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

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

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ABSTRACT

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

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INTRODUCTION

Spread of the *bla*$_{NDM-1}$ 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 *bla*$_{NDM-1}$ 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 *bla*$_{NDM-1}$ 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 *bla*$_{NDM-1}$ 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 *bla*$_{NDM-1}$ gene via intra- or inter-genus conjugational transfer of NDM-1-encoding plasmids; however, successful inter-family transfer of the *bla*$_{NDM-1}$ 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).

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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 $bla_{\text{NDM-1}}$ to these bacteria. The objective of this study was to determine whether plasmid-borne $bla_{\text{NDM-1}}$ genes originating in *Enterobacteriaceae* species could be transferred from an *E. coli* J53-$bla_{\text{NDM-1}}$ transconjugant to *P. aeruginosa* or *A. baumannii* in a biofilm environment.

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MATERIALS AND METHODS

Donor and recipient organisms

Four NDM-1-producing *Enterobacteriaceae* strains with plasmids of different incompatibility types carrying the $bla_{\text{NDM-1}}$ gene were used as the original plasmid donors: a *K. pneumoniae* donor (EKP) carrying the $bla_{\text{NDM-1}}$ gene on a 100 kb plasmid (pEKP) belonging to the FII, L/M, or N2 incompatibility group; an *E. coli* donor (EEC) carrying the $bla_{\text{NDM-1}}$ gene on a 150 kb plasmid (pEEC) belonging to the FII incompatibility group; a *K. pneumoniae* donor (CO-NDM) carrying the $bla_{\text{NDM-1}}$ gene on a 130 kb plasmid (pCO-NDM) of unknown incompatibility type; and a *K. pneumoniae* donor (ATCC BAA-2146) carrying the $bla_{\text{NDM-1}}$ 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_{NDM-1} transconjugants as donors and rifampin-resistant *P. aeruginosa* and *A. baumannii* as recipients. *E. coli* J53-bla_{NDM-1} 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 the bla_{NDM-1} 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_{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, 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_{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 *P. aeruginosa* and *A. baumannii*

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Biofilm mating-out 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* 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 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).

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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 to *P. aeruginosa* or *A. baumannii* are presented in Table 1. Transfer of the *bla*$_{NDM-1}$ plasmid pNDM-US to *P. aeruginosa* from TcNDM-US 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*

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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 *K. pneumoniae*, EKP, and EEC donor strains to *E. coli* J53 are listed in Table 1. ¶
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TcATCC, and in the *A. baumannii-bla*$_{NDM-1}$ transconjugant, *A. baumannii* TcATCC. 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 100kb 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 and E. coli* donor strains into *E. coli* J53, and subsequently from the *E. coli* J53-*bla*$_{NDM-1}$ transconjugants into *P. aeruginosa* and *A. baumannii*. Plasmid analyses by S1 PFGE and *bla*$_{NDM-1}$ ^{32}P labeled probe indicated that the *bla*$_{NDM-1}$ gene remained plasmid-located in the *P. aeruginosa* and *A. baumannii bla*$_{NDM-1}$ transconjugants in our study. To our knowledge, plasmid transfer of the *bla*$_{NDM-1}$ gene into *P. aeruginosa* has not been observed experimentally, and plasmid transfer of *bla*$_{NDM-1}$ 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* J53-*bla*$_{NDM-1}$ 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

Deleted: and ethidium bromide staining (Figure 2 (b)). Probing of the gel with a *bla*$_{CMY-2}$ ^{32}P labeled probe (Figure 2 (a)) indicated that the *bla*$_{CMY-2}$ gene was associated with a 140kb plasmid in the *K. pneumoniae* ATCC donor only and probing of a replicate gel (Figure 2 (c)) indicated that the same 140kb plasmid was associated with the *bla*$_{NDM-1}$ 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-*bla*$_{NDM-1}$ ATCC intermediate donor (Figure 2 (a-c), Figure 3). Interestingly, in one of the *P. aeruginosa* transconjugants the *bla*$_{CMY-2}$ and *bla*$_{NDM-1}$ positive plasmid was slightly smaller (130kb). The probed gel indicated that the *bla*$_{CMY-2}$ and *bla*$_{NDM-1}$ positive plasmid in *P. aeruginosa* was found in various multimeric forms.

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plasmid transfer of *bla*$_{NDM-1}$ 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 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 *P. aeruginosa* and *A. baumannii* recipients had been performed using the original parent *bla*$_{NDM-1}$ donor, rather than via an *E. coli* J53 transconjugant. Mating was performed using an *E. coli* J53- *bla*$_{NDM-1}$ transconjugant to enable comparison of transfer frequency of the different NDM-1 plasmid types and comparison with previous attempts to transfer *bla*$_{NDM-1}$ 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.

Funding Information

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Conflict of Interest

The authors have no conflicts of interest to declare.

Acknowledgements

We wish to thank the Utah Public Health Laboratory for their assistance and the use of their facility for part of the PCR analyses of these isolates. We are also grateful to the Colorado Department of Public Health & Environment Laboratory Services Division for providing the

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In the U.S., healthcare-acquired multidrug-resistant *Acinetobacter* and *P. aeruginosa* are responsible for an estimated 7300 and 6700 infections each year, respectively (Centers for Disease Control and Prevention, 2013). Biofilm-forming *P. aeruginosa* and *A. baumannii* have been implicated in healthcare facility outbreaks traced to environmental sources (Hota *et al.*, 2009, Doidge *et al.*, ...

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

462 **References**

463 Blanc DS, Francioli P & Zanetti G (2007) Molecular Epidemiology of Pseudomonas aeruginosa
464 in the Intensive Care Units - A Review. *The open microbiology journal* **1**: 8-11.

465 Carattoli A (2013) Plasmids and the spread of resistance. *International Journal of Medical*
466 *Microbiology* **303**: 298-304.

467 Chaudhary M & Payasi A (2013) Rising antimicrobial resistance of pseudomonas aeruginosa
468 isolated from clinical specimens in India. *Journal of Proteomics and Bioinformatics* **6**: 5-9.

469 Chen Y, Zhou Z, Jiang Y & Yu Y (2011) Emergence of NDM-1-producing Acinetobacter
470 baumannii in China. *Journal of Antimicrobial Chemotherapy* **66**: 1255-1259.

471 Clermont O, Bonacorsi S & Bingen E (2000) Rapid and simple determination of the Escherichia
472 coli phylogenetic group. *Applied and Environmental Microbiology* **66**: 4555-4558.

473 Davey ME & O'Toole GA (2000) Microbial biofilms: From ecology to molecular genetics.
474 *Microbiology and Molecular Biology Reviews* **64**: 847-867.

475 Donlan RM (2002) Biofilms: Microbial life on surfaces. *Emerging Infectious Diseases* **8**: 881-
476 890.

477 Gurung J, Khyriem AB, Banik A, Lyngdoh WV, Choudhury B & Bhattacharyya P (2013)
478 Association of biofilm production with multidrug resistance among clinical isolates of
479 Acinetobacter baumannii and Pseudomonas aeruginosa from intensive care unit. *Indian journal*
480 *of critical care medicine : peer-reviewed, official publication of Indian Society of Critical Care*
481 *Medicine* **17**: 214-218.

482 Huang TW, Lauderdale TL, Liao TL*, et al.* (2015) Effective transfer of a 47 kb NDM-1-positive
483 plasmid among Acinetobacter species. *The Journal of antimicrobial chemotherapy* **70**: 2734-
484 2738.

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485 Hudson CM, Bent ZW, Meagher RJ & Williams KP (2014) Resistance determinants and mobile
486 genetic elements of an NDM-1-encoding Klebsiella pneumoniae strain. *PLoS ONE* **9**.
487 Janvier F, Jeannot K, Tessé S, Robert-Nicoud M, Delacour H, Rapp C & Mérens A (2013)
488 Molecular Characterization of bla(NDM-1) in a Sequence Type 235 Pseudomonas aeruginosa
489 Isolate from France. *Antimicrobial Agents and Chemotherapy* **57**: 3408-3411.
490 Johnson AP & Woodford N (2013) Global spread of antibiotic resistance: The example of New
491 Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance. *Journal of Medical*
492 *Microbiology* **62**: 499-513.
493 Kumarasamy KK, Toleman MA, Walsh TR*, et al.* (2010) Emergence of a new antibiotic
494 resistance mechanism in India, Pakistan, and the UK: A molecular, biological, and
495 epidemiological study. *The Lancet Infectious Diseases* **10**: 597-602.
496 Madsen JS, Burmølle M, Hansen LH & Sørensen SJ (2012) The interconnection between biofilm
497 formation and horizontal gene transfer. *FEMS Immunol Med Microbiol* **65**: 183-195.
498 Nutman A, Lerner A, Schwartz D & Carmeli Y (2016) Evaluation of carriage and environmental
499 contamination by carbapenem-resistant Acinetobacter baumannii. *Clinical microbiology and*
500 *infection : the official publication of the European Society of Clinical Microbiology and*
501 *Infectious Diseases*.
502 Ou W, Cui L, Li Y, Zheng B & Lv Y (2014) Epidemiological characteristics of blaNDM-1 in
503 Enterobacteriaceae and the Acinetobacter calcoaceticus-Acinetobacter baumannii complex in
504 China from 2011 to 2012. *PLoS One* **9**: e113852.
505 Patzer JA, Walsh TR, Weeks J, Dzierzanowska D & Toleman MA (2009) Emergence and
506 persistence of integron structures harbouring VIM genes in the Children's Memorial Health
507 Institute, Warsaw, Poland, 1998-2006. *The Journal of antimicrobial chemotherapy* **63**: 269-273.

508 Poirel L, Walsh TR, Cuvillier V & Nordmann P (2011) Multiplex PCR for detection of acquired
509 carbapenemase genes. *Diagnostic microbiology and infectious disease* **70**: 119-123.
510 Potron A, Poirel L & Nordmann P (2011) Plasmid-mediated transfer of the blaNDM-1 gene in
511 Gram-negative rods. *FEMS Microbiology Letters* **324**: 111-116.
512 Qi L, Li H, Zhang C, Liang B, Li J, Wang L, Du X, Liu X, Qiu S & Song H (2016) Relationship
513 between antibiotic resistance, biofilm formation, and biofilm-specific resistance in Acinetobacter
514 baumannii. *Frontiers in Microbiology* **7**.
515 Rahman M, Shukla SK, Prasad KN, Ovejero CM, Pati BK, Tripathi A, Singh A, Srivastava AK
516 & Gonzalez-Zorn B (2014) Prevalence and molecular characterisation of New Delhi metallo-
517 beta-lactamases NDM-1, NDM-5, NDM-6 and NDM-7 in multidrug-resistant Enterobacteriaceae
518 from India. *Int J Antimicrob Agents* **44**: 30-37.
519 Roberts AP, Mullany P & Wilson M (2001) Gene transfer in bacterial biofilms. Vol. 336 p.^pp.
520 60-65.
521 Savage VJ, Chopra I & O'Neill AJ (2013) Staphylococcus aureus biofilms promote horizontal
522 transfer of antibiotic resistance. *Antimicrobial Agents and Chemotherapy* **57**: 1968-1970.
523 Sowmiya M, Umashankar V, Muthukumaran S, Madhavan HN & Malathi J (2012) Studies on
524 New Delhi Metallo-Beta-Lactamse-1 producing Acinetobacter baumannii isolated from donor
525 swab in a tertiary eye care centre, India and structural analysis of its antibiotic binding
526 interactions. *Bioinformation* **8**: 445-452.
527 Stalder T & Top E (2016) Plasmid transfer in biofilms: a perspective on limitations and
528 opportunities. *Npj Biofilms And Microbiomes* **2**: 16022.

529 Walsh TR, Weeks J, Livermore DM & Toleman MA (2011) Dissemination of NDM-1 positive
530 bacteria in the New Delhi environment and its implications for human health: An environmental
531 point prevalence study. *The Lancet Infectious Diseases* **11**: 355-362.
532 Zhang C, Qiu S, Wang Y*, et al.* (2013) Higher Isolation of NDM-1 Producing *Acinetobacter*
533 *baumannii* from the Sewage of the Hospitals in Beijing. *PLOS ONE* **8**: e64857.

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