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Research paper

NDM-1-encoding plasmid in *Acinetobacter chengduensis* isolated from coastal water

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ABSTRACT

Background: *Acinetobacter* spp. may cause difficult-to-treat nosocomial infections due to acquisition of carbapenemases, including New Delhi metallo-β-lactamase (NDM). This genus has been pointed out as a possible actor in the early dissemination of bla_{NDM}, and this gene has been documented in a variety of species.
Objective: Here we describe an *Acinetobacter chengduensis* (isolate FL51) carrying bla_{NDM} recovered from coastal water in Brazil.
Methods: *In vitro* techniques included antimicrobial susceptibility and minimum inhibitory concentration tests, PCR, plasmid profile and matting-out/transformation assays. *In silico* approaches comprised comparative genomic analyses using appropriate databases.
Results: FL51 grew at room temperature in a variety of culture media, excluding MacConkey. It showed resistance to all beta-lactams tested and to ciprofloxacin. bla_{NDM-1} was identified, and a single replicon was observed in plasmid profile. *In silico* DNA hybridization revealed *Acinetobacter* FL51 as being *Acinetobacter chengduensis*. bla_{NDM-1} was flanked upstream by IS*Aba14-aphA6*-IS*Aba125* and downstream by ble_{MBL}-*trpF*-Δ*tat*, inserted in a 41,068 bp non typeable plasmid named pNDM-FL51. This replicon showed high coverage and identity with other sequences present in plasmids deposited on the GenBank database, recovered almost exclusively from *Acinetobacter* spp., associated with hospital settings and animal sources.
Conclusion: We described a recently described environmental *Acinetobacter* species carrying a plasmid-borne bla_{NDM} associated with a Tn*125-like* structure. Our findings suggest that replicon may play an important role in bla_{NDM} dissemination among distinct settings within this genus and may support the theory of bla_{NDM} emergence from an environmental *Acinetobacter*.

1. Introduction

The genus *Acinetobacter* comprises 65 species with validly published and correct names (http://www.bacterio.net/acinetobacter.html) that are ubiquitous in nature, some of which may cause difficult-to-treat infections due to acquisition of resistance determinants against a variety of antimicrobial agents (Wu et al., 2019). The emergence and dissemination of carbapenemase-producing bacteria have threatened the use of last resort antimicrobials for treating infections (Wu et al., 2019). For this reason, these microorganisms are classified as urgent threats, thus demanding aggressive attempts to control their spread (Centers for Disease Control and Prevention, 2019).

NDM is one of the most disseminated carbapenemase. NDM-producing *Acinetobacter* species occur worldwide more frequently in (but not limited to) hospital settings (Wu et al., 2019). Interestingly, its encoding gene was likely originated in *Acinetobacter* and then spread among a variety of gram-negative bacilli (Toleman et al., 2012). The genetic context of bla_{NDM} usually includes an upstream intact or

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truncated insertion sequence IS*Aba125*, and a downstream bleomycin resistance (ble_{MBL}) gene, followed by a complete or remaining set of other genes (including *trpF, dsbC, cutA1, and groES-groEL),* and by the insertion sequence IS*CR27* (Partridge and Iredell, 2012). An additional IS*Aba125* element downstream IS*CR27* forms the composite transposon Tn*125*. Moreover, bla_{NDM-1} is often carried by plasmids among *Acinetobacter* spp., a characteristic that enhances its potential for horizontal spread (Wu et al., 2019).

The study of genetic structures enrolled in the dissemination of antimicrobial resistance genes of great medical importance among distinct settings may provide clues on key mechanisms and routes for such a spread. Here, we analyzed the whole sequence of a bla_{NDM-1}-carrying plasmid found in an *Acinetobacter chengduensis* that was recovered from the superficial water of a touristic beach in Rio de Janeiro, Brazil.

2. Material and methods

2.1. Bacterial isolate and in vitro analysis

The isolate *Acinetobacter* sp. FL51 was recovered from Flamengo Beach, in Rio de Janeiro, Brazil, in October 2017, following the methodology described by Paschoal et al. (2017), except the incubation, also attempted at 25 °C. Microbial identification was initially conducted by MALDI-TOF/MS (Bruker Daltonics, Germany) and *rpo*B sequencing (La Scola et al., 2006). The isolate's antimicrobial resistance profile was verified by CLSI disk diffusion (CLSI, 2017) (Clinical and Laboratory Standards Institute, 2005),and carbapenems minimum inhibitory concentration MIC) by *E*-test. bla_{NDM} was identified by PCR that also targeted bla_{KPC}, bla_{BKC}, bla_{GES}, bla_{OXA-48}, bla_{SPM}, bla_{IMP} bla_{VIM}, bla_{SIM}, and bla_{GIM} (Paschoal et al., 2017).

Transfer of bla_{NDM-1} was attempted by mating-out assays using the azide-resistant *Escherichia coli* J53 as the recipient, and transconjugants selection under imipenem (1 μg/mL) and sodium azide (200 μg/mL). Plasmid DNA was extracted (Kieser, 1984), analyzed after conventional electrophoresis, and used to transform chemically competent *E. coli* Top10 cells by heat shock.

Total DNA was extracted using the QIAamp DNA Kit (Qiagen, USA). Genomic library prepared with Nextera XT Library Prep Kit (Illumina Inc., USA) was sequenced using MiSeq v2 Reagent Kit (500 cycles, paired-end) on the Illumina MiSeq platform (Illumina Inc., USA).

2.2. In silico analysis

Reads were trimmed by Trimmomatic v0.36 after quality checking using FastQC v0.11.4 (Bolger et al., 2014). *De novo* assembly was performed using SPAdes genome assembler v3.11.1 on the Unicycler pipeline v0.4.5 and the assembly quality was verified by Quast web interface (http://quast.bioinf.spbau.ru/) (Wick et al., 2017). Resistome, plasmidome and virulome were verified using ResFinder, CARD, PlasmidFinder and VFDB databases by ABRIcate v0.8.13 (https://github.com/tseemann/abricate).

For species identification genome sequences of *Acinetobacter* species downloaded from the NCBI GenBank database were compared using the Average Nucleotide Identity based on BLAST (ANIb) and on Mummer (ANIm) by the JSpecies software v1.2.1 (https://imedea.uib-csic.es/jspecies/download.html). *In silico* DNA-DNA hybridization (isDDH) was performed based on Blast using Genome-to-Genome Distance Calculator (GGDC) web interface (http://ggdc.dsmz.de/ggdc.php#).

3. Results and discussion

FL51 grew at room temperature (25 °C) as dry opaque small colonies in CHROMagar Orientation (BD Diagnostics, France), Müeller-Hinton (DifcoTM, France), tryptic soy agar (DifcoTM) and nutrient agar (DifcoTM), whereas it could neither grow in MacConkey nor at 35–37 °C. MALDI-TOF/MS species identification yielded poor results, revealing different *Acinetobacter* species with scores lower than 1.7. In parallel, *rpoB* sequencing was inconclusive to achieve identification at the species level, showing 90% identity with *Acinetobacter bohemicus* (zone 1) and 94% identity with *Acinetobacter gandensis* (zone 2). Although both MALDI-TOF/MS and molecular techniques can be efficient tools for accurate identification of *Acinetobacter* at the species level, the absence of query species on databases may have hampered identification. To overcome this issue, whole genome sequencing was performed. WGS generated 1,5 million paired-end reads, with an average size of 251 bp, sequencing depth of 221×, and genome coverage estimated at 99.83%. Reads were assembled into 70 contigs, with a genome length of 3,6 Mb, N50 of 317.935 bp, and 39.97% of GC content. Pairwise ANIb, ANIm, and dDDH values for *Acinetobacter chengduensis* were 98.77%, 99.63% and 96.30% respectively. Considering the recommended threshold values of ANIb (95–96%) (Richter and Rosselló-Móra, 2009) and dDDH (70%) (Meier-Kolthoff et al., 2013) for species prediction, our results indicate that FL51 is an *Acinetobacter chengduensis*. Other genomes numbers were $\leq$ 87.27%, 88.77% and 33.80% (Table S1). *A. chengduensis* was recently described in China, after recovery and characterization of two bacterial strains isolated from a hospital sewage in China (Qin et al., 2020). It is probably a predominantly environmental species, considering its lower growth temperature.

FL51 showed resistance to all beta-lactams and fluoroquinolones and susceptibility to amikacin, gentamicin, tobramycin, and trimethoprim/sulfamethoxazole. MIC values for imipenem and meropenem were >256 mg/L and >32 mg/L, respectively. Considering that FL51 has a lower growth temperature all tests were carried out at 25 °C. Since standard methodologies are usually performed at 35 °C ± 2 °C, changing temperature could compromise the results.

Besides bla_{NDM-1}, our strain's resistome was composed by the bleomycin resistance determinant ble_{MLB}, an *aph(3′)-VI* (aminoglycoside 3′-phosphotransferase), a *tet*39 (class C tetracycline resistance protein); *adeJ* and *adek*, both efflux pump membrane transporters. Like FL51, the two isolates described by Qin and colleagues were resistant to ciprofloxacin and susceptible to amikacin, gentamicin and trimethoprim/sulfamethoxazole. However, one strain was susceptible to beta-lactams while the other was highly resistant. This was due to the absence of beta-lactamase genes in the former and the presence of bla_{NDM-1} and bla_{OXA-58} in the later. Different from our findings both strains from China had *mph(E)* and *msr(E)* mediating resistance to macrolides (Qin et al., 2020).

A single plasmid was visible in FL51's plasmid profile. Although conjugation seems to be the main mechanism involved in bla_{NDM} horizontal transfer, and some studies evidenced bla_{NDM-1} exchange between *Acinetobacter* spp. and other species by mating-out assays or by transformation (Khalid et al., 2020), we did not succeed in obtaining neither transconjugants nor transformants.

In Brazil, most bla_{NDM} reports concentrate in *Enterobacteriales* clinical strains (da Silva et al., 2019). Regarding *Acinetobacter* species, national studies show its identification in *Acinetobacter baumannii, Acinetobacter pitti* and *Acinetobacter bereziniae,* all from clinical origin (Rossi et al., 2021; Deglmann et al., 2019; Brasiliense et al., 2019; Schuelter-Trevisol et al., 2016; Chagas et al., 2015; Pagano et al., 2015; Pillonetto et al., 2014). Environmental detection of the gene is restricted to a *Klebsiella pneumoniae* isolated from recreational, a *Stenotrophomonas maltophilia* isolated from soil, and a water sample collected from a river (Campana et al., 2017; Furlan et al., 2018; Sanchez et al., 2018).

Node 29, in which bla_{NDM-1} was located, exhibited 100% coverage and identity with part of pNDM-BJ02 from *Acinetobacter lwoffii* (GenBank accession number: JQ060896.1). Trimmed reads from FL51 WGS were aligned against this plasmid using BWA v0.7.12 generating two plasmid contigs (Li and Durbin, 2009). PCR and amplicon sequencing using primers flanking these contigs confirmed the assembly and yielded a circular structure (Table S2). The pNDM-FL51 sequence presented 41,068 bp and aligned with more than 70% coverage with 35 plasmids

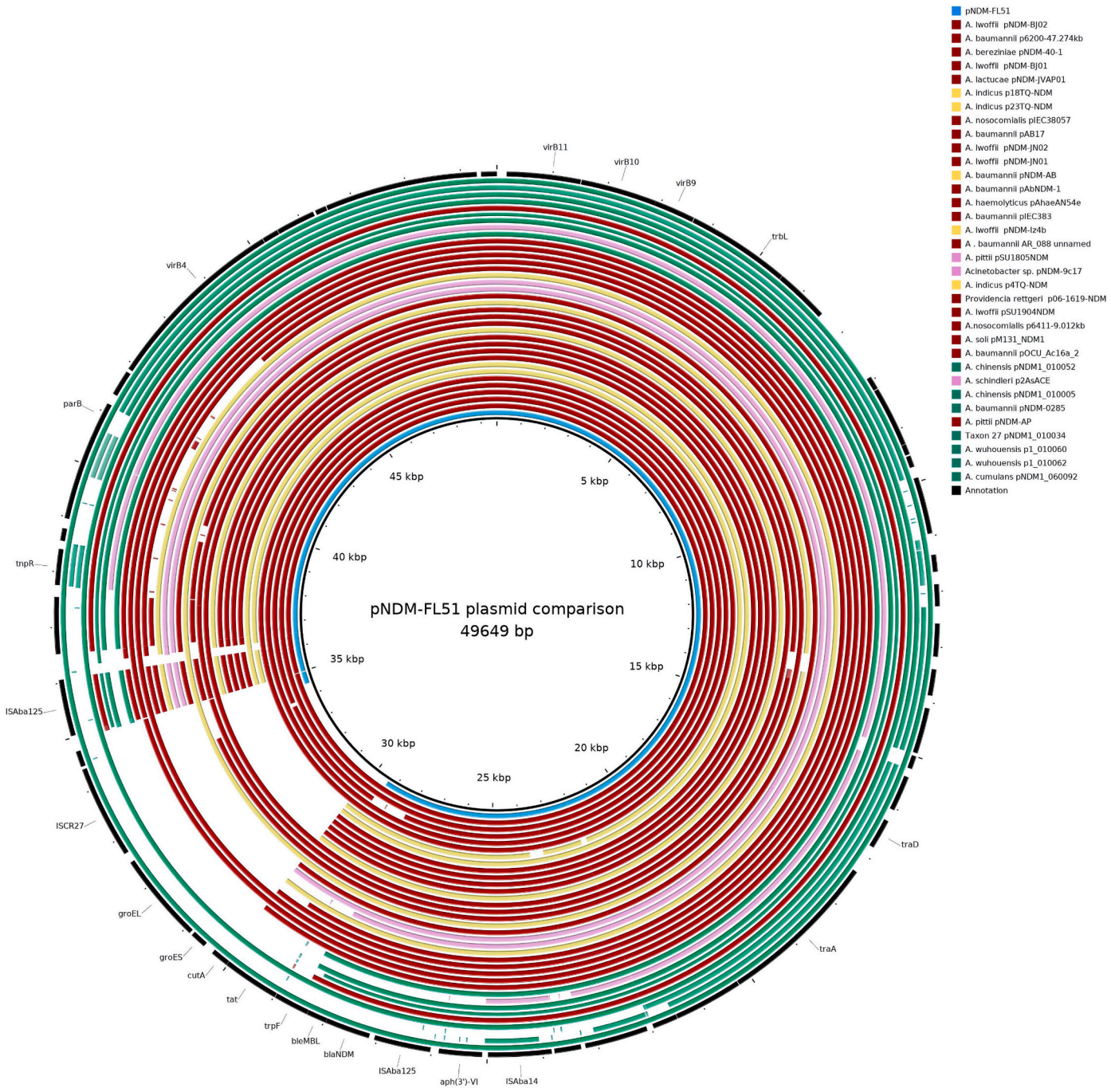

Fig. 1. Comparison of pNDM-FL51 with high identity plasmids using Blast Ring Image Generator (BRIG) v0.95. Different colors indicate distinct sources: green – hospital sewage; yellow – animal; red – human; pink – hospital environment; blue – coastal water.

deposited in GenBank, with identity ranging from 92 to 100% (Fig. 1). These genetic elements were almost exclusively identified in *Acinetobacter* species ($n = 34$), suggesting their narrow host range. This is most likely the reason why we failed to transfer pNDM-FL51 to *E. coli* during conjugation and transformation assays. In addition, the records showed these plasmids were recovered from diverse sources, including clinical specimens, hospital sewage, hospital environment and animals (Fig. 1). No plasmid sequence could be retrieved from the previously described *A. chengduensis* for comparison. Additionally, no plasmid typing sequences or virulence genes were found.

In FL51 $bla_{\text{NDM-1}}$ was flanked upstream by the IS*Aba14-aphA6*-IS*Aba125* gene cluster and downstream by ble_{MBL} and *trpF* genes. Downstream *trpF* we found a truncated tat gene and a *tnpR* frequently identified downstream an IS*CR3* transposase in *Acinetobacter* spp. Although we did not detect either IS*CR27* or a second copy of IS*Aba125*, we found a truncated inverted repeat (IR) sequence downstream Δ*tat* and upstream *tnpR* (Fig. 2). This finding suggests that a complete Tn*125* was likely responsible for mobilizing $bla_{\text{NDM-1}}$ into the replicon and subsequent events led to the deletion of its internal regions. A comparison with other $bla_{\text{NDM-1}}$ genetic contexts of different *Acinetobacter* isolates showed that these IR can also be observed in other plasmid sequences (Fig. 2). Yet, we cannot disregard that similar events might also have occurred in structures missing IR remnants. In these cases, larger deletions could have completely eliminated the whole IS*Aba125*

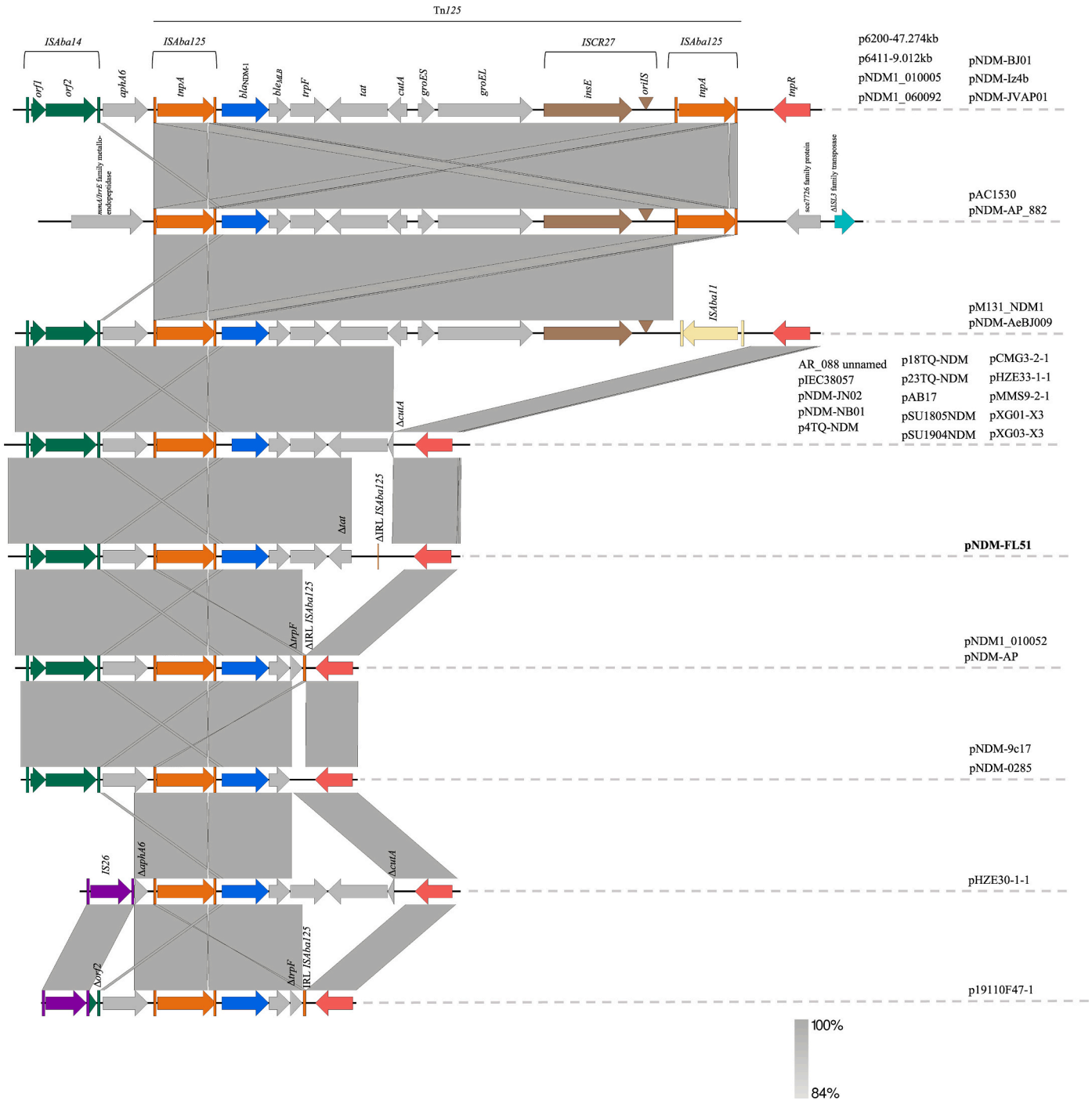

Fig. 2. Comparison between bla_{NDM-1} genetic context found in pNDM-FL51 and in different *Acinetobacter* spp. using Easyfig. Genes are denoted by arrows, inverted repeats by frames and *oriIS* by arrow heads. Gray shading regions designate regions of homology. Plasmids accession numbers in order of appearance: NZ_CP010399, NZ_CP010370, NZ_CP032132, NZ_CP035935, NZ_JQ001791, NZ_KJ547696, NZ_KM923969, CP045561, CP014478, JX072963, CM016430, CP027532, NZ_MK053934, KM210088, MH445382, CP045130, CP045133, CP045197, MT002974, LC483156, LC537594, CP044446, CP044475, CP044451, CP045136, CP045128, MW073138, NZ_CP032142, NZ_KJ003839, NZ_CP026425, NZ_CP026127, CP044484, CP046043.

second copy.

Acinetobacter sp. are naturally competent and prone to transformation. This trait enables genetic material exchange between unrelated members within the genus (Domingues et al., 2019), what may include species found in nature with those of clinical relevance.

Tolemann and cols. postulated that bla_{NDM} could have emerged from an environmental *Acinetobacter* (Toleman et al., 2012). The presence of an NDM-1-carrying plasmid in an environmental species that grows poorly at 37 °C could provide a strong support for such hypothesis. However, our analyses do not enable determining the direction of resistance transmission between clinical and environmental settings, nor the exact role of unknown environmental microorganisms in this particular context. Since similar plasmids are widely disseminated in *Acinetobacter*, it is possible that some environmental samples acquired these replicons due to the spread of resistance multiplied in the human sphere. In both cases the study of these structures is highly relevant so that, in the future, we can fulfill existing gaps in scientific knowledge.

4. Conclusion

We described an *Acinetobacter chengduensis* carrying a bla_{NDM} inserted in a Tn*125-like* structure located in a plasmid found to be widespread among this ubiquitous genus. Therefore, this replicon may be an important platform for bla_{NDM} dissemination among diverse environments. Given that aquatic matrices are considered important bacterial niches and reservoirs of the intrinsic resistome, our findings may support the theory of bla_{NDM} emergence from an environmental *Acinetobacter*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2021.104926.

Accession numbers

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JADBFZ000000000. The version described in this paper is version JADBFZ010000000.

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Transparency declarations

The authors declare no conflict of interest regarding the publication of this article.

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