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MECHANISMS OF RESISTANCE

Spread of Plasmid-Encoded NDM-1 and GES-5 Carbapenemases among Extensively Drug-Resistant and Pandrug-Resistant Clinical *Enterobacteriaceae* in Durban, South Africa

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ABSTRACT Whole-genome sequence analyses revealed the presence of bla_{NDM-1} ($n = 31$), bla_{GES-5} ($n = 8$), $bla_{OXA-232}$ ($n = 1$), or bla_{NDM-5} ($n = 1$) in extensively drug-resistant and pandrug-resistant *Enterobacteriaceae* organisms isolated from inpatients in 10 private hospitals (2012 to 2013) in Durban, South Africa. Two novel NDM-1-encoding plasmids from *Klebsiella pneumoniae* were circularized by PacBio sequencing. In p19-10_01 [IncFIB(K); 223.434 bp], bla_{NDM-1} was part of a Tn*1548*-like structure (16.276 bp) delineated by IS*26*. The multireplicon plasmid p18-43_01 [IncR_1/IncFIB(pB171)/IncFII(Yp); 212.326 bp] shared an 80-kb region with p19-10_01, not including the bla_{NDM-1}-containing region. The two plasmids were used as references for tracing NDM-1-encoding plasmids in the other genome assemblies. The p19-10_01 sequence was detected in *K. pneumoniae* ($n = 7$) only, whereas p18-43_01 was tracked to *K. pneumoniae* ($n = 4$), *Klebsiella michiganensis* ($n = 1$), *Serratia marcescens* ($n = 11$), *Enterobacter* spp. ($n = 7$), and *Citrobacter freundii* ($n = 1$), revealing horizontal spread of this bla_{NDM-1}-bearing plasmid structure. Global phylogeny showed clustering of the *K. pneumoniae* (18/20) isolates together with closely related carbapenemase-negative ST101 isolates from other geographical origins. The South African isolates were divided into three phylogenetic subbranches, where each group had distinct resistance and replicon profiles, carrying either p19-10_01, p18-10_01, or pCHE-A1 (8,201 bp). The latter plasmid carried bla_{GES-5} and *aacA4* within an integron mobilization unit. Our findings imply independent plasmid acquisition followed by local dissemination. Additionally, we detected $bla_{OXA-232}$ carried by pPKPN4 in *K. pneumoniae* (ST14) and bla_{NDM-5} contained by a pNDM-MGR194-like genetic structure in *Escherichia coli* (ST167), adding even more complexity to the multilayer molecular mechanisms behind nosocomial spread of carbapenem-resistant *Enterobacteriaceae* in Durban, South Africa.

KEYWORDS carbapenemases, *Enterobacteriaceae*, GES-5, NDM-1, plasmid-mediated resistance

The global dissemination of carbapenemase-producing *Enterobacteriaceae* (CPE) has reached African countries (1). Clinical isolates of CPE, including *Klebsiella pneumoniae*, *Enterobacter* spp., *Escherichia coli*, *Serratia marcescens*, and *Citrobacter* spp., have been described in South Africa, Gabon, Angola, Senegal, Nigeria, Kenya, and

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Tanzania, as well as in North African countries, including Morocco, Algeria, Tunisia, Libya, and Egypt. Specifically, NDM-1 and OXA-48-like are the most commonly reported carbapenemases in Africa (2–7).

In South Africa, NDM was first detected in *Enterobacter cloacae* in 2011 and subsequently in *K. pneumoniae* and *S. marcescens* (1, 8, 9). The NDM-positive *K. pneumoniae* strains of African origin have been multiclonal (1). Similar to the global situation, GES-carbapenemases are less prevalent and were first described in *Enterobacteriaceae* in South Africa in 2013 (1).

The rapid spread of CPE is supported by intra- and interspecies plasmid-mediated transfer of carbapenemase-encoding genes embedded in transposons and integrons (10–12). As part of class 1 integrons, bla_{GES-5} seems to be widespread worldwide and detected on different plasmid backbones (12–14). As reviewed previously (15), plasmids of several incompatibility groups (Inc) can mediate spread of carbapenem resistance in clinically relevant *Enterobacteriaceae*. While epidemic plasmids encoding KPC and OXA-48-like predominantly belong to IncF or IncL, a diversity of plasmid backbones, including IncA/C, IncF, IncL/M, IncN, IncR, and IncX, are associated with NDM (12, 16–19).

Here, we have explored the molecular epidemiology of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) *Enterobacteriaceae* isolated from the private hospital sector in Durban, South Africa, by whole-genome sequence (WGS) analyses focusing on the carbapenem resistance-encoding determinants and their genetic support.

RESULTS

Phenotypic and genotypic analyses of the *Enterobacteriaceae* collection. Table S1 in the supplemental material summarizes relevant patient data, source of specimen, and relevant phenotypic and genotypic characteristics for the 45 collected carbapenem-resistant *Enterobacteriaceae* (CRE), which included *K. pneumoniae* ($n = 21$), *K. michiganensis* ($n = 1$), *S. marcescens* ($n = 12$), *Enterobacter* spp. ($n = 9$), and *C. freundii* ($n = 1$). Antimicrobial susceptibility testing (Table S2) categorized them as multidrug resistant (MDR), extensively drug resistant (XDR), or pandrug resistant (PDR) according to standard definitions (20). As shown, PDR isolates were found among the *S. marcescens* strains ($n = 6$) only, while the XDRs included *K. pneumoniae* ($n = 5$), *S. marcescens* ($n = 4$), and *Enterobacter* spp. ($n = 4$). The PDR *S. marcescens* organisms belong to a distinct phylogenetic branch that is comprised of all except two (no. 33 and 34) of the included isolates, as evident in the NCBI Genome Tree report consisting of 341 genome assemblies (https://www.ncbi.nlm.nih.gov/genome/tree/1112?; accessed 29 September 2017). This branch included both XDR and PDR isolates from the same intensive care unit (ICU) as well as a bla_{NDM-1}-negative MDR isolate (no. 35) from a different ward, which implicate bla_{NDM-1} acquisition as well as nosocomial spread and development of a PDR genetic lineage.

The WGS analyses (Data Set S1) revealed the presence of carbapenemase-encoding genes in 41 of the 45 isolates: bla_{NDM-1} ($n = 31$), bla_{GES-5} ($n = 8$), bla_{NDM-5} ($n = 1$), and $bla_{OXA-232}$ ($n = 1$). All except three of these were positive in the Carba NP test (Table S1). Four of the CRE contained no known carbapenemase-encoding gene, although one was positive in the Carba NP test.

Phylogenetic analyses of carbapenemase-encoding *K. pneumoniae*. Among the 41 carbapenemase-encoding isolates (Table 1), *K. pneumoniae* was the most prevalent species ($n = 20$), dominated by ST101 ($n = 14$). The available demographic data showed that they were obtained from six hospitals and 14 wards, from different clinical sources, and from both sexes between the ages of 2 months and 82 years (Table S1).

To investigate the global phylogeny and likely origins of these isolates, we added genome assembly data sets ($n = 1148$) downloaded from the PATRIC database (https://www.patricbrc.org/) to our analyses as well as country of origin and multilocus sequence type (MLST). In the phylogenetic tree shown in Fig. S1, all of the South African isolates with the exception of ST323 (bla_{NDM-1}) and ST14 ($bla_{OXA-232}$) clustered together

TABLE 1 Genetic and phenotypic characteristics of the carbapenemase-encoding *Enterobacteriaceae* ($n = 41$)

Bacterial isolate					Carbapenemase-encoding genetic structure				
No.	Strain ID	Taxonomy	MLST	Group[a]	Gene	Plasmid structure[b]	Replicon type	Size (bp)	GenBank accession no.
1	947385799	*E. coli*	ST167		bla_{NDM-5}	pNDM-MGR194-like	IncX3	46,253	NC_022740.1
2	944535499	*K. pneumoniae*	ST14		$bla_{OXA-232}$	PittNDM01 plasmid4	ColPK3	6,141	NZ_CP006802.1
3	939996824	*K. pneumoniae*	ST101	I	bla_{GES-5}	pCHE-A1	IncQ	8,201	KX244760.1
4	945154233	*K. pneumoniae*	ST101	I	bla_{GES-5}	pCHE-A1			
5	945165838	*K. pneumoniae*	ST101	I	bla_{GES-5}	pCHE-A1			
6	945169659	*K. pneumoniae*	ST101	I	bla_{GES-5}	pCHE-A1			
7	957083320	*K. pneumoniae*	ST101	I	bla_{GES-5}	pCHE-A1-like			
8	U44822	*K. pneumoniae*	ST101	I	bla_{GES-5}	pCHE-A1			
9	957083896	*K. pneumoniae*	ST101	I	bla_{GES-5}	pCHE-A1-like			
10	957089165	*K. pneumoniae*	ST101	I	bla_{GES-5}	pCHE-A1			
11	960186733	*K. pneumoniae*	ST101	II	bla_{NDM-1}	p19-10_01	IncFIB(K)	223,434	CP023488.1
12	950171785	*K. pneumoniae*	ST101	II	bla_{NDM-1}	p19-10_01-like			
13	950173000	*K. pneumoniae*	ST101	II	bla_{NDM-1}	p19-10_01-like			
14	951373950	*K. pneumoniae*	ST101	II	bla_{NDM-1}	p19-10_01-like			
15	951362657	*K. pneumoniae*	ST2016	II	bla_{NDM-1}	p19-10_01-like			
16	951384356	*K. pneumoniae*	ST101	II	bla_{NDM-1}	p19-10_01-like			
17	951363981	*K. pneumoniae*	ST101	II	bla_{NDM-1}	p19-10_01-like			
18	950142398	*K. pneumoniae*	ST2017	III	bla_{NDM-1}	p18-43_01	IncR_1, IncFIB(pB171), IncFII(Yp)	212,326	CP023554.1
19	941530379	*K. pneumoniae*	ST323		bla_{NDM-1}	p18-43_01-like			
20	950117510	*K. pneumoniae*	ST2017	III	bla_{NDM-1}	p18-43_01-like			
21	950118422	*K. pneumoniae*	ST2017	III	bla_{NDM-1}	p18-43_01-like			
23	939742031	*K. michiganensis*	ST170		bla_{NDM-1}	p18-43_01-like			
24	950005607	*S. marcescens*	NA[c]	1	bla_{NDM-1}	p18-43_01-like			
25	950196656	*S. marcescens*	NA	1	bla_{NDM-1}	p18-43_01-like			
26	950163360	*S. marcescens*	NA	1	bla_{NDM-1}	p18-43_01-like			
27	950164094	*S. marcescens*	NA	1	bla_{NDM-1}	p18-43_01-like			
28	950165859	*S. marcescens*	NA	1	bla_{NDM-1}	p18-43_01-like			
29	950166381	*S. marcescens*	NA	1	bla_{NDM-1}	p18-43_01-like			
30	950174583	*S. marcescens*	NA	1	bla_{NDM-1}	p18-43_01-like			
31	950172946	*S. marcescens*	NA	1	bla_{NDM-1}	p18-43_01-like			
32	9501453777	*S. marcescens*	NA	1	bla_{NDM-1}	p18-43_01-like			
33	945154301	*S. marcescens*	NA	2	bla_{NDM-1}	p18-43_01-like			
34	945174350	*S. marcescens*	NA	2	bla_{NDM-1}	p18-43_01-like			
36	19870317	*E. asburiae*	ST108		bla_{NDM-1}	p18-43_01-like			
37	939705067	*E. asburiae*	ST435		bla_{NDM-1}	p18-43_01-like			
40	950180354	*E. cloacae* complex	ST145		bla_{NDM-1}	p18-43_01-like			
41	953102574	*E. cloacae* complex	ST433		bla_{NDM-1}	p18-43_01-like			
42	941713674	*E. kobei*	ST54		bla_{NDM-1}	p18-43_01-like			
43	953099839	*E. kobei*	ST434		bla_{NDM-1}	p18-43_01-like			
44	950178628	*Enterobacter* spp.	ST121		bla_{NDM-1}	p18-43_01-like			
45	944526466	*C. freundii*	ST63		bla_{NDM-1}	p18-43_01-like			

[a]Phylogenetic subgroup as determined for *K. pneumoniae* and *S. marcescens* isolates.
[b]Referred to as "-like" when plasmid sequence is not circularized but the carbapenemase-encoding contig revealed 100% nucleotide identity to the given plasmid.
[c]NA, MLST is not available for this species.

in a distinct branch. These findings are in accordance with the NCBI Genome Tree report for *K. pneumoniae* ($n = 2,814$) based on genomic BLAST (https://www.ncbi.nlm.nih.gov/genome/tree/815? shows the phylogenetic branch containing the main cluster of the South African isolates, including ST101 as well as the novel ST2016 and ST2017; accessed 29 September 2017). As revealed by the metadata, the other ST101 isolates constituting the branch come from several different countries. It is worth noting that these do not contain carbapenemase-encoding genes. The exception is the Norwegian isolate (GCA_00143615; bla_{NDM-1}), which has a different content of plasmid replicons and resistance-encoding genes than the South African isolates.

The South African isolates divide into three distinct subbranches, which correlate with the presence of bla_{GES-5} (group I) or bla_{NDM-1} (groups II and III), as marked in Fig. 1. In addition, the plasmid replicon and resistance gene profiles were specific for each of these groups (Data Set S1).

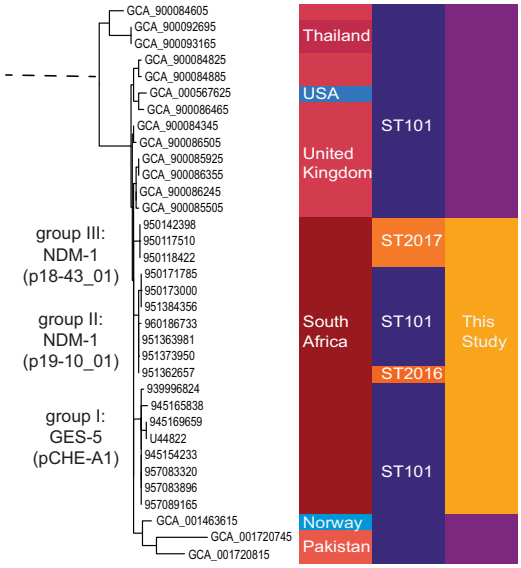

FIG 1 Phylogenetic branch of *K. pneumoniae* containing the South African isolates (n = 18). Global phylogeny from genome assembly data sets (n = 1,148) downloaded from the PATRIC database (https://www.patricbrc.org/) was revealed by rapid core genome multialignment (https://github.com/marbl/parsnp) and metadata (isolation country and MLST) coupled with the use of Phandango (https://github.com/jameshadfield/phandango/wiki). The selected branch shows the isolate assembly identifier (ID) for the downloaded samples (lilac) and isolate ID for the samples from this study (yellow), for which subgroup (I, II, or III) and carbapenemase-encoding determinants are indicated. Color codes show isolation country and MLST for each isolate.

The *bla*$_{NDM-1}$-containing genetic structures. To investigate the genetic backbone of the carbapenemase-encoding determinants, we performed alignments and BLAST analyses. For the assembled *bla*$_{NDM-1}$-containing contigs, we detected two distinct genetic structures, corresponding to phylogenetic groups II and III. The *K. pneumoniae* group II isolates revealed extensive homology to the *bla*$_{NDM-1}$-encoding regions of the completely sequenced *E. coli* plasmid pNDM-HK (NC_019063.1) as well as *K. pneumoniae* plasmids pNDM-OM (NC_019889.1) and pNDM-1-Saitama (NC_021180.1). All seven *bla*$_{NDM-1}$-containing contigs started at precisely the same nucleotide, and five had the exact same length of 16.276 bp (Data Set S1), explained by contig break caused by the surrounding insertion sequence (IS) elements described below.

For the three *K. pneumoniae* group III isolates, the *bla*$_{NDM-1}$-containing contigs showed a different DNA sequence and gene synteny. It is noteworthy that alignment and BLAST analyses grouped them together with 21 other isolates in our CRE collection, including the *K. pneumoniae* isolate of ST323, all of the *S. marcescens* and *Enterobacter* species isolates, and the single isolates of *K. michiganensis* and *C. freundii* (listed in Table 1). The aligned *bla*$_{NDM-1}$-containing contigs showed extensive homology to the conjugative 110-kb pRJF866 *K. pneumoniae* plasmid (NC_025184.1) reported in China (21). Except for base pair substitutions at pRJF866 positions 69130 (G to A) and 73304 (C to G), 100% nucleotide identity was found. Although the contig length varied from 2,268 bp to 27,908 bp (Data Set S1), the high DNA identity strongly suggests a common origin of their *bla*$_{NDM-1}$-encoding determinants.

PacBio circularizing of two novel NDM-1-encoding plasmids. The genetic support of *bla*$_{NDM-1}$was further investigated by PacBio sequencing of two *K. pneumoniae* isolates, from group II (isolate 960186733) and III (isolate 950142398). Circularizing of the DNA sequences revealed two novel NDM-1-encoding plasmids: p19-10_01 (223.434 bp) and p18-43_01 (212.434 bp). Both plasmids encode a heavy-metal efflux system, multiple resistance determinants, and transposable element, as well as type IV secretion systems (T4SS), as depicted in Fig. 2 and 3. While p19-10_01 belongs to the IncFIB(K) replicon type, p18-43_01 is a multireplicon plasmid that includes IncR_1, IncFIB(pB171),

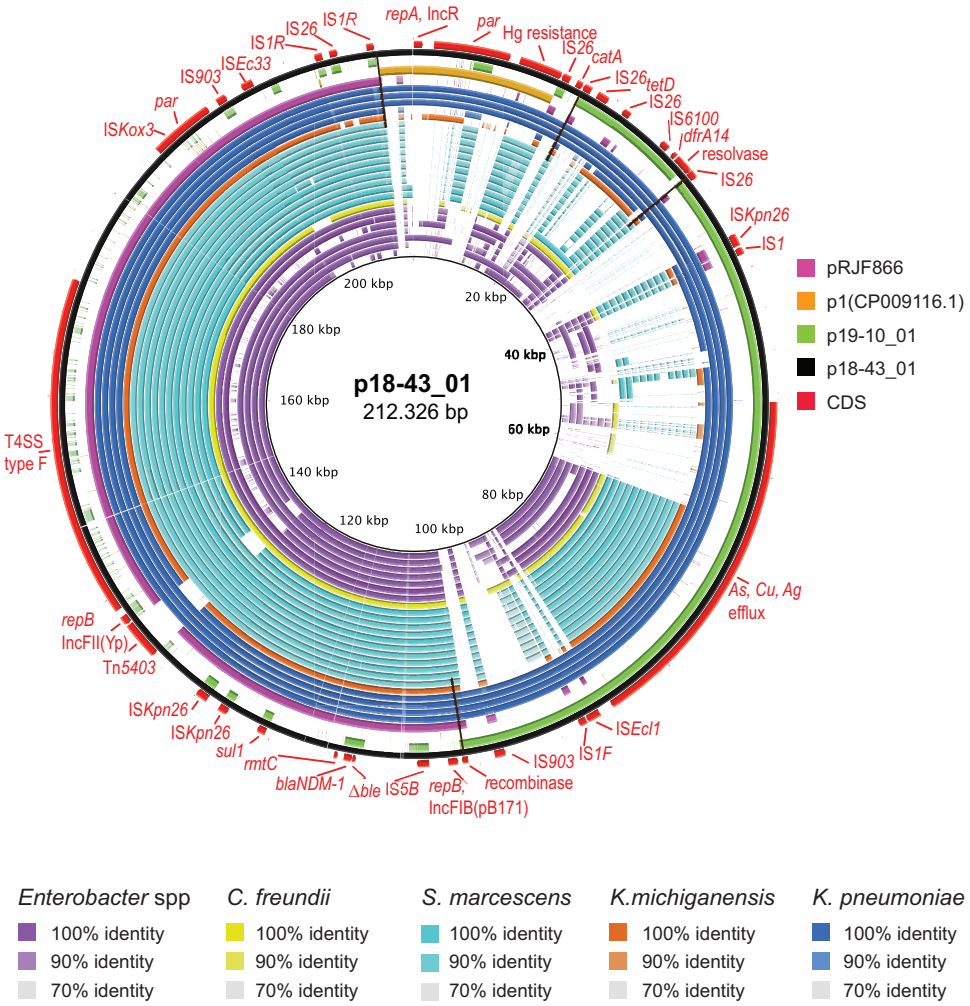

FIG 2 Tracking of plasmid p18-43_01 in NDM-1-encoding CPE isolates (n = 24). The map was constructed using BRIG software. The concentric circles represent comparisons between p18-43_01 and, starting with the inner circle, genome assemblies from *Enterobacter* species (strain ID 950178628, 953099839, 941713674, 950180354, 953102574, 939705067, and 19870317), *C. freundii* (944526466), *S. marcescens* (945174350, 945154301, 950145377, 950172946, 950174583, 950166381, 950165859, 950164094, 950163360, 950196656, and 950005607), *K. michiganensis* (939742031), and *K. pneumoniae* (941530379, 950118422, 950117510, and 950142398). Color codes are given for each species and for DNA identity, ranging from 70 to 100%, as indicated. Plasmids with extensive homology to p18-43_01, including pRJF866 (NC_025184.1), plasmid1 (CP009116.1), pKPC_CAV1217 (CP018675.1), and p19-10_01 (this study), were included in the BLAST comparisons and are represented as circles according to the given color codes. The outer black and red circles represent the p18-43_01 reference sequence and its annotated coding DNA sequence (CDS), respectively. Black transverse lines mark the ends of homology between p18-43_01 and p19-10_01 or pRJF866.

and IncFII(Yp). In p19-10_01, a Tn*1548*-like element delineated by IS*26*, contained bla_{NDM-1} as well as *armA* and other resistance-encoding genes, while the bla_{NDM-1}-containing region in p18-43_01 had a completely different gene synteny.

BLAST comparisons (Fig. S2) show that the two plasmids share regions of high sequence identity, although they are inverted and rearranged. Tree regions (99% identity) were detected, with the largest (~72 kb; positions 1728 to 73963 in p19-10_01) delineated by a putative recombinase (green box) and IS*26*. The alignment also reveal a partly overlapping 8-kb region (positions 73111 to 81049 in p19-10_01), delineated by the IS*26* element and a putative integrase (green box), and an ~5-kb region spanning from positions 82193 to 87068, which is enclosed by IS*26*.

Horizontal spread and evolution of bla_{NDM-1}-encoding plasmids. By using the BLAST Ring Image Generator (BRIG) (22), the two circularized plasmids were references for tracking of similar plasmids in the bla_{NDM-1}-positive isolates. BLAST comparisons

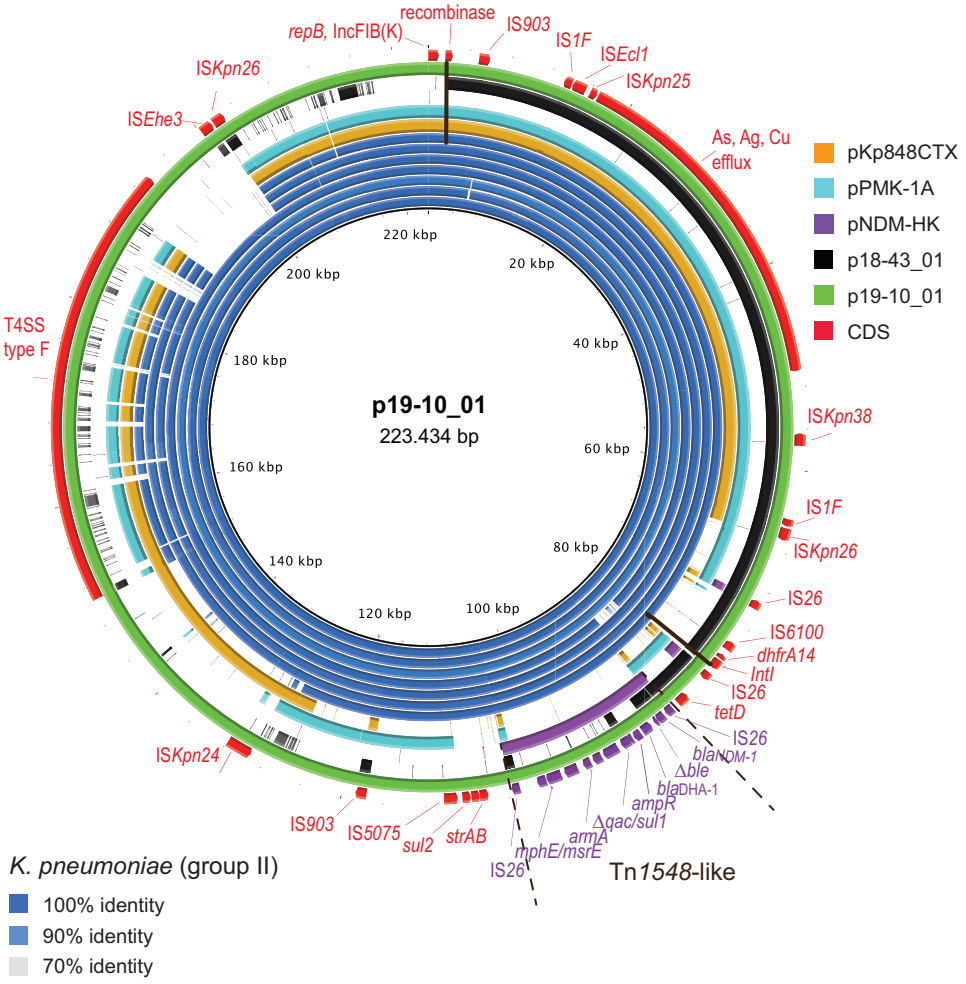

FIG 3 Tracking of plasmid p19-10_01 in subgroup II *K. pneumoniae*. The map was constructed using BRIG software. The concentric blue circles represent BLAST comparisons between p19-10_01 and genome assemblies from *K. pneumoniae* 960186733, 951362657, 951363981, 951373950, 950173000, 950171785, and 951384356, starting with the innermost circle. Color codes are for DNA identity, ranging from 70 to 100%, as indicated. Plasmids with regions homologous to the reference, including pKp848CTX (NC_024992.1), pPMK-1A (NZ_CP008930.1), pNDM-HK (NC_019063.1), and p18-43_01 (this study), were included in the BLAST comparisons and are represented as circles according to the given color codes. The outer black and red circles represent the p18-43_01 reference sequence and its annotated CDS, respectively. The Tn*1548* region is marked, with the CDS shown in purple. The black transverse lines mark the ends of homology between p19-10_01 and p18-43_01.

using p18-43_01 as a reference (Fig. 2) revealed regions with 70 to 100% DNA identity (color codes as indicated for each species) in 24 of the isolates. The concentric circles represent (i) group III *K. pneumoniae* and the phylogenetically distant ST323; (ii) *K. michiganensis* (ST170); (iii) different *Enterobacter* species, comprising ST252, ST54, ST121, and ST145 and the novel sequence types ST433, ST434, and ST435; (iv) *S. marcescens* isolates from two phylogenetic distant branches; and (v) *C. freundii* (novel ST63), in the order given in the figure legend.

Included in the comparison are plasmids with homology to the reference, including p19-10_01 (circle colors as indicated). The regions shared between p18-43_01 and p19-10_01 or pRJ866 are delineated by black lines. A large part corresponding to the pRJF866 sequence was common for all 24 isolates. Of interest, Tn*5403* had inserted onto the pRJF866 part in the reference plasmid but was absent from some of the isolates, including *K. michiganensis* and the *K. pneumoniae* ST323 (isolate 941530379), one *E. kobei* isolate (isolate 9953099839), and two *S. marcescens* isolates (isolates 94517435 and 945154301, which belong to a phylogenetically distant branch).

K. pneumoniae isolates from group III were positive for the three *rep* genes carried by p18-43_01. The IncR_1 replicon type was also detected in one *Enterobacter* isolate, while IncFII(Yp) and IncFIB(pB171) were present in the whole group of 24 isolates. The resistance-encoding genes *rmtC* and *sul1*, closely linked to bla_{NDM-1} in p18-43_01, were present in all isolates, while *catA*, *tetD*, and *dhfrA14* were found in the *Klebsiella* isolates but only sporadically in the others. Taken together, these findings indicate that bla_{NDM-1} is carried by plasmids with a common origin and partly common backbone structure, including T4SS, which has enabled local horizontal transfer between *Enterobacteriaceae* organisms. Further insight into the evolution of the p18-43_01-like plasmids in *Enterobacteriaceae* would require circularizing of their DNA sequences.

Transfer of bla_{NDM-1}-containing transposon. The BRIG analyses (Fig. 3) showed that major parts of p19-10_01, including the bla_{NDM-1}-containing region, were present in all *K. pneumoniae* isolates from group II. The observed differences indicate local evolution of the bla_{NDM-1}-containing plasmids after acquisition. Genome assemblies from isolates 951373950, 951362657, and 951363981 reveal the most similarity to p19-10_10 (960186733), which correlates with their phylogenetic relatedness (Fig. 1). Circularization of additional plasmids would be required for further investigation of plasmid changes. Notably, the only part of pNDM-HK present in these isolates was the Tn*1548*-like structure delineated by IS*26*, which has 100% sequence identity. Using pNDM-HK, pNDM-OM, or pNDM-1-Saitama as the reference plasmid in the BRIG analyses resulted in the same observation (data not shown): the group II *K. pneumoniae* isolates contained no other parts of these plasmids except from the Tn*1548*-like structure, which strongly suggests movement of this putative bla_{NDM-1}-containing transposon between different replicons followed by horizontal transfer.

bla_{GES-5} is colocated with *aacA4* on a pCHE-A-like plasmid. For the *K. pneumoniae* group I isolates, BLAST analyses of the bla_{GES-5}-containing contigs revealed extensive genetic homology to the *E. cloacae* pCHE-A (NC_012006.1) plasmid (11). For six of the isolates, we identified a complete, circular pCHE-A-like plasmid (8,201 bp), named pCHE-A1 (KX244760). Except for three base pair changes and a 641-bp insertion downstream of bla_{GES-5}, it had 100% identity to pCHE-A. The inserted DNA showed 100% identity to a class I integron bearing an aminoglycoside 6′-*N*-acetyltransferase (*aacA4*) found in *K. pneumoniae* (JN108899.1) and in other *Enterobacteriaceae*. Interestingly, *aacA4* was inserted into the described integron mobilization unit (IMU) of pCHE-A (11) within the proposed consensus sequence of the conserved core for site-specific recombination of gene cassettes into integrons (23). The inserted DNA interrupted the GTTAG-ATGC sequence of pCHE-A, resulting in GTTAG-GC (5′ end), which is identical to the consensus sequence. The insertion site was conserved in the 3′ end of the inserted DNA.

BRIG analyses (Fig. S3) confirmed the presence of DNA with 100% coverage and identity to the pCHE-A1 reference (strain 957089165) in all *K. pneumoniae* group I isolates.

Other plasmid-borne carbapenemase-encoding genes. We detected bla_{NDM-5} in an *E. coli* ST167 strain. The bla_{NDM-5}-positive contig (~9 kb) revealed 100% nucleotide identity to the bla_{NDM-5}-containing region of the 46.3-kb *K. pneumoniae* plasmid pNDM-MGR194 (KF220657.1). The plasmid was not circularized, but BLAST analyses using pNDM-MGR194 as a reference showed the presence of the complete plasmid DNA in the *E. coli* strain (data not shown).

The single *K. pneumoniae* ST14 isolate not belonging to the main phylogenetic cluster encoded OXA-232. The $bla_{OXA-232}$-containing contig of 6,348 bp shared 100% sequence identity with the *K. pneumoniae* PittNDM01 plasmid4 (CP006802.1), named pPKPN4 (24), and circularization of the 6,141-bp plasmids was enabled. BRIG comparisons between pPKPN4 and the assembled sequences from this isolate confirmed the finding (data not shown).

DISCUSSION

The molecular characterization of clinical CRE from the private hospital sector in Durban, South Africa, revealed complex patterns for the dissemination of carbapenem resistance. In this collection of MDR, XDR, and PDR *Enterobacteriaceae*, we identified four different carbapenemase-encoding genes contained by five different plasmid-associated genetic supports. The overall WGS data indicate plasmid acquisition into an established local *K. pneumoniae* clone of ST101 as well as horizontal transfer between different genera of *Enterobacteriaceae*, accompanied by clonal dissemination. Our results are in line with those observed in the Jiaxingin Zhejiang Province in China, where cross-species transfer and clonal spread were suggested to contribute synergistically to the rapid increase in prevalence of CRE in hospital settings (25).

Reports from several European and Mediterranean countries suggest a continental spread of ST101 associated with OXA-48 (26–28). The ST101 strains in this study were isolated within the same hospital environment but encoded NDM-1 or GES-5 on three different plasmids, further demonstrating the capability for adaptation and spread of this genetic lineage.

Resistance-encoding plasmids can be extremely dynamic due to nested genetic elements that enable short-term evolution as well as rapid dissemination of resistance genes between multiple species, strains, and plasmids (29). In our study, the circularization of two NDM-1-encoding plasmids revealed two different plasmids structures, including replicon types and $bla_{\text{NDM-1}}$-containing regions. Nevertheless, we identified large regions with high sequence identity, although the regions were rearranged. Their presence in the same hospital niche and within closely related *K. pneumoniae* isolates strongly point to a common source of these plasmids, which then have evolved in their host by recombination events.

The NDM-1-encoding part of p18-43_01, corresponding to pRJF866 (21) and including the IncFIB and IncFII(Yp) replicons, was detected in four different genera of *Enterobacteriaceae*, which implicates a common ancestor. In p18-43_01, we observed a Tn*5403* insertion in the pRJF866-homologous part. This genetic marker was absent from five of the isolates, including the *K. pneumoniae* ST232 isolate, the *K. michiganensis* isolate, one *E. kobei* isolate, and two *S. marcescens* isolates, which belong to a different branch of the phylogenetic tree than the others. This implies that independent transfer events of two different plasmid variants had occurred for all three genera. However, further analyses of the phylogeny and host adaptation for this versatile NDM-1-encoding plasmid structure would require circularizing of plasmids from the different isolates.

The p19-10_01 plasmid carried $bla_{\text{NDM-1}}$ on a putative mobile element with 100% nucleotide identity to a segment of the completely sequenced pNDM-HK, pNDM-OM, and pNDM-1-Saitama plasmids (30–32). The region contains multiple resistance determinants, including *armA* flanked by IS*26*, and structurally resembles a Tn*1548*-type composite element (33, 34). In our study, we detected no other parts of these plasmids in the Tn*1548*-like containing *K. pneumoniae* strains, which strongly suggests a mobilization of this element from one plasmid to the other. IS*26* has been shown to mediate the formation of transposons that carry antibiotic resistance genes (35, 36) and to significantly reorganize plasmids by replicative transposition or by homologous recombination between preexisting IS*26* structures (37, 38). Here, transfer by IS*26* activity would explain the finding of Tn*1548*-like in a new genetic context. IS*26* could also offer an explanation for the differences in plasmid structure observed between the isolates due to homologous recombination and merging of plasmids.

The pCHE-A plasmid is a mobilizable IncQ-type plasmid first described in an *E. cloacae* strain isolated in Canada (11). The $bla_{\text{GES-5}}$ gene is part of a novel IMU, which could be mobilized by providing transposase activity in *trans* (11). Here, we detected a homologous plasmid with an additional resistance-encoding gene, *aacA4*. The insertion of *aacA4* into the conserved core sequence for site-specific recombination (23) supports an integron activity, although the *IntI* gene is partially deleted in pCHE-A. These

findings substantiate the potential for accumulation and spread of resistance genes by pCHE-A, as suggested previously (11).

K. pneumoniae ST14 has been associated with CTX-M-15, FOX-7, and NDM-1 outbreaks in Tanzania, Italy, and other parts of the world (1, 39–41). An OXA-232-producing *K. pneumoniae* ST14 outbreak clone was recently detected in South Korea (42) and traced to India, where it is reported to be dominant (43). The 6.1-kb OXA-232-encoding pPKNPN4 detected in our study was initially described in the *K. pneumoniae* isolate PittNDM01 (24) and also corresponds to an OXA-232-encoding plasmid reported in *K. pneumoniae* and *E. coli* (42, 44), which accentuates its dissemination.

NDM-5 was first identified in an *E. coli* strain from the United Kingdom in 2011 (45). In recent years, there has been widespread occurrence of NDM-5 in *K. pneumoniae* (45–47). Here, bla_{NDM-5} detected in *E. coli* was harbored by pNDM_MGR194, which was found to circulate in *K. pneumoniae* in India (48, 49). The finding of this broad-host-range IncX3 plasmid in *Proteus mirabilis* as well (50) further extends its role in enhancing the spread of bla_{NDM-5}.

The presence of bla_{NDM-1} in different species and STs of *Klebsiella*, as well as in other genera of the *Enterobacteriaceae* family, emphasizes the broad-host-range dissemination of mobile NDM-encoding elements. In addition to *K. pneumoniae*, *E. cloacae* is known as a major host for NDM-1 both in South Africa and in other African countries (1, 2). Outbreaks of NDM-1-producing *E. cloacae* have also been reported (51), and epidemiological analyses have revealed specific NDM-1-associated STs (52, 53). In our study, however, the NDM-1-producing *Enterobacter* spp. encountered several species. *S. marcescens* isolates are mostly associated with neonatal outbreaks in ICUs worldwide (54, 55). Dissemination of NDM-1-producing *S. marcescens* in ICUs has not, to our knowledge, been reported before. The development and nosocomial spread of a PDR genetic lineage of *S. marcescens* is of great concern.

In conclusion, acquisition of different resistance-encoding plasmids, horizontal transfer, and clonal dissemination facilitate the spread of carbapenemase genes in Durban, South Africa. The overall observations emphasize the importance of early detection of CRE and targeted infection control measures to prevent dissemination.

MATERIALS AND METHODS

Ethical considerations. Ethical approval was obtained from the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (reference number BE040/14).

Bacterial strains. Forty-five clinical CRE (nonsusceptible to ertapenem and/or meropenem) collected by Lancet Laboratories, Durban, South Africa, between 2012 and 2013 from patients hospitalized in 10 different private hospitals (represented by the letters A to J) and wards (represented by digits after the letters) in Durban, South Africa, were included in the study (Table S1 in the supplemental material). Duplicate isolates from the same patients were excluded. Species identification and antimicrobial susceptibility testing were undertaken using matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker Daltonic Gmbh, Bremen, Germany) and broth microdilution using in-house-designed, premade Sensititre microtiter plates (Thermo Fisher Scientific, East Grinstead, UK), respectively. Interpretation was according to EUCAST breakpoints, version 7.1 (www.eucast.org). Nonsusceptibility included both the intermediate and resistant categories. Carbapenemase production was examined by the Carba NP test as previously described (56, 57).

DNA analysis. Genomic DNA for Illumina sequencing was purified using a GenElute bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Paired-end libraries were generated using the Nextera kit (Illumina, San Diego, CA, USA), followed by sequencing on an Illumina MiSeq platform at the Norwegian Sequencing Centre or at the Centre for Bioinformatics at UiT–The Arctic University of Norway.

For PacBio sequencing, genomic DNA was purified by a Genomic-tip 100/G kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was subjected to the 20-kb library preparation protocol and 6-kb cutoff BluePippin (Sage Sciences, Beverly, MA, USA) size selection, followed by sequencing with the Pacific Biosciences RSII sequencer using P6-C4 chemistry, a 360-min movie time, and one SMRT-cell per sample at the Norwegian Sequencing Centre.

Bioinformatic analysis. Illumina sequence reads were adaptor- and quality-trimmed using Trimmomatic (58) and subsequently assembled with Spades v.3.6.0 (59) using the "–careful" flag. PacBio long-read sequences were assembled and polished at The Norwegian Sequencing Centre (http://www.sequencing.uio.no/) using HGAP, v3, in SMRT analysis software, v2.3.0 (Pacific Biosciences) (60). Minimus2 from AMOS (61) circularized unitigs, and the *dnaA* (chromosome) or *repA* (plasmids) gene was set as the first nucleotide position using the Circulator (62).

For in-house analysis purposes, assemblies were annotated using prokka v.1.11 (63) with further NCBI BLAST searches and annotation of resistance and plasmid replicon genes by the Resfinder, NCBI β-lactamase, and PlasmidFinder databases found in ABRicate (https://github.com/tseemann/abricate). Annotation of NDM-1-containing plasmids additionally included ISfinder (64) searches for IS elements and identifying T4SS using T346Hunter (65). Assemblies deposited in GenBank were annotated using the PGAP pipeline provided by NCBI (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/), with additional manual curation of resistance gene and mobile genetic element annotations.

To visualize presence/absence of specific plasmid DNA, fully sequenced plasmids were used as reference input to BRIG (22) together with the Illumina sequence reads.

To investigate the global phylogeny and identify the likely origins of the *K. pneumoniae* isolates, genome assembly data sets were downloaded from the PATRIC database (https://www.patricbrc.org/), identifying all isolates with "country isolated" metadata. Genomes with fewer than 400 contigs were selected and run through MinHASH (66) using standard settings, and genomes above a MASH distance threshold of 0.05 excluded to remove isolates genetically distant from the main phylogroup. The data sets were then run through parsnp v.1.2 (61) with "–c –x" flags enabled and random reference selection among the included samples. FigTree (http://tree.bio.ed.ac.uk/software/figtree/) was used to edit the phylogenetic trees. Phylogeny, country origin metadata, and MLST type were determined by MLST software (https://github.com/tseemann/mlst) coupled with Phandango (67).

The housekeeping genes of new or unknown STs were sent for curation and assignment of new ST numbers at the *K. pneumoniae* MLST database at the Pasteur Institute, the *Enterobacter cloacae* MLST website (https://pubmlst.org/ecloacae/), and the *Citrobacter freundii* MLST website (https://pubmlst.org/cfreundii/).

Accession number(s). The raw read sequences and the assembled whole-genome contigs have been deposited in GenBank under Bioproject PRJNA287968. The plasmids pCHE-A1, p19-10_01, and p18-43_01 have accession numbers KX244760.1, CP023488.1, and CP023554.1, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02178-17.

SUPPLEMENTAL FILE 1, PDF file, 4.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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