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Research Letter

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Horizontal transfer of the bla_{NDM-1} gene to *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in biofilms

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One sentence summary: Interfamily transfer of NDM-1-encoding plasmids to *Pseudomonas aeruginosa* and *Acinetobacter baumannii* by conjugation within biofilms.

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ABSTRACT

Horizontal gene transfer has contributed to the global spread of the bla_{NDM-1} gene. Multiple studies have demonstrated plasmid transfer of bla_{NDM-1} between Gram-negative bacteria, primarily *Enterobacteriaceae* species, but conjugational transfer of natural bla_{NDM-1} plasmids from *Enterobacteriaceae* into *Pseudomonas aeruginosa* and *Acinetobacter baumannii* has not previously been shown. As *P. aeruginosa* and *A. baumannii* are both typically strong biofilm formers, transfer of natural bla_{NDM-1} plasmids could potentially occur more readily in this environment. To determine whether natural bla_{NDM-1} plasmids could transfer to *P. aeruginosa* or *A. baumannii* in biofilms, three clinical and environmental *Enterobacteriaceae* strains carrying NDM-1-encoding plasmids of different incompatibility types were mated with *E. coli* J53, producing *E. coli* J53- bla_{NDM-1} transconjugants. Subsequently, dual-species biofilms were created using the *E. coli* J53 transconjugants as plasmid donors and either *P. aeruginosa* or *A. baumannii* as recipients. Biofilm transfer of NDM-encoding plasmids to *P. aeruginosa* and *A. baumannii* was successful from one and two *E. coli* J53- bla_{NDM-1} transconjugants, respectively. This demonstrates the potential for the spread of bla_{NDM-1}, genes to *P. aeruginosa* and *A. baumannii* in clinical and environmental settings.

Keywords: NDM-1; biofilm; horizontal gene transfer; *Pseudomonas aeruginosa*; conjugation; *Acinetobacter baumannii*

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INTRODUCTION

The recent increase in carbapenem-resistant infections is a global public health concern, and treatment options for the associated pathogens have become significantly limited (Gupta *et al.* 2011). The bla_{NDM-1} carbapenemase gene, which confers resistance to carbapenem antibiotics, has spread rapidly across the world since it was first reported in 2009 (Yong *et al.* 2009; Johnson and Woodford 2013). The gene is typically mobile, commonly carried on plasmids of diverse sizes and incompatibility types that are capable of interspecies, intergenus and interfamily transfer (Carattoli 2013). Successful dissemination of the bla_{NDM-1} gene is most commonly attributed to conjugational transfer of NDM-1-encoding plasmids to other Gram-negative bacteria (Carattoli 2013; Johnson and Woodford 2013).

The bla_{NDM-1} gene is typically found in *Enterobacteriaceae* species, but has also been detected in a variety of non-fermenting Gram-negative bacteria including *Aeromonas caviae, Stenotrophomonas maltophilia, Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Walsh *et al.* 2011; Zhang *et al.* 2013). Yet, previous attempts at interfamily transfer of bla_{NDM-1} harboring plasmids to *P. aeruginosa* or *A. baumannii* have not been successful in the laboratory (Potron, Poirel and Nordmann 2011; Huang *et al.* 2015).

Surface mating approaches, such as filter or biofilm mating methods, are often used for Gram-negative and Gram-positive conjugation experiments (Roberts, Mullany and Wilson 2001; Kumarasamy *et al.* 2010; Walsh *et al.* 2011). However, because pili often assist in gene transfer in Gram-negative bacteria, broth mating has also been a commonly used method in Gram-negative mating experiments (Potron, Poirel and Nordmann 2011). Many bacterial species, such as *P. aeruginosa* and *A. baumannii*, are frequently found in the environment 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 (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; Gurung *et al.* 2013), which could play a role in facilitating transfer of bla_{NDM-1} to these bacteria. Serious hospital outbreaks of carbapenemase-producing bacteria have previously been traced to biofilms, where intergenus transfer of carbapenemase genes has been identified (Conlan *et al.* 2014). Similarly, NDM-1 (Lowe *et al.* 2013), *P. aeruginosa* (Hota *et al.* 2009) and *A. baumannii* (La Forgia *et al.* 2010) have all been implicated in biofilm-associated outbreaks in healthcare facilities. The objective of this study was to determine whether natural plasmids harboring bla_{NDM-1} genes originating in *Enterobacteriaceae* species could be transferred from an *Escherichia coli* J53-bla_{NDM-1} transconjugant to *P. aeruginosa* or *A. baumannii* in a biofilm environment. Understanding and demonstrating the role of biofilms in bla_{NDM-1} transfer to these organisms is critical for implementing proper interventions to minimize further dissemination of this resistance factor in environmental and clinical settings.

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 *Klebsiella 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 *Escherichia 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 water and sewage samples as part of a study previously conducted in Southeast Asia by one of the authors, and the CO-NDM and ATCC strains were isolated from clinical samples and were obtained from the Colorado Department of Public Health and Environment Laboratory Services Division and from the American Type Culture Collection, respectively. Azide-resistant *E. coli* J53 was used as a recipient in mating experiments with the original plasmid donor strains. Subsequent conjugation assays were performed using the *E. coli* J53-bla_{NDM-1} transconjugants as donors and rifampin-resistant *Pseudomonas aeruginosa* (PA01UOB7) and *Acinetobacter baumannii* (AB01CPI1) as recipients. The azide-resistant *E. coli* J53 and rifampin-resistant *P. aeruginosa* isolates were from the private collection of one of the authors. The *A. baumannii* recipient strain was isolated from a clinical sample and kindly provided by Dr Mark Fisher. *E. coli* J53-bla_{NDM-1} transconjugants were subsequently used as donors to allow for comparison of transfer frequencies between each of the NDM-encoding plasmid types, as previously described (Potron, Poirel and Nordmann 2011).

Broth conjugations into azide-resistant *Escherichia coli* J53

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

Transfer of bla_{NDM-1} into *E. coli* J53 was confirmed by PCR with a previously described primer set (Poirel *et al.* 2011) and on CHROMagar Orientation (DRG International, Springfield, NJ) 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, Bonacorsi and Bingen 2000). Three of the bla_{NDM-1} 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 *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

Biofilm conjugation assays were performed using NDM-1-positive *E. coli* J53 transconjugants TcEKP, TcEEC and TcNDM-US as bla_{NDM-1} plasmid donors and *P. aeruginosa* PA01UOB7 and *A. baumannii* AB01CPI1 as recipients. Using optical density measurements, log-phase donor and recipient LB broth cultures were combined in a 1:4 donor-to-recipient ratio in 1 mL LB.

Table 1. Conjugation frequencies of NDM-1-encoding plasmids from *E. coli* J53-bla_{NDM-1} transconjugants into rifampin-resistant *P. aeruginosa* or *A. baumannii*.[a]

bla_{NDM-1} donor strain	*E. coli* J53- bla_{NDM-1} transconjugant[b]	*P. aeruginosa* PA01UOB7[c] 72-h biofilm	*A. baumannii* AB01CPI1[d] 24-h biofilm
ATCC BAA-2146	TcNDM-US	7.6×10^{-7}	1.2×10^{-5}
EKP	TcEKP	Not detectable	2.0×10^{-5}

[a]Donor-to-recipient ratio 1:4. Conjugation frequencies given as number of *P. aeruginosa* or *A. baumannii*-bla_{NDM-1} transconjugants per *E. coli* J53-bla_{NDM-1} transconjugant.
[b]No transconjugants were detected in the 24 or 72-h *P. aeruginosa* or *A. baumannii* biofilms using *E. coli* J53-bla_{NDM-1} transconjugant TcEEC as the plasmid donor.
[c]No transconjugants were detected in the 24-h *P. aeruginosa* biofilm using any of the *E. coli* J53-bla_{NDM-1} transconjugants as the plasmid donor.
[d]No transconjugants were detected in the 72-h *A. baumannii* biofilm using any of the *E. coli* J53-bla_{NDM-1} transconjugants as the plasmid donor.

The 1 mL dual-species conjugation mixtures were placed in a 48-well plastic plate and incubated for either 24 or 72 h, allowing the culture to form a mixed biofilm on the sides of the wells. Conjugation mixtures were incubated at 30°C for the *P. aeruginosa* conjugations and 37°C for the *A. baumannii* conjugations. LB was exchanged every 24 h 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°C for 48 h.

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

Minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) of all bla_{NDM-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-labeled bla_{NDM-1} probes using methods described by Patzer *et al.* (2009). Probes were prepared by PCR using primers pairs: NDMF/R TGGCTTTTGAAACTGTCGCACC, CTGTCACATCGAAATCGCGCGA.

RESULTS

Conjugations into *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

Biofilm transfer frequencies of the bla_{NDM-1} plasmids from the *Escherichia coli* J53-bla_{NDM-1} transconjugants to *P. aeruginosa* or *A. baumannii* are presented in Table 1. Transfer of the bla_{NDM-1} plasmid to *P. aeruginosa* from *E. coli* TcNDM-US was successful in a 72-h biofilm at a frequency of 7.6×10^{-7} transconjugants per donor but was not detected in a 24-h biofilm. Transfers of the bla_{NDM-1} plasmids from *E. coli* TcNDM-US and *E. coli* TcEKP to *A. baumannii* were successful in 24-h biofilms at frequencies of 1.2×10^{-5} and 2.0×10^{-5} transconjugants per donor, respectively, but transfer was not observed in the 72-h biofilms. Transfer of bla_{NDM-1} in broth was only detected with the *E. coli* J53 TcEKP donor and *A. baumannii* recipient, and was observed at a frequency of 2.3×10^{-5} transconjugants per donor. Transfer of pEEC to *P. aeruginosa* or *A. baumannii* was not detected.

Minimum inhibitory concentrations

All parental bla_{NDM-1} donor strains had meropenem MICs >32 μg/mL. Prior to bla_{NDM-1} transfer into *P. aeruginosa* and *A. baumannii*, the meropenem MICs of these recipients were 1 and 0.125 μg/mL, respectively. All *E. coli* J53, *P. aeruginosa* and *A. baumannii* bla_{NDM-1} 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 an NDM-encoding plasmid of ~140 kb. An NDM-encoding plasmid of the same size was found in the *P. aeruginosa*-bla_{NDM-1} transconjugant, *P. aeruginosa* TcNDM-US and in the *A. baumannii*-bla_{NDM-1} transconjugant (*A. baumannii* TcNDM-US). Analysis of the EKP bla_{NDM-1} donor by S1 PFGE and bla_{NDM-1} ^{32}P labeled probe showed that the bla_{NDM-1} gene was located on a 100-kb plasmid, and a bla_{NDM-1} plasmid of the same size was found in the *A. baumannii*-bla_{NDM-1} transconjugant (*A. baumannii* TcEKP). S1 PFGE analysis did not show insertion of the bla_{NDM-1} gene into the chromosome of either *P. aeruginosa* or *A. baumannii*.

DISCUSSION

We successfully transferred plasmids carrying the bla_{NDM-1} gene from *Klebsiella pneumoniae* and *Escherichia coli* donor strains into *E. coli* J53, and subsequently from the *E. coli* J53-bla_{NDM-1} transconjugants into *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Transfer of natural bla_{NDM-1} plasmids from *Enterobacteriaceae* into these species by conjugation has previously been attempted, but has been unsuccessful prior to this study (Potron, Poirel and Nordmann 2011). Transfer of the bla_{NDM-1} gene into *P. aeruginosa* has not been observed experimentally, regardless of donor species. Plasmid transfer of bla_{NDM-1} into *A. baumannii* has previously only been demonstrated by electroporation (Potron, Poirel and Nordmann 2011), or by using an *Acinetobacter* donor agar and surface mating techniques (Huang *et al.* 2015).

We did not directly mate the original bla_{NDM-1} donors with the *P. aeruginosa* and *A. baumannii* recipient strains, limiting the results of this study. 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 *P. aeruginosa* and *A. baumannii* recipients had been performed using the original parent bla_{NDM-1} donors, rather than via an *E. coli* J53 transconjugant. An *E. coli* J53- bla_{NDM-1} transconjugant was used as the donor to compare transfer frequency of the different NDM-1 plasmid types and to compare these results with previous attempts to transfer bla_{NDM-1} plasmids into *P. aeruginosa* and *A. baumannii* using this methodology (Potron, Poirel and Nordmann 2011).

Our results suggest that biofilms could be an important factor in the interfamily transfer and spread of plasmids carrying bla_{NDM-1} in nature, informing potential surveillance and mitigation strategies to reduce further environmental and clinical dissemination.

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Conflict of interest. None declare.

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