Title: Horizontal transfer of the \textit{bla}_{NDM-1} gene to \textit{Pseudomonas aeruginosa} and \textit{Acinetobacter baumannii} within a biofilm

Running title: Transfer of \textit{bla}_{NDM-1} into \textit{P. aeruginosa} and \textit{A. baumannii}

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ABSTRACT

Horizontal gene transfer has contributed to the global spread of the *bla*<sub>NDM-1</sub> gene. Studies have demonstrated plasmid transfer of *bla*<sub>NDM-1</sub> into various Gram-negative bacterial species, but attempts to demonstrate transfer of *bla*<sub>NDM-1</sub> 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 *bla*<sub>NDM-1</sub> 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-*bla*<sub>NDM-1</sub> 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-*bla*<sub>NDM-1</sub> transconjugant into *P. aeruginosa* was successful in a 72-hour biofilm, and transfer of NDM-encoding plasmids from two *E. coli* J53-*bla*<sub>NDM-1</sub> transconjugants to *A. baumannii* was successful in 24-hour biofilms.
**INTRODUCTION**

*Spread of the bla*<sub>NDM-1</sub> 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 bla*<sub>NDM-1</sub> 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 bla*<sub>NDM-1</sub> 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 bla*<sub>NDM-1</sub> 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 *bla*<sub>NDM-1</sub>-gene via intra- or inter-genus conjugational transfer of NDM-1-encoding plasmids; however, successful inter-family transfer of the *bla*<sub>NDM-1</sub>-harboring 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 conjugational 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 (Poiron et al., 2011).

**Broth conjugations into azide-resistant *E. coli* J53**

Log phase Luria-Bertani (LB) broth cultures of each of the bla<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 of bla<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 the bla<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***
Biofilm mating-out assays were performed using NDM-1-positive E. coli J53 transconjugants TeKp, 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 x 10^7 cells from an E. coli J53 transconjugant donor and 1.0 x 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 bla_{NDM-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 $^{32}$P labelled bla_{NDM-1} and bla_{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, CTGTCACATCGAAATCGCGCGA; CMY2F/R AAATCGTTATGCTGCGCTCT, GACACGGACAGGGTTAGGAT, respectively.

RESULTS

Biofilm conjugations into P. aeruginosa and A. baumannii

Transfer frequencies of the bla_{NDM-1} plasmids from the E. coli J53-bla_{NDM-1} transconjugants into P. aeruginosa or A. baumannii are presented in Table 1. Transfer of the bla_{NDM-1} plasmid pNDM-US to P. aeruginosa was successful in a 72-hour biofilm, but was not detected in a 24-hour biofilm. Transfer of the bla_{NDM-1} plasmids pNDM-US and pEKP from the TcNDM-US and TcEKP to A. baumannii was successful in a 24-hour biofilm, but was not detected in a 72-hour biofilm.

Minimum inhibitory concentrations

All parental bla_{NDM-1} donor strains had meropenem MICs greater than 32 µg/mL. All transconjugants had meropenem MICs of 24 µg/mL or greater.

Plasmid analysis

S1 PFGE and bla_{NDM-1} $^{32}$P labeled probe showed that the ATCC BAA-2146 bla_{NDM-1} donor harbored a NDM-encoding plasmid of the approximately 140 kb. An NDM-encoding plasmid of the same size was found in the P. aeruginosa-bla_{NDM-1} transconjugant, P. aeruginosa...
DISCUSSION

We successfully transferred plasmids carrying the 
NDM-1 gene from Klebsiella and E. coli donor strains into P. aeruginosa and A. baumannii. Plasmid analyses by S1 PFGE and blaNDM-1 
labeling probe indicated that the blaNDM-1 gene remained plasmid-located in the

Agar and filter 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 J53-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

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Deleted: and ethidium bromide staining (Figure 2 (b)). Probing of the gel with a blaCMY-2 labeled probe (Figure 2 (a)) indicated that the blaCMY-2 gene was associated with a

140kb plasmid in the K. pneumoniae ATCC donor only and probing a replicate gel (Figure 2 (c)) indicated that the

same 140kb plasmid was associated with the blaCMY-2 gene. Probed gels also indicated that transfer of the 140kb plasmid to P. aeruginosa and A. baumannii occurred as a result of

mating the recipient strains with the

E. coli J53-blaNDM-1 ATCC intermediate donor (Figure 2 (a-c), Figure 3).

Interestingly, in one of the P. aeruginosa transconjugants the

blaCMY-2 and blaNDM-1 positive plasmid was slightly smaller (130kb). The probed gel indicated that the

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plasmid transfer of \textit{blaNDM-1} to \textit{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 \textit{A. baumannii} (data not shown). Transfer of pEEC to either recipient was not detected. It should be noted that transfer of pEEC or higher transfer rates of pEKP and pNDM-US donors, might have been observed if mating with the \textit{P. aeruginosa} and \textit{A. baumannii} recipients had been performed using the original parent \textit{blaNDM-1} donor, rather than via an \textit{E. coli} J53 transconjugant. Mating was performed using an \textit{E. coli} J53- \textit{blaNDM-1} transconjugant to enable comparison of transfer frequency of the different NDM-1 plasmid types and comparison with previous attempts to transfer \textit{blaNDM-1} plasmids into \textit{P. aeruginosa} and \textit{A. baumannii} (Potron et al., 2011). Prior studies have been unable to detect transfer of NDM-encoding plasmids to \textit{P. aeruginosa} or \textit{A. baumannii} from \textit{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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\textbf{Conflict of Interest}

The authors have no conflicts of interest to declare.

\textbf{Acknowledgements}

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\textit{NDM-1}bla-encoded plasmids into \textit{P. aeruginosa} and \textit{A. baumannii} (Centers for Disease Control and Prevention, 2013). Biofilm-implicated healthcare outbreaks traced to environmental sources (Hota et al., 2009, Doidge et al., 2014). The plasmid was of identical size (140 kb) to the ATCC donor in one \textit{P. aeruginosa} transconjugant but a second transconjugant was slightly smaller (approx. 130kb). This is likely due to a deletion event, which appears to be a common event during plasmid transfer of NDM-encoding plasmids (Kumarasamy et al., 2010). Interestingly, in both \textit{P. aeruginosa} transconjugants the NDM-encoding plasmids were found in multiple forms which are most likely multimeric plasmid forms, which was also partially visible in the donor strain (Figure 2). Multiple copies of the \textit{blaNDM-1} gene on the \textit{P. aeruginosa} chromosome have been reported previously (Jovicic et al., 2014), and similar multimeric plasmid forms have also been seen in NDM-encoding plasmids in \textit{Acinetobacter} species (Jones et al., 2014). In the U.S., healthcare-acquired multidrug-resistant \textit{Acinetobacter} and \textit{P. aeruginosa} are responsible for an estimated 7300 and 6700 infections each year, respectively (Centers for Disease Control and Prevention, 2013).
CO-NDM donor strain, and Dr. Mark Fisher for providing the clinical A. baumannii recipient strain.
References


