistant species identified in other studies on artificial environment, for example swimming pools (Papadopoulou *et al.* 2008), bottled mineral water (Falcone-Dias *et al.* 2012), or wastewater (Sigala & Unc 2013).

The resistance profiles of the two antibiotic resistant populations to other antibiotics were generally similar, but tetracycline-resistant strains exhibited broader multiple antibiotic resistance (Figure 3). Interestingly, the majority of the closest matches to multiple resistant strains, namely *Aeromonas punctata* subsp. *caviae*, two subspecies of *Alcaligenes faecalis*, *Chryseobacterium arthrosphaerae*, *Chryseobacterium indologenes*, *E. coli*, *Morganella morganii* subsp. *sibonii*, *Raoultella ornithinolytica*, *Serratia nematodiphila* and *Shigella flexneri*, are known as fecal or human- or animal-associated bacteria (Brenner & Farmer III 2005; Busse & Auling 2005; Martin-Carnahan & Joseph 2005; Kämpfer *et al.* 2010; Bernardet *et al.* 2011). In most cases, none of these taxa were among the major constituents of both antibiotic resistant communities, although most of them appeared as minor constituents in both communities.

Ciprofloxacin and novobiocin, both known as DNA gyrase inhibitors, rendered least resistance among the tested antibiotics, together with rifampicin, known as an RNA polymerase inhibitor (Figure 3). In contrast, most strains were resistant to penicillin and ampicillin, known as cell wall synthesis inhibitors. Antibiotics known to act on translation levels caused varying degrees of resistance. For example, a low degree of resistance was observed against aminoglycosides (amikacin, gentamycin and kanamycin) to both populations, whereas a high degree of resistance was observed against erythromycin. The degree of antibiotic resistance by the action mechanism was in the order of RNA polymerase inhibitor and DNA gyrase inhibitors, translation inhibitors, metabolic inhibitor (tetrahydrofolate synthesis), and cell wall synthesis inhibitors. The degree of resistance by structural category of antibiotics was in the order of macrolide (erythromycin) and penicillins, trimethoprim, phenocol (chloramphenicol) and aminocoumarin (novobiocin), aminoglycosides, rifampicin, and quinolone (ciprofloxacin) (Figure 3). In general, this pattern of resistance agrees well with a previous observation by Moore *et al.* (2010a, b). The high degree of resistance to penicillins by antibiotic resistant bacteria is also in line with previous observations (Moore *et al.* 2010a, b; Mudryk *et al.* 2010; Walsh & Duffy 2013).

CONCLUSIONS

Through this study, resistance to antibiotics among diverse groups of planktonic bacteria in freshwater environments was confirmed. The bacterial taxa encompass environmental strains for which antibiotic resistance or pathogenicity has not been reported, as well as potential pathogens and also those well known for resistance. The level of viable counts of penicillin-resistant bacteria are notable, since penicillins have been virtually non-detectable in the freshwater environments of Korea. In addition, the low proportion of enteric bacteria in penicillin-resistant populations compared to that in tetracycline-resistant populations also implies the presence of the natural antibiotic resistant bacterial community in the aquatic environment.

Members of *Pseudomonas* were found as a prominent antibiotic resistant group for both antibiotics, and thus

obviously forming the core freshwater resistome, together with other common genera including *Acinetobacter*, *Aeromonas*, *Chryseobacterium*, *Klebsiella* and *Serratia*. However, variations were found at species level, and no particular species could be identified as the core.

The resistance to multiple antibiotics having different action mechanisms may be intrinsic among some environmental microbes in general. However, the strains exhibiting broad multiple antibiotic resistance were mostly affiliated to species that have been frequently found in association with human or animal sources, and thus the extensive study on the multiple antibiotic resistant strains might provide an insight on the effect of anthropogenic activities to the microbial community in the aquatic environment.

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