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The Soil Microbiota Harbors a Diversity of Carbapenem-Hydrolyzing β-Lactamases of Potential Clinical Relevance

Dereje Dadi Gudeta, Valeria Bortolaia, Greg Amos, Elizabeth M. H. Wellington, Kristian K. Brandt, Laurent Poirel, Jesper Boye Nielsen, Henrik Westh, Luca Guardabassi

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The origin of carbapenem-hydrolyzing metallo-β-lactamases (MBLs) acquired by clinical bacteria is largely unknown. We investigated the frequency, host range, diversity, and functionality of MBLs in the soil microbiota. Twenty-five soil samples of different types and geographical origins were analyzed by antimicrobial selective culture, followed by phenotypic testing and expression of MBL-encoding genes in Escherichia coli, and whole-genome sequencing of MBL-producing strains was performed. Carbapenemase activity was detected in 29 bacterial isolates from 13 soil samples, leading to identification of seven new MBLs in presumptive Pedobacter roseus (PEDO-1), Pedobacter borealis (PEDO-2), Pedobacter kyungheensis (PEDO-3), Chryseobacterium piscium (CPS-1), Epilithonimonas tenax (ESP-1), Massilia oculi (MSI-1), and Sphingomonas sp. (SPG-1). Carbapenemase production was likely an intrinsic feature in Chryseobacterium and Epilithonimonas, as it occurred in reference strains of different species within these genera. The amino acid identity to MBLs described in clinical bacteria ranged between 40 and 69%. Remarkable features of the new MBLs included prophage integration of the encoding gene (PEDO-1), an unusual amino acid residue at a key position for MBL structure and catalysis (CPS-1), and overlap with a putative OXA β-lactamase (MSI-1). Heterologous expression of PEDO-1, CPS-1, and ESP-1 in E. coli significantly increased the MICs of ampicillin, ceftazidime, cefpodoxime, cefoxitin, and meropenem. Our study shows that MBL producers are widespread in soil and include four genera that were previously not known to produce MBLs. The MBLs produced by these bacteria are distantly related to MBLs identified in clinical samples but constitute resistance determinants of clinical relevance if acquired by pathogenic bacteria.

Soil is an important reservoir of antibiotic resistance determinants (1, 2). Several studies have shown that specific antibiotic resistance genes of high clinical relevance may have originated from environmental bacteria (3–6). However, the origins of resistance genes encoding carbapenem-hydrolyzing metallo-β-lactamases (MBLs) are largely unknown. MBLs are currently the most critical β-lactamases in clinical settings because they are insensitive to β-lactamase inhibitors and confer resistance to carbapenems, a β-lactam class of last-resort drugs used for treatment of severe bacterial infections (7).

Based on amino acid sequences, MBLs are classified as molecular classes B β-lactamases and are further divided into B1, B2, and B3 subclasses (8). Subclass B1 and B3 MBLs bind two zinc ions (Zn1 and Zn2) in their active sites, employ different metal binding amino acids (for the Zn2 ligand), and exhibit broad-spectrum activity (9). Subclass B2 MBLs are mono-Zn enzymes whose activity is inhibited upon binding the second Zn (10) and have strong preferences for carbapenem substrates (11). B1 and B3 enzymes exist across different bacterial genera, whereas B2 enzymes have been described only in members of the genera Serratia and Aeromonas (10, 11).

The most common MBLs in clinical bacteria are NDM, VIM, and IMP (12). In addition, new types of MBLs are continuously emerging in pathogens from unknown reservoirs (13, 14). Identification of unknown MBL-encoding genes occurring in the environment is important for assessment of the human health risks associated with environmental development and transfer of carbapenem resistance (15, 16). The aim of this study was to investigate the frequency, host range, diversity, and functionality of MBLs in soil bacteria. Phenotypic and genotypic characterization of the MBLs detected in the soil microbiota revealed the presence of an environmental reservoir of new carbapenem-hydrolyzing MBLs of potential clinical relevance.

MATERIALS AND METHODS

Soil samples. A total of 25 soil samples recovered from six different geographical locations (Algeria, United Kingdom, Germany, Denmark, Norway, and Spain) and soil uses (agricultural and nonagricultural) were analyzed, including 10 samples collected in previous studies (17–23) (Table 1). Fifteen samples were collected in this study from 0- to 15-cm depth using sterile plastic bags and stored at −20°C. All soil samples were thawed at room temperature before processing.

Selective isolation of carbapenem-resistant Gram-negative bacteria. A combined approach, including direct plating and selective enrichment in media with different nutrient contents, was used for isolation of carbapenem-resistant Gram-negative bacteria.
enem-resistant bacteria. All media (here defined as “selective” media) were supplemented with 8 μg/ml of vancomycin for inhibition of Gram-positive bacteria, 100 μg/ml of cycloheximide for inhibition of fungi, and 4 μg/ml of meropenem (MP) for selection of carbapenem-resistant bacteria (24). After sieving and dispersion by stirring and sonication (25), soil samples were serially diluted up to 10⁻³ in sterile distilled water. These suspensions were used for direct plating of 200-μl aliquots per plate on selective brain heart infusion agar (BHI-A) (nutrient-rich medium) and on selective diluted nutrient broth agar (DNB-A) optimized for growth of taxonomically diverse oligotrophic soil bacteria (25). In parallel, selective enrichment of meropenem-resistant bacteria was performed by adding aliquots of 1 ml undiluted soil bacterial suspension separately to 99 ml of selective brain heart infusion broth (BHI-B) and 99 ml of selective diluted nutrient broth (DNB-B). After 48 h of incubation at 25°C under shaking at 150 rpm, the enriched cultures were serially diluted up to 10⁻³, and each dilution was plated as described above. All the agar plates were incubated at 25°C and read daily for 2 weeks. Subsequently, all presumptive carbapenem-resistant isolates displaying unique colony morphology (relative to size, surface texture, color, and margins) and different microscopic appearances (relative to shape and size) were subcultured on selective BHI-A or DNB-A prior to storage in 15% glycerol at −80°C.

**Media and antibiotics.** Media and antibiotics were purchased from Difco (Le Pont-de-Claux, France) and Sigma-Aldrich (Steinheim, Germany), respectively.

**Phenotypic and genotypic characterization of carbapenem-resistant bacteria.** The modified version of the CarbaNP test proposed by Dortet et al. (26) was used for detection of carbapenem-resistant activity in presumptive carbapenem-resistant isolates from soil. Isolates not lysing in Tris-HCl buffer (Thermo Scientific, Rockford, IL, USA) were sonicated (two cycles of 30 s at 40% amplitude) in 200 μl of Tris-HCl lysis buffer using a Sonopuls Ultrasonic homogenizer (Bandelin Electronic GmbH & Co. KG). Imipenem-hydrolyzing isolates were identified by 16S rRNA gene sequencing using universal primers (see Table S1 in the supplemental material). Isolates were assigned to bacterial species based on the 97% cutoff value of nucleotide identity proposed by Stackebrandt and Goebel (27).

**Phenotypic characterization.** Phenotypic testing was performed by the disk-diffusion method using a 20-μg disc of meropenem (MP) and 4 mg/ml of EDTA on Luria-Bertani plates (Difco, Le Pont-de-Claux, France). Media were incubated for 18 h, and the presence or absence of a zone of inhibition was recorded. Synergy between meropenem and EDTA was considered indicative of MBL production. Synergy was defined as zones of inhibition of >18 mm around the disk with meropenem and >10 mm around the disk with the combination of meropenem and EDTA.

**Expression of carbapenemase activity.** Carbapenemase activity was determined using a modified version of the CarbaNP test (28). Escherichia coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used as the recipient for plasmid DNA transformation. The MBL producers were used as donors for conjugation into E. coli TOP10 by the filter mating method (29). The conjugates were selected on Luria-Bertani plates containing 5 μg/ml of amoxicillin and 5 μg/ml of tetracycline (pCF430 resistance determinant).

**Identification of MBL-encoding genes.** To identify MBL-encoding genes, plasmid DNA was isolated from the MBL producers by the miniprep method, and the plasmid inserts were sequenced. Sequences were compared with those belonging to species or genera in which MBLs have been described. The modified version of the CarbaNP test proposed by Dortet et al. (26) was used for detection of carbapenem-resistant activity in presumptive carbapenem-resistant isolates from soil. Isolates not lysing in Tris-HCl buffer (Thermo Scientific, Rockford, IL, USA) were sonicated (two cycles of 30 s at 40% amplitude) in 200 μl of Tris-HCl lysis buffer using a Sonopuls Ultrasonic homogenizer (Bandelin Electronic GmbH & Co. KG). Imipenem-hydrolyzing isolates were identified by 16S rRNA gene sequencing using universal primers (see Table S1 in the supplemental material). Isolates were assigned to bacterial species based on the 97% cutoff value of nucleotide identity proposed by Stackebrandt and Goebel (27). MBL phenotypic detection was done using MBL MP/MPi Etest strips (bioMérieux, Marcy l’Etoile, France) containing MP at one extremity and MP plus EDTA (MPI) at the other extremity and under conditions reported below. Synergy between meropenem and EDTA was considered indicative of MBL production.

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Marker) or 50 μg/ml of kanamycin (pZE21MCS resistance marker). Amoxicillin was used for screening libraries to avoid false-negative results (30). Amoxicillin-resistant transformants were screened for carbapenemase production by the modified CarbaNP test as described above. DNA inserts from recombinant plasmids harbored by carbapenemase-producing transformants (recombinant clones) were amplified by PCR (see Table S1 in the supplemental material) and Sanger sequenced (Macrogen, Seoul, Republic of Korea).

**WGS and bioinformatics analysis.** Whole-genome sequencing (WGS) was performed on soil MBL-producing strains belonging to species for which MBLs have not been described. DNA was extracted using a DNeasy blood and tissue kit (Qiagen, Düsseldorf, Germany), and normalization of the DNA concentration was performed using Qubit (Invitrogen, Paisley, United Kingdom). Libraries were generated using a Nextera XT DNA Sample Preparation kit (Illumina, CA, USA), and sequencing was performed on an Illumina MiSeq benchtop sequencer (Illumina, CA, USA) using a MiSeq Reagent Kit v2 (300 cycles) and two 150-bp paired-end reads. Reads from sequencing were assembled using Velvet v1.0.11 (http://www.ebi.ac.uk/~zerboin/velvet/) and VelvetOptimiser (http://bioinformatics.net.au/software.velvetoptimiser.shtml). Putative MBL-encoding genes in the WGS data were detected by ARG-ANNOT (31). The MBL three-dimensional (3D) structure was predicted with the protein-modeling server Phyre2 v2.0 (32). In the contigs containing the putative MBL-encoding genes, open reading frames (ORFs) were predicted and annotated using CLC main workbench v6.8.2 (Qiagen, Aarhus, Denmark), and each predicted protein was used as a query sequence (BLASTP) to search similar conserved domains in the NCBI sequence database. MBLs displaying maximum identity to the query sequences were used for amino acid alignments (ClustalW2) and phylogenetic-tree construction (FigTree v1.4.2 [http://tree.bio.ed.ac.uk/software/figtree/]).

**Screening of MBL production in reference strains.** MBL production was screened in 17 validated reference strains displaying close 16S rRNA gene sequence identity (>99%) to selected MBL producers isolated from soil. To determine whether it was an intrinsic property within MBL-producing strains, meropenem MIC values of Meropenem-resistant Gram-negative bacteria were isolated from all 25 soil samples analyzed in this study, leading to a total of 130 isolates. Carbapenemase activity was detected by CarbaNP test in 29 of these isolates, originating from 13 soil samples, including both agricultural and nonagricultural soils (Table 1). These carbapenemase-producing isolates belonged to the genera *Pedobacter*, *Epilithonimonas*, *Sphingomonas*, *Massilia*, *Chryseobacterium*, *Janthinobacterium*, and *Stenotrophomonas* (Table 1). All carbapenemases were classified as MBLs based on synergy between meropenem and EDTA. Based on 16S rRNA gene sequence identity, seven strains were assigned to species or genera that were not known to produce MBLs prior to this study: *Pedobacter roseus* strain SI-33 (*n* = 1), *Pedobacter borealis* strain ALS-14 (*n* = 1), *Pedobacter kyungheensis* strain Stok-3 (*n* = 1), *Chryseobacterium piscium* strain Stok-1 (*n* = 1), *Epilithonimonas tenax* strain Stok-2 (*n* = 1), *Massilia oculi* strain SB1-3 (*n* = 1), and *Sphingomonas* sp. strain ALS-13 (*n* = 1) (Table 2). The *Sphingomonas* strain was not assigned to any species, as the closest species (*Sphingomonas hakookensis*) displayed 16S rRNA gene sequence identity below the cutoff value of 97% (95.71%). The remaining 22 strains were related to *Stenotrophomonas maltophilia* and *Janthinobacterium lividum*, which are known MBL producers (28).

**Genetic context of MBL-encoding genes in soil bacteria.** New MBL-encoding genes were identified in presumptive *C. piscium* strain Stok-1 (annotated as *bla*<sub>CMY-1</sub>), *E. tenax* strain Stok-2 (*bla*<sub>Er</sub>), and *P. roseus* strain SI-33 (*bla*<sub>SPG</sub>) by using shotgun cloning in *E. coli* TOP10 cells, and their genetic context was further examined by WGS (see Table S2 in the supplemental material). The *bla*<sub>CMY-1</sub> gene was located between two genes encoding putative piri-C protein (Orfa2) and NADPH-dependent FMN reductase (Orfa3) (Fig. 1A). The *bla*<sub>SPG</sub> gene was identified on an operon encoding a putative diphosphomevalonate decarboxylase (Orfb1), two hypothetical proteins (Orfb2 and Orfb3), and a putative bleomycin resistance protein (Orfb4) (Fig. 1B; see Table S3 in the supplemental material). The *bla*<sub>CMY-1</sub> gene was located between 275-bp (Nr1) and 440-bp (Nr2) noncoding DNA sequences (Fig. 1C). An operon encoding putative glh2 tail sheath proteins (Ptp1 and Ptp2), glh2 tail tube protein (Ptp2), and asparagine-binding protein (Ptbp) was identified upstream of *bla*<sub>CMY-1</sub> stop codon, followed by two genes encoding putative YTVN β-propellin repeat-containing proteins (YTVN1 and YTVN2). An additional operon encoding acrilavine resistance protein (AcrB), resistance nodulation cell division protein (RND), and RND family efflux transporter MFP subunit (MFP) was identified downstream of the β-propellin repeats (Fig. 1D; see Table S3 in the supplemental material).

**MIC determination.** The MIC of meropenem was determined by Etest (bioMérieux, Marcy l’Etoile, France) in MBL-producing soil isolates, reference strains, and recombinant clones. The Etest was performed according to the manufacturer’s instructions, with the exception of the incubation temperature, set at 25°C for MBL-producing soil isolates and reference strains. The MICs of a broader range of β-lactams were determined in the recombinant clones by broth microdilution using the Sensititre ESBL plate format (Trek Diagnostic Systems, OH, USA) according to standard procedures (http://www.eucast.org/).

**Nucleotide sequence accession numbers.** The nucleotide sequences described in this study were deposited in the GenBank nucleotide sequence database with accession numbers KP109675 to KP109681 and in the Comprehensive Antibiotic Research Database (CARD) (http://aac.asm.org/content/577/73484.full/) to enhance early detection in clinical isolates by WGS, which is becoming a routine tool for strain typing in clinical microbiology.

**RESULTS**

**Frequency and classification of carbapenemase-producing bacteria isolated from soil.** Meropenem-resistant Gram-negative bacteria were isolated from all 25 soil samples analyzed in this study, leading to a total of 130 isolates. Carbapenemase activity was detected by CarbaNP test in 29 of these isolates, originating from 13 soil samples, including both agricultural and nonagricultural soils (Table 1). These carbapenemase-producing isolates belonged to the genera *Pedobacter*, *Epilithonimonas*, *Sphingomonas*, *Massilia*, *Chryseobacterium*, *Janthinobacterium*, and *Stenotrophomonas* (Table 1). All carbapenemases were classified as MBLs based on synergy between meropenem and EDTA. Based on 16S rRNA gene sequence identity, seven strains were assigned to species or genera that were not known to produce MBLs prior to this study: *Pedobacter roseus* strain SI-33 (*n* = 1), *Pedobacter borealis* strain ALS-14 (*n* = 1), *Pedobacter kyungheensis* strain Stok-3 (*n* = 1), *Chryseobacterium piscium* strain Stok-1 (*n* = 1), *Epilithonimonas tenax* strain Stok-2 (*n* = 1), *Massilia oculi* strain SB1-3 (*n* = 1), and *Sphingomonas* sp. strain ALS-13 (*n* = 1) (Table 2). The *Sphingomonas* strain was not assigned to any species, as the closest species (*Sphingomonas hakookensis*) displayed 16S rRNA gene sequence identity below the cutoff value of 97% (95.71%). The remaining 22 strains were related to *Stenotrophomonas maltophilia* and *Janthinobacterium lividum*, which are known MBL producers (28).

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lapped the 5’ end of a gene encoding a putative OXA β-lactamase, which was annotated as bla_MS1-OXA (Fig. 1G). Genes encoding a putative hypothetical protein and a putative argininosuccinate synthase were detected upstream of bla_MS1 and downstream of bla_MS1-OXA, respectively (data not shown).

**Sequences of the new MBLs identified in soil bacteria.** The predicted amino acid sequences of the seven new MBLs from soil bacteria were analyzed, and their evolutionary relationship with previously described MBLs was shown by phylogenetic-tree analysis (Fig. 2).

**TABLE 2 Phenotypic and genotypic traits of metallo-β-lactamase-producing soil strains and closely related reference strains**

<table>
<thead>
<tr>
<th>Soil strain</th>
<th>Meropenem MIC (μg/ml)</th>
<th>Name (accession no.)</th>
<th>16S rRNA identity (%)</th>
<th>CarbaNP test result</th>
<th>Meropenem MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. roseus</em> strain SI-33</td>
<td>8</td>
<td><em>P. roseus</em> CL-GP80* (DQ112353)</td>
<td>99.78</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>P. kyangheensis</em> strain Stok-3</td>
<td>&gt;32</td>
<td><em>P. kyangheensis</em> THGT17 (IN196132)</td>
<td>99.72</td>
<td>Negative</td>
<td>8</td>
</tr>
<tr>
<td><em>P. bargaei</em> DSM19626</td>
<td>&gt;32</td>
<td><em>P. bargaei</em> DSM19626 (EU030687)</td>
<td>97.52</td>
<td>Negative</td>
<td>8</td>
</tr>
<tr>
<td><em>E. tenax</em> strain Stok-2</td>
<td>4</td>
<td><em>E. tenax</em> DSM16811 (AF493969)</td>
<td>98.24</td>
<td>Positive</td>
<td>3</td>
</tr>
<tr>
<td><em>C. piscium</em> strain Stok-1</td>
<td>4</td>
<td><em>C. piscium</em> CCUG 51923 (AM404049)</td>
<td>98.58</td>
<td>Positive</td>
<td>3</td>
</tr>
<tr>
<td><em>S. hankookensis</em> strain SI-3</td>
<td>0.5</td>
<td><em>M. oculi</em> CCUG 43427A (FR773700)</td>
<td>98.94</td>
<td>Negative</td>
<td>0.25</td>
</tr>
</tbody>
</table>

a ND, not determined, as the strain was not available or did not grow on Mueller-Hinton medium.

116 (standard numbering system) β-lactamases PEDO-1, PEDO-2, CPS-1, and ESP-1, which was previously reported in GOB β-lactamases (34) (Fig. 3). Similarly to all GOB β-lactamases (GOB-1 to GOB-18), a Met residue was present at position 221 in PEDO-1, PEDO-2, and ESP-1, which was previously reported in GOB β-lactamases instead of the Ser residue found at this position in other subclass B3 β-lactamases (8). Remarkably, CPS-1 β-lactamase displayed a previously unobserved Leu residue at position 221 (Fig. 3). Based on multiple amino acid sequence alignment, PEDO-1, PEDO-2, CPS-1, and ESP-1 β-lactamases showed maximum identity (56 to 75%) to GOB-1 β-lactamase from *Elizabethkingia meningoseptica* (formerly *Chryseobacterium meningosepticum*) (34) (see Table S4 in...
the supplemental material). SPG-1 showed the highest amino acid identity (47%) to CAU-1 β-lactamase from "Caulobacter crescentus" (35), and MSI-1 β-lactamase displayed approximately 40% identity to SMB-1 and AIM-1 β-lactamases from "Serratia marcescens" (13) and "Pseudomonas aeruginosa" (36), respectively (see Table S4 in the supplemental material). MSI-1 (class B β-lactamase homologue) and MSI-OXA (class D β-lactamase homologue) displayed the highest amino acid identity (64 to 66%) to putative domains in "Massilia timonae" strain CCUG 45783, where the two domains were fused to form a putative bifunctional MBL-OXA enzyme. The two domains in the reference strain were annotated as MT-1 and MT-OXA (Fig. 1H). MT-1 displayed a predicted 3D structure similar to that of SMB-1 from "S. marcescens", whereas MT-OXA resembled FUS-1 (OXA-85), a narrow-spectrum class D β-lactamase identified in "Fusobacterium nucleatum" subsp. *polymorphum* (37). All metal-coordinating amino acid residues of subclass B1 MBLs were conserved in PEDO-3 (data not shown). PEDO-3 showed the highest amino acid identity (55%) to JOH1-1 from *Flavobacterium johnsoniae* (38) (see Table S5 in the supplemental material).

**Carbapenem resistance in reference strains.** Among the 17 purchased reference strains, carbapenemase activity was detected in five out of six *Sphingomonas* species and in all *Chryseobacterium* (n = 3) and *Epilithimonas* (n = 2) species tested. All these reference strains produced class B β-lactamase, as demonstrated by inhibition of carbapenemase activity by EDTA using CarbaNP test II. In contrast, no carbapenemase activity was found in the five *Pedobacter* species and the *M. timonae* reference strain (Table 2). The MICs of meropenem were determined for only 14 strains, since three *Sphingomonas* reference strains did not grow on Mueller-Hinton agar. The MICs ranged between 1.5 and 32 μg/ml in strains displaying carbapenemase activity, whereas lower MICs (0.047 to 0.5 μg/ml) were observed for strains without carbapenemase activity, with the exception of *Pedobacter agri* PB92, for which the MIC was 8 μg/ml.

**PCR screening of the reference strains using primers targeting the seven new MBL-encoding genes revealed the presence of (i) bla*CPSt-1* in *Chryseobacterium balustinum* and *Chryseobacterium scophthalmum* but not in the *C. piscium* reference strain CCUG 51923; (ii) bla*ESPl-1* in both *E. tenax* and *Epilithimonas lactis* reference strains; and (iii) bla*MSI-1* in the *M. timonae* reference strain CCUG 45783. The remaining four genes (bla*PEDO-1*, bla*PEDO-2*, bla*PEDO-3*, and bla*SPG-1*) were not detected in any reference strain.

**Heterologous expression of MBL-encoding genes in *E. coli*.** The effects of heterologous expression of ESP-1, CPS-1, and PEDO-1 on β-lactam susceptibility were assessed by comparing the MICs of a broad range of β-lactams between the three carbapenemase-producing recombinant clones and the wild-type *E. coli* TOP10. Expression of these MBL-encoding genes conferred resistance to ampicillin, cefoxitin, cefpodoxime, and ceftazidime alone.
or combined with clavulanic acid and determined an increase in the MICs of cefazolin, cefotaxime (with or without clavulanic acid), and meropenem below the clinical breakpoints (Table 3). The MICs of ceftriaxone and piperacillin-tazobactam were increased by expression of ESP-1, but not by expression of CPS-1 and PEDO-1. None of the MBLs isolated from soil bacteria affected the levels of susceptibility to cefepime.

**DISCUSSION**

This study shows that carbapenem-hydrolyzing MBLs are widespread in soil, as indicated by the recovery of carbapenemase-producing bacteria in 52% of the samples tested. Seven new carbapenemases were detected in a wide range of bacterial hosts ranging from Bacteroidetes (Pedobacter, Chryseobacterium, and Epilithonimonas) to Proteobacteria (Sphingomonas and Massilia). Our study is the first to report MBLs in members of the genera Pedobacter, Sphingomonas, Epilithonimonas, and Massilia. Although the occurrence of presumptive MBLs has been sporadically reported in environmental bacteria (3, 28, 39), previous studies were based on either metagenomics, which does not allow identification of the host bacteria, or culture on nutrient-rich media, which are known to inhibit growth of a large proportion of soil bacteria (25). Moreover, most of the studies did not show the hydrolytic activity of presumptive MBLs toward carbapenems.

Knowledge of the bacterial hosts, the genetic organization of MBL-encoding genes, and the activities of the enzymes is useful to predict the likelihood of horizontal gene transfer to clinically relevant species, as well as to facilitate detection of the possible emergence of new resistance genes in clinical settings (15, 16).

Phylogenetic-tree and amino acid sequence analyses showed that the seven MBLs detected in soil belonged to either subclass B1 (PEDO-1) or B3 (PEDO-1, PEDO-2, CPS-1, ESP-1, MSI-1, and SPG-1). These new MBLs were distantly related to the most frequently detected MBLs in clinical isolates, with predicted amino acid identities ranging from 40 to 69%. Closely related Pedobacter species produced different MBL subclasses, highlighting the diversity of MBLs within the genus. MSI-1 in the M. oculi strain was phylogenetically related to THIN-B, a resident carbapenemase produced by J. lividum (28). These two MBLs shared common ancestry with acquired SMB-1 and AIM-1 carbapenemases produced by S. marcescens (13) and P. aeruginosa (35), respectively (Fig. 2). This may suggest that bla₅SMB-1 and bla₅AIM-1 could have...
FIG 3 Alignment of amino acid sequences of subclass B3 metallo-β-lactamases newly described in this study with previously described MBLs. Metal-binding amino acids (12) are indicated with arrowheads. Position 221 is indicated with a star. Leu 221 in CPS-1 is boxed. GenBank/EMBL sequence database accession numbers are as follows: GOB-1, AAF04458; L1, ABO60992; FEZ-1, CAB96921; CAU-1, AJ308331; BJP-1, NP772870; and AIM-1, M998375.
been acquired from members of Oxalobacteraceae, to which both Massilia and Janthinobacterium belong.

Remarkable features were observed in some of the new MBLs. CPS-1 contains an unusual, not previously reported Leu residue at position 221. Position 221 is critical for MBL structure and catalysis. CPS-1 contains an unusual, not previously reported Leu residue at position 221. Position 221 is critical for MBL structure and catalysis.

Most (five out of six) of the Massilia and Janthinobacterium reference strains did not show carbapenemase activity. Meropenem, Cefotaxime-clavulanic acid, and Piperacillin-tazobactam were detected by examining the published sequence of the Massilia strain Stok-1 (bla_{CPSP}), respectively. This suggests heterogeneity of the MBLs within these genera, likely including as-yet-undescribed MBL types. Although none of the Pedobacter reference strains produced carbapenemase and harbored bla_{PedO1} and P. agri PB92 showed a high (8 μg/ml) meropenem MIC, and analysis of available WGS data (accession number AJLG00000000) indicated that it harbored a putative MBL (GenBank protein accession number WP_010603234.1, displaying 57% and 73% amino acid identity to putative MBL is not optimally expressed under the conditions of X-ray structure of FEZ-1 carbapenemase produced by Fluoribacter (Legionella) gormanii (47). However, no carbapenemase activity was detected in P. agri PB92, suggesting that the putative MBL is not optimally expressed under the conditions of the CarbaNP test. Alternatively, other mechanisms of resistance may be responsible for the high meropenem MIC value observed for the strain.

No mobile genetic elements were detected in the regions adjacent to the MBL-encoding genes, except in bla_{PedO1}. Similarly, progenitors of genes encoding CTM-X and OXA-48 β-lactamases, now widespread in clinical Gram-negative isolates worldwide, were detected on the chromosome of environmental isolates of Klyvera and Shewanella in the absence of mobile genetic elements (5, 6). Interestingly, downstream of thebla_{ESP}, gene from E. tenax Stok-2 there was a gene encoding a putative bleomycin resistance protein that has been previously reported downstream of bla_{ESP} (48, 49), suggesting a possible evolutionary relationship between ESP-1 and NDM-1. It has been suggested that environmental opportunistic pathogens may facilitate transfer of resistance genes between the environment and clinical settings (3). Based on the recent resistance risk ranking proposal (16),bla_{PedO1} could qualify for resistance readiness condition 2 (RESCon 2), i.e., potentially.

### Table 3: Effects of heterologous expression of metallo-β-lactamase-encoding genes from E. tenax strain Stok-2 (bla_{ESP}), C. piscium strain Stok-1 (bla_{CPSP}), and P. roseus strain SI-33 (bla_{PedO1}) on MICs of β-lactams in E. coli TOP10

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>EUCAST clinical breakpoint (μg/ml)</th>
<th>MIC (μg/ml)</th>
<th>E. coli</th>
<th>E. coli pESP-1</th>
<th>E. coli pCPSP-1</th>
<th>E. coli pPEDO-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meropenem*</td>
<td>&gt;8</td>
<td>0.032</td>
<td>0.5</td>
<td>0.094</td>
<td>0.094</td>
<td>0.094</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;4</td>
<td>≤0.25</td>
<td>32</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>≥8</td>
<td>≤1</td>
<td>&gt;16</td>
<td>≤8</td>
<td>≤8</td>
<td>16</td>
</tr>
<tr>
<td>Cefepine</td>
<td>≥4</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>≤1</td>
<td>16</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>-</td>
<td>≥8</td>
<td>&gt;64</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Cefpodoxime</td>
<td>&gt;1</td>
<td>0.5</td>
<td>32</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>&gt;2</td>
<td>&lt;0.25</td>
<td>2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>≥2</td>
<td>≤1</td>
<td>2</td>
<td>≤1</td>
<td>≤1</td>
<td>16</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&gt;8</td>
<td>≤4</td>
<td>256</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>&gt;16</td>
<td>≤4</td>
<td>16</td>
<td>4</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Cefazidime-clavulanic acid</td>
<td>-</td>
<td>≤0.25</td>
<td>16</td>
<td>4</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Cefotaxime-clavulanic acid</td>
<td>-</td>
<td>0.12</td>
<td>2</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*a MIC was determined by Etest.

b –, data could not be obtained.
transferable resistance determinants with known mechanisms harbored by a nonpathogen. The remaining MBL-encoding genes could be grouped as RESCOn5, a group conferring resistance to currently used antibiotics but not located on mobile genetic elements.

The new metallo-β-lactamase-encoding genes isolated from soil bacteria displayed poor activity against meropenem after cloning into E. coli (Table 3). Previous studies have shown that most of the clinically important MBLs, such as VIM (50, 51) and IMP (52), confer reduced susceptibility, but not resistance, to meropenem on E. coli laboratory strains despite their high catalytic efficiency toward carbapenems (7, 9). This could be due to additional mechanisms (e.g., porin loss and efflux pumps) or β-lactamases that may correspond to the carbapenem resistance phenotype in the original host (53) or to the high permeability coefficient of carbapenems in E. coli (54).

In conclusion, this study expands the current understanding of the occurrence, host range, diversity, and functionality of MBL-encoding genes in the soil microbiota. MBL production may have the occurrence, host range, diversity, and functionality of MBL-encoding genes in the soil microbiota. MBL production.


http://dx.doi.org/10.1038/nrmicro3067.


http://dx.doi.org/10.1016/j.mib.2009.06.013.


Erratum for Gudeta et al., The Soil Microbiota Harbors a Diversity of Carbapenem-Hydrolyzing β-Lactamases of Potential Clinical Relevance

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Volume 60, no. 1, p. 151–160, 2016. Page 156, Figure 2 legend, line 1: “subclass B1 (red lines) and subclass B3 (blue lines) MBLs” should read “subclass B1 (blue lines) and subclass B3 (red lines) MBLs.”

Page 156, right column, lines 10 and 11: “B1 (PEDO-1)” should read “B1 (PEDO-3).”