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Characterization of a Novel Putative Xer-Dependent Integrative Mobile Element Carrying the bla_{NMC-A} Carbapenemase Gene, Inserted into the Chromosome of Members of the *Enterobacter cloacae* Complex

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An *Enterobacter ludwigii* strain was isolated during routine screening of a Japanese patient for carriage of carbapenem-resistant *Enterobacteriaceae*. PCR analysis revealed the bla_{NMC-A} carbapenemase gene. Whole-genome sequencing revealed that bla_{NMC-A} was inserted in the chromosome and associated with a novel 29.1-kb putative Xer-dependent integrative mobile element, named EludIMEX-1. Bioinformatic analysis identified similar elements in the genomes of an *Enterobacter asburiae* strain and of other *Enterobacter cloacae* complex strains, confirming the mobile nature of this element.

Members of the *Enterobacter cloacae* complex (ECC) are an important cause of health care-associated infections, with a notable propensity to acquire antibiotic resistance determinants (1). The ECC includes at least six species, namely, *E. cloacae*, *Enterobacter asburiae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii*, and *Enterobacter nimipressuralis* (1). The epidemiology of these species, however, remains poorly defined since conventional phenotypic methods and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry, which are routinely used in the clinical microbiology laboratory, are unreliable for identification of ECC isolates at the species level (1, 2). Even 16S rRNA gene sequencing is unreliable for identification of ECC isolates at the species level, which requires sequence analysis of additional targets such as the *hsp60* and *rpoB* genes (3, 4).

Carbapenems are among the most reliable drugs for treatment of infections caused by multidrug-resistant ECC strains. Acquired resistance to carbapenems remains uncommon among isolates of these species and is generally due to overexpression of the resident AmpC β-lactamase in combination with permeability defects or to the production of acquired carbapenemases of different types (VIM, IMP, NDM, OXA-48, KPC, and NMC-A/IMI) (5, 6).

NMC-A is a serine carbapenemase originally detected in a carbapenem-resistant *Enterobacter* strain (NOR-1, initially identified as *E. cloacae* and subsequently reidentified as *E. asburiae*) isolated from a soft tissue infection in France in 1990 (7, 8). The enzyme is able to hydrolyze a broad spectrum of β-lactam substrates, with preference for penicillins, narrow-spectrum cephalosporins, and carbapenems (7, 9), and together with IMI-type enzymes belongs to a well-defined lineage of molecular class A β-lactamases (10). In the NOR-1 index strain, the bla_{NMC-A} gene was integrated in the chromosome, and expression was regulated by a LysR-type transcriptional regulator encoded by the *nmcR* gene, located upstream of the β-lactamase gene (11).

Since the first description, NMC-A has occasionally been reported to occur in *E. cloacae* isolates from Europe, the United States, and South America (12–15), while closely related enzymes of the IMI-type (IMI-1 to IMI-8, with 98% to 86% amino acid sequence identity to NMC-A) have also been detected in isolates of *E. cloacae*, *E. asburiae*, and *Escherichia coli* from the United States, Europe, the Far East, and South Africa (16–23). Nevertheless, NMC-A and IMI carbapenemases have remained overall uncommon in the clinical setting, unlike other class A serine carbapenemase such as the KPC-type enzymes (24).

Apart from the chromosomal location and the linkage with the *nmcR* regulatory gene, the genetic context of bla_{NMC-A} has not been further elucidated.

In this work, we investigated an NMC-A-positive isolate of *E. ludwigii* by whole-genome sequencing (WGS) and identified a novel 29.1-kb genetic element carrying the bla_{NMC-A} gene, which was putatively integrated in the chromosome by a Xer-mediated recombination mechanism.

E. ludwigii AOUC-8/14 was isolated in August 2014 from a surveillance rectal swab taken from a patient upon admission to the Neurointensive Care Unit of Careggi University Hospital of Florence, Italy. The patient was a Japanese tourist admitted to the hospital due to a ruptured brain aneurysm. The isolate grew on the carba section (selective for carbapenemase-producing *Enterobacteriaceae*) of the chromID Carba Smart dual medium (bioMérieux, Marcy l'Étoile, France) and was initially identi-

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TABLE 1 Antimicrobial susceptibility of *E. ludwigii* AOUC-8/14

Antibiotic	MIC (μg/ml)
Ampicillin	>256
Piperacillin	8
Piperacillin-tazobactam[a]	1
Amoxicillin-clavulanate[b]	256
Cefotaxime	0.125
Cefepime	0.125
Ceftazidime	0.25
Aztreonam	4
Amikacin	2
Gentamicin	0.25
Tobramycin	0.5
Imipenem	128
Meropenem	32
Ertapenem	8
Doripenem	8
Colistin	0.125
Chloramphenicol	16
Ciprofloxacin	≤0.015

[a] Tazobactam at fixed concentration of 4 μg/ml.
[b] Clavulanate at a 1:2 proportion to amoxicillin.

fied as *E. cloacae* or *E. asburiae* by MALDI-TOF mass spectrometry (Vitek-MS; bioMérieux, France). By reference broth microdilution (25), the isolate was resistant to carbapenems and chloramphenicol but susceptible to piperacillin, piperacillin-tazobactam, expanded-spectrum cephalosporins, fluoroquinolones, aminoglycosides, and colistin (Table 1).

A crude extract, prepared by sonic disruption of an overnight culture grown in LB medium, revealed the presence of carbapenemase activity (imipenem-hydrolyzing specific activity, 261 ± 3 nmol/min/mg protein) that was not inhibited by EDTA when tested in a spectrophotometric assay as described previously (26). In PCR assays, the isolate tested negative for bla_{KPC}, bla_{OXA-48}, bla_{NDM}, and bla_{VIM} carbapenemase genes (27) and positive for the bla_{NMC-A} gene (13).

The AOUC-8/14 isolate was investigated by draft WGS using an Illumina MiSeq platform and a 2 × 300-bp paired-end approach (Illumina Inc., San Diego, CA, USA). The WGS reads were assembled with A5-miseq (28) and analyzed by BLAST searches using the nr and WGS databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

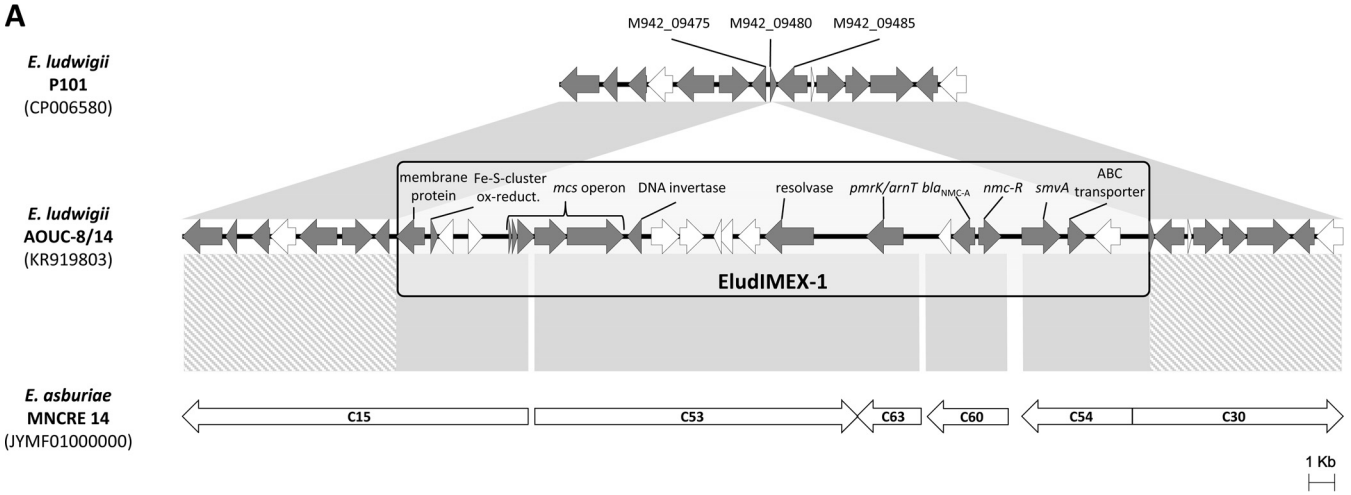
Data from WGS identified AOUC-8/14 as *E. ludwigii* based on sequence analysis of the *rpoB* and *hsp60* genes (4). Identification at the species level was further confirmed by comparison of the WGS data with the genomes of type strains of *E. ludwigii* and of other species of the ECC using GGDC software, version 2.0 (29) (Table 2). Analysis of the WGS data detected the resident *ampC* gene, encoding a class C β-lactamase of the ACT lineage identical to that of *E. cloacae* strain ARC4540 (GenBank accession no. KJ949114), and confirmed the presence of a bla_{NMC-A} gene identical to that of *E. asburiae* NOR-1 (11).

In *E. ludwigii* AOUC-8/14, the bla_{NMC-A} gene was located in a contig of 888 kb that, in a BLAST comparison, revealed high similarity (>99% nucleotide identity) to the complete genomes of *Enterobacter* strains P101 (GenBank accession no. CP006580) and EcWSU1 (GenBank accession no. CP002886), reported as *E. cloacae* in the database entries but reidentified here as *E. ludwigii* based on analysis of their *rpoB* and *hsp60* genes and on comparisons with the genomes of the *E. ludwigii* type strain and of strains of other ECC species by the GGDC software (Table 2). A high level of similarity (>99% nucleotide identity) of the contig containing the bla_{NMC-A} gene was also observed with the draft genome of the *E. ludwigii* EN-119 type strain (accession no. JTLO01000001) (30). Sequence alignment of these regions also revealed that in AOUC-8/14, bla_{NMC-A} was associated with a 29.1-kb DNA insertion that was not present in the other strains and was flanked by two 29-bp imperfect inverted repeats homologous to the XerC/XerD binding sites (31, 32) (Fig. 1). Such sites are present on the chromosome of most bacteria and are targeted by the XerC/XerD tyrosine recombinases to resolve chromosome dimers (31). Xer-mediated recombination events are also involved in the topological maintenance of several plasmids and in integration of mobile DNA elements that are indicated as IMEX (integrative mobile genetic elements exploiting the Xer-mediated recombination mechanism) (31). As such, the DNA insertion associated with the bla_{NMC-A} gene was identified as a putative IMEX and named EludIMEX-1. The element was inserted into

TABLE 2 Results of comparative analysis of the genomes of *E. ludwigii* AOUC-8/14 and other ECC strains carried out using GGDC software, version 2.0 (29)[a]

Strain (accession no.)	*E. ludwigii* EN-119^T	*E. ludwigii* **P101**	*E. ludwigii* **EcWSU1**	*E. asburiae* MNCRE14	*E. asburiae* **L1**	*E. cloacae* **ATCC 13047^T**	*E. hormachei* ATCC 49162^T
E. ludwigii AOUC-8/14 (LGIV00000000)	90	90	91	36	35	34	31
E. ludwigii EN-119^T (JTLO01000001)		90	90	35	35	34	31
E. ludwigii **P101** (CP006580)			91	36	36	34	31
E. ludwigii **EcWSU1** (CP002886)				36	36	34	31
E. asburiae MNCRE14 (JYMF01000000)					73	36	34
E. asburiae **L1** (NZ_CP007546)						36	34
E. cloacae **ATCC 13047^T** (NC_014121)							33
E. hormachei ATCC 49162^T (AFHR01000000)							

[a] The value for each comparison represents the percentage of DNA-DNA relatedness. Values of >70% indicate belonging to the same species. Sequences are from the WGS database (draft genomes) or from the nr database (complete genomes). Complete genomes are boldfaced.



B

		XerC	XerD		XerD	XerC	
AOUC-8/14		ACGGCAAGCCTAATGGTCAATTATTCGCAGT	29093 bp		ACTATGAATAATGGTTCATTAGGCAAACCTGCTAATACCCGCT		
P101	99%	..			..........C..........		99%
MNCRE 14	87%	.G.....	29093 bp?		.....G.....C..........		89%
N12-1563	99%	.....	29093 bp		.....C..........		99%
N12-1562	93%	.G.....	29093 bp		.....G.....C..........		87%
N11-1141	99%	.....	29093 bp		.....C..........		99%
N10-3276	99%	.....	29093 bp		.....C..........		99%
N11-1168	92%	.A.....CA.....T....	28088 bp		.....G..C..C..........		86%
WCHECl-1060	87%	.G.....CA.....T....	28063 bp		...G.....G.....C..........		89%

FIG 1 (A) Comparison of the genomic region of *E. ludwigii* AOUC-8/14 carrying the bla_{NMC-A} gene and the corresponding chromosomal region of *E. ludwigii* P101 (region 1872587 to 1888317 of GenBank accession no. CP006580), revealing the chromosomal insertion of the EludIMEX-1 element in AOUC-8/14. Comparisons with the corresponding regions of the EcWSU1 and EN-119 *E. ludwigii* strains yielded identical results and are not shown. ORFs are indicated by arrows, showing direction of transcription. Proteins of unknown function are filled in white. Regions connected by gray areas exhibit >98% nucleotide sequence identity. Comparison with the draft genome of *E. asburiae* MNCRE14 (GenBank accession no. JYMF01000001) is shown at the bottom. In this case, contigs from WGS of MNCRE14 (indicated by C and contig number) were aligned and ordered according to homology with the corresponding parts of the AOUC-8/14 genome. The regions connected by striped areas exhibit <91% nucleotide sequence identity. (B) Nucleotide sequences at the junctions of EludIMEX-1 with the *E. ludwigii* AOUC-8/14 chromosome, revealed by alignment with the corresponding *E. ludwigii* P101 chromosomal region. The imperfect repeats homologous to the XerC/XerD binding sites are boxed, the conserved consensus sequence is boldfaced (32), and the size of the intervening DNA segment (bp) is indicated. A comparison is also shown with the presumptive bla_{NMC-A}-carrying element of strain MNCRE14, with the elements associated with bla_{NMC-A} from ECC strains N12-1563 (GenBank accession no. KR057496.1), N12-1562 (GenBank accession no. KR057495.1), N11-1141 (GenBank accession no. KR057493.1), and N10-3276 (GenBank accession no. KR057492.1), and with the elements associated with bla_{IMI} from ECC strain N11-1168 (GenBank accession no. KR057494.1) and *E. cloacae* WCHECl-1060 (GenBank accession no. LFDQ01000001). Percentages at the left and right sides of the sequences represent the nucleotide identity with the corresponding region of AOUC-8/14 (for a 10-kb region, except for N12-1563, N12-1562, N11-1141, N10-3276, and N11-1168, which have shorter flanking regions).

the gene defined by locus tag M942_09480 of *E. ludwigii* P101, encoding a hypothetical protein of unknown function and containing a region of homology to part of the XerC binding site (Fig. 1). The EludIMEX-1 element showed a lower GC content than that of the *E. ludwigii* AOUC-8/14 genome (39.1% versus 54.6%), further confirming its acquisition by horizontal gene transfer.

EludIMEX-1 carried 23 open reading frames (ORFs), including the bla_{NMC-A} and *nmcR* genes, a microcin S-like operon similar to that from plasmid pSYM1 from *Escherichia coli* G3/10 (33), a *pmrK* (*arnT*)-like homolog possibly involved in the decoration of bacterial lipopolysaccharide with 4-amino-4-deoxy-L-arabinose, putative recombinase, and resolvase genes, and additional ORFs encoding hypothetical proteins of unknown function (Fig. 1; see also Table S1 in the supplemental material).

Interestingly, a BLAST search against the WGS database detected the presence of an element very similar to EludIMEX-1 (>98% nucleotide sequence identity) in the draft genome of *Enterobacter* strain MNCRE14 (GenBank accession no. JYMF01000001), although fragmented in different contigs (Fig. 1). In MNCRE14, reported as *E. cloacae* in the database but reidentified here as *E. asburiae* based on analysis of *rpoB* and *hsp60* genes and on genome comparisons by the GGDC analysis (Table 2), this element was inserted at the same genomic position as in AOUC-8/14, in an overall conserved but more divergent genomic context (Fig. 1), in agreement with its belonging to a different species. This observation suggested an independent acquisition of the two elements by recombination at the same chromosomal site and further supported the involvement of a site-specific recombination mechanism in the mobilization of this element.

While this paper was under revision, the sequences of genomic regions associated with bla_{NMC-A} and bla_{IMI} from ECC strains isolated in Canada and China were released in public databases. The regions associated with bla_{NMC-A} (GenBank accession numbers KR057492.1, KR057493.1, KR057495.1, and KR057496.1) were very similar to EludIMEX-1 (>99% nucleotide identity), while those associated with bla_{IMI} (accession numbers KR057494.1 and LFDQ01000001) were more divergent but very similar to each other and clearly related to Elu-

dIMEX-1; all were apparently inserted in similar genetic contexts (Fig. 1B).

Altogether, present findings identified a novel putative IMEX that may be responsible for integration of the bla_{NMC-A} carbapenemase gene into the chromosome of *Enterobacter* spp., apparently via a Xer-dependent recombination mechanism. Related elements may be responsible for chromosomal integration of bla_{IMI} genes. The site specificity requirement and the dependence on the XerC/XerD recombination system may explain the lower promiscuity of this element and the more limited diffusion observed for the bla_{NMC-A}- and bla_{IMI}-type genes compared with that observed for other carbapenemase genes associated with different types of mobile genetic elements.

Nucleotide sequence accession numbers. The nucleotide sequence of EludIMEX-1 was deposited in the INSDC database under the accession no. KR919803. The whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. LGIV00000000. The version described in this paper is version no. LGIV01000000.

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