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Functional metagenomic analysis reveals rivers are a reservoir for diverse antibiotic resistance genes

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ABSTRACT

The environment harbours a significant diversity of uncultured bacteria and a potential source of novel and extant resistance genes which may recombine with clinically important bacteria disseminated into environmental reservoirs. There is evidence that pollution can select for resistance due to the aggregation of adaptive genes on mobile elements. The aim of this study was to establish the impact of waste water treatment plant (WWTP) effluent disposal to a river by using culture independent methods to study diversity of resistance genes downstream of the WWTP in comparison to upstream. Metagenomic libraries were constructed in *Escherichia coli* and screened for phenotypic resistance to amikacin, gentamicin, neomycin, ampicillin and ciprofloxacin. Resistance genes were identified by using transposon mutagenesis. A significant increase downstream of the WWTP was observed in the number of phenotypic resistant clones recovered in metagenomic libraries. Common β-lactamases such as bla_{TEM} were recovered as well as a diverse range of acetyltransferases and unusual transporter genes, with evidence for newly emerging resistance mechanisms. The similarities of the predicted proteins to known sequences suggested origins of genes from a very diverse range of bacteria. The study suggests that waste water disposal increases the reservoir of resistance mechanisms in the environment either by addition of resistance genes or by input of agents selective for resistant phenotypes.

1. Introduction

The growing number of bacteria resistant to multiple antibiotics pose a great risk to both animal and human health, yet despite this the role of the environment in the dissemination of antibiotic resistance genes is still largely unknown (Wellington et al., 2013). Anthropogenic activities such as agriculture increase the load of environmental antibiotic resistant bacteria with recent reports of diverse resistance genes present in farm environments (Zhu et al., 2013) and increased levels of antibiotic resistance genes in soil following the application of manure (Byrne-Bailey et al., 2011). Waste water treatment plants (WWTPs) are a hotspot for resistance gene transfer between bacteria from different origins due to mixing of urban, industrial, clinical and agricultural waste (Rizzo et al., 2013). The subsequent disposal of effluent and solids from WWTPs can increase loads of antibiotic resistant genes in the environment for

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example via the application of sludge to land as fertilizer (Gaze et al., 2011) and effluent to rivers which could act as a reservoir of resistance genes (Korzeniewska et al., 2013). The overall impact of WWTPs on environmental resistome is poorly studied and has potentially far reaching impacts of escalating resistance gene dissemination.

The application of metagenomics has revealed antibiotic resistance gene diversity in the uncultured bacterial fraction of environmental samples is much higher than observed in cultured isolates and resistance genes associated with pathogens can be found in soil metagenomes (Forsberg et al., 2012; Pehrsson et al., 2013). The mobilisation of $bla_{CTX\text{-}M}$ from *Kluyvera* sp. and *qnr* genes from *Shewanella* sp. provide evidence of resistance gene flow from non-clinical populations to clinical environments where they have had a significant role in conferring antibiotic resistance to animal and human pathogens (Nordmann and Poirel, 2005; Olson et al., 2005). Functional metagenomics has been used to investigate antibiotic resistance in a variety of environments including Alaskan soil (Allen et al., 2009), apple orchards (Donato et al., 2010), seabirds (Martiny et al., 2011), and sludge (Parsley et al., 2010). Research is needed to quantify the impacts of anthropogenic activities on the environmental resistome.

In this study we aimed to investigate the impact of WWTP effluent on the resistome of a river, with the hypothesis that antibiotic resistance gene abundance and diversity would be significantly impacted by the effluent outflow. River sediment samples were taken from downstream and upstream of an effluent pipe and functional metagenomics was used to investigate resistance gene abundance and diversity.

2. Materials and methods

Sediment core samples were taken on January 23rd 2011 in triplicate from a river 300 m, 600 m and 900 m upstream and downstream of a large WWTP in the UK Midlands. The WWTP served a large urban catchment of approximately 500,000 people.

2.1. Construction of metagenomic libraries

DNA was extracted from samples using FASTDNA Spin kit for soil (MP biomedicals, UK) as per the manufacturer's instructions. DNA concentration was measured using spectrophotometry and standardised to the same concentration across all samples. Downstream samples (DS) were pooled into 1 ml final volume to create the library. Upstream samples (US) were also pooled into 1 ml final volume to create the library. DS and US DNA was initially purified using gel fractionation; 1% agarose (Helena Laboratories, UK) was prepared, samples were loaded and gel electrophoresis was performed at 40 V for 18 h. DNA was selected from the size range 5 kb–20 kb and recovered via electroelution. Gel purified DNA was blunt-ended and phosphorylated using an End-Repair kit (Epicentre, USA), then purified and concentrated using Microcon centrifugal filters (Millipore, UK). Blunt DNA was ligated into vector pCC1 (Epicentre, USA) using Fast-link DNA ligase with an extended 16 °C incubation overnight. The ligation was desalted via drop dialysis using 'V' series membranes (Millipore, UK), and electroporated into Transformax Epi300 *Escherichia coli* (Epicentre, USA). Libraries were titred using a dilution series.

2.2. Metagenomic library analysis

Both libraries were amplified in LB broth amended with chloramphenicol (12 mg l^{-1}) by incubating overnight at room temperature. Chloramphenicol was used to maintain the pCC1 vector. Resulting amplified libraries were stored at 4 °C for analysis. Ten clones were selected from each library and plasmid extractions were performed using a Miniprep kit (Qiagen, UK). Plasmids were cut with BamHI (New England Biolabs, USA) to excise inserts with resulting digests analysed using gel electrophoresis. Banding patterns were analysed to calculate the mean insert size for each library.

2.3. Quantification of total bacterial numbers

The total quantity of bacteria in the DS and US samples was analysed using qPCR for the 16S rRNA gene as previously described (Gaze et al., 2011).

2.4. Metagenomic library screening for antibiotic resistance phenotype

Both entire libraries were screened on the aminoglycoside antibiotics amikacin, gentamicin, neomycin, the fluoroquinolone ciprofloxacin and the β-lactam ampicillin. Concentrations for screening were selected by testing the minimum inhibitory concentrations (MICs) in *E. coli* Epi300 strain with an empty pCC1 vector, and choosing a concentration a tenth higher than this MIC to ensure a minimal amount of false positives. The Epi300 strain with an empty pCC1 vector was subsequently used as a negative control. Positive clones were selected and plasmid extractions were performed (Qiagen, UK). Restriction fragment length polymorphism (RFLP) utilising restriction enzymes BamH1, EcoRI and EcoRV was used for analysis of clones to determine the positive number of clones resistant to each antibiotic.

2.5. Characterisation of resistant clones

A selection of unique clones were chosen for further characterisation. Unique positive clones were tested for resistance at the clinical breakpoints of each selected antibiotic using published methods (Andrews, 2001). Resistance genes were elucidated using transposon mutagenesis with the EZ-Tn5 kit (Epicentre, USA) which inactivated the resistance gene allowing for selection of clones by loss of phenotype. Clones were then sequenced using the Kan-3 Fp-2 primer forward primer and Kan-3 RP reverse primer. Sequence data was analysed with NCBI blast (Camacho et al., 2009) and NCBI ORF finder (Available at: http://www.ncbi.nlm.nih.gov/gorf/.html [last accessed 31/01/14]) to identify the resistance gene and flanking regions.

Table 1
Summary of metagenomic libraries made from DS and US samples.

Sample	Average insert size	Average number of clones	Library coverage
DS	4.2 kb	2×10^6	8.4 Gb
US	4.7 kb	2×10^6	9.4 Gb

2.6. Statistical analysis

Statistical analyses were performed using MedCalc for Windows, version 9.5.0.0 (MedCalc Software, Mariakerke, Belgium). Differences in gene prevalence were tested for significance using a χ^2-test for the comparison of two proportions (from independent samples). The average *E. coli* genome size (4.6 Mb) was used to estimate how many genomes were in each library by dividing number of DNA Mb in the library by 4.6. The estimated proportion of bacteria in the sediment resistant to each antibiotic was calculated by dividing the number of resistant unique clones by the average number of genomes in the library, this was then expressed as a percentage.

2.7. Phylogenetic analysis of resistance genes

DNA sequences were trimmed and aligned with ClustalW (Thompson et al., 1994) using MEGA 5.2 (Kumar et al., 1994). Phylogenetic trees were constructed using the Neighbour-Joining method (Saitou and Nei, 1987). A bootstrap consensus tree inferred from 1000 replications was generated, evolutionary distances were computed using the *p*-distance method (Tamura et al., 2011). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed.

3. Results

3.1. Metagenomic libraries

Estimated sizes of the libraries were 8.4 Gb for DS and 9.4 Gb US (Table 1). Using the average genome size of *E. coli* (4.6 Mb) it can be estimated that the DS library consisted of approximately 1826 bacterial genomes and the US library consisted of approximately 2043 bacterial genomes. The total bacterial abundance in each sediment sample was calculated by qPCR to be 9.11×10^7 bacteria g^{-1} sediment DS and 6.78×10^7 bacteria g^{-1} sediment US. From this we can calculate that the DS library captured approximately 0.002% of the bacteria in a gram of river sediment downstream of the WWTP, and the US library captured approximately 0.003% of the bacteria in a gram of river sediment upstream of the WWTP.

3.2. Resistance gene abundance

Antibiotic resistant clones were selected and RFLP was performed on extracted plasmid DNA to determine the number of unique clones conferring resistance to each antibiotic (Table 2). Several hundred clones conferring ampicillin resistance were recovered in both the DS and US libraries; analysis of a subset of these revealed more than 50 unique clones indicating that at least one in every 36 genomes carried an ampicillin resistance gene downstream. There was a significantly higher number of antibiotic resistant clones from the DS library compared to the US library for antibiotics neomycin (14 DS vs. 6 US) (Chi Square 4.232 $P = 0.0397$), amikacin (4 DS vs. 0 US) (Chi Square 23.040 $P < 0.0001$) and ciprofloxacin (4 DS vs. 1 US) (Chi Square 18.181 $P < 0.0001$). All unique clones were resistant at clinically significant breakpoints (Table 2) using published methods (Andrews, 2001).

3.3. Antibiotic resistant gene identities

Transposon mutagenesis was performed on a subset of antibiotic resistant clones selected at random in order to investigate the genes responsible for conferring resistance (Table 3). The β-lactamase gene bla_{TEM} was identified in multiple ampicillin resistant clones in both DS and US libraries. The flanking regions of each bla_{TEM} were unique proving they were from different host backgrounds. Resistance to gentamicin was conferred by genes bearing similarity to clinically important genes such as aminoglycoside 3′-adenylytransferase (90%) found in clinical pathogens such as *Yersinia pestis*. Genes previously not associated with gentamicin resistance were also recovered (Table 3). Ciprofloxacin resistance was attributed to the proteins RecA (74%) and RecX (33%), which have to date not been associated with resistance to fluroquinolones. Other unusual genes were recovered for neomycin and amikacin resistance.

3.4. Diversity of aminoglycoside resistance genes

Three clones conferring resistance to aminoglycosides contained highly divergent acetyltransferases with 36–59% protein similarity to known proteins. Phylogenetic

Table 2
Analysis of resistant clones in DS and US libraries.

Antibiotic	MIC of *Epi*300 strain (mg L^{-1})	Number of resistant clones DS	Number of resistant clones US	Proportion of bacteria carrying resistant gene downsteam (%)	Proportion of bacteria carrying resistant gene upstream (%)	MIC tested for resistant clones (mg L^{-1})
Ampicillin	8	>50	>50	2.74	2.45	16
Gentamicin	3	15	9	0.82	0.44	6
Neomycin	8	14	6	0.76	0.29	16
Amikacin	4	4	0	0.22	0	16
Ciprofloxacin	0.25	4	1	0.22	0.05	1

The estimated proportion of bacteria in the sediment resistant to each antibiotic was calculated by dividing the number of resistant unique clones by the average number of genomes in the library, this was then expressed as a percentage.

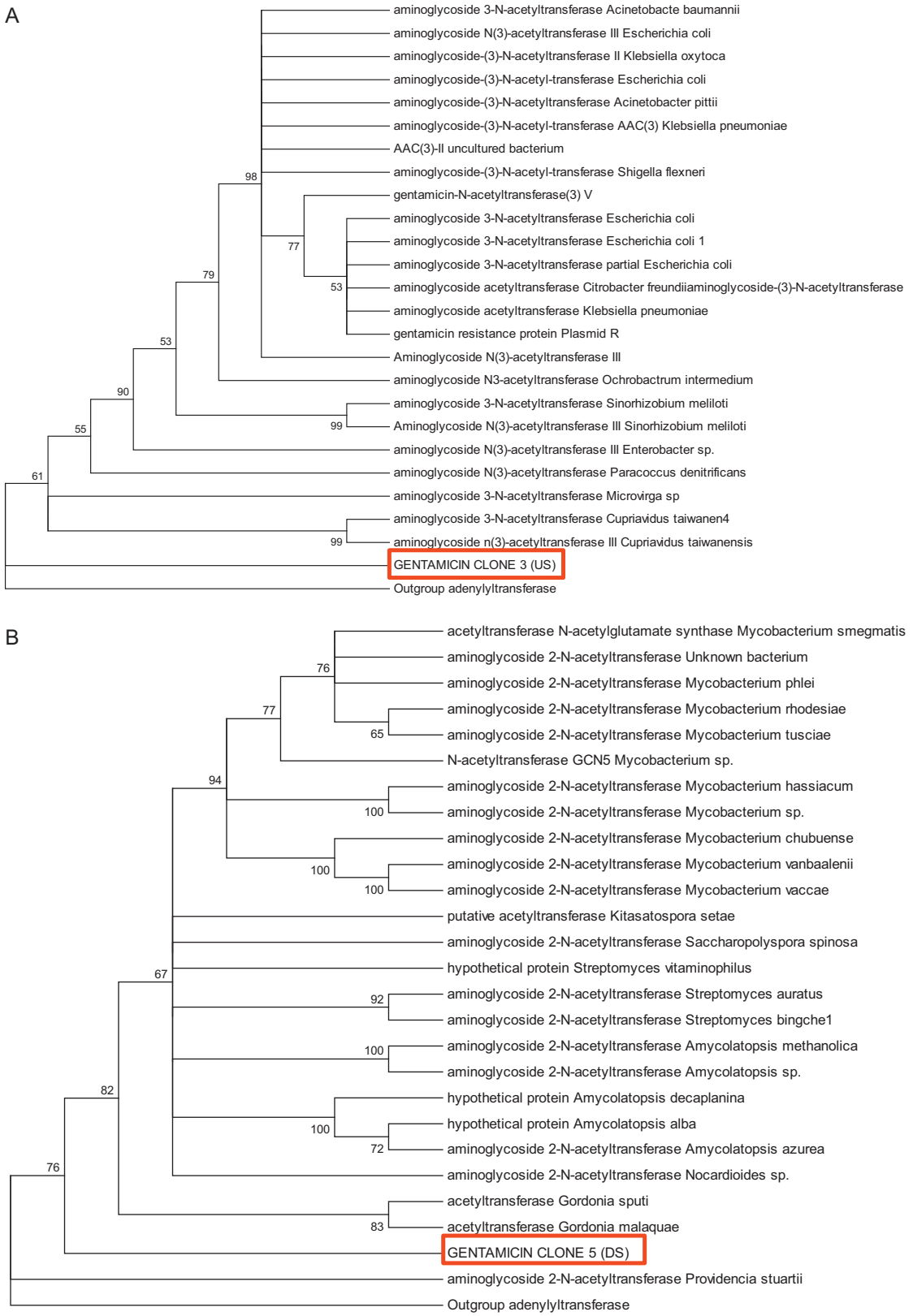

Fig. 1. Phylogenetic relationships of acetyltransferases recovered US and DS. (A) Gentamicin clone 3 US. (B) Gentamicin clone 5 DS and (C) Amikacin clone 1 DS. Bootstraps inferred from 1000 replications. Branches < 50% bootstraps were collapsed. The evolutionary distances were computed using the *p*-distance method (Tamura et al., 2011) and are in the units of the number of amino acid differences per site.

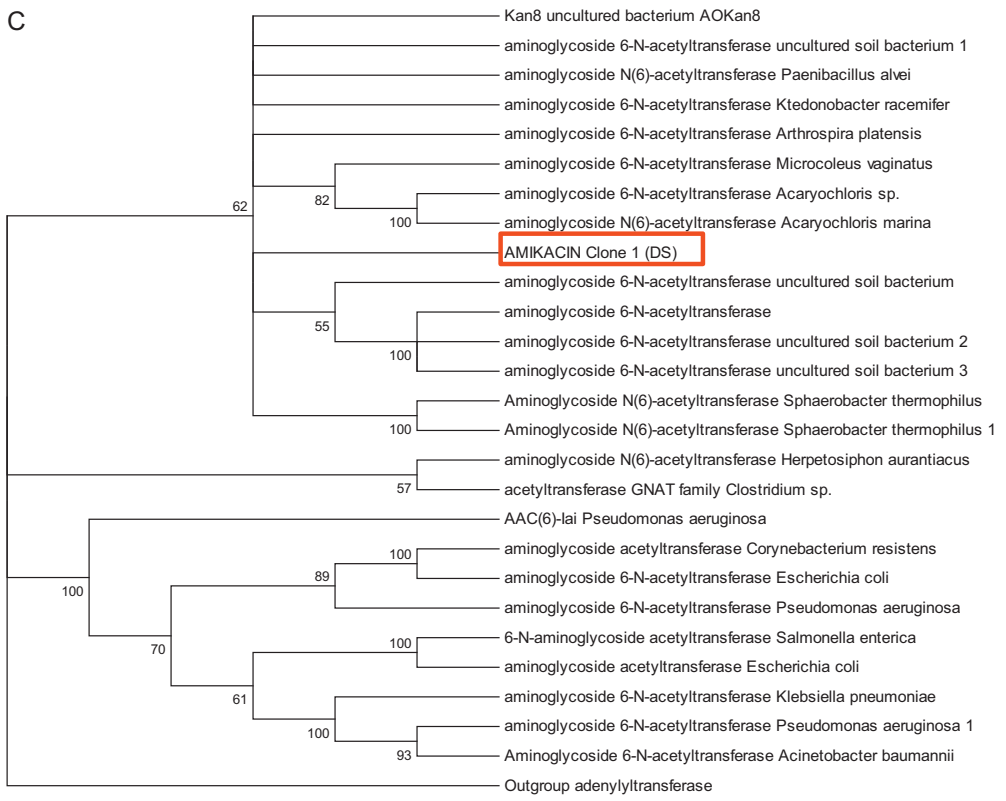

Fig. 1. (*Continued*).

analysis revealed the three genes encoded for proteins related to aminoglycoside 3′-N-acetyltransferases (Gentamicin clone 3 US), aminoglycoside 2′-N-acetyltransferases (Gentamicin clone 5 DS) and aminoglycoside 6′-N-acetyltransferases (Amikacin clone 1 DS) (Fig. 1). Gentamicin clone US3 was not recovered in the aminoglycoside 3′-N-acetyltransferase clade and formed an outlier with the root. The two other clones were similar to genes present in environmental bacteria such as actinobacteria and cyanobacteria.

4. Discussion

This study revealed that WWTP effluent increased the abundance of bacterial resistance to clinically important antibiotics in river sediments. The presence of clinically relevant resistance genes in the environment has previously been reported (Forsberg et al., 2012), however this study illustrates a potential dissemination route via WWTPs. Although sequence based studies have suggested that area's impacted by WWTP effluent contain elevated numbers of sequences of resistance genes (Port et al., 2012), this is the first comparative study of the functional river resistome before and after the addition of effluent.

The number of unique resistance clones in DS libraries and US libraries were recorded, and the most prevalent phenotype was ampicillin resistance. We can estimate a prevalence of $\sim 2 \times 10^5$ ampicillin resistance genes in the downstream sediment and 1.65×10^5 ampicillin resistance genes in the upstream sediment which indicates the widespread resistance to this antibiotic. This is not unexpected as a number of studies have illustrated the abundance of resistance to semisynthetic β-lactams (Allen et al., 2009). The amikacin resistance population showed the largest amplification in numbers for downstream samples, with an estimated sevenfold increase of resistance genes at 2×10^4 DS and 3×10^3 US. This level of amplification was closely followed by ciprofloxacin and neomycin. Prevalence data here suggests an input of resistance determinants or selective agents through the WWTP effluent.

Aminoglycoside resistance genes recovered were extremely diverse, however some encoded for acetyltransferases, the same family of genes conferring resistance in clinical bacteria (Chevereau et al., 1974). All these genes recovered when expressed in the current study gave clinically relevant MICs to at least one aminoglycoside antibiotic. A number of environmental studies concerned with reservoirs of resistance genes have recovered similarly diverse genes (McGarvey et al., 2012) however none have done estimates of prevalence or investigated potential routes of dissemination. In addition our study revealed a novel gentamicin resistance gene which appeared to have an independent phylogeny to other acetyltransferases. This study supports previous research which indicated resistance gene diversity in uncultured environmental bacteria is much higher than that studied in clinically relevant bacteria (Forsberg et al., 2012).

Table 3
Identities of resistance genes and predicted proteins from clones analysed by transposon mutagenesis.

Antibiotic resistance conferred (library)	Predicted size of protein (amino acids)	Predicted domains	Nearest sequence identity (bacteria identity)
Gentamicin clone 1 (US library)	420	Potassium transporter superfamily	77% Potassium transport protein (*Janthinobacterium* sp.)
Gentamicin clone 2 (US library)	58	None	75% Hypothetical protein (*Escherichia coli*)
Gentamicin clone 3 (US library)	264	Aminoglycoside 3-N-acetyltransferase	59% Aminoglycoside-(3)-N-acetyltransferase (*Escherichia coli*)
Gentamicin clone 4 (US library)	329	Thiamine pyrophosphate family	88% Pyruvate dehydrogenase subunit E1 (*Janthinobacterium* sp.)
Gentamicin clone 5 (DS library)	178	Aminoglycoside 3-N-acetyltransferase	36% Acetyltransferase (GNAT) family protein (*Providencia rettgeri*)
Gentamicin clone 6 (DS library	77	DUF4111	90% Aminoglycoside 3′-adenylytransferase (*Yersinia pestis*)
Gentamicin clone 7 (DS library)	88	Aminoglycoside 3′-phosphotransferase (APH)	100% Aminoglycoside 3′-phosphotransferase (*Pseudomonas putida*)
Amikacin clone 1 (DS library	185	Aminoglycoside 3-N-acetyltransferase	58% Aminoglycoside N(6′)-acetyltransferase (*Gloeocapsa* sp.)
Amikacin 2 (DS library)	119	Nucleotidyl transferase superfamily	62% Methionyl-tRNA synthetase (*Haliscomenobacter hydrossis*)
Ampicillin clone 1 (US library	289	Beta-lactamase2 superfamily	99% Beta-lactamase TEM (*Bacillus subtilis*)
Ampicillin clone 2 (DS library)	289	Beta-lactamase2 superfamily	99% Beta-lactamase TEM (*Bacillus subtilis*)
Ampicillin clone 3 (DS library)	289	Beta-lactamase2 superfamily	99% Beta-lactamase TEM (*Bacillus subtilis*)
Neomycin clone 1 (DS library)	107	TroA like superfamily FepB BC-type Fe3+ -hydroxamate transport system, periplasmic component	48% Hypothetical protein (*Streptomyces* sp.)
Neomycin clone 2 (DS library)	162	Glycosyltransferase family 25	36% Glycosyltransferase 25 family member 1 (*Agrobacterium* sp.)
Ciprofloxacin clone 1 (DS library)	145 154	RecX (recombination regulator) RecA (bacterial DNA recombination protein)	33% Regulatory protein RecX (*Listeria seeligeri*) 74% Recombinase A (*Geobacter lovleyi*)

Identities of resistance genes and predicted proteins from clones analysed by transposon mutagenesis. Predicted size of proteins were generated using ORF finder by NCBI. Translated nucleotide identities were used to generate protein sequence from which predicted domains and sequence identity were obtained using NCBI BLASTP.

Significant increases in the number of aminoglycoside resistant clones observed DS compared to US combined with the sequence data is the first evidence that anthropogenic pollution such as WWTP effluent increases the abundance of environmentally diverse resistance genes.

Sequence analysis of some resistance genes showed similarity to genes assigned to housekeeping functions. This may be evidence for evolutionary origins of resistance genes (Aminov and Mackie, 2007). One mechanism for ciprofloxacin resistance involved the recombination system RecA and RecX, RecX facilitates recombination repair by modulating RecA, thus could be a possible mechanism to repair the damage done by ciprofloxacin inhibition of the DNA gyrase (Cardenas et al., 2012). Amongst the other genes recovered, transporters were evident, but more obscure mechanisms of resistance attributed to enzymes such as glycosyltransferase and pyruvate dehydrogenase were also recovered, for both of which the resistance function is unknown. Our data demonstrates the diversity of genes recovered by expression analysis which can confer antibiotic resistance and indicates that there is a significant environmental reservoir of diverse mechanisms encoding protection against antibiotics (Martiny et al., 2011).

The impact of effluent discharge on the number of clones conferring resistance to clinically important antibiotics such as ciprofloxacin and amikacin is a cause for concern. Rivers are a vital part of the ecosystem and provide an essential source of drinking water for wild animals as well as being used for crop irrigation and recreational activities. High levels of antibiotic resistance in rivers resulting from WWTP pollution may explain why an increasing number of studies are reporting resistance genes in both wild and domestic animals. Several studies have demonstrated resistance genes in livestock such as the clinical $bla_{\text{CTX-M-15}}$ in UK cattle (Watson et al., 2012), chickens, turkeys (Randall et al., 2011) and dogs (Timofte et al., 2011). Increasing levels of antibiotic resistant genes in wild animals such as wolves (Goncalves et al., 2013) and birds (Dolejska et al., 2011; Ewers et al., 2010) could be through drinking contaminated water, particularly for seagulls which often feed and drink near sewage treatment plants (Fricker, 1984).

In conclusion, functional metagenomics provides a valuable resource when analysing resistance mechanisms in bacteria often revealing genes which sequenced-based analysis would not detect. WWTPs create a large reservoir of resistance genes which potentially can contribute to clinical cases of resistant bacteria in animals and humans through

direct exposure from contaminated river water. Further research is needed to improve waste disposal methods in order to reduce environmental reservoirs of antibiotic resistance and ultimately lower the clinical burden.

Conflicts of interest

We declare that we have no conflicts of interest.

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