Title: Horizontal transfer of the blaNDM-1 gene to *Pseudomonas aeruginosa* and *Acinetobacter baumannii* within a biofilm

Running title: Transfer of blaNDM-1 into *P. aeruginosa* and *A. baumannii*

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ABSTRACT

Horizontal gene transfer has contributed to the global spread of the blaNDM-1 gene. Studies have demonstrated plasmid transfer of blaNDM-1 into various Gram-negative bacterial species, but attempts to demonstrate transfer of blaNDM-1 plasmids into Pseudomonas aeruginosa and Acinetobacter baumannii have either been unsuccessful or only observed with a donor of the same genus. There is evidence that plasmid transfer frequency may increase when conjugation occurs within a biofilm versus between planktonic cells. To determine whether blaNDM-1 gene transfer to P. aeruginosa or A. baumannii could be facilitated in a biofilm environment, one E. coli and two Klebsiella pneumoniae strains carrying NDM-1-encoding plasmids of different incompatibility types were mated with an E. coli J53 strain to produce E. coli J53- blaNDM-1 transconjugant plasmid donors. Dual-species biofilms were then created using the E. coli J53 transconjugants and a P. aeruginosa or A. baumannii recipient strain and incubated for 24 or 72 hours. Transfer of an NDM-encoding plasmid from one E. coli J53- blaNDM-1 transconjugant into P. aeruginosa was successful in a 72-hour biofilm, and transfer of NDM-encoding plasmids from two E. coli J53- blaNDM-1 transconjugants to A. baumannii was successful in 24-hour biofilms.
INTRODUCTION

Spread of the blaNDM-1 carbapenemase gene is a global public health concern (Kumarasamy et al., 2010). The gene is typically mobile, commonly carried on plasmids of diverse sizes and incompatibility types that are capable of inter-species, inter-genus, and inter-family transfer. (Carattoli, 2013). Successful dissemination of the blaNDM-1 gene is more commonly attributed to conjugational transfer of NDM-1-encoding plasmids to other Gram-negative bacteria, rather than clonal spread (Carattoli, 2013, Johnson & Woodford, 2013).

The blaNDM-1 gene is typically found in Enterobacteriaceae species, but has also been detected in a variety of non-fermenting Gram-negative bacteria such as Aeromonas caviae, Stenotrophomonas maltophilia, Acinetobacter baumannii, and several Pseudomonas species, including Pseudomonas aeruginosa (Walsh et al., 2011, Zhang et al., 2013). Dissemination of the blaNDM-1 gene in P. aeruginosa and A. baumannii isolates has now been reported globally in both clinical and environmental samples, and treatment options have become significantly limited (Chen et al., 2011, Chaudhary & Payasi, 2013). Many of these isolates likely acquired the blaNDM-1 gene via intra- or inter-genus conjugational transfer of NDM-1-encoding plasmids, however, successful inter-family transfer of the blaNDM-1 harbozing plasmids to P. aeruginosa or A. baumannii has not yet been demonstrated in the laboratory (Potron et al., 2011, Janvier et al., 2013, Huang et al., 2015).

Conjugation experiments using Gram-positive donors and recipients typically use surface mating approaches such as filter mating or biofilm formation (Roberts et al., 2001, Savage et al., 2013). However, because pili can assist in gene transfer in Gram-negative bacteria, broth mating has been a commonly used method for conjugal transfer of NDM-encoding plasmids in Gram-negative species (Potron et al., 2011, Sowmiya et al., 2012, Rahman et al., 2014),
Bacterial species such as *P. aeruginosa* and *A. baumannii*, can frequently be recovered from hospital or natural environments (Blanc et al., 2007, Walsh et al., 2011, Nutman et al., 2016), and are often found in a sessile or biofilm state (Donlan, 2002, Gurung et al., 2013). Studies have demonstrated that horizontal gene transfer can occur at higher frequencies in biofilms versus planktonic cells in Gram-negative bacterial species (Madsen et al., 2012). The high density and close spatial proximity of the cells create an ideal environment for interspecies transfer of genetic information (Donlan, 2002, Madsen et al., 2012). *P. aeruginosa* and *A. baumannii* are commonly associated with biofilm formation (Donlan, 2002, Qi et al., 2016), which could potentially facilitate the transfer of *bla*NDM-1 to these bacteria. The objective of this study was to determine whether plasmid-borne *bla*NDM-1 genes originating in *Enterobacteriaceae* species could be transferred from an *E. coli* J53-*bla*NDM-1 transconjugant to *P. aeruginosa* or *A. baumannii* in a biofilm environment.

**MATERIALS AND METHODS**

**Donor and recipient organisms**

Four NDM-1-producing *Enterobacteriaceae* strains with plasmids of different incompatibility types carrying the *bla*NDM-1 gene were used as the original plasmid donors: a *K. pneumoniae* donor (EKP) carrying the *bla*NDM-1 gene on a 100 kb plasmid (pEKP) belonging to the FII, L/M, or N2 incompatibility group; an *E. coli* donor (EEC) carrying the *bla*NDM-1 gene on a 150 kb plasmid (pEEC) belonging to the FII incompatibility group; a *K. pneumoniae* donor (CO-NDM) carrying the *bla*NDM-1 gene on a 130 kb plasmid (pCO-NDM) of unknown incompatibility type; and a *K. pneumoniae* donor (ATCC BAA-2146) carrying the *bla*NDM-1 gene on a 140 kb plasmid (pNDM-US) belonging to the A/C incompatibility group (Hudson et al., 2014). EKP and EEC were recovered from environmental samples in Southeast Asia, and the...
CO-NDM and ATCC strains were isolated from clinical samples. Azide-resistant *E. coli* J53 was used as a recipient for mating experiments with the original plasmid donor strains. Subsequent mating-out assays were performed using the *E. coli* J53-bla<sub>NDM-1</sub> transconjugants as donors and rifampin-resistant *P. aeruginosa* and *A. baumannii* as recipients. *E. coli* J53-bla<sub>NDM-1</sub> transconjugants were used as donors to allow for comparison of transfer frequencies between each of the NDM-encoding plasmid types, as previously described (Potron et al., 2011). Broth conjugations into azide-resistant *E. coli* J53

Log phase Luria-Bertani (LB) broth cultures of each of thebla<sub>NDM-1</sub> donors and the *E. coli* J53 recipient were combined in a 10:1 donor-to-recipient ratio in fresh LB. Mating-out assays were performed as described by Walsh et al. (Walsh et al., 2011) using LB rather than nutrient broth. Conjugation mixtures were incubated overnight at 30 and 37 degrees C, then serially diluted and plated on LB agar containing 0.5 µg/mL meropenem and 100 µg/mL sodium azide.

Transfer ofbla<sub>NDM-1</sub> into *E. coli* J53 was confirmed by PCR with a previously described primer set (Poirel et al., 2011) and on CHROMagar Orientation<sup>TM</sup> (DRG International, Springfield, New Jersey) containing 0.5 µg/mL meropenem. Putative NDM-1-positive *E. coli* J53 transconjugants from the EEC donor were differentiated from the parent EEC strain by PCR detection of the yja-A gene found in *E. coli* J53 but absent in the EEC strain (Clermont et al., 2000). Three of thebla<sub>NDM-1</sub> donors, EKP, EEC, and ATCC-BAA-2146 produced *E. coli* J53 transconjugants, designated *E. coli* TcEKP, *E. coli* TcEEC, and *E. coli* TcNDM-US, respectively.

Biofilm conjugations into rifampin-resistant *P. aeruginosa* and *A. baumannii*

Detected: Details on the four NDM-1-producing *Enterobacteriaceae* strains used as bla<sub>NDM-1</sub> donors in the first phase of the conjugation assays are given in Table 1.

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Biofilm mating-out assays were performed using NDM-1-positive *E. coli* J53 transconjugants. TeEKP, TeEEC, and TeNDM-US as *blaNDM-1* plasmid donors and *P. aeruginosa* and *A. baumannii* as recipients. Using optical density measurements, log-phase donor and recipient LB broth cultures were combined in a 1:4 donor-to-recipient ratio, with approximately $2.5 \times 10^7$ cells from an *E. coli* J53 transconjugant donor and $1.0 \times 10^8$ cells from the *P. aeruginosa* or *A. baumannii* recipient in 1 mL LB.

The 1 mL dual-species conjugation mixtures were placed in a 48-well plastic plate and incubated for either 24 or 72 hours, allowing the culture to form a biofilm on the sides of the wells. Conjugation mixtures were incubated at 30 degrees for the *P. aeruginosa* conjugations and 37 degrees for the *A. baumannii* conjugations. LB was exchanged every 24 hours to maintain nutrient levels. At the end of the incubation period the broth was again exchanged, and biofilms were scraped from the well sides using a sterile metal scraper. The LB containing the biofilm scrapings was pulse-vortexed to break apart the cells, and serial dilutions of the biofilm suspension were plated on tryptic soy agar containing 75 µg/mL ticarcillin and 50 or 100 µg/mL rifampin for *A. baumannii* or *P. aeruginosa*, respectively. Plates were incubated at 37 degrees C for 48 hours.

Colonies from ticarcillin-rifampin selection plates were subcultured to CHROMagar™ with 0.5 µg/mL meropenem. *P. aeruginosa* or *A. baumannii* colony lysates from the CHROMagar™ plates were tested for the *blaNDM-1* gene by PCR, as described above.

Minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) of all *blaNDM-1* donors, recipients, and transconjugants were determined by meropenem Etest® (bioMérieux Clinical Diagnostics, Marcy l'Etoile, France).
Plasmid analysis

Genetic location (plasmid or chromosome) of the blaNDM-1 gene after conjugational transfer was determined by a combination of Pulsed Field Gel Electrophoresis (PFGE) of S1 digested macro DNA of the various plasmid donor strains and transconjugants followed by detection using \( ^{32} \text{P} \) labelled bla\(_{\text{NDM-1}} \) and bla\(_{\text{CMY-2}} \) probes using methods described by Patzer et al. 2009 (Patzer et al., 2009). Probes were prepared by PCR using primers pairs: NDMF/R TGGCTTTTGAAACTGTCGCACC, CTGTCACATCGAAATCGCGAG; CMY2F/R AAATCGTTATGCTGCGCTCT, GACACGGACAGGGTTAGGAT, respectively.

RESULTS

Biofilm conjugations into \( P. \) aeruginosa and \( A. \) baumannii

Transfer frequencies of the bla\(_{\text{NDM-1}} \) plasmids from the \( E. \) coli J53-bla

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Figure 1 shows the overall sequence of the mating-out assays and results. Transfer rates of NDM-encoding plasmids from the ATCC BAA-2146 \( E. \) coli, EKP, and EEC donor strains to \( E. \) coli J53 are listed in Table 1.

Broth and

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DISCUSSION

We successfully transferred plasmids carrying the blaNDM-1 gene from *Klebsiella* and *E. coli* donor strains into *P. aeruginosa* and *A. baumannii*. Plasmid analyses by S1 PFGE and blaNDM-1 32P labeled probe indicated that the blaNDM-1 gene remained plasmid-located in the *P. aeruginosa* and *A. baumannii* transconjugants in our study. To our knowledge, plasmid transfer of the blaNDM-1 gene into *P. aeruginosa* has not been observed experimentally, and plasmid transfer of blaNDM-1 into *A. baumannii* has previously only been demonstrated by electroporation (Potron et al., 2011) or using agar surface mating techniques and an *Acinetobacter* donor (Huang et al., 2015). Agar and filter surface mating methods, like biofilm, provide a stable, spatially-structured environment for gene transfer; however, sites of nutrient uptake and gas exchange differ, and less of the protective extracellular polymeric substance is produced in agar colonies compared to biofilms (Davey & O'Toole, 2000, Stalder & Top, 2016).

Additionally, the plasmids donors in our assays were *Enterobacteriaceae* species and inter-family transfer via *E. coli* 153 blaNDM-1 transconjugants to *P. aeruginosa* and *A. baumannii* has not previously been demonstrated with other NDM-1-encoding plasmids.

NDM-1 mating assays described in the peer-reviewed literature frequently use broth mating techniques (Potron et al., 2011, Zhang et al., 2013, Ou et al., 2014). We did not observe
plasmid transfer of blaNDM-1 to P. aeruginosa in planktonic broth cultures, similar to other studies (Potron et al., 2011, Janvier et al., 2013), and only pEKP transferred in broth to A. baumannii (data not shown). Transfer of pEKP to either recipient was not detected. It should be noted that transfer of pEKP, or higher transfer rates of pEKP and pNDM-US donors, might have been observed if mating with the P. aeruginosa and A. baumannii recipients had been performed using the original parent blanDM-1 donor, rather than via an E. coli J53 transconjugant. Mating was performed using an E. coli J53- blanDM-1 transconjugant to enable comparison of transfer frequency of the different NDM-1 plasmid types and comparison with previous attempts to transfer blanDM-1 plasmids into P. aeruginosa and A. baumannii (Potron et al., 2011). Prior studies have been unable to detect transfer of NDM-encoding plasmids to P. aeruginosa or A. baumannii from Enterobacteriaceae donors by conjugation under broth conditions. This work demonstrates that conjugative inter-family transfer of these plasmids can be successful when mating occurs in a biofilm environment.

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Conflict of Interest
The authors have no conflicts of interest to declare.

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Moved up [2]: While studies of clinical NDM-1-producing P. aeruginosa isolates have found the blaNDM-1 gene located on the chromosome in P. aeruginosa (Janvier et al., 2013, Jovicic et al., 2014), the gene remained plasmid-located in both the P. aeruginosa and A. baumannii transconjugants in our study.

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CO-NDM donor strain, and Dr. Mark Fisher for providing the clinical A. baumannii recipient strain.
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