



Novel clinically relevant antibiotic resistance genes associated with sewage sludge and industrial waste streams revealed by functional metagenomic screening

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ABSTRACT

A growing body of evidence indicates that anthropogenic activities can result in increased prevalence of antimicrobial resistance genes (ARGs) in bacteria in natural environments. Many environmental studies have used next-generation sequencing methods to sequence the metagenome. However, this approach is limited as it does not identify divergent uncharacterized genes or demonstrate activity. Characterization of ARGs in environmental metagenomes is important for understanding the evolution and dissemination of resistance, as there are several examples of clinically important resistance genes originating in environmental species. The current study employed a functional metagenomic approach to detect genes encoding resistance to extended spectrum β -lactams (ESBLs) and carbapenems in sewage sludge, sludge amended soil, quaternary ammonium compound (QAC) impacted reed bed sediment and less impacted long term curated grassland soil. ESBL and carbapenemase genes were detected in sewage sludge, sludge amended soils and QAC impacted soil with varying degrees of homology to clinically important β -lactamase genes. The flanking regions were sequenced to identify potential host background and genetic context. Novel β -lactamase genes were found in Gram negative bacteria, with one gene adjacent to an insertion sequence *ISPme1*, suggesting a recent mobilization event and/ the potential for future transfer. Sewage sludge and quaternary ammonium compound (QAC) rich industrial effluent appear to disseminate and/or select for ESBL genes which were not detected in long term curated grassland soils. This work confirms the natural environment as a reservoir of novel and mobilizable resistance genes, which may pose a threat to human and animal health.

1. Introduction

Antimicrobial resistance (AMR) increasingly compromises the efficacy of modern medicine in treating infectious diseases with significant health, societal and economic impacts (Chaired by O'Neill, 2014; Gaze et al., 2008; Gullberg et al., 2011; Wellington et al., 2013). Resistance to β -lactam antibiotics, which constitute > 50% of worldwide antibiotic usage, is one of the most important medical problems (Bonomo, 2017). There are two main mechanisms harboured by bacteria that confer resistance to β -lactam antibiotics, mutation of penicillin binding

proteins (PBPs) (mostly Gram positives), and production of β -lactamases (mostly Gram negatives) (Ozturk et al., 2015; Palzkill, 2013). PBP gene mutation or the acquisition of a new PBP gene (PBP2a) is an important resistance mechanism in *Streptococcus pneumoniae* and methicillin-resistant *Staphylococcus aureus* (MRSA). β -lactamases are divided into four classes (Class A–D) based on their sequence similarity. Classes A, C and D possess a serine at the active site. The most prevalent ESBLs are the serine- β -lactamases, encoded by gene families such as *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}, which belong to class A; the *AmpC* and *bla*_{CMY} gene families which belong to class C; and the *bla*_{OXA} gene family

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which belongs to class D (Ambler, 1980; Bebrone, 2007; Canton et al., 2012; Hawkey, 2015).

Class B β -lactamases, also known as metallo- β -lactamases (MBLs), require a zinc ion for catalysis. MBLs are divided into three subgroups (B1 to B3) based on their sequence and metal requirements. Subclasses B1 and B3 contain two zinc ion binding sites, while subclass B2 contains only one, thus explaining the narrower substrate specificity of the latter. Most of the well-known clinically important MBLs belong to subgroup B1 including *bla*_{IMP}, *bla*_{VIM}, *bla*_{SPM} and *bla*_{NDM} that confer resistance to almost all known β -lactam antibiotics, including the last resort subclass of β -lactams, the carbapenems (Cornaglia et al., 2011; Walsh et al., 2011). Most B1 MBLs are encoded by plasmid localized genes, whereas most B2 and B3 MBLs are chromosomally located, such as the B2 subclass *bla*_{CPHA} gene from *Aeromonas hydrophila* and the clinically relevant *bla*_{AIM-1}, which has been identified recently in multi-drug resistant *Pseudomonas aeruginosa* (Cornaglia et al., 2011; Meini et al., 2014; Walsh et al., 2011).

The class B MBLs form a sub-family of the MBL superfamily, which has > 6000 members. Most of them share five conserved motifs and similar $\alpha\beta\alpha$ type folding. The MBL super group possesses various enzymatic activities to hydrolyse thiol-ester, phosphodiester and sulfuric ester bonds (Bebrone, 2007). The majority of these members have not been characterized functionally, except for Subclass B3 (Salimraj et al., 2016). It has been speculated that recent therapeutic use of carbapenems and other β -lactam antibiotics has selected for ancestral MBL genes and driven their evolution and subsequent emergence as AMR genes in pathogenic bacteria (Bebrone, 2007).

Next generation sequencing (NGS) has accumulated metagenome data from human/animal gut microbiomes and environmental bacterial populations. One drawback of NGS is that it merely describes “who is there” but not “what are they doing?” (Culligan et al., 2014). Functional metagenomics is a powerful approach to identify targeted phenotypes and potentially novel genes, without prior knowledge of sequence (Allen et al., 2009; Gudeta et al., 2015; Gudeta et al., 2016).

The aim of this study was to determine the impact of sewage and industrial waste streams on abundance of clinically important ARGs in soils. Libraries were prepared from sewage cake, human impacted and pristine soils and used to screen for ARGs using *E. coli* as an expression host. Prevalence of phenotypes was related to anthropogenic pollution and ARGs recovered were characterized. We discovered a group of uncharacterized novel B3 MBLs, novel ESBL and PBP genes associated with sewage sludge and industrial waste streams.

2. Materials and methods

2.1. Sample description

Sewage cake (SC) was fully digested, dehydrated, limed and was collected from a large WWTP. Soil samples were taken from a farm land with SC being applied as fertiliser one month or one year before sampling time; or without SC application. The reed bed sediment (RB) received effluent from a textile mill with high QAC usage (Gaze et al., 2005). The grass land soils were taken from the Rothamsted Park Grass experimental plots with and without farm yard manure amendment (FYM) (Nesme et al., 2014).

2.2. Metagenomic DNA extraction and purification

Five sub-samples from each soil were pooled prior to DNA extraction followed by extraction using FastDNA Spin Kit for Soil (MP Biomedical) and sonicated for 1 s before being loaded onto 2% low-melting agarose gel. The gel was run for 2 h at 120 V. The gel band of 4–6 kb DNA fragments were cut and digested with gelase (Epicentre). The digested DNA solutions were purified and concentrated with Amicon Centrifugal Filter Units (cut-off 100, ThermoFisher).

2.3. Construction of metagenomic libraries

The purified DNA was treated with End-It End Repair kit (Epicentre) and was ligated to the plasmid PCF430 (Newman and Fuqua, 1999) that had been previously cleaved with Hind III plus BamH1 and made blunt-ended. The ligated DNA was transformed to TransforMax EC100 competent cells (Epicentre). The transformed cells were recovered in 1 ml Super Optimal broth with Catabolite repression (SOC) broth at 37 °C for 1 h and were plated out on Luria Agar (LA) plates supplemented with either cefotaxime (0.25 μ g/ml), ceftazidime (1 μ g/ml) or imipenem (1.0 μ g/ml) in addition to 10 μ g/ml tetracycline (for plasmid maintenance). The total number of clones was estimated by plating out a small fraction of the libraries on selective LA plates with 10 μ g/ml tetracycline. 30 clones from each library were randomly picked, their plasmids were extracted and the DNA inserts were excised with BamH1 enzymes. The sizes of the inserts were estimated on agarose gel and the sizes of the libraries were calculated based on the average insert size and total number of clones. Library screening was performed on LA agar plates supplemented with 10 μ g/ml tetracycline plus cefotaxime (0.25 μ g/ml), ceftazidime (1 μ g/ml) or imipenem (1.0 μ g/ml). Resistant colonies were picked for further characterization.

2.4. Gene inactivation by transposon mutagenesis

EZ-Tn5 Kan-2 Insertion Kit (Epicentre) was used to create gene-inactivation mutants in the resistant clones according to manufacturer's instructions. The mutant plasmids were extracted and were sequenced to obtain the resistance gene sequences and their flanking DNA regions by Sanger sequencing. The whole genes and flanking sequences were obtained by primer walking into the flanking regions of the ESBL, MBL or PBP like genes.

2.5. DNA sequence analysis

The genes, operons and transcriptional units were predicted using the Bacterial Genome Annotation Pipeline, Fgenesb of Softberry (www.softberry.com). The closest known related sequences were identified via standard Protein-Protein BLAST (NCBI). Bacterial taxonomy was searched with the NCBI Taxonomy Browser.

2.6. Gene cloning and expression in expression vector

The entire ORF of novel β -lactamase genes were amplified by PCR (for primer sequences, see Table S2) and ligated into *Nde*I + *Bam*H1 or *Nde*I + *Sac*I sites of the pET26b expression vector. The ligation mix was transformed to *E. coli* BL21 (DE3) and *E. coli* EC100, respectively. The resistant transformants were confirmed by sequencing the plasmid inserts.

2.7. Determination of minimum inhibitory concentrations (MIC)

MICs were determined using the Agar Dilution Method as previously described (Andrews, 2001).

2.8. Presentation and visualization of genes and operon prediction

For the third generation cephalosporins (3GC) resistant clones, the context of genes and operons were drawn with IBS Illustrator (Liu et al., 2015).

2.9. Phylogenetic analysis

An unrooted phylogeny tree was built by using the Neighbour-Joining Tree method with Mega6 software (Tamura et al., 2013).

Table 13GC resistant clone numbers per Gb metagenomics library and their association with *intl1* gene prevalence in their source samples.

Library ID	Samples names	Library sizes (Gb)	Names (and number) of 3GC clones	3GC clone no per Gb library
1	Reed bed	0.63	RM3 (1)	1.59
2	Sewage cake	1.03	CX1 (1), CM1 (1)	1.94
3	1 month post application	1.87	H33 (1)	0.53
4	1 year post application	3.60	H5 (1)	0.28
5	No sewage cake (adjacent to application plots)	2.63	AM1 (1)	0.38
6	Park grass soil	1.53	(0)	0
7	Park grass soil with FYM	1.47	(0)	0

2.10. Gene accession numbers

Reference gene sequences were obtained from GenBank and their accession numbers are: AJ250876 (*bla*_{THIN-B}); AB636283 (*bla*_{SMB-1}); AB294542 (*bla*_{L1}); NG_048709 (*bla*_{BJP-1}); Y17896 (*bla*_{FEZ-1}); AF090141 (*bla*_{GOB-1}); KT175899 (*bla*_{OXA-48}); KJ151293 (*bla*_{KPC-2}); MF477011 (*bla*_{CTX-M-15}); DQ478720 (*bla*_{SHV-1}); KP634895 (*bla*_{TEM-1}); AM051149 (*bla*_{OKP-A}); AF197943 (*bla*_{SFH-1}); X57102 (*bla*_{CHP-A}); Y01415 (*bla*_{IMI-S}); KX999121 (*bla*_{NDM-1}); M11189 (*bla*_{BCL1}); Y18050 (*bla*_{VIM-1}); AJ620678 (*bla*_{GIM-1}); NG_049239 (*bla*_{JOHN-1}); AF244145 (*bla*_{IMP-4}).

The sequences of the six clones were deposited in GenBank. The accession numbers for clone RM3, CM1, H33, CX1, H5 and AM1 are from KF485391 to KF485396, respectively.

3. Results

3.1. Identification of novel β -lactamase genes

Seven metagenomic DNA libraries were constructed from soils with different management histories with a total coverage of approximately 12.7 Gbp (Table 1 and Table S1). Screening the seven libraries on LA agar plates supplemented with 3GCs (cefotaxime or ceftazidime) or the carbapenem imipenem resulted in six unique resistant clones, five from plates with ceftazidime and one with cefotaxime.

The six unique clones contained inserts of between 3 and 9 Kbp in size, which encoded multiple genes. To identify the gene conferring resistance, transposon insertion inactivation mutagenesis was used. This revealed six novel genes with various degrees of homology to known ARGs, four of which were homologous to MBL genes with an identity of 55% to 95%. One was homologous to SHV-1 β -lactamase with 65% identity, and one was homologous to the PBP2a gene with 64% identity (see Fig. 1). Subsequent primer walking sequencing enabled flanking regions to be obtained.

3.2. Susceptibility profile to β -lactam antibiotics

The β -lactamase encoding sequences were predicted with ORFfinder (NCBI) and cloned into expression vector PET26B, which was transformed into *E. coli* BL21 (DE3) and EC100 cells, respectively. The MICs of transformed cells, with and without resistance genes, against a panel of β -lactam class antibiotics were determined. The MICs of BL21 (DE3) cells with cloned resistance genes showed no difference from the control cells with the empty plasmid when expression was induced with IPTG. However, MICs of EC100 *E. coli* cells with the cloned β -lactamase genes were significantly higher than in the cells without the cloned genes. As summarised in Table 2, all five β -lactamase genes conferred elevated resistance to penicillin derivatives and 3GC antibiotics. *bla*_{CM1} conferred the highest level of resistance to various types of penicillins. Unlike *bla*_{CTX-M}, which hydrolyses cefotaxime more efficiently than ceftazidime (Walther-Rasmussen and Hoiby, 2004) *bla*_{CX1}, *bla*_{CM1}, *bla*_{RM3}, *bla*_{AM1} and *bla*_{H33} all conferred higher activity towards ceftazidime than cefotaxime, with *bla*_{CX1} having the highest activity. Both *bla*_{CX1} and *bla*_{CM1} conferred resistance comparable to clinically important β -lactamases. Furthermore, three of the five β -lactamase genes

(*bla*_{CX1}, *bla*_{RM3} and *bla*_{H33}) conferred reduced susceptibility to imipenem.

3.3. Phylogenetic analysis

To determine the phylogenetic relationships of the novel β -lactamase genes to well-characterized genes, a phylogenetic tree was constructed (Fig. 2) showing clustering of novel genes within class A and class B subclass B1 and B3.

3.4. Prediction of bacterial host based on the genetic context of resistance genes

Clone CX1 from limed and dewatered sewage cake was predicted to contain seven genes within two operons (Fig. 1) showing highest similarity to amino acid sequences in members of the Bacteroidetes phylum including *Chitinophaga sancti*, *Chryseobacterium antarcticum*, *Arcticibacter eurypsychrophilus* and *Rufibacter tibetensis*. Interestingly, gene *bla*_{CX1} and an ORF with similarity to *ble*_{MBL} (resistance to the anti-cancer drug belomycin) are likely to form a single operon (Fig. 1).

The CM1 clone, also from limed and dewatered sewage cake, contained seven genes that formed three operons illustrating similarity to ORFs found in *Paracoccus*, *Gemmobacter* and *Rhodobacteraceae* spp., within the phylum Proteobacteria. As this clone also contained plasmid related genes such as those for a replication initiation protein and an addiction-antitoxin module, it is apparent that the clone is from a novel plasmid (Fig. 2). Remarkably, there is an *ISPme1* transposase gene, a member of the *IS1380* family, immediately upstream of the gene *bla*_{CM1}, suggesting that *bla*_{CM1} may be mobilizable by *ISPme1*.

Clone H33, from soil one-month after amendment of limed and dewatered sludge, contained three genes forming two operons. The first gene showed high similarity to a subclass B3 MBL *bla*_{LRA-3}, which was isolated from an environmental bacterium in remote Alaskan soil with 95% identity (Allen et al., 2009). The other two genes demonstrated similarity to hypothetical proteins from an *Acidobacteria* sp., which is ubiquitous and abundant in soils.

RM3 was identified from a clone library constructed from soil with high levels of biocide exposure. It contained eight genes within two operons. All of the eight genes were homologous to those from *Janthinobacterium* sp., (Betaproteobacteria). Gene 2 through to gene 8 formed the second operon, including the gene *bla*_{RM3}. The intergenic distances within the operon were all < 77 base pairs, strongly suggesting they formed a single transcriptional unit and had a similar biological function. As the three genes upstream of *bla*_{RM3} were all transporter related proteins and the gene downstream was a heat shock protein, we predict that *bla*_{RM3} had an original function relating to transportation across the cell membrane or the stress response.

Clone AM1, from unamended soil adjacent to sludge amended soil, contained three operons, which comprised of five genes. The first and second genes showed similarity to genes from *Candidatus solibacter*, which is classified within the Acidobacteria phylum. However, the third gene showed similarity to a sequence from *Rubrobacter aplysinae*, which is a member of the Actinobacteria phylum, although the identity was low (30%). The fourth gene showed highest similarity to a B3 subclass gene

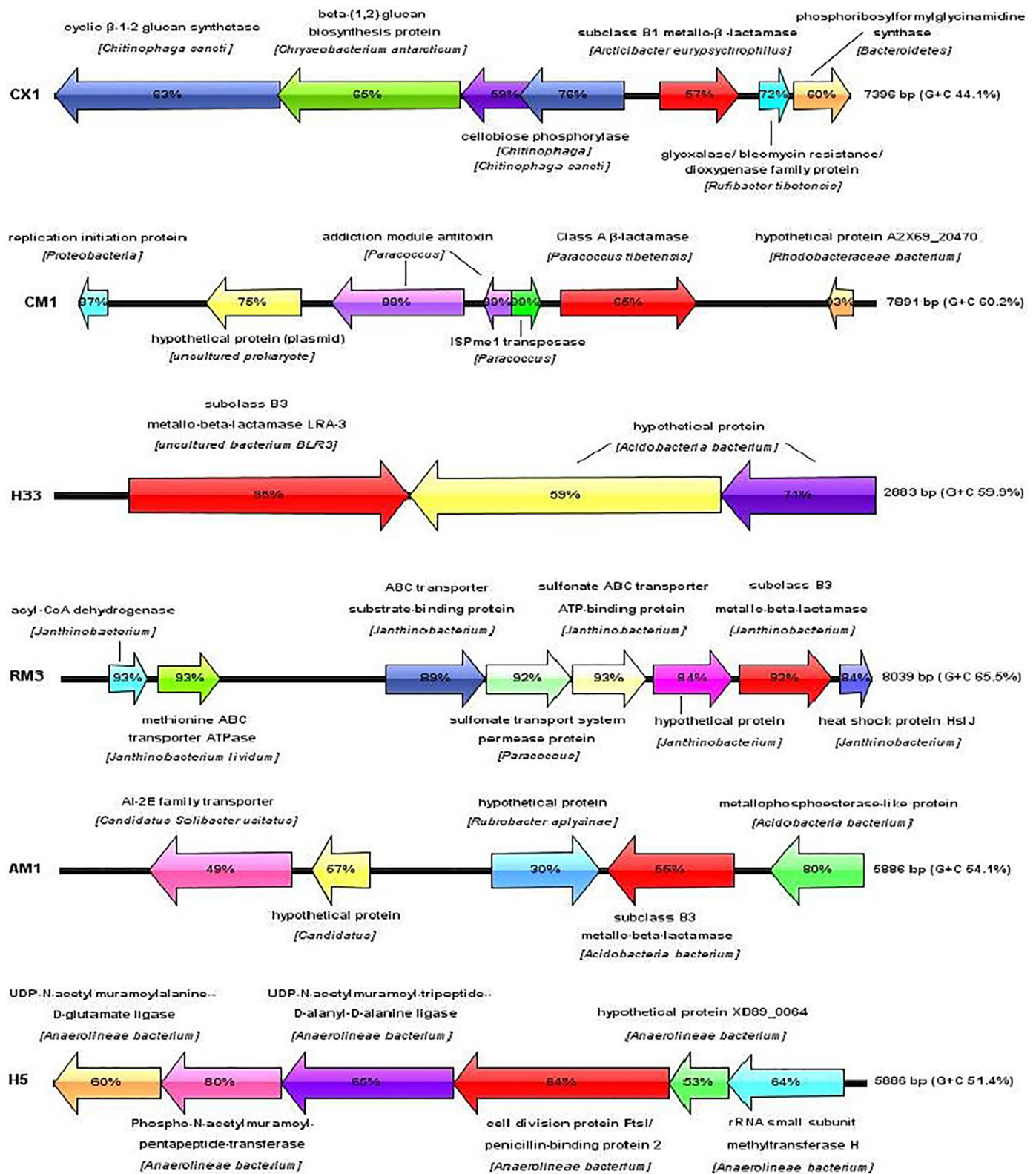


Fig. 1. Gene and operon prediction for the 3GC resistant clones (CX1, CM1, H33, RM3, AM1 and H5). Identity percentage for each predicted gene is shown within each arrow. Possible host species for each predicated gene are italicised in square brackets beneath the gene name.

from an *Acidobacteria* sp. (55%). The *bla*_{AM1} gene was immediately upstream of a gene encoding a metallo-phosphoesterase-like protein, with an intergenic region of 70 base pairs. It is likely that they co-transcribe into a single mRNA, implying they have related functions.

Clone H5, from a soil DNA library one-year after sludge amendment,

contained six genes forming a single operon. The closest sequence homologs of all the genes were from the class Anaerolineae in the phylum Chloroflexi, which contains many environmental 16S rRNA gene sequences with only a few cultured representatives in GenBank. In addition clone H5 encoded a PBP-like protein, which conferred an

Table 2

Minimal inhibitory concentrations (MICs, $\mu\text{g/ml}$) of the novel β -lactamase gene expression clones in *E. coli* against some commonly used β -lactams. Abbreviations: AMP, Ampicillin; AMO, Amoxicillin; CAR, Carbenicillin; TEM, Temocillin; AZT, Aztreonam; 3rd generation cephalosporin (TAX, Cefotaxime; ZIM, Ceftazidime); carbapenem (IMI, Imipenem).

	AMP	AMO	CAR	TEM	AZA	TAX	ZIM	IMI	Gene classification
pCF430	8	8	32	16	0.25	0.25	0.5	0.5	Control
<i>bla</i> _{CX1}	128	128	256	32	0.5	1	32	1	MBL B1
<i>bla</i> _{RM3}	16	8	32	32	0.5	0.25	8	1	MBL B3
<i>bla</i> _{H33}	8	8	32	16	0.25	0.25	4	1	MBL B3
<i>bla</i> _{AM1}	8	8	32	16	0.25	0.25	4	0.5	MBL B3
<i>bla</i> _{CM1}	> 256	> 256	> 256	32	2	0.25	8	0.5	Class A

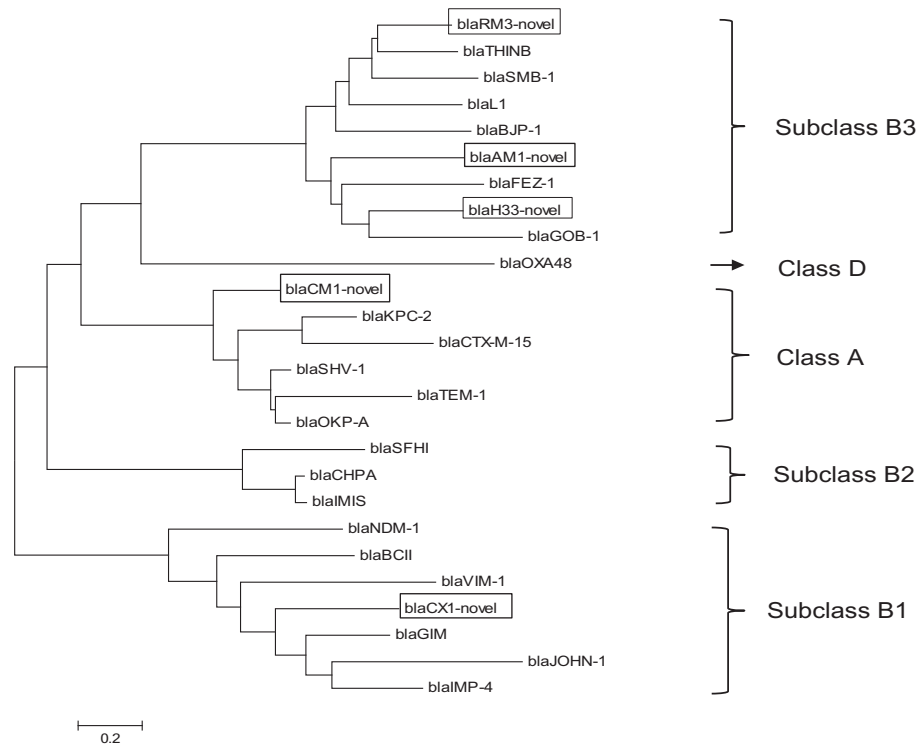


Fig. 2. An unrooted phylogeny tree showing phylogenetic relationship of newly identified and well characterized β -lactamase genes. Boxed genes are novel genes identified in this study.

elevated resistance to ceftazidime.

4. Discussion

Gene expression in BL21 (DE3) was ineffective in this study, probably due to high promoter efficiency and overproduction of enzymes that are toxic to the cells (Rosano and Ceccarelli, 2014). However, the genes were expressed in EC100 cells, possibly because *E. coli* RNA polymerase also transcribes the genes from the pET26b T7 promoter though at much lower efficiency than in BL21(DE3) (Wiggs et al., 1979). The novel resistance genes were clearly expressed both from their own promoter (as in the cloning vector pCF430) and from the T7 promoter (as in the expression vector pET26B) in *E. coli* EC100.

Novel resistance genes were found in limed and dewatered sewage cake, cake amended soils and biocide impacted reed bed sediments. No ESBL or MBL genes were found in the libraries from Rothamsted Park Grass soils amended with or without farm yard manure (predominantly sourced from organic beef cattle). However, one novel resistance mechanism was identified from control soil adjacent to the sewage cake amended soil, which may have been due to cross-contamination at the same farm.

*Bla*_{CM1} is a new class A β -lactamase gene. It is most closely related to SHV-1 and OKP-A enzymes, both of which were found in variety of

bacterial pathogens. *Bla*_{CX1} is a new member of the B1 subclass, which includes *bla*_{NDM}, and is closely related to the environmental *bla*_{JOHN-1} gene, and the clinically prevalent *bla*_{GIM} and *bla*_{IMP} gene families. *Bla*_{H33}, *bla*_{RM3} and *bla*_{AM1} are all new members of the B3 subclass, which were predominantly chromosomally located but were associated with resistance in some opportunistic pathogens (Horsfall et al., 2011).

*Bla*_{CX1} was located directly upstream of a bleomycin resistance-like gene (*ble*_{MBL}). The association is similar to that of another B1 subclass gene, *bla*_{NDM}, and a close associated *ble*_{MBL} gene. The *bla*_{NDM} genes and *ble*_{MBL} gene are co-expressed from the same promoter (Dortet et al., 2012). Bleomycin was approved by the Food and Drug Administration in 1975 for anti-cancer therapy. Its resistance genes (e.g. *ble*_{MBL}) have been found in number of pathogenic as well as environmental bacterial species. It was speculated that bleomycin could therefore co-select for *bla*_{NDM} carrying bacteria in cancer patients (Dortet et al., 2012; Dortet et al., 2017). Since *bla*_{CX1} and the *ble*_{MBL}-like genes are separated by 97 base pairs and are transcribed in the same direction, they are likely to be expressed from the same promoter, implying they could be mobilized together.

*Bla*_{CM1} was located adjacent to an insertion sequence *ISPme1*. This IS element can mobilize adjacent genes onto plasmids and was able to drive the transcription of downstream genes, such as *dhlB2*, a dehalogenase that can biodegrade a groundwater pollutant 1,2-

Dichloroethane (Munro et al., 2016). A truncated *ISPme1* was located immediately upstream of the *bla_{OXA-45}* gene from *Xanthobacter autotrophicus* and provides the promoter for its expression (Li et al., 2009). Furthermore, another member of IS1380 family, *ISEcp1*, mobilized adjacent *bla_{CTX-M}* genes in a manner similar to that of *ISPme1* (Bartosik et al., 2008). This demonstrates that *bla_{CM1}* is likely to be highly mobile and that sewage application to soil may introduce highly mobile ESBLs to agricultural soil.

The gene *bla_{RM3}* was shown to encode a MBL with activity against most β -lactam classes including cephalosporins in vitro in *E. coli*, and thus has the potential to evolve into a clinically important gene. A RM3 MBL crystal structure revealed the MBL superfamily $\alpha\beta/\beta\alpha$ fold, which more closely resembled that in mobilized B3 MBLs (*bla_{AIM-1}* and *bla_{SMB-1}*) than other chromosomal enzymes (*bla_{L1}* or *bla_{FEZ-1}*) (Salimraj et al., 2016).

The fourth gene in clone H5 encodes a PBP-like protein, which conferred an elevated resistance to ceftazidime. This result was similar to a previous study in that some *E. coli* strains were capable of resistance to imipenem due to chromosomal PBP2 mutations (Aissa et al., 2016).

It is likely that most of the novel ARGs identified in this study originate from the chromosome of environmental bacteria, however ARGs identified in CX1 and CM1 (and other genes in these clones) show similarity to genes from gut associated bacteria as well as environmental bacteria suggesting selection could have occurred in the human gut or during waste water treatment. Although the gene contexts for most clones appear to be chromosomal, clone CM1 appears to be plasmid borne (Fig. 1).

This study illustrates the power of functional metagenomic screening for identification and characterization of novel antibiotic resistance genes and inferring the phylogenetic origin of these genes. It further confirmed that anthropogenic activities, such as sewage cake and industrial effluent amendments, disseminate and/or select for antibiotic resistant bacteria in soils (Moore et al., 2011).

5. Conclusion

In this study uncharacterized members of the β -lactamase gene family with similarity to previously described MBL and ESBL resistance genes reported in clinical pathogens were identified. Genes cloned directly from sewage cake showed highest similarity to clinically important resistance genes including *bla_{NDM1}*. Phenotypic characterization of clones (possible with functional metagenomic approaches) demonstrated resistance to 3GCs and reduced susceptibility to carbapenems in some cases. Application of digested sewage cake to farm land is likely to introduce large numbers of these genes to soil as high abundance is necessary for detection with functional metagenomics given the low coverage relative to metagenome size. Biocide rich waste may also co-select for and introduce antibiotic resistance to the environment as suggested by previous studies (Gaze et al., 2011). While most genes appeared to be located chromosomally, this study demonstrated that novel genes have the potential to be mobilized by insertion sequences or through mobilization onto plasmids facilitating spread. No novel ESBL or MBLs were detected in long term curated grassland soils suggesting that their potential as reservoirs of abundant clinically significant resistance is relatively low. However, a previous study of the same soils reported a high diversity of ARG-like sequences inferred from NGS analysis (Nesme et al., 2014). Most of the novel genes characterized in this study would not have been identified as ARGs using commonly applied search strategies based on high sequence similarity. This demonstrates the value of phenotypic characterization of clinically significant resistance genes in functional metagenomic libraries.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2019.105120>.

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