Mobile genetic elements associated with *bla*$_{\text{NDM-1}}$ in *Acinetobacter* spp. and *Vibrio cholerae*.

by

Lim Stephen Jones

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Summary

NDM-producing bacteria are associated with extensive antimicrobial resistance (AMR). This thesis reports on detailed molecular analysis, including whole genome sequencing, of *Acinetobacter* spp. and *Vibrio cholerae* isolates.

A study of clinical *Acinetobacter baumannii* isolates from India, demonstrated spread of a single strain containing *bla*$_{NDM-1}$ but with evidence of significant genetic plasticity between isolates. A novel plasmid, pNDM-32, was fully characterised in isolate CHI-32. This contained multiple AMR genes including *bla*$_{NDM-1}$ and the aminoglycoside methyltransferase gene *armA*. A *repAci10* replicase gene was identified but no conjugation machinery and the plasmid could not be transferred in conjugation experiments.

A single isolate of *Acinetobacter bereziniae* from India contained plasmid, pNDM-40-1, harbouring *bla*$_{NDM-1}$, which was closely related to plasmids from NDM-producing Acinetobacter spp. isolated in China, and was readily transferred into *Escherichia coli* and *Acinetobacter pittii* by conjugation. Five *bla*$_{NDM-1}$ positive *Acinetobacter* spp. isolated from a faecal screening study in Pakistan also included three, clonal, *Acinetobacter haemolyticus* isolates harbouring a similar plasmid.

Three environmental *V. cholerae* strains from India and a blood isolate from a traveller returning to the UK from India were found to include three distantly related strains. 2 isolates of a single strain contained an IncA/C plasmid, pNDM-116-17, harbouring AMR genes including *bla*$_{NDM-1}$. In one isolate pNDM-116-17 had become integrated into a chromosomal region containing a SXT-like element. In the other isolates *bla*$_{NDM-1}$ and other AMR determinants were localised to a large plasmid, pNDM-116-14, with a novel replicase and a full complement of conjugative transfer genes, and a novel genomic island, SGI-NDM-1.

Most previous studies have focused on Enterobacteriaceae. The current work contributes to an understanding of the full extent of the genetic diversity of *bla*$_{NDM-1}$ contexts, and their dissemination. Such knowledge should help to infer factors which contribute to the spread of AMR in bacterial pathogens.

Publications and Presentations

Publications resulting from data presented in this thesis:

Jones L.S., Carvalho M.J., Toleman M.A., White P.L., Connor T.R., Mushtaq A., Weeks J.L., Kumarasamy K.K., Raven K.E., Török M.E., Peacock S.J., Howe R.A., Walsh T.R. (2015). Characterization of plasmids in extensively drug-resistant Acinetobacter strains isolated in India and Pakistan. *Antimicrobial Agents and Chemotherapy*, 59(2): 923-9.

Jones L.S., Toleman M.A., Weeks J.L., Howe R.A., Walsh T.R., Kumarasamy K.K. (2014). Plasmid carriage of *bla*$_{NDM-1}$ in clinical *Acinetobacter baumannii* isolates from India. *Antimicrobial Agents and Chemotherapy*, 58(7): 4211-3.

Darley, E. Weeks, J., Jones, L., Daniels, V., Wootton, M., MacGowan, A. and Walsh, T.R. Overseas traveller presenting with NDM-1 positive polymicrobial infactions including Vibrio cholerae: an increased need for vigilance. *The Lancet,* 380(9850): 1358.

Toleman, M.A., Spencer, J, Jones, L. and Walsh, T.R. (2012). *bla*$_{NDM-1}$ Is a Chimera Likely Constructed in *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy,* 56(5):2773-6.

Manuscripts in preparation:

Jones, L., Connor, T., Toleman, M., Mutreja, A., Thomson, N., Howe, R. and Walsh, T. *Vibrio cholerae*: an environmental reservoir for mobile elements and antimicrobial resistance genes including *bla*$_{NDM-1}$? Planned for submission to *PLOS Pathogens*.

Jones L.S., Perry, B., Kumarasamy K.K., Török M.E., Peacock S.J., Howe R.A. and Walsh T.R. Complete sequence of plasmid pNDM-32, harbouring *bla*$_{NDM-1}$ and *armA*, from *A. baumannii* and sequence variation of plasmids and resistance islands in closely related isolates. Planned for submission to *Antimicrobial Agents and Chemotherapy*.

Talks resulting from data presented in this thesis:

Origins and spread of New Delhi metallo-β-lactamase 1 (NDM-1): lessons from genomic analysis of *Acinetobacter* spp. and *Vibrio cholera*. At the WCAT Annual Training Day, Cardiff University, 18/10/14.

Vibrio cholerae: an environmental reservoir for mobile elements and antibiotic resistance genes including *bla*$_{NDM-1}$? At the South West & South Wales Microbiology Forum, Bath University, 12/09/13.

Vibrio cholerae: an environmental reservoir for mobile elements and antibiotic resistance genes including *bla*$_{NDM-1}$? The 2013 Panceltic Summer Meeting, Holland House, Cardiff, 22/06/13.

Mobile genetic elements associated with *bla*$_{NDM-1}$ in multi-drug resistant *Acinetobacter* species and *Vibrio cholerae*. Talk given at WCAT annual training day, Glamorgan Building, Cardiff University, 19/10/12.

The Genetic Context of *bla*$_{NDM-1}$ in *Acinetobacter baumannii* and *Acinetobacter guillouiae* from Chennai, India. Talk given at MITReG: Interdisciplinary Postgraduate Research Day, 21/09/12.

Poster presentations resulting from data presented in this thesis:

Jones, L., Toleman, M., Weeks, J., Kumarasamy, K., Howe, R. and Walsh, T. A broad host range conjugative plasmid carrying *bla*$_{NDM-1}$ in an *Acinetobacter bereziniae* isolate from India: Conjugation experiments and genetic stability. Presented at 23rd ECCMID, Berlin, 28th April 2013, P1296.

Jones, L., Toleman, M., Connor, T., Weeks, J., Kumarasamy, K., Torok, E., Howe, R., Peacock, S. and Walsh, T. A broad host range conjugative plasmid carrying *bla*$_{NDM-1}$ in an *Acinetobacter bereziniae* isolate from India: Complete sequence and comparison with related genetic contexts. Presented at 23rd ECCMID, Berlin, 28th April 2013, P1295.

Jones, L., Toleman, M., Mutreja, A., Weeks, J., Connor, T., Thomson, N., Howe, R. and Walsh, T. The genetic context of *bla*$_{NDM-1}$ in *Vibrio cholerae*. Presented at 23rd ECCMID, Berlin, 28th April 2013, P1292.

Jones, L., Toleman, M.A., Weeks, J, Howe, R., Walsh, T.R. and Kumarasamy, K. The genetic context of *bla*$_{NDM-1}$ in *Acinetobacter baumannii* and *Acinetobacter guillouiae* from Chennai, India. Presented at the Cardiff Institute of Infection and Immunity Annual Meeting 2012.

Jones, L., Toleman, M.A., Weeks, J, Howe, R., Walsh, T.R. and Kumarasamy, K. The genetic context of *bla*$_{NDM-1}$ in *Acinetobacter baumannii* from clinical isolates dating back to 2005 from Chennai, India. Presented at 22nd ECCMID, London 2nd April 2012, P1713.

Jones, L., Toleman, M.A., Berry, N., Weeks, J., Daniels, V.E., Dickson, W.A., Davies, D., Wootton, M., Howe, R., Walsh, T.R. and Kumarasamy, K. The genetic context of *bla*$_{NDM-1}$ in *Acinetobacter baumannii* from a burns unit outbreak in Swansea, Wales. Presented at 22nd ECCMID, London 2nd April 2012, P1705.

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Declaration

I declare that this thesis represents my own work, except where otherwise acknowledged. The opinions given are my own and not those of Cardiff University or Public Health Wales.

No portion of this thesis has been submitted for any other degree or award at this or any other university or place of learning, nor is it being submitted concurrently in candidature for any degree or other award.

Dr Lim S. Jones MBBCh, FRCPath

Clinical Lecturer
Institute of Infection and Immunity
Medical Microbiology and Infectious Diseases
Cardiff University

Honorary Registrar,
Medical Microbiology and Virology
Public Health Wales
Cardiff

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List of abbreviation:

AA	Amino acid
ABC	*Acinetobacter baumannii* complex
AFLP	Amplified fragment length polymorphism
AMR	Antimicrobial resistance
bla	β-lactamase gene
CBA	Colombia Blood Agar
CDC	Centre for disease control and prevention
CPE	Carbapenemase-producing Enterobacteriaceae
CRE	Carbapenem-resistant Enterobacteriaceae
3'CS	Three prime conserved sequence (of class 1 integrons)
CS	Cell suspension (buffer)
Ct	Cycle threshold value(s)
CTAB	Cetyltrimethylammonium bromide
DR(s)	Direct repeat(s)
EDTA	Ethylenediaminetetraacetic acid
ES	EDTA-sarkosyl (buffer)
GEI(s)	Genomic island(s)
GNB	Gram-negative bacilli
HGT	Horizontal gene transfer
HCCA	α-cyano-4-hydroxycinnamic acid (HCCA)
ICE(s)	Integrative and conjugative element(s)
IME(s)	Integrative and mobilizable element(s)
Inc	Incompatibility (referring to plasmid typing)
IS(s)	Insertion sequence(s)
LB	Luria Bertani (agar/ broth)
LMP	Low melting point agar
MDR	Multi-drug resistant
MFS	Major facilitator superfamily
MGE(s)	Mobile genetic element(s)
MH	Mueller Hinton (agar)
ML	Maximum likelihood (phylogenetic tree)
MLST	Multilocus sequence typing
NPAB	NDM-producing *Acinetobacter baumannii*

NDM	New Delhi metallo-β-lactamase
NPE	NDM-producing Enterobacteriaceae
NPGNB	NDM-producing GNB
NPVC	NDM-producing *Vibrio cholerae*
ORF	Open reading frame
PBP(s)	Penicillin binding protein(s)
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
qPCR	Quantitative PCR
SNP(s)	Single nucleotide polymorphism(s)
SSC	Saline-Sodium Citrate (solution)
TBE	Tris-borate-EDTA (buffer)
TCBS	Thiosulphate Citrate Bile Salts Sucrose (agar)
TCP	Toxin co-regulated pillus
TE	Tris-EDTA (buffer)
Tn(s)	Transposon(s)
T4SS	Type IV secretion system
WGS	Whole genome sequencing
XDR	Extensively-drug resistant

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Chapter 1

General Introduction

1.1 Introductory notes

The New Delhi metallo-β-lactamase (NDM) enzymes are amongst the most recently described carbapenemases; bacterial enzymes capable of hydrolysing and inactivating antimicrobials of the carbapenem class (Yong *et al.* 2009b; Dortet *et al.* 2014). Production of these enzymes in many species is associated with multi-drug resistant (MDR) phenotypes and seriously compromises the treatment options for infections with these organisms. Despite not being described in bacteria isolated prior to 2005 (Castanheira *et al.* 2011; Berrazeg *et al.* 2014; Jones *et al.* 2014a), *bla*$_{\text{NDM-1}}$ has since been described in many species, strain backgrounds and located on a diverse range of mobile genetic elements (MGE) (Kumarasamy *et al.* 2010; Walsh *et al.* 2011; Johnson and Woodford 2013; Dortet et al. 2014). A brief review is provided here of some of the key areas of background relevant to this thesis namely; antimicrobial resistance (AMR); NDM-producing bacteria; mechanisms of horizontal gene transfer (HGT); MGE associated with dissemination of AMR genes and plasticity of their genetic contexts; and the species of Gram-negative bacilli (GNB) harbouring *bla*$_{\text{NDM-1}}$ studied in this thesis.

1.2 Antimicrobial Chemotherapy

1.2.1 Antimicrobial drug discovery

The introduction into clinical practice in the 1930's and 1940's of the sulphonamides and penicillin, respectively, resulted in a transformation in the prognosis of many bacterial infections, from being predominantly fatal, to being readily treatable (Powers 2004). A period of rapid and extensive drug discovery and development followed, with new classes of antimicrobials being identified and chemically modified, in order to alter their spectra of activity and/ or their pharmacological properties (Figure 1.1) (Powers 2004; Livermore 2011; Silver 2011). Figure 1.1 demonstrates how the pipeline of drug discovery has slowed considerably. No representative of a novel class of antimicrobials has been licensed for systemic use since daptomycin in 2003, although new derivatives of existing antimicrobial classes continue to be introduced into clinical practice (Pucci and Bush 2013). Theoretically if resistance has already emerged to a class of antimicrobials, there is a good chance that resistance will develop readily against any new agent of the same class, as has occurred in the past.

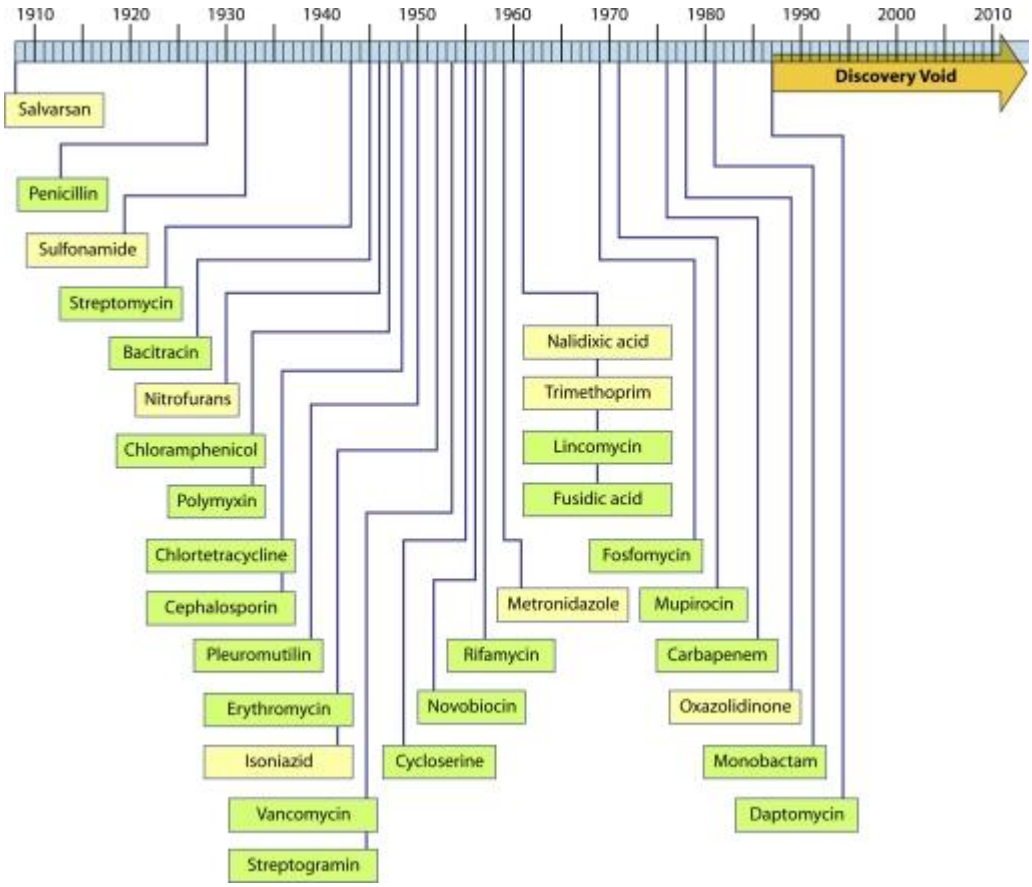


Figure 1.1 – Illustration of the "discovery void" of antimicrobial agents. Dates indicated are those of reported initial discovery or patent. Reproduced with permission from Silver LL, Clin Microbiol Rev. 2011;24:71-109 (Silver 2011).

1.2.2 AMR: intrinsic and acquired.

Bacteria can be intrinsically resistant to a drug or class of antimicrobials, but soon after the introductions of sulphonamides and penicillins the capacity of these organisms to develop acquired resistance to antimicrobials became apparent (McDonald 2006; Toleman and Walsh 2011). Today the global spread of AMR amongst a diverse range of bacterial pathogens is of major public health concern. In recent years the extent of

resistance and its rapid dissemination amongst various species of GNB has been particularly worrisome (Magiorakos *et al.* 2012; Tzouvelekis *et al.* 2012).

Acquired resistance to an antimicrobial can occur because of a small number of general mechanisms. An altered drug target site can reduce the affinity of the drug for its target, for example, an altered penicillin binding protein (PBP) which renders the organisms resistant to some β-lactams. Alternatively mechanisms exist which reduce the access of the drug to its target site, as with reduced permeability of Gram-negative outer membrane (see Figure 1.2), for example by outer membrane porin loss, or increased efflux of drug across the outer membrane (Blair et al. 2015). Bacteria can also produce enzymes which breakdown or modify the active drug and render it inactive, as with β-lactamases and aminoglycoside modifying enzymes (Bush and Jacoby 2010; Ramirez and Tolmasky 2010).

In some cases the extent of resistance depends on a balance of many different factors. For β-lactam resistance in Gram-negative organisms, for example, the permeability of the outer bacterial membrane, drug efflux mechanisms, the affinity of any β-lactamase enzymes produced to the antibiotic in question, and the level of expression of the enzyme(s) will all affect the level of resistance (Livermore 2012; Blair et al. 2015). Permeability and efflux in particular vary greatly with the bacterial species in question and can be affected by mutational changes, interruption of genes and the acquisition of genes on MGE (Hancock 1998).

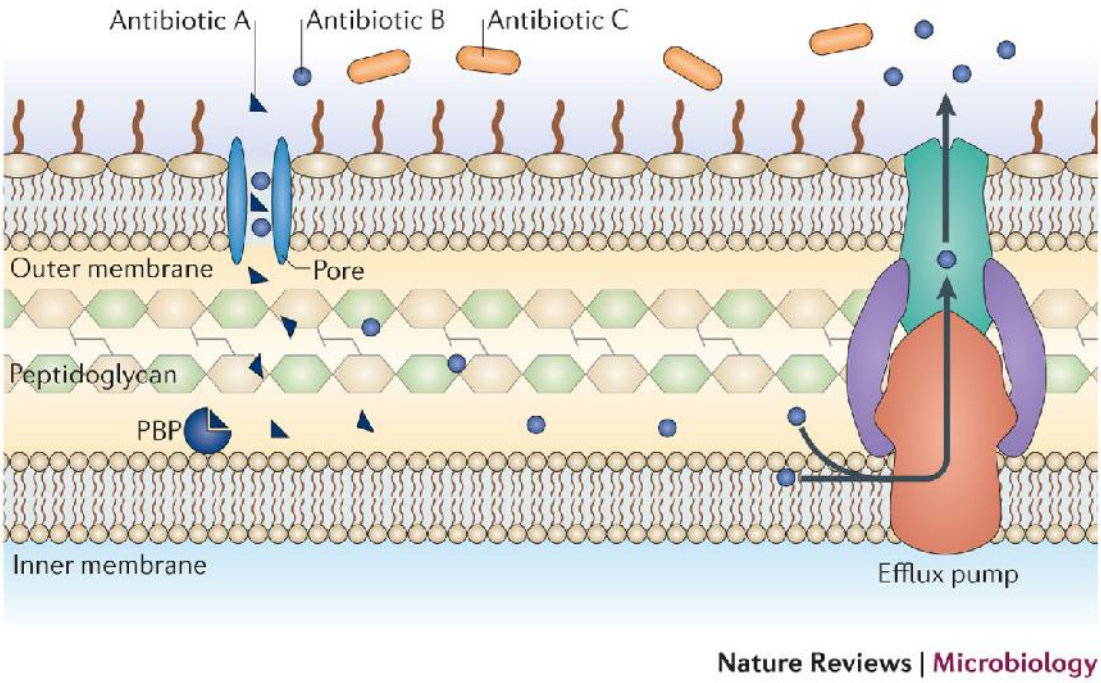


Figure 1.2 – Normal structure of the Gram-negative outer membrane and interaction with antimicrobials. Antimicrobials A and B are able to pass through the outer membrane. A is an example of a β-lactamase which is then able to interact with the PBP at the inner membrane. B is able to pass through the porin channel but is efficiently removed by an efflux pump. C is unable to pass through the available porin channel. Reproduced with permission from Blair JM *et al.*, Nat Rev Microbiol. 2015 13(1):42-51 (Blair *et al.* 2015).

It is important to note that the clinical impact of resistance to β-lactams is greater because of resistance to other agents. β-lactamase (*bla*) genes, including *bla*$_{\text{NDM-1-like}}$ genes are commonly found in bacteria with multiple other mechanisms of resistances and resistance genes are often clustered within MGEs, integrons or genomic islands (GEI) (Tzouvelekis et al. 2012). An important implication of this is that the competitive advantage of resistant bacteria on exposure to antimicrobials is likely to result in co-selection, where MDR populations of bacteria are selected for by exposure to multiple agents

(Cantón and Ruiz-Garbajosa 2011). This explains why in some studies cycling of antimicrobials does not apparently result in any benefit.

1.2.3 Resistance to β-lactams in GNB

The β-lactams are a large group of antibiotics, structurally related to penicillin. These bind to penicillin binding proteins (PBPs) at the cytoplasmic membrane, and by a complex variety of mechanisms, probably including interference with cell wall biosynthesis and release of autolytic enzymes, result in inhibition of growth and eventually cell lysis and death (Ogawara 1981). Whilst most GNB are relatively resistant to the earlier penicillins, a large number of β-lactams of the penicillin, cephalosporin and carbapenem subclasses have been developed with clinically relevant activity against these organisms. As these agents are well tolerated and efficacious they are amongst the most widely prescribed, and are prominent in treatment guidelines for many infections, including Gram-negative sepsis (Koliscak *et al.* 2013; Cooke *et al.* 2014; Paul *et al.* 2014). Despite the multifactorial nature of β-lactam resistance it is widely believed that the production of β-lactamase enzymes are the most epidemiologically significant mechanisms of resistance in many species of GNB (Hawkey 2008; Bush 2010; Livermore 2012).

β-lactamases can be classified according to molecular or functional classification schemes (Bush and Jacoby 2010). The phenotypic properties, molecular and functional groups of a few of the key groups of β-lactamases found in GNB are summarised in Table 1.1. The molecular class A, C and D enzymes all have a serine residue at their active site involved in hydrolysis of the β-lactam ring. The class B, metallo-β-lactamases (MBLs), invariably have

zinc residues at their active site. Enzymes of different classes have varying hydrolytic profiles and epidemiology. Particularly significant, for example, amongst the Enterobacteriaceae has been the dissemination, probably by HGT, of extended spectrum β-lactamases (ESBLs) (Hawkey 2008; Livermore 2012). In particular CTX-M type ESBLs spread globally amongst *E. coli* and *K. pneumoniae*, and have been associated with particularly successful pathogenic strains of these bacteria.

Functional group	Molecular class (subclass)	Distinctive substrate	Inhibited by		Defining characteristics	Representative enzymes
			CA or TZB	EDTA		
1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	E. coli AmpC, ACT-1, CMY-2, FOX-1, DHA-1
2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15,
2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2df	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-58, OXA-40, OXA-48
2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2
3a	B	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	NDM-1, VIM-1, IMP-1

Table 1.1 – Classification schemes for bacterial β-lactamases. Adapted with permission from Bush K and Jacoby GA AAC 2010, 54(3): 969-976. CA – clavulanic acid; TZB – Tazobactam.

Enzymes capable of hydrolysing carbapenems with sufficient efficiency to result in clinically relevant resistance are often referred to collectively as carbapenemases. These include the molecular class A and D enzymes of the KPC and OXA groups, and MBLs (class B), most notably of the IMP, VIM and NDM groups (Walsh 2010; Tzouvelekis et al. 2012). KPC producing Enterobacteriaceae have probably had the greatest clinical impact so far, with the enzyme particularly associated with the successful ST258 strain of *K. pneumoniae*, which has caused significant outbreaks in several countries (see Figure 1.3) and with evidence of increased mortality associated with bacteraemia caused by KPC producing *K. pneumoniae* (Munoz-Price *et al.* 2013). Most OXA carbapenemases are associated with carbapenem resistance in *A. baumannii*, although OXA-48 and OXA-181 have emerged as important causes of reduced carbapenem susceptibility in Enterobacteriaceae (Poirel *et al.* 2012b). Carbapenem resistance caused by MBLs in Enterobacteriaceae, especially associated with VIM and NDM enzymes, is now increasingly identified and of greater clinical concern.

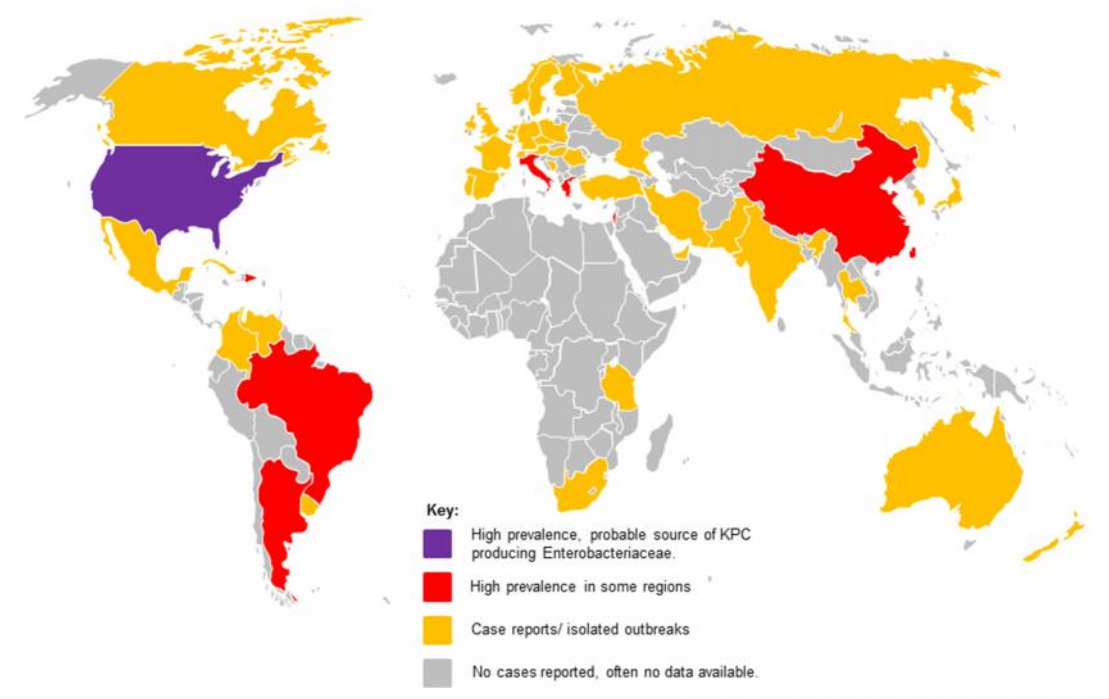


Figure 1.3 – Worldwide dissemination of Enterobacteriaceae producing KPC. Based on published data as of February 2015.

Although the epidemiology of carbapenem-resistant Enterobacteriaceae (CRE) is poorly established there is considerable evidence that some geographical regions have a significant problem, for example in the Indian subcontinent (see section 1.3). In Europe, where there are established monitoring systems, CRE, predominantly *K. pneumoniae*, is more frequent in some Southern European countries such as Greece and Italy (Cantón *et al.* 2012; European Centre for Disease Prevention and Control 2014), as shown in Figure 1.4. Even in countries like the United Kingdom where the prevalence of CRE remains low, this number has risen rapidly in recent years (Cantón et al. 2012), as shown in Figure 1.5 .

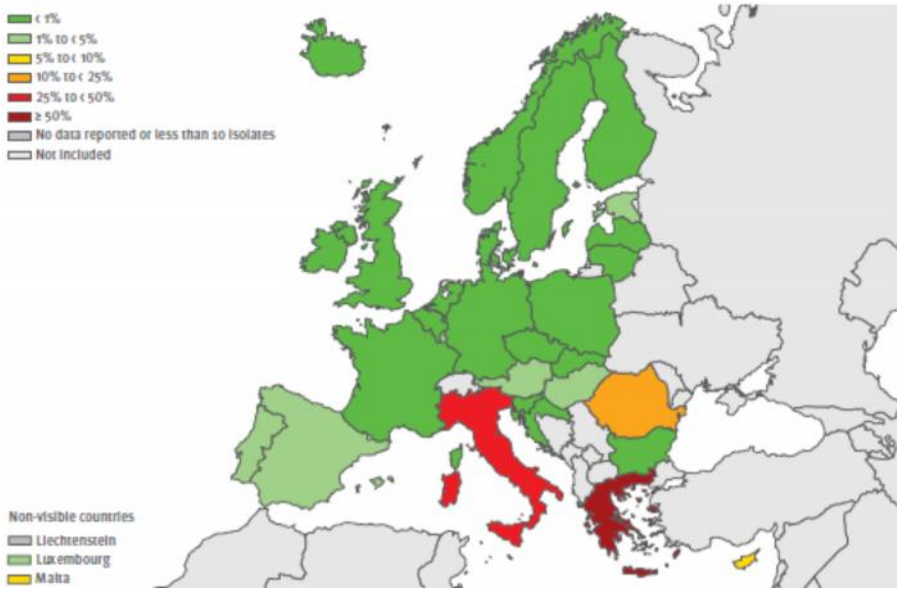


Figure 1.4 – Percentage of invasive isolates of *K. pneumoniae* resistant to carbapenemases, by country, EU/EEA countries, 2013. Reproduced from the Annual report of the European antimicrobial resistance surveillance network, 2013 (European Centre for Disease Prevention and Control 2014).

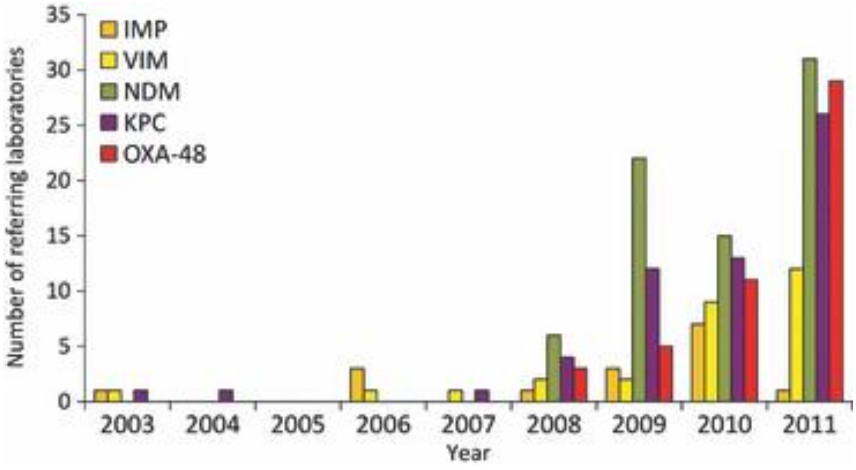


Figure 1.5 – Numbers of UK laboratories referring at least one carbapenemase-producing Enterobacteriaceae (CPE) isolate to the Antibiotic Resistance Monitoring and Reference Laboratory (ARMRL) (Health Protection Agency). Reproduced with permission from Cantón R *et al.* Clin Microbiol Infect. 2012, 18: 413-431 (Cantón et al. 2012).

1.3 NDM-producing bacteria

1.3.1 First identification of NDM-1, properties of NDM enzymes and their host species

The first description of the NDM-1 enzyme was from a urinary *K. pneumoniae* isolate in 2008 from a patient in Sweden, following transfer from a hospital in New Delhi, India (Yong et al. 2009b). The patient was later found to have gut colonisation with an NDM-1 producing *E. coli*. The enzyme is only distantly related to other MBLs sharing closest amino acid (AA) identity (~32%) with the VIM-1 and VIM-2 enzymes (Yong et al. 2009b). NDM-1 has a hydrolytic profile similar to other MBLs with relatively efficient hydrolysis which includes meropenem, imipenem and most other β-lactams, with the exception of aztreonam. As of 5th January 2015 the Lahey clinic website listed 13 variants of NDM-1, all of which are very closely related. Studies on the kinetics of NDM variants have not, so far, consistently shown any significant differences in kcat/Km values for carbapenems (Makena *et al.* 2014; Tada *et al.* 2014; Wang *et al.* 2014a).

In general NDM-producing GNB (NPGNB) are resistant to most antimicrobials, with many Enterobacteriaceae being resistant to all antimicrobials with the exception of colistin, tigecycline and fosfomycin, and *Acinetobacter* spp. often only being susceptible to colistin (Kumarasamy et al. 2010; Johnson and Woodford 2013; Dortet et al. 2014). Susceptibility profiles amongst other NPGNB, including to the carbapenems, are more unpredictable than for Enterobacteriaceae and *Acinetobacter* spp (Nordmann *et al.* 2011b; Walsh et al. 2011). Of NDM-producing Enterobacteriaceae (NPE) the predominant species are *K. pneumoniae* and *E. coli*, with *Citrobacter* spp.

and *Enterobacter* spp. being less frequent, and other species including *Salmonella* spp. and *Shigella* spp. being encountered uncommonly (Kumarasamy et al. 2010; Walsh et al. 2011; Johnson and Woodford 2013; Berrazeg et al. 2014; Dortet et al. 2014). The majority of NPE are resistant to aztreonam because of the co-production of ESBL or plasmidic AmpC type β-lactamases (Kumarasamy et al. 2010; Dortet et al. 2014).

1.3.2 Epidemiology of NPGNB

Although the naming of the enzyme after New Delhi proved to be controversial (Walsh and Toleman 2011b), this first report was followed by further evidence of the enzyme being prevalent in India and other parts of South Asia, including Pakistan, Bangladesh and Nepal (Kumarasamy et al. 2010; Castanheira et al. 2011; Perry *et al.* 2011; Bharadwaj *et al.* 2012; Tada *et al.* 2013; Datta *et al.* 2014; Islam *et al.* 2014; Rahman *et al.* 2014; Stoesser *et al.* 2014). A study published in 2010 reported on a survey of CRE from multiple sites from around India and Pakistan (see Table 1.2) from 2009 and from the UK from 2003-2009 (Kumarasamy et al. 2010). In the UK the first NPE was identified in 2008 and through to the end of 2009, 37 NPE were referred to the UK reference laboratory. Of these at least 17 had a history of travel to India or Pakistan within the last 12 months (Kumarasamy et al. 2010). The bacterial isolates from all of the study centres were from a range of clinical sample types including urine, blood and respiratory secretions but many patients from Chennai and Haryana had community acquired infections.

Country	Year	Source of isolates	No. NPGNB (prevalence)	Species of NPGNB (No.)	Reference
IND	2006-2007	Clinical ENT isolates.	15 (1% of 1,443 isolates)	EC (6), KP (6), ECL (3)	(Castanheira et al. 2011)
IND	2007-2011	ENT isolates from neonatal blood.	15 (14% of 105 isolates)	EC (6), KP (6), ECL (3)	(Datta et al. 2014)
IND and PAK	2009	Clinical ENT isolates.	CHE 44 (1.2% of 3521), HAR 26 (13% of 198). 73 other sites.	EC (19), KP (40), ECL (7), PRSP (2), CF (1), KO (1)*	(Kumarasamy et al. 2010)
IND	2009	CRE from intra-abdominal infections.	33 isolates	EC (8), KP (18), ECL (5), PR (1), MM (1)	(Lascols *et al.* 2011)
IND	2009	CRE clinical isolates.	22 (out of 24 CRE)	EC (9), KP (9), MM (1), ESP (2), KOZ (1)	(Deshpande *et al.* 2010)
IND	2010	Clinical isolates of GNB.	20 (2.3% of 885 isolates)	ASP (13), PSE (7)	(Bharadwaj et al. 2012)
BGD	2010	Clinical isolates of GNB.	14 (3.5% of 403 isolates)	EC (2), KP (9), AB (3), PR (1), CF (1).	(Islam *et al.* 2012)
PAK	2012	Clinical Isolates of GNB.	31 (8.7% of 356 isolates)	PSE (15), KP (13), EC (3)	(Nahid *et al.* 2013)
IND	2012	EC isolates from urinary samples.	45 (15% of 300 isolates)	EC (45)	(Khajuria *et al.* 2014)
BGD	2007-2009	1789 EC and 90 SHG spp. isolated from diarrhoeal faeces.	0	-	(Islam *et al.* 2013)
IND	2008-2012	3GCR isolates from faeces of 40 volunteers + 150 paediatric pts.	0	-	(Shahid *et al.* 2012)
PAK	2010	ENT on selective isolation from faecal screening.	37 samples (18.5% of 200), 64 isolates.	EC (30), KP (3), ECL (21), CSP (8), PR (2)	(Perry et al. 2011)

Country	Year	Source of isolates	No. NPGNB (prevalence)	Species of NPGNB (No.)	Reference
BGD	2012	ENT on selective isolation from diarrhoeal faeces.	9 samples (9% of 100), 13 isolates.	EC (6), KP (4), ECL (1), PAN (1), AB (1)	(Islam et al. 2014)
PAK	2011	ENT on selective isolation from diarrhoeal faeces.	13 samples (8.6% of 152), 16 isolates.	EC (8), KP (5), ECL (1), CF (1), KG (1)	(Day *et al.* 2013b)
PAK	2011	GNB on selective isolation from faecal screening.	32 samples (18.3% of 175), 37 isolates.	EC (21), KP (11), ECL (4), CF (8)	(Day *et al.* 2013a)
IND	2010	New Delhi tap water and seepage water samples.	12 (7% of 171) seepage samples, 2 (4% of 50) water, 20 isolates.	11 genera inc. 12 PSE (6), EC (3), SHG (1), VC (2)	(Walsh et al. 2011)
IND	2012	GNB from 77 drinking water, 10 drain and 3 sewage samples	0	-	(Shahid et al. 2012)

Table 1.2 – Summary of literature on prevalence of NPGNB in South Asia. Isolated case reports, small case series and outbreak reports not included. Highlighted in colour according to source of study isolates: Blue – clinical; Green – faecal colonisation; Pink – environmental contamination. ENT – Enterobacteriaceae; CHE – Chennai; HAR - Haryana. Country codes: BDG – Bangladesh; IND – India; PAK – Pakistan. Organism codes: AB – *A. baumannii*; CF – *C. freundii*; CSP – *Citrobacter* spp.; EC – *E. coli*; ECL – *E. cloacae*; ESP – *Enterobacter* spp.; KOZ – *Klebsiella ozonae*; KO – *Klebsiella oxytoca*; KP – *K. pneumoniae*; KG - *Kluyvera georgiana* MM – *Morganella morganii*; PR - *Providencia rettgeri*; PRSP – *Proteus* spp.; ASP – *Acinetobacter* spp.; PAN – *Pantoea* sp.; PSE – *Pseudomonas* spp.; SHG – *Shigella* spp.; VC – *Vibrio cholerae.*

The data that has since been published on clinical infections and colonisation with NPGNB from South Asia provides a limited picture of their epidemiology. Studies vary markedly in their methodologies, the prevalence and predominant species of NPGNB, and contain relatively little data on clinical outcomes. Some of the key studies are summarised in Table 1.2. It is noteworthy that, although Enterobacteriaceae represent the majority of isolates, NDM-producing *Acinetobacter* spp. and *P. aeruginosa* predominate in some clinical studies. Several other case series not shown in Table 1.2 have also reported NDM producers of these species from clinical isolates, especially in intensive care unit patients (Karthikeyan *et al.* 2010; Khajuria *et al.* 2013; Sartor *et al.* 2014; Shanthi *et al.* 2014; Jones *et al.* 2015).

A further environmental study sampled river water at two sites in February and June 2012 (Ahammad *et al.* 2014), but is not shown in Table 1.2 as it is methodologically very different to the other studies. In the Yamuna river, as it passes through Delhi, $bla_{\text{NDM-1}}$ was consistently detectable at high levels based on quantitative PCR. By contrast, in the upper Ganges, at a site which experiences an influx of pilgrims from across India in May/June, the abundance of $bla_{\text{NDM-1}}$ increased significantly in June. Considered as a whole the studies cited in this section suggest that in parts of South Asia, the source of NDM producing pathogens may be community acquisition and the environment rather than healthcare acquisition, as is commonly felt to be the case for other MDR pathogens.

Since the first report of NDM in 2009 NPGNB have been reported from around the world, as shown in Figure 1.6. In many countries the findings have been similar to those from the UK, in that cases have been mainly sporadic

and have included a significant number in which patients have a history of travel to South Asia, not always with healthcare contact (Centers for Disease Control and Prevention (CDC) 2010; Chen *et al.* 2011; Poirel *et al.* 2011d; Cantón et al. 2012; Escobar Pérez *et al.* 2013; Govind *et al.* 2013; Johnson and Woodford 2013; Berrazeg et al. 2014; Jain *et al.* 2014; Peirano *et al.* 2014; Qin *et al.* 2014). As a result it has been proposed by many authors that the worldwide dissemination of NPGNB is probably largely attributable to spread from South Asia. However, as more NPGNB have been identified worldwide it has become increasingly evident that there is significant complexity to their epidemiology.

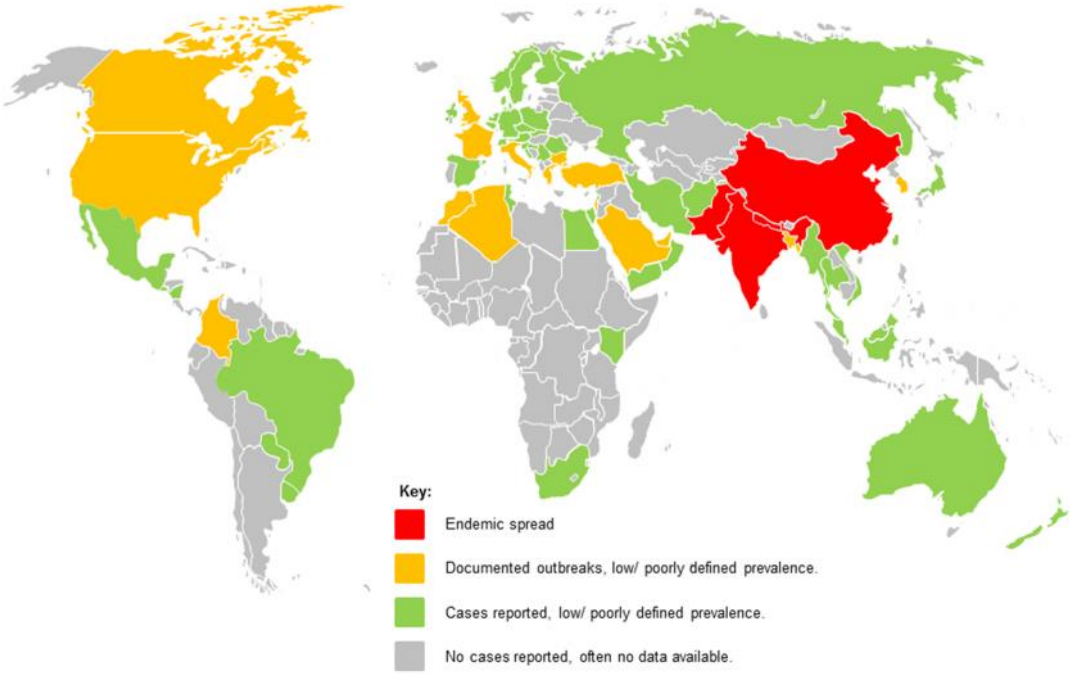


Figure 1.6 – Worldwide distribution of NPGNB. Based on published data as of February 2015.

More recent UK data shows that for 250 patients with NPE, travel histories were available for only 101 (40%), but that of these 52% (53/101) had a history of travel to India (Jain et al. 2014). More than 40% had no history of foreign travel outside the UK and some cases were being managed in General Practice, suggesting the possibility of community spread. Similar findings, although with smaller numbers of isolates are reported from several European countries, America, Canada and Australasia (Centers for Disease Control and Prevention (CDC) 2010; Cantón et al. 2012; Williamson *et al.* 2012; Decousser *et al.* 2013; Johnson and Woodford 2013; Rasheed *et al.* 2013; Dortet et al. 2014; Peirano et al. 2014; Shoma *et al.* 2014).

Although the numbers are modest in comparison with those imported from South Asia, sporadic cases of colonisation or infection with NPGNB from around Europe have travel histories to countries in the Balkans, the Middle East or North Africa (Struelens *et al.* 2010; Bonnin *et al.* 2012b; Johnson and Woodford 2013; Dortet et al. 2014; Meletis *et al.* 2014). Data from these regions themselves are currently limited, although a study from Serbia identified seven *P. aeruginosa* producing NDM-1 in 2010 (Jovcic *et al.* 2011). Subsequently there have been reports of clinical NPE isolates from Croatia, Romania and Serbia, as well as a significant outbreak of *K. pneumoniae* producing NDM in Greece (Mazzariol *et al.* 2012; Mirovic *et al.* 2012; Szekely *et al.* 2013; Deshpande *et al.* 2014; Voulgari *et al.* 2014; Zujic-Atalic *et al.* 2014). Several papers have also reported NPGNB, mostly *A. baumannii*, from the Middle East and North Africa (Espinal *et al.* 2011; Kaase *et al.* 2011; Poirel *et al.* 2011c; Ghazawi *et al.* 2012; Bakour *et al.* 2014). In keeping with this, many of the cases presumed to have been imported to Europe from North

Africa and the Middle East were *A. baumannii* (Decousser et al. 2013; Johnson and Woodford 2013).

In China most of the patients from which NPGNB have been isolated have no apparent travel history to other parts of the world (Chen et al. 2011; Fu *et al.* 2012; Ho *et al.* 2012; Yang *et al.* 2012; Hu *et al.* 2013; Qin et al. 2014). Initial studies suggested that NPE were rare in China but they have been recognised increasingly frequently since then (Hu *et al.* 2014; Qin et al. 2014). However, most reports of NPGNB have been *Acinetobacter* spp (Chen et al. 2011; Fu et al. 2012; Hu *et al.* 2012; Wang *et al.* 2012; Yang et al. 2012; Zhou *et al.* 2012; Sun *et al.* 2013b; Zhang *et al.* 2013a; Wang *et al.* 2014b; Zhang *et al.* 2014). These have included a number of *A. baumannii* isolates (Chen et al. 2011; Zhang *et al.* 2013b; Wang et al. 2014b) but have mainly been other species. In addition one study demonstrated that PCR for *bla*$_{\text{NDM-1}}$ of faecal samples was positive in 14.8% and that *bla*$_{\text{NDM-1}}$ positive bacteria could be isolated for 7.9 % of patients, with most isolates being *Acinetobacter* spp. (Wang *et al.* 2013).

1.3.3 Clinical impact of NPGNB

There is no evidence that any of the plasmids and other MGE which harbour *bla*$_{\text{NDM-1-like}}$ genes contain significant virulence determinants (Sekizuka *et al.* 2011; Carattoli *et al.* 2012; Hu et al. 2012; Dolejska *et al.* 2013; Hishinuma *et al.* 2013; Dortet et al. 2014). Therefore the species and strain backgrounds of NPGNB probably determine their virulence potential (Peirano *et al.* 2013; Dortet et al. 2014). So far *bla*$_{\text{NDM-1-like}}$ genes have not established a strong association with strains which have had significant clinical impact.

However, pathogenic strains previously associated with resistant phenotypes have been found to harbour *bla*$_{\text{NDM-1-like}}$ genes, including ST101 and ST131 *E. coli*, ST11 and ST14 *K. pneumoniae* and ST1 *A. baumannii* (Mushtaq *et al.* 2011; Peirano *et al.* 2011c; Giske *et al.* 2012; Jones et al. 2014a).

Despite the paucity of information, an increasing number of papers reporting clinical outcomes of patients with NPGNB have been published. Results of key publications are summarised in Table 1.3. The combined crude mortality rates from these studies are 48.5% (32/66) and 27.4% (23/84) for neonates and adults (excluding data from Greece for which crude mortality is not stated) respectively. These figures should be interpreted with caution given that no meaningful comparator groups are available and the heterogeneity of the studies. Of note, whilst most of the reported neonatal cases had blood stream infections, many of the adult cases had isolates from other sites such as urine and respiratory secretions, and so the two figures are not comparable with one another.

Country	Year	Pt group	Study type	Source	Species (strains)	Clinical Outcome	Reference
IND	2007-2011	NEO	Case-control	Blood	EC, KP, ESP (clonally diverse)	Crude mortality NPE 13.3% (2/15) vs non-NPE 22.2% (20/90), p=0.66.	(Datta et al. 2014)
IND	2009	NEO	Outbreak	Blood	EC (clonal by PFGE)	Crude mortality 100% (4/4).	(Roy *et al.* 2011)
NPL	2011-2012	NEO	Outbreak	Blood	KP (ST15)	Crude mortality NPE 64% (16/25). Unit wide 46% (45/98) during outbreak, 27% (32/117) following year.	(Stoesser et al. 2014)
COL	2011-2012	NEO	Outbreak	Blood	KP (ST1043).	Crude in hospital mortality 33.3% (2/6).	(Escobar Pérez et al. 2013)
NPL	2012-2013	NEO	Outbreak	Blood	ECL (clonal by WGS)	Crude mortality 46% (6/13)	(Stoesser *et al.* 2015)
IND	2010	Adult/ Child	Case series	Blood, Pus, RS, PF, CSF	ASP, PSE	Crude mortality: Adult 13.3% (2/15), Child 0% (0/5).	(Bharadwaj et al. 2012)
CHN	2011-2012	Adult/ Child/ NEO	Case series	Urine, Blood, RS, WND	EC, KP, KO, ECL, CF (Clonally diverse)	Crude mortality: Adult 18.2% (2/11), Child 50% (1/2), Neonate 66.7% (2/3)	(Qin et al. 2014)
KEN	2007-2009	Adult	Case series	Urine, Pus	KP (ST14)	Crude mortality 14.3% (1/7) Attributable mortality 0% (0/7).	(Poirel et al. 2011d)
GRC	2010-2013	Adult	Outbreak	Blood	KP (ST11)	Attributable mortality 35% (6/17).	(Voulgari et al. 2014)

Country	Year	Pt group	Study type	Source	Species (strains)	Clinical Outcome	Reference
BGR	2012	Adult	Outbreak	Urine, RS, Stool, Blood	EC (ST101)	Crude mortality 80% (4/5)	(Poirel *et al.* 2014)
IND	2012	Adult	Case series	Urine, Blood, Pus, RS, CSF	EC, KP, CSP, ESP, PRO (Clonally diverse)	Crude mortality 24.6% (14/57).	(Rahman et al. 2014)
FRA	2013	Adult	Outbreak	RS, Blood	AB (ST85)	Crude mortality 50% (2/4)	(Decousser et al. 2013)

Table 1.3 – Summary of papers reporting clinical outcome for NPGNB. Case reports and small case series not included. Studies highlighted in colour according to study type: Pink – Case-control study; Blue – Outbreak investigation; Green – Case series. Country codes: BGR – Bulgaria; CHN – China; COL – Colombia; FRA – France; GRC – Greece; IND – India; KEN – Kenya; NPL – Nepal. Organism codes: AB – *A. baumannii*; CF – *C. freundii*; CSP – *Citrobacter* spp.; EC – *E. coli*; ECL – *E. cloacae*; ESP – *Enterobacter* spp.; KO – *Klebsiella oxytoca*; KP – *K. pneumoniae*; PRO – *Providencia* spp. Other abbreviations: NEO – neonate; PFGE – Pulsed field gel electrophoresis; CSF – cerebrospinal fluid; PF – pleural fluid; RS – respiratory secretions; WND – Wound swab.

Although several other outbreaks are reported involving adults, in many cases most or all patients were colonised rather than infected (Gaibani *et al.* 2011; Poirel et al. 2011d; Borgia *et al.* 2012; Kim *et al.* 2012; Koo *et al.* 2012; Yang et al. 2012; Lowe *et al.* 2013; Pisney *et al.* 2014). All other reports of patient outcomes are from isolated case reports or small case series (Bogaerts *et al.* 2011; Chan *et al.* 2011; Peirano *et al.* 2011a; Stone *et al.* 2011; Darley *et al.* 2012; Oteo *et al.* 2012; Karczewski *et al.* 2014; Qin et al. 2014; Seija *et al.* 2015).

1.3.4 Genetic contexts of *bla*$_{\text{NDM-1-like}}$ genes

It should be noted that the terminology with regards to genetic contexts is often not defined and can lead to confusion. The term “genetic context” is used in this thesis as a general term to refer to the strain background, MGE(s) and DNA sequences immediately flanking the gene(s) referred to. To refer to only the sequences immediately flanking the gene(s) the term “immediate genetic context” will be used. Where a specific bacterial species, strain or mobile element is referred to these will be named in the text.

The genetic contexts in terms of species, strain background and MGE harbouring *bla*$_{\text{NDM-1-like}}$ genes are varied. In many cases *bla*$_{\text{NDM-1-like}}$ genes are found on plasmids of differing size and incompatibility type. The most common incompatibility types identified have been IncA/C, IncF, IncN, IncL/M and IncH, although in many cases the type could not be identified (Kumarasamy et al. 2010; Walsh et al. 2011; Carattoli 2013; Johnson and Woodford 2013; Sartor et al. 2014; Stoesser et al. 2014; Stoesser et al. 2015). Strikingly, most of the *Acinetobacter* spp. isolates for which plasmids have been characterised in

detail all contain conjugative plasmids, which are very closely related, but have no identifiable replicase or origin of replication (Hu et al. 2012; Zhang et al. 2013b; Jones et al. 2015). These plasmids are discussed in detail in Chapter 4.

Many isolates have also been described in which *bla*$_{\text{NDM-1-like}}$ genes are apparently located on the chromosome (Kumarasamy et al. 2010; Walsh et al. 2011; Poirel *et al.* 2012a). However, most of these contexts have not been sequenced, or only a limited amount of the flanking sequence has been defined. As a result it is not known whether *bla*$_{\text{NDM-1-like}}$ genes are associated with GEIs. There are few exceptions to this so far, one of which is described in Chapter 5. A *Proteus* GEI has also been described that harbours *bla*$_{\text{NDM-1}}$ (Girlich *et al.* 2014).

There is quite significant variation in the immediate genetic contexts found associated with *bla*$_{\text{NDM-1-like}}$ genes (Nordmann et al. 2011b; Poirel *et al.* 2011b; Partridge and Iredell 2012; Poirel et al. 2012a; Toleman *et al.* 2012; Dortet et al. 2014). The immediate contexts are discussed in some detail in the results chapters; however, some general points should be noted and examples of immediate contexts intended to illustrate them are shown in Figure 1.7. As discussed in Chapter 3, it is believed that *bla*$_{\text{NDM-1-like}}$ genes were established in *Acinetobacter* spp. prior to dissemination into other GNB, and that sequences from these species represent the earliest known *bla*$_{\text{NDM-1-like}}$ contexts. In many *Acinetobacter* spp. NDM is found in a composite transposon (Tn), Tn*125*, formed by two copies of IS*Aba125*, as represented by the *A. baumannii* and *A. lwoffii* contexts shown in Figure 1.7 (Pfeifer *et al.* 2011; Partridge and Iredell 2012; Poirel et al. 2012a).

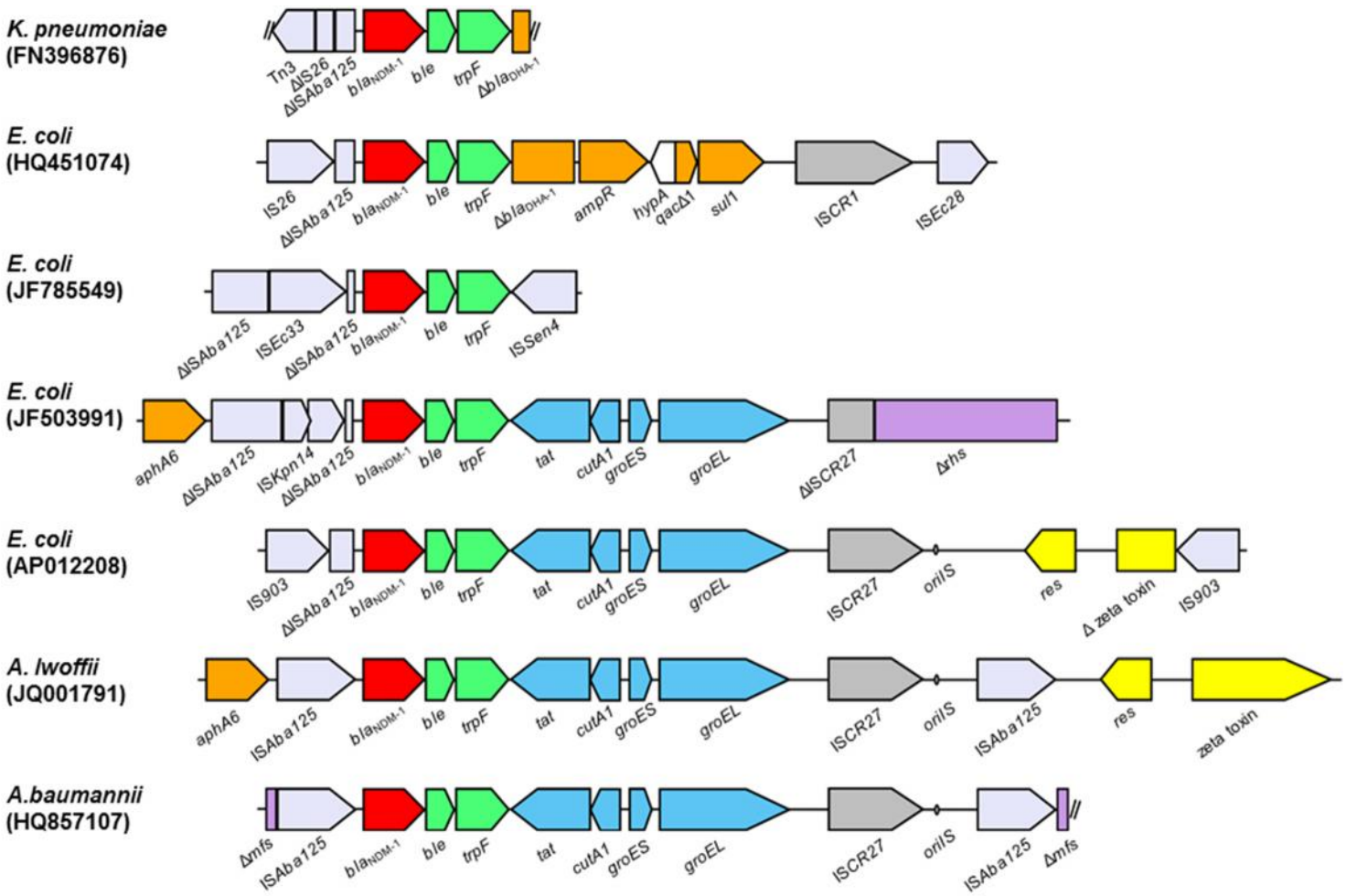

K. pneumoniae
(FN396876)
Tn3
ΔIS26
ΔISAba125
blaNDM-1
ble
trpF
ΔblaOXA-1
E. coli
(HQ451074)
IS26
ΔISAba125
blaNDM-1
ble
trpF
ΔblaOXA-1
ampR
hypA
qacΔ1
sul1
ISCR1
ISEc28
E. coli
(JF785549)
ΔISAba125
ISEc33
ΔISAba125
blaNDM-1
ble
trpF
ISSen4
E. coli
(JF503991)
aphA6
ΔISAba125
ISKpn14
ΔISAba125
blaNDM-1
ble
trpF
tat
cutA1
groES
groEL
ΔISCR27
Δrhs
E. coli
(AP012208)
IS903
ΔISAba125
blaNDM-1
ble
trpF
tat
cutA1
groES
groEL
ISCR27
oriIS
res
Δ zeta toxin
IS903
A. lwoffii
(JQ001791)
aphA6
ISAba125
blaNDM-1
ble
trpF
tat
cutA1
groES
groEL
ISCR27
oriIS
ISAba125
res
zeta toxin
A.baumannii
(HQ857107)
Δmfs
ISAba125
blaNDM-1
ble
trpF
tat
cutA1
groES
groEL
ISCR27
oriIS
ISAba125
Δmfs

Figure 1.7 – Examples of immediate genetic contexts of *bla*$_{NDM-1}$ in Enterobacteriaceae and *Acinetobacter* spp. The examples chosen are not representative of the full range of variation in terms of immediate contexts but are intended to illustrate key points highlighted in the main text. ORFs are colour coded with the direction of transcription indicated by arrow heads, truncated remnants of ORFs are shown as rectangles. Red – *bla*$_{NDM-1}$; lime green – usually immediately downstream of *bla*$_{NDM-1}$; blue – from a common context in *Xanthomonas* and *Pseudoxanthomonas*; dark grey – IS*CR27* transposase; light grey – IS transposases, note transposases are labelled with IS name; orange – other AMR genes; yellow – genes commonly found as part of the backbone of pNDM-BJ01-like plasmids. *ble* – bleomycin resistance gene; *trpF* – phosphoribosylanthranilate isomerase gene; *tat* – twin-arginine translocation pathway signal sequence domain gene; *cutA1* – periplasmic divalent cation tolerance gene; *groES* – co-chaperonin gene; *groEL* – chaperonin gene; IS*CR27* – insertion sequence common repeat 27 transposase gene; *oriIS* – origin of insertion of IS*CR27*; *Δmfs* – interrupted major facilitator superfamily (MFS) metabolite/H+ symporter gene; *aphA6* – aminoglycoside *O*-phosphotransferase, aminoglycoside resistance gene; Δr*hs* – interrupted gene coding for a type IV secrtion protein; Δ*bla*$_{DHA-1}$ – fragment of AmpC type β-lactamase gene; *ampR* – lysR family *bla*$_{DHA-1}$ regulator gene; *sul1* – dihydropteroate synthase gene mediating sulphonamide resistance; *qacEΔ1* – multidrug resistance exporter gene; hypA – gene coding for hypothetical protein.

There is much greater variety in immediate *bla*$_{\text{NDM-1-like}}$ contexts in other species, with most of those which have been characterised so far being from Enterobacteriaceae (Nordmann et al. 2011b; Partridge and Iredell 2012; Toleman et al. 2012; Dortet et al. 2014). Some Enterobacteriaceae sequences contain an intact IS*Aba125* but in many others IS*Aba125* is truncated by other ISs (Toleman et al. 2012). However, in all known examples a fragment of IS*Aba125* is preserved which contains the -35 sequence motif of the *bla*$_{\text{NDM-1-like}}$ promoter sequence. This promoter has been shown to result in strong expression of *bla*$_{\text{NDM-1-like}}$ genes and also acts as the promotor for *ble*, which codes for a bleomycin resistance protein and is immediately downstream of *bla*$_{\text{NDM-1-like}}$ genes.

In most contexts in GNB, other than *Acinetobacter* spp., *ble* and *trpF* are still found downstream of NDM but the rest of the contents of Tn*125* are often absent (Poirel et al. 2011b; Dortet *et al.* 2012; Partridge and Iredell 2012). A relatively common finding downstream of *trpF* is of a truncated *bla*$_{\text{DHA-1}}$ gene and its transcriptional regulator gene, *ampR* followed by conserved region of a complex class 1 integron, including an IS*CR* element. In general immediate *bla*$_{\text{NDM-1-like}}$ contexts contain multiple AMR genes, ISs and complete or partial class 1 integrons (Ho *et al.* 2011; Poirel *et al.* 2011a; Sekizuka et al. 2011; Carattoli et al. 2012; Hu et al. 2012; Dolejska et al. 2013; Hishinuma et al. 2013; Huang *et al.* 2013; Girlich et al. 2014).

Although it is a common finding that AMR genes are associated with a number of genetic backgrounds and have variability in their genetic contexts, many of the most successful ones are predominantly associated in clinical practice with a limited number of successful strains and/ or MGE responsible

for their acquisition (Higgins *et al.* 2010; Woodford *et al.* 2011; Tzouvelekis et al. 2012; D'Andrea *et al.* 2013; Munoz-Price et al. 2013). This has not yet occurred with *bla*$_{\text{NDM-1-like}}$ genes and so the genetic variation associated with these genes is somewhat greater than has commonly been observed with other successful AMR genes.

1.4 Integrons, MGE and HGT

The following section introduces the MGE that are responsible for HGT between bacteria and plasticity of the immediate genetic contexts associated with *bla*$_{\text{NDM-1-like}}$ genes and other AMR genes. HGT describes the transfer of DNA sequences between living cells followed by integration into and stable maintenance in the new host. There are three main mechanisms by which HGT between prokaryotes occurs; transformation, transduction and conjugation.

The importance of HGT between bacteria and archaea to prokaryotic evolution, including clinically relevant features, like AMR and virulence factors, is increasingly recognised, despite the fact that it challenges conventional views of gradual, step-wise evolutionary change (Boto 2010; Stokes and Gillings 2011). Thus prokaryotic organisms can be seen as having access to a "communal" gene pool, which is an economical way of maintaining access to a wide range of phenotypic characteristics, allowing the population as a whole to remain more responsive to changing ecological circumstances (Stokes and Gillings 2011).

Although HGT events are often difficult to prove conclusively, several lines of evidence suggest that HGT has had a significant impact on

prokaryotic evolution. Thus divergent phylogenies, regions of altered codon usage or GC % and the presence of gene/ protein sequences, where the closest homologues are from different species or genera, all suggest the influence of HGT (Koonin *et al.* 2001; Boto 2010). The presence of complete operons, GEIs or plasmids with significant regions of synteny, found in multiple species or genera, strongly suggests that HGT events have occurred at some time in evolution (Koonin et al. 2001; Stokes and Gillings 2011).

1.4.1 Conjugation – plasmids and integrative and conjugative elements (ICE)

Plasmids are MGE which usually exist in an extrachromosomal form and replicate autonomously (Carattoli *et al.* 2005; Frost *et al.* 2005). Many can be transferred to a new host by conjugation, either utilising conjugation machinery coded for by the plasmid itself (self-conjugative), or along with another plasmid which provides the conjugation machinery (mobilizable) (Smillie *et al.* 2010). Some plasmids are not capable of conjugative transfer (non-mobilizable), although they may occasionally be horizontally transferred by transformation or transduction (see below).

Plasmidic conjugation requires mating pair formation by direct physical contact between donor and recipient cells (Frost et al. 2005; Smillie et al. 2010). This is most often achieved by a type IV secretion system (T4SS). These form a conjugation pilus, produced by the donor cells. Single stranded plasmid DNA is produced after nicking at the origin of transfer by a relaxase enzyme and a nucleoprotein complex, called the relaxosome, is formed. This structure docks with the T4SS and is transferred from the donor cell to the

recipient. Plasmid complementary strands are then synthesised in both cells so that the donor and recipient both harbour the plasmid, as shown in Figure 1.8 (Frost et al. 2005).

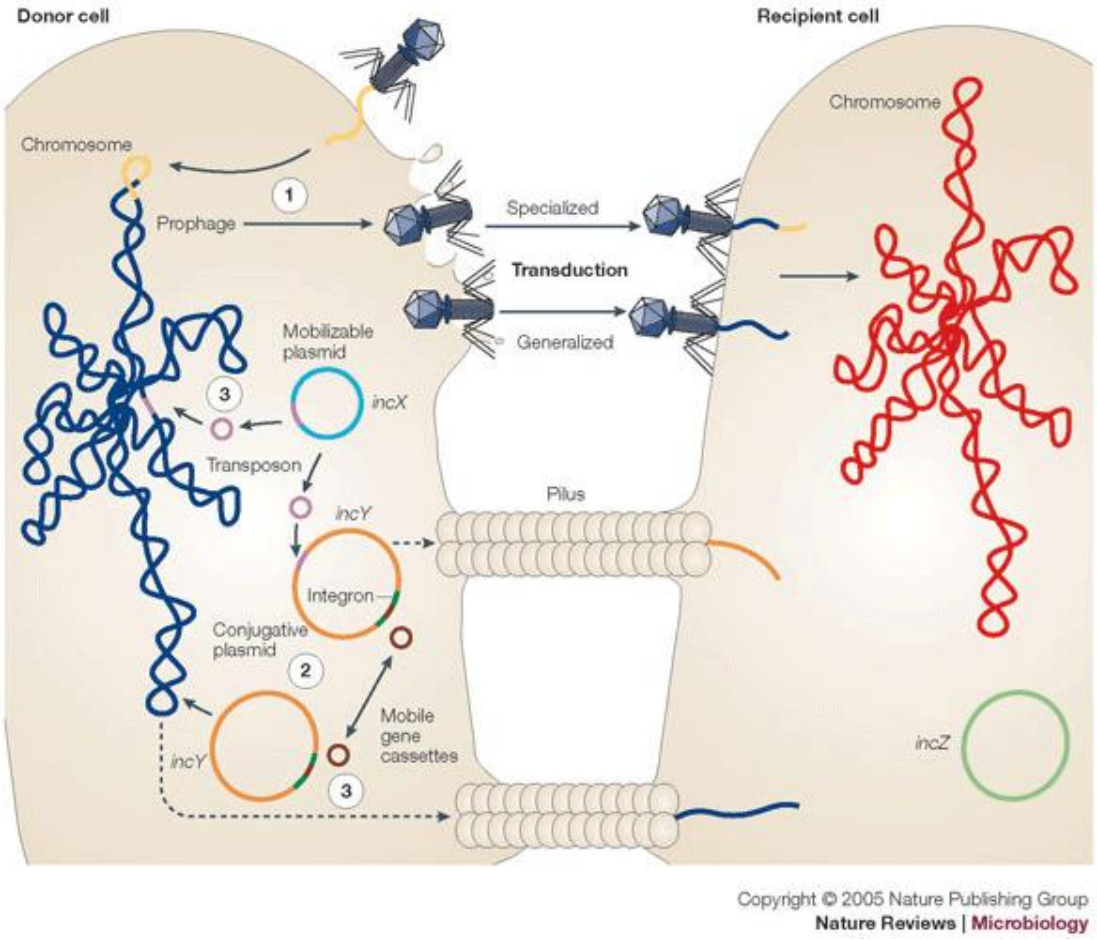


Figure 1.8 – Schematic representing mechanisms of HGT between bacterial cells and genetic plasticity associated with MGEs. Details of these processes are described in the main text of section 1.4. Figure shows transduction by phages (1); conjugation (2); transposition and transfer of gene cassettes between integrons (3). Phage genome – yellow; Bacterial chromosome of donor – dark blue; Bacterial chromosome of recipient – red; Conjugative plasmids – orange; Tns – pink; Integrons – dark green; gene cassettes – brown. Reproduced with permission from Frost LS *et al.*, Nature Rev Microbiol. 2005, 3: 722-732 (Frost et al. 2005).

The complexity of the conjugation machinery means that conjugative plasmids are large and because of the metabolic requirements imposed on the host, require additional genes to help stably maintain themselves (Frost et al. 2005; Sengupta and Austin 2011). All plasmids possess functional modules of genes which mediate replication of the plasmid in close co-ordination with the growth cycle of the host. This is necessary to ensure that plasmids are stably maintained, rather than lost from cells at the time of cell division, but with control of the copy number of the plasmid present. Many plasmids also code for “addiction systems.” These usually take the form of a toxin-antitoxin, which result in the killing of cells which fail to maintain the plasmid (Sengupta and Austin 2011).

Plasmids have traditionally been classified according to their replicon, comprising of the origin of replication, replicase and its regulatory factors (Carattoli et al. 2005). Plasmids which share very closely related replicons cannot usually be maintained alongside each other in a bacterial cell and are thus said to be incompatible (Novick 1987). This is the basis of incompatibility (Inc) typing. In practice Inc typing is increasingly based on sequence analysis or PCR based methods because of the laborious nature and technical limitations of incompatibility typing (Carattoli et al. 2005; Bertini *et al.* 2010; Carattoli 2011). A growing number of MGE are being recognised which incorporate both phage and conjugative plasmid related genes, arranged in functional modules (Wozniak *et al.* 2009; Wozniak and Waldor 2010; Guglielmini *et al.* 2011; Toleman and Walsh 2011). Various terms have been used to describe these elements including integrative and conjugative elements (ICE), conjugative Tns and integrative plasmids. The similarities in

terms of structure and function suggest that these terms all describe a single class of MGE, which will be referred to here as ICEs. The model proposed for the properties of ICEs is that they normally replicate integrated into the host chromosome but are capable of excision and replication, forming an extra-chromosomal circular intermediate (Wozniak and Waldor 2010). They can independently of other MGEs initiate conjugation between the host and a suitable donor. The double stranded extra-chromosomal intermediate is nicked at the origin of transfer and a single strand is transferred by the conjugation machinery to the recipient. Thus both donor and recipient now harbour the ICE, which is then capable of reintegration into the chromosome of the host and the recipient. Integrative and mobilizable elements (IMEs) are similar to ICEs, but lack the ability to mediate conjugative transfer independently, and thus rely on the conjugation machinery of another MGE (Douard *et al.* 2010).

Plasmids, ICEs and IMEs frequently harbour a large number of genes which have phenotypically important functions, including genes conferring resistance to antimicrobials, disinfectants or heavy metals (Frost et al. 2005; Wozniak and Waldor 2010; Carattoli 2013). Frequently these are found in common “hot spots” for the insertion of accessory genes. These regions often contain many of the genetic elements discussed later in this section such as insertion sequences (ISs), IS common regions (IS*CR*s), Tns and class 1 integrons, which are likely to have been responsible for the acquisition of the genes of phenotypic importance.

1.4.2 Transduction

Transduction describes the transfer of DNA by bacteriophages (viruses which infect bacteria, commonly referred to as phages) or phage like particles (Lang *et al.* 2012; Penadés *et al.* 2014). The main life cycles of successful bacteriophage infection are the lytic and lysogenic cycles. The lytic cycle results in the “hijacking” of the cellular machinery to produce new phages, which are released following cell lysis. The lysogenic cycle results in integration of phage DNA into the host chromosome as a prophage (see Figure 1.8). These replicate along with the host DNA unless the lytic cycle is triggered. Phages play a significant role in the acquisition of some key bacterial virulence functions, including toxins. However, phages are thought to be capable of transferring any bacterial gene (Lang et al. 2012), and recent evidence suggests they could also play a significant role in the HGT of AMR genes (Modi *et al.* 2013; Quirós *et al.* 2014).

This HGT can occur by generalised transduction, where during the lytic cycle bacterial DNA is mispackaged into the phage capsid (Lang et al. 2012; Arber 2014). Infection with other phages may result in “specialized transduction,” where host genes near to the phage insertion site on the bacterial chromosome are packaged in the phage capsid along with the phage genome (Lang et al. 2012; Arber 2014). When compared to other characterised mechanism of HGT, transduction does not require the close cell to cell interactions involved in conjugation and the transducing DNA is protected from destruction by the phage capsid (Penadés et al. 2014). A major limitation of transduction has been thought to be the narrow host ranges of most phages, but transducing particles may be capable of HGT to a broader

range of hosts than have been shown to support phage replication (Chen and Novick 2009; Penadés et al. 2014).

1.4.3 Transformation

Transformation describes the uptake by bacteria of free DNA into the cytoplasm, which is then integrated into the new host (Chen and Dubnau 2004; Mell and Redfield 2014). DNA can be abundant in the external environment either because of release at the time of cell death or due to active excretion of DNA by some organisms. In order for this to take place the organism must be “competent,” that is with the necessary machinery to actively uptake and regulate the extracellular DNA. A number of bacterial species are naturally competent but most only become so under certain physiological conditions. A few bacteria have been shown to limit uptake of DNA unless certain sequence motifs are present, favouring transformation with DNA of related bacteria.

Once the DNA has been taken into the cytoplasm recombinases can integrate the DNA into the chromosome of the new host, or free plasmids introduced by transformation can be maintained extrachromosomally. Integration is either by homologous recombination, where DNA is integrated at sites with a significant degree of sequence homology (Chen and Dubnau 2004; Mell and Redfield 2014), or rarely by illegitimate recombination with sequences of limited homology or no homology (Hülter and Wackernagel 2008). A limitation of this form of HGT may be that in the environment DNA is vulnerable to degradation, although experiments suggest that extracellular

DNA is abundant in all environmental sources tested (Lorenz and Wackernagel 1994) .

1.4.4 Integrons

Integrons are gene capture and expression systems (Cambray et al. 2010; Gillings 2014), the structure and function of which are summarised in Figure 1.9. An integrase is coded for by *intI*, which mediate recombination between the *attI* site of the integron with the *attC* recombination sites of gene cassettes. Gene cassettes are circular genetic elements, which in most cases comprise a single open reading frame (ORF) and an *attC* site. Integrons usually contain arrays of multiple gene cassettes. A promoter sequence within the integrase coding sequence, or between the integrase and *attI*, results in strong expression of the adjacent gene cassette, with weaker expression of cassettes further downstream (Cambray et al. 2010; Toleman and Walsh 2011; Gillings 2014).

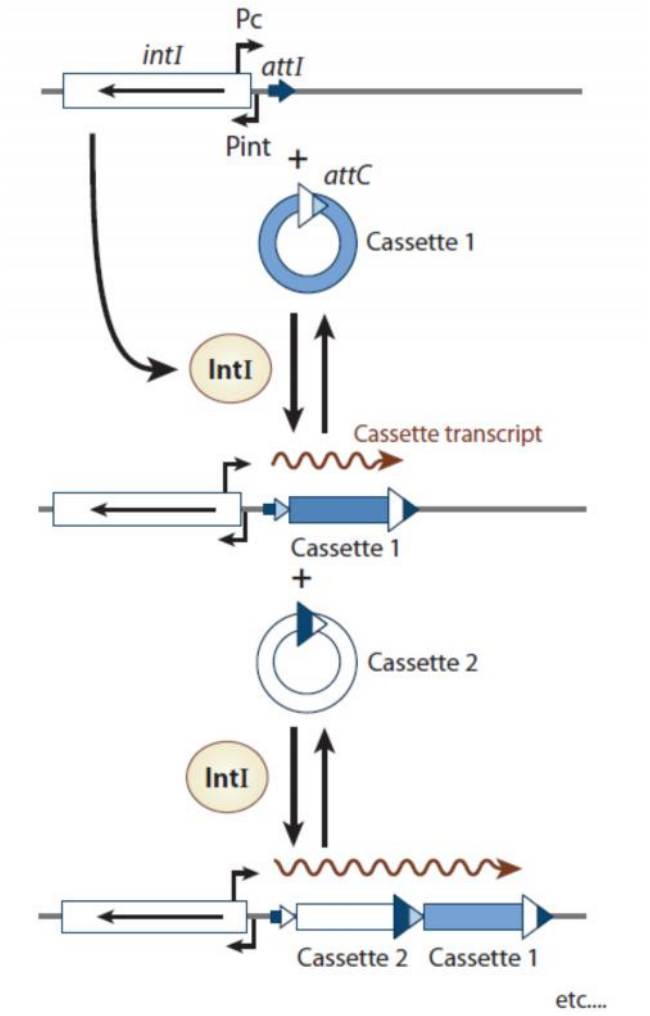


Figure 1.9 – Schematic representing the structure of integrons and a model of the incorporation of circular gene cassettes at the *attI* site. Reproduced with permission from Cambray G *et al.* Ann Rev Genet. 2010, 44: 141-66 (Cambray *et al.* 2010).

Integrons have access to a vast array of ORFs, most of which code for proteins of unknown function, and are thought to have a significant impact on the genetic plasticity of bacteria in many environments. This has been exemplified by a small number of classes of integrons (class 1, 2 and 3), which have played an important part in the evolution of AMR in bacteria (Toleman and Walsh 2011; Gillings 2014). Most widespread in clinically

relevant bacteria are class 1 integrons. These usually have a small number of gene cassettes (0-8). The ancestors of modern class 1 integrons probably conferred significant selective advantage in clinical environments because they contained *qacE*, coding for an efflux pump conferring resistance against quaternary ammonium compounds, which were widely used in disinfectants (Toleman and Walsh 2011; Gillings 2014). This structure then became associated with Tn*402*, a Tn which targets *res* sites of plasmids, favouring dissemination to new genetic backgrounds (Minakhina *et al.* 1999).

The 3' conserved sequence (3'CS) of modern class 1 integrons was formed by an event which deleted the 3' end of *qacE* including its associated *attC* site, and placed a *sulI* gene downstream, which codes for a sulphonamide resistant dihydropteroate synthase (Gillings 2014). This same insertion event probably resulted in the deletion of some of the transposition genes of Tn*402* (Toleman and Walsh 2011). Class 1 integrons have subsequently become associated with other genetic elements, for example in some IS*CR1* is found next to the 3'CS (see below) (Toleman *et al.* 2006; Toleman and Walsh 2011).

1.4.5 ISs, Tns and IS*CR*s

A growing number of genetic elements have been identified which are capable of transposition to new genetic locations within a genome, including to new replicons, independently of homologous recombination (Darmon and Leach 2014). Insertion sequences (IS) are short genetic elements which usually contain one or two ORFs coding for a transposase. Intact ISs have terminal inverted repeats and on insertion into the host DNA usually result in

direct repeats (DRs) of the insertion target flanking the IS. Excision from the original location at the IS boundaries and insertion at the new genetic location is catalysed by the elements transposase(s) (Mizuuchi 1992).

IS can have important effects as they may interrupt and inactivate genes or directly affect the expression of adjacent genes. Altered expression occurs if the IS sequence contains a complete promoter or -35 promoter motif the correct distance from a -10 promoter motif (Darmon and Leach 2014), as occurs with IS*Aba125* and *bla*$_{\text{NDM-1-like}}$ genes (Poirel et al. 2011a). ISs can also lead to genetic changes in the host sequence in a number of ways. IS transposition may only mobilise the IS sequence, but a few elements, such as IS*Ecp1*, IS*911* and IS*CR* elements, can also transpose flanking DNA sequences (Toleman et al. 2006; Toleman and Walsh 2011). However, two similar ISs near to one another within a replicon can form a composite Tn, where both ISs and any intervening host sequence are mobilised by a single transposition event (Darmon and Leach 2014). Alternatively, IS sequences can lead to significant genetic changes because of homologous or illegitimate recombination between similar ISs, resulting in large deletions or chromosomal re-arrangements.

Tns are larger genetic elements which are structurally and functionally diverse. They usually harbour genes not related to their own transposition which have important phenotypic properties, such as AMR genes. Like ISs they usually have flanking IRs and DRs (Darmon and Leach 2014). The simplest Tns, including composite Tns, function similarly to ISs and have a limited number of ORFs coding for transposases and sometimes other proteins thought to be involved in initiating and regulating transposition

between genetic locations, limited to the host cell, but not to a single replicon. Other much larger and more complex elements are considered by some authors to be Tns, for example the ICEs.

IS*CR* were first described as “common regions” adjacent to the 3’CS of class 1 integrons (Toleman et al. 2006). It was later proposed that these elements were novel ISs, which transposed by a rolling circle replication mechanism as previously characterised for IS*91*-like elements. Importantly, unlike IS*91*-like elements, IS*CR* elements are frequently found associated with AMR genes. A single ORF codes for a transposase but IS*CR*s do not contain IRs characteristic of most IS. Rolling circle transposition is thought to be mediated by the transposase and to commence at the origin of replication, *ori*IS. Replication normally terminates at a termination signal, *ter*IS, upstream of the ORF. In IS*91*-like elements a proportion of the time the *ter*IS signal is not recognised and instead a DNA sequence upstream of the element is transposed. It is hypothesised that IS*CR*s behave in a similar manner. In the case of IS*CR1*, all of the sequences described are linked to the 3’CS of the class 1 integrons, and there is no sequence with identity to *ter*IS from other IS*CR* or IS*91*-like elements, suggesting that earlier in evolution the *ter*IS sequence was deleted. This being the case, it would suggest that IS*CR1* transposition would then more frequently result in the transposition of a variable length of the class 1 integron upstream of it.

1.4.7 Genomic islands (GEIs)

The term GEIs has been used to refer to regions of DNA which differ between closely related strains, often have nucleotide characteristics, like the

GC content, which vary from that of the strain in which they reside and which may in some cases represent elements which are mobile by some mechanism, or have been mobile in the past (Juhas *et al.* 2009). Many GEIs harbour regions coding for genes of significant phenotypic importance, so they are often referred to according to the predominant function of these genes as pathogenicity islands, resistance islands, symbiosis islands and so on. To make the nomenclature more confusing a proportion of these elements are likely to be ICEs. Other features which many GEIs share is that they are often flanked by base pair direct repeats and integrated at tRNA gene sites, features that they share with some ICEs. ISs, Tns and integrons are frequently found within GEIs.

1.5 Gram-negative species analysed in this thesis

The following section introduce the species of GNB in which *bla*$_{\text{NDM-1}}$ contexts were investigated in this thesis. An overview of these species is offered in terms of their clinical significance, AMR, mechanisms of HGT and MGEs associated with these species.

1.5.1 *Acinetobacter* spp.

The genus *Acinetobacter* includes a growing number of species of non-fermentative Gram-negative cocco-bacilli, including 37 named species at the time of writing (http://www.bacterio.cict.fr/a/acinetobacter.html, see Table 1.4). Identification to species level by traditional biochemical methods is unreliable, with species now defined by a variety of molecular methods (Towner 2009). *Acinetobacter baumannii* has emerged in recent years as an important

pathogen. Other members of the *A. baumannii* complex (ABC), *Acinetobacter pittii* (previously *Acinetobacter* genomospecies 3) and *Acinetobacter nosocomialis* (previously *Acinetobacter* genomospecies 13TU) are also strongly associated with hospital infections (Nemec *et al.* 2011; McConnell *et al.* 2013). These species are biochemically indistinguishable from each other and it is likely that studies of *A. baumannii* have often included other members of the ABC. Other *Acinetobacter* spp. are probably infrequent causes of opportunistic infections.

Acinetobacter sp.	Proposed reservoir
A. apis	Unknown – initial isolation honey bee intestine.
A. baumannii	**Unknown – hospital associated coloniser & pathogen.**
A. baylyi	Unknown – initial isolates from activated sludge.
A. beijerinckii	Unknown – early isolates all human/ clinical source.
A. bereziniae	**Early isolates clinical and hospital environment.**
A. boissieri	Unknown – initial isolates from floral nectar.
A. bouvetii	Unknown – initial isolates from activated sludge.
A. brisouii	Unknown – initial isolate from peat.
A. calcoaceticus	Soil or wastewater.
A. gandensis	Unknown – initial isolates from horses and cattle.
A. gerneri	Unknown – initial isolates from activated sludge.
A. grimontii	Unknown – initial isolates from activated sludge.
A. guangdongensis	Unknown – initial isolates from lead-zinc ore mine site.
A. guillouiae	Unknown – initial isolates sewage, soil and clinical.
A. gyllenbergii	Unknown – early isolates all human/ clinical source.
A. haemolyticus	**Unknown – early isolates all human/ clinical source.**
A. harbinensis	Unknown – initial isolate from river water.
A. indicus	Unknown – initial isolate hexachlorocyclohexane dump site.
A. johnsonii	Human and animal flora, food spoilage.
A. junii	Unknown – early isolates all human/ clinical source.
A. kookii	Unknown – initial isolates isolated soil and sediment.

Acinetobacter sp.	Proposed reservoir
A. lwoffii	Human and animal skin flora, food spoilage flora.
A. nectaris	Unknown – initial isolates from floral nectar.
A. nosocomialis	Unknown – hospital associated coloniser & pathogen.
A. parvus	Unknown – most initial isolates clinical.
A. pittii	Unknown – hospital associated coloniser & pathogen.
A. puyangensis	Unknown – initial isolates from tree bark.
A. qingfengensis	Unknown – initial isolates from tree bark.
A. radioresistens	Human and animal skin flora, food spoilage flora.
A. rudis	Unknown – initial isolates from milk and wastewater.
A. schindleri	**Unknown – early isolates all human/ clinical source.**
A. soli	Unknown – initial isolation soil, clinical isolates reported.
A. tandoii	Unknown – initial isolates from activated sludge.
A. tjernbergiae	Unknown – initial isolates from activated sludge.
A. towneri	**Unknown – initial isolates from activated sludge.**
A. ursingii	Unknown – early isolates all human/ clinical source.
A. venetianus	Unknown – initial isolates from lagoon water.

Table 1.4 – Named *Acinetobacter* spp. and their sources of isolation (Bouvet and Grimont 1986; Di Cello *et al.* 1997; Nemec *et al.* 2001; Carr *et al.* 2003; Kim *et al.* 2008; Nemec *et al.* 2009; Towner 2009; Anandham *et al.* 2010; Nemec *et al.* 2010; Vaz-Moreira *et al.* 2011; Malhotra *et al.* 2012; Choi *et al.* 2013; Li *et al.* 2013; Álvarez-Pérez *et al.* 2013; Feng *et al.* 2014; Kitanaka *et al.* 2014; Li *et al.* 2014a; Li *et al.* 2014b; Smet *et al.* 2014)**.** Species names in bold indicate that NDM-producing isolates studied in this thesis.

Although ABC have been identified in some studies as colonisers in the community and from environmental, animal samples and lice, these reports are infrequent, and vary considerably between studies and between geographic locations (Eveillard *et al.* 2013). Thus the nature of any environmental reservoir for the ABC is controversial. Other *Acinetobacter* spp. are probably largely environmental in origin, although for some species their reservoirs are poorly established (Towner 2009). Others, like *A. johnsonii*, *A. lwofii* and *A. radioresistens*, are most often associated with human or animal skin colonisation, as well as food spoilage. Several species, most notably *A. calcoaceticus* (which is sometimes grouped with members of the ABC as the *Acinetobacter calcoaceticus* - *baumannii* complex) and *A. johnsonii*, are found mainly in soil or wastewater (Towner 2009; Evans *et al.* 2013).

Infections caused by *A. baumannii* predominantly occur in hospitalised patients and particularly in the most vulnerable patients, such as those on intensive care or burns units (McConnell et al. 2013). These organisms have been found to cause many, often quite difficult to control outbreaks of infection on such units (Dijkshoorn *et al.* 2007; Zarrilli *et al.* 2013). Reasons for this probably include their resistance to desiccation and disinfectants, allowing persistence in hospital environments. Due to the frequency of MDR phenotypes, antimicrobial selection pressure is also likely to be an important driver of nosocomial spread.

A. baumannii has been associated with a wide range of infections but is most commonly associated with pneumonia, blood stream and burn wound infections (McConnell et al. 2013). Pneumonia is most often ventilator associated, although community acquired pneumonia caused by *A. baumannii*

is reported. Blood stream infections are most likely to be secondary to intravascular device infections, pneumonia or burn wound infections. For several years *A. baumannii* infections were encountered in soldiers returning with injuries sustained during fighting in Iraq and Afghanistan. Unusually in this context it has been associated with significant skin and soft tissue infections, including cases of necrotising fasciitis, and with osteomyelitis.

Significant crude mortality has been associated with VAP (40-70%) and blood stream infections (28%-43%) caused by *A. baumannii* (McConnell et al. 2013). However, as *A. baumannii* infections are most common in patients who are severely unwell, defining the attributable mortality is not straight forward. Although there are limitations to the evidence, meta-analysis of available observational studies have concluded that *A. baumannii* infections are associated with significant attributable mortality (Falagas *et al.* 2006; Falagas and Rafailidis 2007). A review of outcomes of treatment of infections with carbapenem susceptible versus non-susceptible *A. baumannii* also suggested that mortality was higher in the latter group (Lemos *et al.* 2014).

Acinetobacter spp. are not intrinsically resistant to many of the antimicrobials used to treat GNB, and it remains the case that most isolates of the “environmental” species have low MICs to most of these antimicrobials (Towner 2009). *Acinetobacter* spp., in particular *A. baumannii* have proven to be particularly adept at acquiring resistance mechanisms. Rates of resistance to carbapenems, which had become the drugs of choice for treating most *A. baumannii* infections, have risen alarmingly in many parts of the world including the USA, parts of Asia and in Southern Europe (Higgins et al. 2010; Evans et al. 2013; Lemos et al. 2014). A recent report looking at a global

collection of *A. baumannii* isolates showed that the only antimicrobials for which more than 50% of isolates were susceptible were colistin, doxycycline and minocycline (Castanheira *et al.* 2014).

Globally the nosocomial spread of resistant *A. baumannii* is strongly associated with certain strains (Higgins et al. 2010; Karah *et al.* 2012; Zarrilli et al. 2013). These have been able to acquire multiple AMR determinants, which have then probably been selected for by the extensive use of broad spectrum antimicrobials in healthcare. Terminology has become relatively confusing as our understanding, and the techniques employed for strain typing, have developed. The European clones I-III were initially defined by amplified fragment length polymorphism (AFLP) analysis. Subsequently these were found to be globally distributed, and thus renamed global clones, and found to closely correspond to strain types and clonal complexes as defined by multilocus sequence typing (MLST) typing schemes and pulsed field gel electrophoresis (PFGE) types.

More recently analysis of WGS data for core genome single nucleotide polymorphisms (SNPs) and indels has been utilised to provide a finer level of differentiation (Zarrilli et al. 2013). This has allowed the tracking of different patterns of transmission of closely related isolates. In the near future WGS will probably replace these other techniques for the epidemiological investigation of *A. baumannii*. In the meantime more extensive investigation with other typing methods has led to the proposal of further global clones, although these are not recognised as frequently as the original three (Higgins et al. 2010; Karah et al. 2012).

The predominant β-lactamases of clinical importance in *A. baumannii* are the OXA enzymes (Poirel and Nordmann 2006a). Many of the OXA β-lactamases of *A. baumannii* and other *Acinetobacter* spp. have some activity against carbapenems and are associated with clinically relevant resistance to carbapenems. However, the hydrolysis of carbapenems by OXAs is not particularly potent, and so clinically relevant carbapenem resistance probably requires the presence of other mechanisms. This is supported by experimental evidence from gene knockouts and transfer of various *bla*$_{OXA}$ genes to *A. baumannii* recipients, in whom levels of resistance are modest unless mechanisms such as AdeABC efflux pumps are also present (Evans et al. 2013).

There are five groups of OXA β-lactamases described in *A. baumannii* to date (Evans et al. 2013). The OXA-23-like and OXA-58-like enzyme producers have caused outbreaks of carbapenem-resistant *A. baumannii* in many countries. OXA-40-like (OXA-40 was originally named OXA-24) enzymes have been reported in various geographic locations but seem to be particularly important in Spain and Portugal (Poirel *et al.* 2010; Evans et al. 2013). Recently the enzymes OXA-143 and the related enzyme OXA-182 have been described from *A. baumannii* isolates from Brazil and Korea, respectively. The genes coding for these enzymes were probably acquired by HGT in *A. baumannii*.

Most *A. baumannii* in addition harbour a *bla*$_{OXA\text{-}51\text{-}like}$ carbapenemase gene on their chromosome (Turton *et al.* 2006b; Evans et al. 2013). There remains uncertainty as to how significant these enzymes may be to carbapenem resistance because of the limited hydrolytic profiles of the

variants which have so far been tested (Evans et al. 2013). However, it has been proposed that if the gene has an IS*Aba1* element upstream, and the promoter associated with it, the increase in expression can result in clinically relevant levels of resistance (Turton *et al.* 2006a). Of note the acquired OXA type β-lactamases like *bla*$_{\text{OXA-23}}$ are very often associated with ISs and their promoters (Corvec *et al.* 2007).

In addition to the OXA type β-lactamases most *A. baumannii* harbour ADCs (*Acinetobacter*-derived cephalosporinsases), which are AmpC type chromosomal enzymes (Lopes and Amyes 2012; Evans et al. 2013). At intrinsic levels of expression these are associated with resistance to narrow spectrum penicillin and cephalosporins. However, insertion of certain ISs upstream of these genes can enhance their expression and result in resistance to extended spectrum cephalosporins (Evans et al. 2013). Less frequent in *A. baumannii*, but associated with high level carbapenem resistance, are MBL enzymes, a number of which have been described in *A. baumannii*, including NDM-1 and NDM-2.

More recent genetic investigations of *A. baumannii* facilitated by WGS have demonstrated that there is evidence of extensive recombination between strains and significant variation in genetic determinants of resistance, even between closely related strains, suggesting that HGT has played a significant part in shaping this species' evolution (Adams *et al.* 2010; Snitkin *et al.* 2011; Wright *et al.* 2014). ISs and plasmids are intimately associated with AMR determinants in *A. baumannii*. Plasmid replicon types in *Acinetobacter* spp. are distinct from those in Enterobacteriaceae (Bertini et al. 2010).

Also strongly associated with AMR genes in *A. baumannii* are several GEIs. In particular a family of closely related GEIs is found inserted within the *comM* gene, especially in global clones I and II (Adams et al. 2010; Hamidian and Hall 2011; Karah et al. 2012; Nigro and Hall 2012). These islands are called AbaR islands and consist of a backbone Tn, with additional Tns, ISs, class 1 integrons and AMR determinants, making up their variable regions.

Competence has been extensively studied in *A. baylyi* but has not been widely observed in other members of the genus (Young *et al.* 2005). A naturally competent *A. baumannii* strain, A118, has been recently sequenced (Traglia *et al.* 2014). Most of the proposed competence genes of *A. baumannii* showed high levels of AA identity between A118 and a set of other *A. baumannii* strains. However, the proposed competence gene, *comM*, was not interrupted in A118, unlike in many clinical *A. baumannii* isolates (Adams et al. 2010; Karah et al. 2012; Traglia et al. 2014). An intriguing study has recently shown that when grown on semi-solid agar several *A. baumannii* isolates are able to utilise their type 4 pilus for twitching motility and are transformable under these conditions (Wilharm *et al.* 2013). A separate mechanism of HGT from *A. baumannii* has also been proposed secondary to the release of outer membrane vesicles, which have been experimentally shown to be able to transform an *A. baumannii* recipient with DNA containing $bla_{\text{OXA-24/40}}$ (Rumbo *et al.* 2011).

1.5.2 *Vibrio cholerae*

V. cholerae is a Gram-negative, spiral shaped bacillus found in coastal and estuarine waters and closely associated with copepods and shellfish

(Harris *et al.* 2012). It is best known for causing the disease cholera, characterised by profuse watery diarrhoea and vomiting. In severe cases cholera can result in profound dehydration and multi-organ failure and death (Harris et al. 2012). However, there is a range of disease severity, with most cases of *V. cholerae* infections with epidemic strains having either mild or asymptomatic disease (Morris 2003).

Rapid fluid and electrolyte replacement is the mainstay of treatment (Morris 2003; Harris et al. 2012). Antimicrobials may be of some benefit as adjunctive therapy but treatment guidelines currently advise restricting their use to severe cases, in which they may reduce the duration of illness and excretion of the organism (Kitaoka *et al.* 2011). The WHO advises doxycycline or tetracycline as first line treatment for adults and erythromycin for children or pregnant women (Global Task Force on Cholera Control 2010). Resistance to all agents currently recommended for *V. cholerae* has been reported, predominantly in South Asia (Kitaoka et al. 2011; Harris et al. 2012). Notably β-lactams are not advised but NDM-producing *V. cholerae* (NPVC) have been identified from environmental and clinical samples (Walsh et al. 2011; Darley et al. 2012; Mandal *et al.* 2012).

V. cholerae is regarded as an important re-emerging pathogen. Cholera remains endemic in many countries in Asia and Africa, and epidemic outbreaks of disease continue to emerge. Worldwide, reported cases of cholera fluctuate year on year. According to WHO data, the most recent peak occurred in 2011, when there were 589,854 cases and 7816 deaths reported (World Health Organization 2012). However, estimates suggest that in reality there are likely to be in the region of 3 – 5 million cholera cases each year,

associated with 100,000 – 130,000 deaths (World Health Organization 2010). The 7th worldwide pandemic of cholera is still ongoing. It commenced in Indonesia in 1961, before spreading to other parts of Asia, Africa, Europe and Latin America (Mutreja *et al.* 2011; Harris et al. 2012), as illustrated by Figure 1.10. The previous 6 pandemics occurred between 1817 and 1925, before which the disease was probably largely confined to the Indian subcontinent (Harris et al. 2012).

V. cholerae strains have traditionally been distinguished by their O antigen serogroup, of which more than 200 have been described (Zo *et al.* 2009). Epidemic cholera is predominantly associated with *V. cholerae* strains of serogroups O1 or O139 (Karaolis *et al.* 1998; Morris 2003; Mutreja et al. 2011; Harris et al. 2012). The O1 serotype is divided into two biotypes, classical and El Tor. The classical biotype was responsible for the 5th, 6th and possibly earlier pandemics. *V. cholerae* 01 El Tor has largely been responsible for the 7th Pandemic (Mutreja et al. 2011; Harris et al. 2012). There are two major serotypes of *V. cholerae* O1, Inaba and Ogawa (Harris et al. 2012). These vary in relative prevalence over time and with geographic region (Harris et al. 2012; World Health Organization 2012, 2013). In 1992 the O139 serogroup was first identified as a cause of a cholera epidemic in South Asia, where it has since remained confined (Ramamurthy *et al.* 2003; Blokesch and Schoolnik 2007; Harris et al. 2012).

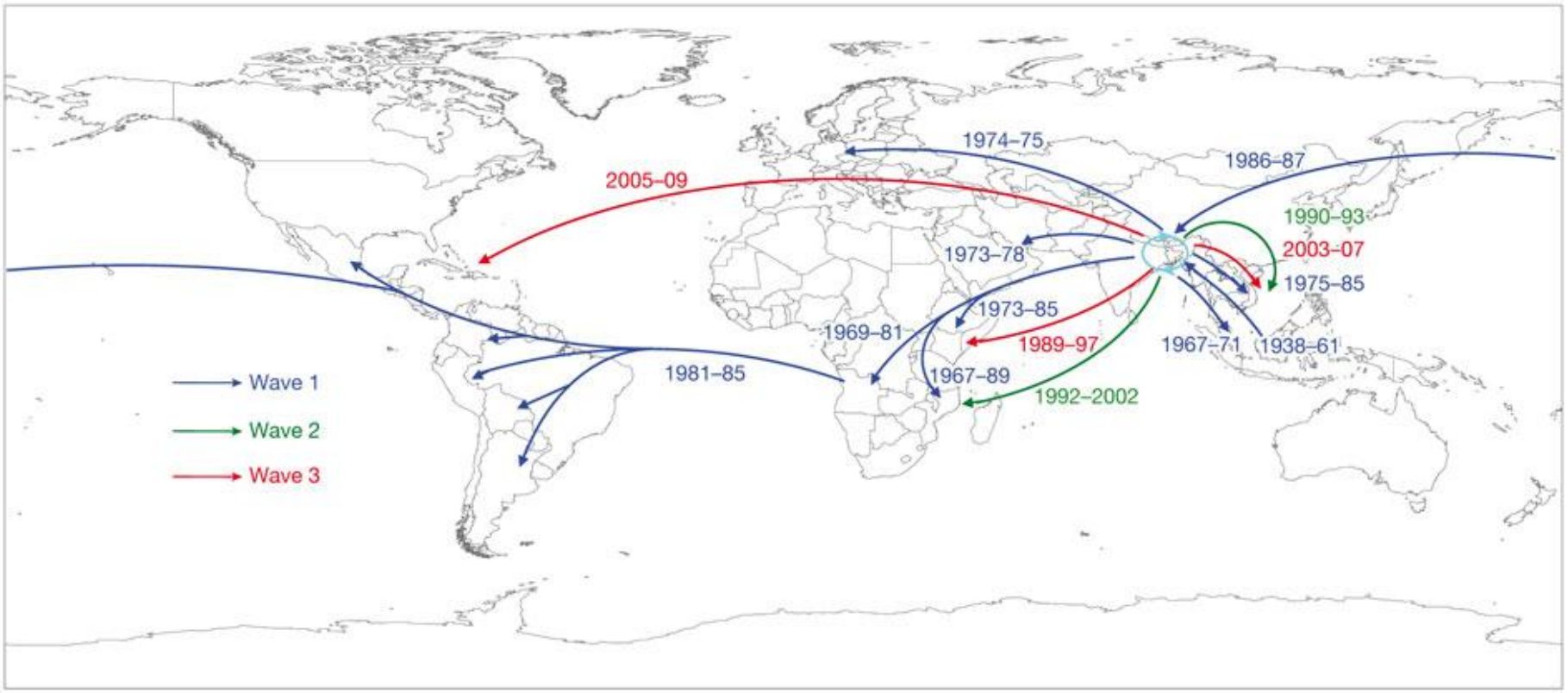


Figure 1.10 - Transmission events inferred for the seventh-pandemic based on a phylogenetic analysis of SNP differences across the whole core genome, excluding probable recombination events, drawn on a global map. Reproduced with permission from Mutreja A *et al.*, Nature 2011, 477: 462-465 (Mutreja et al. 2011).

The epidemic strains of *V. cholerae* all possess two important pathogenicity regions, both of which are absent in most non-O1, non-O139 strains (Karaolis et al. 1998; Morris 2003; Chun *et al.* 2009; Harris et al. 2012). The cholera toxin is a protein exotoxin coded for by genes which are found within the CTXΦ prophage (Waldor and Mekalanos 1996). A second pathogenicity region, which also has features suggestive of a phage origin, is the *Vibrio* Pathogenicity Island-1 (VPI-1) (Karaolis et al. 1998; Karaolis *et al.* 1999). This element contains genes necessary for the colonisation of the gut and the toxin co-regulated pilus (TCP) (Morris 2003).

Other serogroups of *V. cholerae* have been associated with gastrointestinal infection and with occasional outbreaks (Karaolis et al. 1998; Morris 2003). Sometimes these strains can cause invasive infections and sepsis. >90% of non-O1/O139 serogroup strains produce a polysaccharide capsule, unlike 01 serogroup strains which are very rarely associated with sepsis (Morris 2003). In Asia various groups of immunocompromised patients, especially with cirrhosis of the liver, have been identified as being vulnerable to blood steam infections with *V. cholerae* (Ko *et al.* 1998; Morris 2003; Petsaris *et al.* 2010). Although such infections are uncommon they are associated with significant mortality, with studies reporting rates of 24-62%, although estimating the attributable mortality is problematic (Ko et al. 1998; Morris 2003; Petsaris et al. 2010).

WGS has shown that 7th pandemic isolates have conserved core genomes (Mutreja et al. 2011). Epidemic O139 isolates have substantially different O-antigen biosynthetic gene clusters to O1 El Tor isolates, despite being from a common genetic lineage (Ramamurthy et al. 2003; Chun et al. 2009; Mutreja et al. 2011). It is thought, therefore, that this gene cluster was acquired horizontally from another strain (Ramamurthy et al. 2003; Blokesch and Schoolnik 2007; Chun et al. 2009).

That this can occur is supported by experimental data (Blokesch and Schoolnik 2007). Conversely, several strains have been identified which are distantly genetically related despite sharing a common serogroup (Chun et al. 2009; Mutreja et al. 2011). Indeed, classical strains seem to be an independent lineage from El Tor strains (Mutreja et al. 2011). Other differences between 7th pandemic isolates are largely as the result of differences in the presence of various GEIs (Chun et al. 2009). Relatively few environmental and non-O1/O139 isolates have been sequenced but they appear to be genetically diverse (Chun et al. 2009; Mutreja et al. 2011).

Given the right environmental conditions, including the presence of chitin, which is widely present in marine environments and present in the exoskeletons of copepods, with which *Vibrio* spp. are associated, *V. cholerae* are naturally transformable (Lo Scrudato and Blokesch 2012; Sun *et al.* 2013a). This may be one mechanism by which *V. cholerae* strains are able to share genetic information in the environment. Experiments on chitinous surfaces have demonstrated serogroup transformation of O1 to O139 and O37 serogroups (Blokesch and Schoolnik 2007).

Several broad host range MGEs associated with antibiotic resistance genes have been identified in *V. cholerae*, most notably ICEs of the SXT/ R391 family. These elements were absent from the 01 El Tor strains in the first wave of the 7th pandemic, but present in most isolates from the second and third waves of transmission (Pugliese *et al.* 2009; Kitaoka et al. 2011). SXT/ R391 ICEs have also been found in non-O1/O139 strains and in many other species of GNB (Burrus *et al.* 2006; Wozniak et al. 2009; Ceccarelli *et al.* 2013). *In vitro* SXT can be transferred efficiently to various recipients including *E. coli* (Beaber *et al.* 2004). SXT in *V. cholerae* was initially associated with trimethoprim/ sulphamethoxazole and

streptomycin resistance (Kitaoka et al. 2011) but subsequently variants have been identified harbouring a variety of different resistance genes (Burrus et al. 2006; Wozniak et al. 2009; Kitaoka et al. 2011). In addition resistance plasmids, especially IncA/C plasmids, have been described in *V. cholerae* (Pan *et al.* 2008; Pugliese et al. 2009; Kitaoka et al. 2011). IncA/C plasmids are genetically similar to SXT/ R391 ICEs, have a broad host range and are readily transferred to several Gram-negative species *in vitro* (Wozniak et al. 2009; Carattoli et al. 2012; Johnson and Lang 2012).

V. cholerae strains, as well as often harbouring class 1 integrons (Jain *et al.* 2008; Kitaoka et al. 2011; Kumar and Thomas 2011), also have a super integron on chromosome 2 associated with more than 100 gene cassettes (Rowe-Magnus *et al.* 1999; Baharoglu *et al.* 2012). Although most of the gene cassette ORFs code for proteins of unknown function, the variability in super-integrons suggests that they may contribute to adaption and plasticity in response to changing environmental conditions (Rowe-Magnus et al. 1999; Gao *et al.* 2011; Baharoglu et al. 2012). Both types of integron and SXT have been shown to be influenced by *recA* mediated "stress" responses. In the case of SXT exposure to antibiotics like ciprofloxacin are among the SOS response stimulators shown to enhance conjugation rates (Beaber et al. 2004; Kitaoka et al. 2011). Conjugation and natural transformation have also been shown to lead to increased gene cassette recombination secondary to SOS induction (Guerin *et al.* 2009; Baharoglu et al. 2012).

1.6 Concluding remarks

The work reported in this thesis focuses on the genetic contexts associated with bla_{NDM-1} in two very different groups of GNB; namely, *Acinetobacter* spp. and *V. cholerae*. This helps to expand the knowledge regarding the diversity of contexts

associated with *bla*$_{NDM-1}$ beyond the Enterobacteriaceae, in which most work has been conducted. *Acinetobacter* spp. were of interest in that the genus includes important nosocomial pathogens and is proposed to be genus from which *bla*$_{NDM-1-like}$ genes have spread to other GNB. *V. cholerae* is also of clinical importance and was felt to be an interesting model organism in view of its dissemination in the environment and potential for genetic plasticity. In studying the genetic contexts and MGEs associated with the spread of *bla*$_{NDM-1}$ it is hoped to gain insights into how this resistance gene and other AMR determinants disseminate and why certain contexts are more successful than others. Ultimately the objective of such work is to contribute to the debate on necessary action that might stem the spread of MDR pathogens and provide impetus for greater commitment to develop new therapeutic strategies.

Chapter 2

Materials and Methods

2.1 Bacterial isolates studied

All of the bacterial strains used in this thesis are shown in Table 2.1. The 2005 *Acinetobacter* spp. isolates from Tamil Nadu, India, were supplied by K. Kumarasamy from Chennai University. The 2013 *Acinetobacter* spp. were isolated in Cardiff from faecal screening samples collected in Karachi, Pakistan, from March to August 2012. These samples were supplied by A. Mushtaq from Dow Medical College, Karachi. Further details of this study are reported in Chapter 4. The 2010 environmental isolates of *V. cholerae* were isolated by Prof. Walsh's group in Cardiff (Walsh et al. 2011). *V. cholerae* BRV8 was provided by Dr E. Darley (Darley et al. 2012). Further details on the *V. cholerae* isolates are given in Chapter 5.

K. pneumonia 05-506 was used as a *bla*$_{\text{NDM-1}}$ positive control in some experiments. The strains *Escherichia coli* UAB190 (Mata *et al.* 2011) and *A. pittii* AG3528 were used as recipients in mating experiments and were provided by Caterina Mata, of Universitat Autònoma de Barcelona, and Dr Mandy Wootton at the Specialist Antimicrobial Chemotherapy Unit, Cardiff, respectively. The *bla*$_{\text{NDM-1}}$ positive transconjugants used in further experiments are also shown in Table 2.1. The strains *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls for antimicrobial susceptibility testing

Isolate	Species	Source	Location isolated	Year isolated
CHI-16	*Acinetobacter baumannii*	Blood	Tamil Nadu, India	2005
CHI-18	*Acinetobacter baumannii*	Blood	Tamil Nadu, India	2005
CHI-32	*Acinetobacter baumannii*	Blood	Tamil Nadu, India	2005
CHI-34	*Acinetobacter baumannii*	Sputum	Tamil Nadu, India	2005
CHI-40-1	*Acinetobacter bereziniae*	Pus	Tamil Nadu, India	2005
CHI-40-2	*Acinetobacter baumannii*	Pus	Tamil Nadu, India	2005
CHI-41	*Acinetobacter baumannii*	Sputum	Tamil Nadu, India	2005
CHI-44	*Acinetobacter baumannii*	Endotracheal aspirate	Tamil Nadu, India	2005
CHI-45-1	*Acinetobacter baumannii*	Endotracheal aspirate	Tamil Nadu, India	2005
73261-EC	*Acinetobacter haemolyticus*	Faecal screening	Karachi, Pakistan	2012
70114-EC	*Acinetobacter haemolyticus*	Faecal screening	Karachi, Pakistan	2012
69122-EW	*Acinetobacter haemolyticus*	Faecal screening	Karachi, Pakistan	2012
74312-EC	*Acinetobacter schindleri*	Faecal screening	Karachi, Pakistan	2012
73668-ECT	*Acinetobacter towneri*	Faecal screening	Karachi, Pakistan	2012
116-17a	*Vibrio cholerae*	Seepage water	New Delhi, India	2011
116-17b	*Vibrio cholerae*	Seepage water	New Delhi, India	2011
116-14	*Vibrio cholerae*	Seepage water	New Delhi, India	2011
BRV8	*Vibrio cholerae*	Blood	Bristol, England	2011
UAB190	*Escherichia coli*	Laboratory strain derived from HB101	Barcelona, Spain	
AG3528	*Acinetobacter pittii*	Wound swab	Cardiff, Wales	2011
UAB190$_{NDMP1}$	*Escherichia coli*	Transconjugant	Cardiff, Wales	2012
UAB190$_{NDMP2}$	*Escherichia coli*	Transconjugant	Cardiff, Wales	2012
UAB190$_{NDMP3}$	*Escherichia coli*	Transconjugant	Cardiff, Wales	2012
AG3528$_{NDMP1}$	*Acinetobacter pittii*	Transconjugant	Cardiff, Wales	2012
AG3528$_{NDMP2}$	*Acinetobacter pittii*	Transconjugant	Cardiff, Wales	2012
AG3528$_{NDMB1}$	*Acinetobacter pittii*	Transconjugant	Cardiff, Wales	2012
AG3528$_{NDMB2}$	*Acinetobacter pittii*	Transconjugant	Cardiff, Wales	2012
KP 05-506	*Klebsiella pneumonia*	Urine	Őrebro, Sweden	2008
ATCC 25922	*Escherichia coli*	Clinical isolate	Seattle	
ATCC 27853	*Pseudomonas aeruginosa*	Clinical isolate	Boston	

Table 2.1 – List of study isolates.

2.2 Reagents and media

Ready-made reagents supplied directly from the manufacturers are given in the main text of this Chapter. Recipes for reagents made up locally are provided in Appendix 2.1 and a full list of media used is included in Appendix 2.2.

2.3 Bacterial identification

Preliminary identification of *V. cholerae* and 2005 *Acinetobacter* spp. isolates was performed using the BD phoenix automated identification system (Becton Dickinson, Franklin Lakes, USA), according to manufacturer's instructions. In brief, isolates were inoculated in Phoenix ID broth to give a suspension of 0.5-0.6 McFarland. A drop of AST indicator solution and 25 µl of the bacterial suspensions were added to Phoenix AST Broth. These suspensions were poured on to the ID and AST ports of Gram-negative Phoenix panels respectively. Organism identification were then provided by the automated system, with susceptibility results where applicable.

Following the introduction of matrix-assisted laser desorption/ ionization time of flight mass spectrometry (MALDI-TOF, Bruker, Billerica, USA) as a means of bacterial identification in the Specialist Antimicrobial Chemotherapy Unit, this was adopted as the main method of preliminary identification. MALDI-TOF was used to provide preliminary identification for all 2012 Karachi *Acinetobacter* spp. isolates and the 2005, Tamil Nadu *Acinetobacter* spp. isolate CHI-40-1, which earlier experiments had not provided a reliable identification for. It was also used to confirm species background of putative transconjugants obtained by conjugation experiments (see section 2.16). Most samples were tested by the direct transfer method only, following manufacturer's instructions. Single colonies from overnight growth on solid media

were directly applied to a Bruker MSP 96 target, polished steel plate. Samples were overlaid with 1 µl of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (Bruker) and allowed to air dry. The plates were then loaded onto the MALDI biotyper. Scores of reliability of ≥2.0 were required for species level identification. For samples yielding lower reliability scores, MALDI-TOF was repeated following protein extraction.

For the extraction process 300 µl of Sigma molecular water was inoculated with 1 µl loop full of the test isolate and vortexed. 900 µl of absolute ethanol was then added to suspensions and these were vortexed for ~1 min, then centrifuged for 2 min at 13,000 rpm. The supernatants were discarded, the samples centrifuged again for 2 min at 13,000 rpm and then air dried. 10-50 µl of 70% formic acid solution was mixed with each sample, in proportion with the size of the pellet. Equal volumes of 100% acetonitrile were then added and mixed. Samples were centrifuged for 2 min at 13,000 rpm. 1 µl of extract supernatants were then applied to the Bruker MSP 96 target plate and then processed as above.

For *A. baumannii* isolates, support for initial identification was provided by PCR for *bla*$_{\text{OXA-51}}$-like genes (see section 2.7), which are universally present in *A. baumannii* but rarely described in other species (Turton et al. 2006b). Amplicons were sequenced. Isolates which underwent whole genome sequencing (WGS) were also subjected to ribosomal MLST (rMLST) (see sections 2.15 and 2.16). For other *Acinetobacter* spp. the identification provided by MALDI-TOF was supplemented by sequencing of 1378 bps of their 16S rRNA genes (sections 2.7, 2.8 and 2.16). All *V. cholerae* isolates underwent WGS and speciation was supported by rMLST.

2.4 Susceptibility testing

Initial susceptibility testing for all 2005 *Acinetobacter* spp. isolates was provided by BD phoenix (see section 2.2). Additional susceptibility testing of 2005 *Acinetobacter* spp. isolates and testing of all other study isolates was with gradient strip methods using E-test (Biomerieux, LaPlane, France) and MIC test strips (Liofilchem®, Roseto degli Abruzzi, Italy). 0.5 McFarland inoculums of test isolates were spread on Mueller Hinton (MH) agar to give a continuous lawn of growth and then gradient strips applied after allowing to dry. Interpretation of minimum inhibitory concentration (MIC) results was according to manufacturer's advice, using European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (version 3.1) for *Acinetobacter* spp. and transconjugants. For *V. cholerae* interpretation was according to CLSI guidelines for *Vibrio* spp. other than *V. cholerae*, in the absence of any species specific breakpoints available from EUCAST or CLSI (Clinical and Laboratory Standards Institute 2006).

2.5 Restriction digests and S1 nuclease treatment of genomic DNA and pulsed field gel electrophoresis

For all work reported in Chapters 3 and 4, relating to *Acinetobacter* spp. and *bla*$_{\text{NDM-1}}$ positive transconjugants, genomic DNA was prepared in agarose plugs following Antibiotic Resistance: Prevention and Control (ARPAC) guidance for *A. baumannii* strain typing (ARPAC 2009). Strains were grown on Luria Bertani (LB) agar with 1 µg/ml of meropenem for *bla*$_{\text{NDM-1}}$ positive strains, or LB agar without meropenem for *bla*$_{\text{NDM-1}}$ negative strains overnight and checked for purity. Pure cultures were harvested and suspended in 2 ml 1× TE buffer to optical density of 1.8 to 2.0 at a wavelength of 600 nm. 350 µl of cell suspensions were warmed to 50 °C

on a heating block, mixed with an equal volume of LMP agarose and loaded into plug moulds. After setting these were placed in universals containing 3 ml of ES buffer with 1 mg/ml proteinase K (Sigma Aldrich, St. Louis, USA) and incubated for 3 h at 55°C. Plugs were then washed in 5 ml of 1× TE buffer at 4°C 4 times for at least 3 h.

For *V. cholerae* strains the above protocol produced degraded genomic DNA. A new protocol was developed based on the PulseNet *V. cholerae* PFGE SOP available at http://www.cdc.gov/pulsenet/PDF/vibrio_pfge_protocol-508c.pdf but utilising equipment and reagents which were readily available in our laboratory. Strains were grown on LB agar (with 1 µg/ml of meropenem for *bla*$_{NDM-1}$ positive strains) and inoculated into 3 ml of cell suspension buffer (CS buffer) to an optical density of 0.9 to 1.1 at a wavelength of 600 nm. 400 µl of cell suspensions were warmed to 50 °C, mixed with 20 µl of 20 mg/ml proteinase K and then with 400 µl of low melting point (LMP) agarose and loaded into plug moulds. After setting each plug was placed in 2 ml of proteolysis buffer supplemented with 10 µl of 20 mg/ml of proteinase K and incubated at 50 °C overnight. The following day plugs were washed 5 times in 1× TE buffer at 50°C for 30 min for each wash.

For digestion with *Apa*I (Thermo Fisher Scientific, Waltham, USA) plugs were washed once with 1 ml 1× TE buffer for 15 minutes and twice with 1× *Apa*I buffer (Thermo Fisher Scientific) for 20 min, all at 4 °C. 20 units of *Apa*I were applied direct to the plugs and then diluted with 200 µl of fresh 1× *Apa*I buffer and incubated at 30 °C overnight in a moist box. For digestion with *Not*I (Thermo Fisher Scientific), plugs were washed in 0.1× TE with shaking for 30 min at room temperature, then twice for 15 min in 1× buffer O (Thermo Fisher Scientific) at 4 °C. 20 units of *Not*I were applied direct to the plugs and then diluted with 100 µl of fresh 1× buffer O and

incubated at 37 °C overnight in a moist box. For digestion with endonuclease S1, plugs were washed in 0.1× TE with shaking for 30 min at room temperature, twice for 15 minutes each time in 2× S1 buffer and once for 15 min in 1× S1 buffer at 4 °C. Plugs were incubated with S1 nuclease at 37 °C for 45 min, with final concentrations of $5x10^{-3}$ u/µl for *Acinetobacter* and $1.25x10^{-4}$ u/µl for Enterobacteriaceae and *V. cholerae*. Previous experiments had shown that higher concentrations of the enzyme were required to achieve optimal results in *Acinetobacter* compared to Enterobacteriaceae. For all enzymes the following day the enzyme and buffer were aspirated and plugs were washed in 0.5× TBE buffer and loaded onto a 1.5% pulsed field agarose gel made with 0.5× TBE buffer.

Fragments were separated using a CHEF DR III (Bio-Rad Laboratories, Inc., Hercules, USA) apparatus. PFGE conditions varied with the enzyme used to digest genomic DNA and the species being investigated: for *Apa*I an initial switch time of 5 s, a final switch time of 13 s, a field angle of 120°, 6 V/cm for 20 h at 14°C, as per ARPAC guidance (ARPAC 2009); for *Not*I digestion of *V. cholerae* isolates an initial switch time of 2 s, a final switch time of 10 s, a field angle of 120°, 6 V/cm for 13 h, followed by an initial switch time of 20 s, a final switch time of 25 s, a field angle of 120°, 6 V/cm for 6 h at 14 °C; for *Not*I digestion of *A. baumannii* isolates an initial switch time of 2 s, a final switch time of 12 s, a field angle of 120°, 6 V/cm for 13 h at 14 °C; for S1 nuclease an initial switch time of 5 s, a final switch time of 45 s, a field angle of 120°, 6 V/cm for 18 h at 14 °C. The electrophoresis tank was filled with 2L of 0.5× TBE buffer. Gels were stained in molecular grade water (Corning, New York, USA) containing 1 µg/ml of ethidium bromide (Sigma Aldrich) for ~30 min and destained in molecular grade water for ~60 min. Gels were read under UV light and photographed.

2.6 In gel hybridisation with ^{32}P labelled gene probes

PFGE or standard electrophoresis gels were placed on a sheet of filter paper and incubated at 50 °C overnight. When ready to use gels were rehydrated in sterile distilled water for 45 min, placed in denaturing solution for 45 min and then neutralising solution for 45 min. The gel was then transferred to a hybridisation tube with 20 ml of pre-hybridisation solution and incubated at 65 °C overnight.

Gel purified PCR amplicons were used as gene probe templates. A list of the gene probes used in the course of this thesis is given in Table 2.2 along with details of the PCR amplicons and genomic DNA templates used to prepare them. Gene probes were made by the random primer method, incorporating CTP ^{32}P (PerkinElmer, Waltham, USA) using a Prime-It II Random Primer Labelling Kit (Agilent technologies, Santa Clara, USA) as previously described (Patzer *et al.* 2009). 15 µl of template DNA was mixed with 10 µl of random primers and 8 µl of molecular grade water and boiled for 5 min in a water bath. 2.5 µl of CTP ^{32}P, 10 µl dCTP buffer and 1 µl Klenow (Agilent technologies) fragment were rapidly added, prior to incubation for 15 min at 37 °C. The mixture was then added to a Nick column and allowed to run through with 320 µl of 0.1 M Tris HCL buffer at pH 7.5. A further 430 µl of Tris HCL buffer was run through the column, and the labelled probe was collected in a new microcentrifuge tube, boiled again and applied directly to the gel, with pre-hybridisation buffer and incubated overnight at 65 °C in a hybridisation oven. All gels had been pre-treated with pre-hybridisation buffer at 65 °C overnight.

The following day the probe was disposed of and gels were washed twice for 30 min each with 2× SSC (with 0.1% SDS) and with 0.1 SSC (with 0.1% SDS). Probed gels were wrapped in cling film and placed with film in an X-ray cassette.

These were placed at -80°C for at least 24 hours. Finally autoradiographs were developed using Sigma Aldrich Kodack developer and fixer (Sigma Aldrich).

Probe target	PCR Primers*	Template#	Species tested
bla$_{NDM-1}$	NDM 1F NDM 1R	*K. pneumoniae* KP-506	*Acinetobacter* spp. (Chapters 3 and 4), *V. cholerae* (Chapter 5)
IS*Aba125*	ISAba125 5F ISAba125 3R	*A. baumannii* CHI-45-1	*A. baumannii*, *A. bereziniae* (Chapter 3)
IS*15-Δ*	VibIS26F IS26gapR2	*A. baumannii* CHI-45-1	*A. baumannii*, *A. bereziniae* (Chapter 3)
IS*CR27*	insE 5F ISCR27g2R	*A. bereziniae* CHI-40-1	*A. baumannii*, *A. bereziniae* (Chapter 3)
traA	traA F1 traA R2	*A. bereziniae* CHI-40-1	*Acinetobacter* spp. (Chapter 4)

Table 2.2 – List of gene probes, with primers and templates used to produce probes, and species tested. *Primers used in PCR reactions, PCR amplicons were used to make gene probes. #Isolates used as template in PCR reactions.

2.7 Preparation of whole cell genomic DNA

Preparation of genomic DNA as PCR template and for WGS of *Acinetobacter* spp. and *V. cholerae* was performed using a method based on that described by K. Wilson (Wilson 2001). Overnight growths on LB agar (with 1 µg/ml meropenem for *bla*$_{NDM-1}$ positive isolates) were harvested and suspended in 9.5 ml of TE buffer. To these suspensions 50 µl of 20 mg/ml proteinase K was added, followed by 500 µl of

10% SDS. This mixture was then incubated for 1hr at 37 °C. 1.8 ml of 5 M NaCl solution was added and suspensions were left to stand for 10 min. CTAB NaCl solution was added and samples incubated at 65 °C for 20 min. Equal volumes of 24:1 chloroform (Thermo Fisher Scientific): isoamyl alcohol solution (Thermo Fisher Scientific) were added and samples placed on a rotary mixer for approximately 80 min. Samples were centrifuged at 3,600 rpm for 30 min and the top layers transferred to fresh tubes with an equal volume of isopropanol. Precipitated DNA was removed and added to 2 ml of 70% ethanol (Thermo Fisher Scientific). Excess ethanol was discarded and then the containers placed in a 55 °C incubator to evaporate residual ethanol.

For isolates obtained during the passage experiment genomic DNA was prepared using the Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) according to manufactures instructions, and used as template for the real time quantitative PCR experiments. Isolates were grown overnight in LB broth (with or without meropenem, depending on the strain, see section 2.18) on a rotary mixer at 37 °C. 1 ml of overnight cultures was centrifuged for 2 min at 13,000 rpm to pellet the cells and the supernatants discarded. 600 µl of Nuclei Lysis Solution (Promega) was added to the pellets and samples were incubated at 80°C on a heating block for 5 min, then allowed to cool to room temperature. 3 µl of RNase Solution (Promega) was added to the cell lysates. The solutions were incubated at 37 °C for 60 min and allowed to cool to room temperature. 200 µl of Protein Precipitation Solution (Promega) was added to the cell lysates and vortexed vigorously, incubated on ice for 5 min and then centrifuged at 13,000 rpm for 10 min. The supernatants were transferred to clean 1.5 ml Eppendorf tubes containing 600 µl of isopropanol (Thermo Fisher Scientific) and chilled to 4 °C. These were gently mixed by inversion,

centrifuged at 13,000 rpm for 5 min, the supernatants discarded and the tubes drained on absorbent paper. 600 µl of room temperature 70% ethanol was added and samples were centrifuged at 13,000 rpm for 10 min. The ethanol was carefully removed and samples placed in a 55 °C incubator to air dry the pellets for ~10-15 min. Pellets were rehydrated in 100 µl of molecular grade water overnight at 4 °C.

2.8 Polymerase chain reaction (PCR) and standard gel electrophoresis

Many PCR reactions were used in the course of the experiments performed for this thesis. These were used for the detection of resistance genes (eg *bla*$_{\text{NDM-1}}$, *bla*$_{\text{OXA-23}}$), for aiding in identification (eg 16S rRNA gene sequencing), for the detection of pNDM-BJ01-like plasmids (see Chapter 4) and for the closure of wider *bla*$_{\text{NDM-1}}$ contexts, including complete plasmid sequences, from WGS contigs. Immediate *bla*$_{\text{NDM-1}}$ contexts in some *Acinetobacter* isolates were analysed by primer walking, using PCR of overlapping sequences and amplicon sequencing. For *A. baumannii* isolates and *A. bereziniae* CHI-40-1 this data was later supplemented by WGS. The sequence of the immediate *bla*$_{\text{NDM-1}}$ context from *A. haemolyticus* was obtained from primer walking results only. In view of the large number of PCR primers used a full list of primers used for work associated with each chapter is given in Appendices 3.2, 4.2, 5.2, 5.4, 5.6 and 5.8. Most PCRs employed as template 1 µl of genomic DNA, prepared as described above. For initial confirmation of the presence of *bla*$_{\text{NDM-1}}$ in study isolates and confirmation of the presence of *bla*$_{\text{NDM-1}}$ in putative transconjugants, templates were prepared by emulsifying single colonies in 50 µl of molecular grade water. Some of the PCR and amplicon sequencing experiments for the Karachi *Acinetobacter* spp. isolates were performed by Dr Maria

Carvalho, while primer design, planning and analysis was performed by myself for all experiments.

For every 1 µl of template DNA PCR reactions contained 5 µl PCR ReddyMix PCR Master Mix (Thermo Fisher Scientific), 3 µl of molecular grade water and 0.5 µl of forward and reverse primers. PCRs were run on G-Storm GS1 Thermal Cycler (G-storm, Somerton, UK). For some PCRs, including that for *bla*$_{\text{NDM-1}}$, optimum annealing temperatures were determined using temperature gradients. Given the number of different PCR reactions used to achieve closure of WGS, optimisation for all primer combinations was impractical, and a default annealing temperature of 60 °C was employed. For PCRs which failed to amplify a product of the expected size or for which products of multiple lengths were seen, and for which no alternative assembly was confirmed by PCR, optimisation of annealing temperature was attempted. This represented a small minority of the PCRs performed to achieve closure. For all PCRs extension times were determined by expected product size, based on 1 minute per kb of expected size, with a minimum of 1 minute. PCR reactions were as follows 95°C for 5 min followed by 35 cycles of 95°C for 1 min, annealing temperature variable for 1 min, extension 68°C for variable time, 68°C for 10 min after cycles finished. Products were loaded onto 1% HiRes standard agarose gels (AGTC Bioproducts Ltd., Hull, England) stained with ethidium bromide and standard electrophoresis run at 280 V for approximately 45 min.

2.9 Gel purification of PCR amplicons and Sanger sequencing of products

For sequencing, gel fragments were excised and purified using a QIAquick gel extraction kit (Qiagen, Limburg, Netherlands). Excised gel fragments were weighed and 3 volumes of Buffer QG (Qiagen) added to 1 volume of gel. Agarose blocks were

incubated at room temperature in Buffer QG, for approximately 2 hours. For PCR amplicons of <500 bp or >4 kb 1 gel volume of isopropanol was also added to the samples. Samples were applied to QIAquick columns (Qiagen) in 2 ml collection tubes, up to maximum volumes of 800 µl, and centrifuged for 1 min at 13,000 rpm on a table-top microcentrifuge. The flow-through was then discarded. For samples larger than 800 µl columns were then reloaded and centrifuged a second time. 500 µl of fresh QG buffer was then applied to the column and centrifuged again for 1 min at 13,000 rpm, and follow through discarded. 750 µl of Buffer PE (Qiagen) was then applied to the columns to wash. These were left to stand for ~2 min, centrifuged as previously, the flow throughs discarded and then centrifuged again. The columns were then transferred to clean 1.5 ml Eppendorf microcentrifuge tubes. 50 µl of molecular grade water (between pH 7.0 and 8.5) was then applied to the columns, which were centrifuged as previously. 5 µl of the resulting purified DNA samples were analysed for appropriate size and yield by electrophoresis, conditions as above.

DNA concentrations were determined using a Jenway 7315 Spectrophotometer (Bibby Scientific Ltd., Stone, United Kingdom). Purified products were submitted for Sanger sequencing to Euofins MWG operon (Ebersberg, Germany). 15 µl of the purified PCR product was submitted at a concentration determined by Eurofins guidance (2 ng/µl for amplicons 150-300 bp; 5 ng/µl for amplicons 300-1000 bp; 10 ng/µl for amplicons > 1000 bp). Samples were pre mixed with 2 µl of the primer for sequencing at a concentration of 10 µM. Chromatograms were checked and edited using Chromas Lite (Technelysium Pty Ltd, South Brisbane, Australia). Reference sequences were downloaded from NCBI/ ENA databases. Reference sequences and sequenced PCR amplicons were analysed in

Geneious (Drummond *et al.* 2012) to confirm amplification of the expected PCR product.

2.10 Real time quantitative PCR

Real-time quantitative PCR (qPCR) was performed to quantify changes in *bla*$_{NDM-1}$ and *traA* copy number present in bacterial cells over the course of the passage experiment. The single copy chromosomal gene *rpoB* was used as the reference gene. qPCR primers and probes are shown in Appendix 4.2. PCRs were optimised for annealing temperature (50-70°C), $MgCl_2$ concentration (2-5 mM), primer concentration (0.25-0.75 µM) and probe concentration (0.2-0.4 µM). Dual labelled probes with fluorescent dye and quenchers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). PCRs were performed on a Rotorgene Q HRM (Qiagen, Manchester, UK). qPCR for *bla*$_{NDM-1}$ and *traA* were run as duplex reactions at 95°C for 15 min, then 35 cycles of 95°C for 10 s and 60°C for 30 s. The qPCR for the *rpoB* references was different for each strain background. For CHI-40-1 and AG3528$_{NDMP1}$ the same primer pair was used (rpoB Ac F1 and rpoB Ac R1) but the probes differed (*rpoB* 40-1 and *rpoB* AG3, respectively). For UAB190$_{NDMP2}$ passaged isolates the primers *rpoB* Ac F3 and *rpoB* Ac R3 were used with probe *rpoB* Ec. All *rpoB* qPCR runs were performed using the conditions 95°C for 15 min, then 40 cycles of 95°C for 10 s and 58°C for 30 s. All qPCR reactions were performed with a final volume of 20 µl, with 2 µl of Lightcycler FastStart DNA Master HybProbe (Roche, Mannheim, Germany) and 5 µl of template. Final concentrations of primers, probes and $MgCl_2$ were as indicated in Appendix 4.2. *bla*$_{NDM-1}$ and *traA* fluorescence cycle threshold values (Ct) were compared to *rpoB* Ct values and quantification was performed by the ΔΔCt method as previously described (Johnson

et al. 2013). Regression analysis was performed using Excel 2007 (Microsoft, Redmond, USA). A validation experiment showed that ΔCt values were linear over the range of values detected in the passage experiment (see Chapter 4). All experiments were performed in triplicate and the mean values and 2 standard deviations calculated for the regression analysis.

2.11 Whole genome sequencing (WGS) and de novo assembly

A list of the isolates for which WGS was performed is shown in Table 2.3. Isolates were sequenced using an Illumina HiSeq 2000 at the Wellcome Trust Sanger institute, Cambridge. A unique index-tagged insert library was prepared to allow processing of the sample data following multiplex sequencing with other libraries on 8 channels of an Illumina Genome Analyzer GAII cell to give 100bp paired end reads, as previously described (Mutreja et al. 2011). Reads were assembled *de novo* using the Velvet Assembly Tool (Abbott *et al.* 2005; Carver *et al.* 2005; Zerbino and Birney 2008) (Version 1.2.10). *Acinetobacter spp.* assemblies were performed by Dr T. Connor and *V. cholerae* assemblies by Dr A. Mutreja, on the Sanger Institute server.

Sequenced isolates	Reference sequences (Accession number)
A. baumannii CHI-32, CHI-34 and CHI-45-1	*A. baumannii* plasmid pWA3 (JQ241791) *A. baumannii* AYE (CU459140) *E. coli*, pNDM102337 (JF714412) *Citrobacter freundii* plasmid pCTX-M3 (AF550415) *A. baumannii* A85 (KC118540)
A. bereziniae CHI-40-1	*A. lwoffii* plasmid pNDM-BJ01 (JQ001791)
V. cholerae 116-17a and 116-17b	*E. coli* plasmid pNDM-1_Dok01 (AP012208) *V. cholerae* SXT ICE (AY055428)
V. cholerae 116-14	*Vibrio harveyi* ZJ0603 scaffold 7 (JH720477) *C. freundii* plasmid pNDM-CIT (JX182975) *V. cholerae* SXT ICE (AY055428)
V. cholerae BRV8	*Salmonella* Typhimurium DT104 SGI-1 (NC_022569.1) *C. freundii* plasmid pNDM-CIT (JX182975) *V. cholerae* N19161 chromosome 1 (AE003852) *V. cholerae* SXT ICE (AY055428)

Table 2.3 – List of isolates which underwent WGS and reference sequences used in analysis and annotation.

2.12 Identification and closure of WGS contigs associated with *bla*$_{NDM-1}$ contexts

Initially contigs containing *bla*$_{NDM-1}$ (or other gene(s) of interest) were identified using nucleotide BLAST searches, using the MEGABLAST algorithm. Query sequences (e.g. *bla*$_{NDM-1}$ reference sequence from *K. pneumoniae* 05-506F, accession number N396876) were used to search all WGS contigs for an isolate. Once contigs of interest were identified, potentially overlapping contigs could be identified by using that contig as the query sequence to run further BLAST analysis.

In most instances this would identify small overlaps between contigs at the extreme 5' and 3' prime end of the contig used as the query (see Figure 2.1 for example). Potential assemblies with overlapping contigs were then determined using the de novo assembly tool in Geneious version 5.6. Several pieces of information simplified the identification of potentially linked contigs of interest. Contigs from a specific plasmid had sequence coverage which, in many cases, were similar to each other and usually higher than that of chromosomal contigs. Looking at annotation of contigs (see Section 2.14) often made it possible to predict correctly contigs which were linked, on the basis of similarity with reference sequences. Putative links between contigs were confirmed by PCR across contig boundaries, with sequencing of amplicons, as described above.

2.13 Sequence alignment and comparison

Nucleotide and protein sequences were aligned and analysed in Geneious version 5.6 (Drummond et al. 2012). For short, closely related nucleotide sequences the de novo assembly tool was used. For longer nucleotide sequences and protein sequences the MAFFT plugin was used, using the default settings defined in Geneious. The only exception to this was the nucleotide sequence assembly used to align rMLST loci for *Acinetobacter* spp. reported in Chapter 4. This work was done before the MAFFT plugin became available and so was performed using the MUSCLE alignment tool (Edgar 2004) at http://www.ebi.ac.uk/Tools/msa/muscle/, using default settings. All alignments were checked visually in Geneious, and trimmed or corrected manually if necessary.

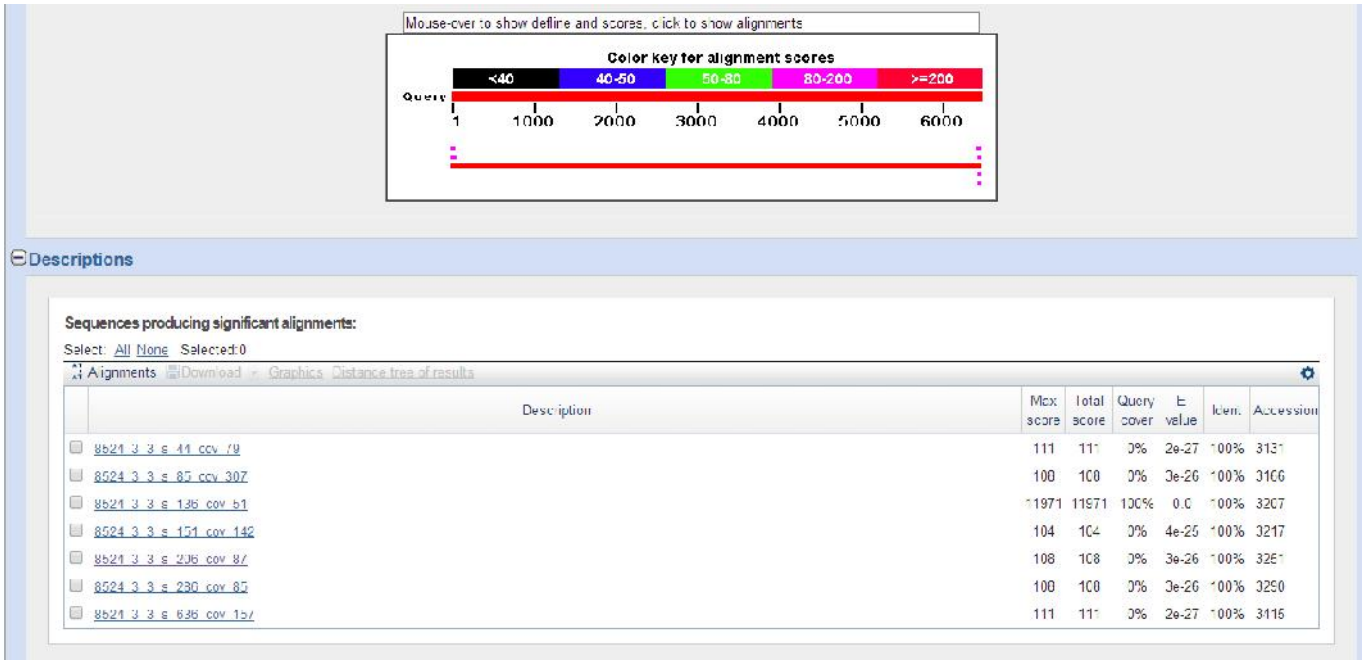


Figure 2.1 – Example of BLAST output used to identify putative contig links. Example shows output of contig 136 from the WGS of *A. bereziniae* CHI-40-1 against all contigs from the same strain, saved as a single FAS file. The graphic shows that a single contig has a high alignment score ≥ 200 (in red), which is contig 136 itself. At either end the contigs shown in pink have alignment scores of between 80 and 200. The graphic and query coverage given in the table below shows that only a small part of these contigs have significant identity. The table also shows, however that they have 100% identity over the sequences with similarity and low E values. The contigs actually linked were inferred by comparison to the reference sequence, pNDM-BJ01 (JQ001791), and the assembly confirmed by the methods stated elsewhere in section 2.12.

2.14 Annotation of nucleotide sequences

WGS and final assemblies of the MGE analysed were submitted to the RAST online annotation pipeline (Aziz *et al.* 2008). Final annotation for some sequences (e.g. pNDM-40-1), for which very closely related reference sequences were available were refined using the transfer annotations tool on aligned sequences in Geneious. Only coding sequences with 98% identity were transferred and were then compared with the RAST gene models. The annotation models were compared, edited and finalised using Artemis. Details of the reference sequences, including their Accession numbers, are given in Table 2.3. For pNDM-116-14, the plasmid from *V. cholerae* 116-14, and *A. baumannii* CHI-32 no useful references were identified for the majority of the plasmid sequences. In these cases the final annotations were refined from the RAST output with reference to BLASTP analysis of translated protein sequences, and for the relaxase and T4SS proteins of pNDM-116-14 by the output of ConjScan online tool available at http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::CONJscan-T4SSscan., which identifies conserved domains using a hidden Markov model as described by Guigliumani et al (Guglielmini et al. 2011). The ResFinder 2.0 online tool available at http://cge.cbs.dtu.dk/services/ResFinder/ was used to identify acquired AMR genes in the WGS assemblies of all isolates, using a threshold identity of 70% and a minimum length of 60% (Zankari *et al.* 2012).

2.15 Multilocus sequence typing (MLST) and ribosomal MLST (rMLST)

MLST of *A. baumannii* isolates was initially performed according to the method described by Turton et al (Turton *et al.* 2007). PCR was performed for the genes *csuE*, *ompA* and *bla*$_{\text{OXA-51}}$-like, amplicons were sequenced (see sections 2.9

and 2.10) and sequences used to identify matching loci. For *A. baumannii* isolates which underwent WGS the full 7 loci Pasteur MLST scheme was applied to confirm results. A BLASTN search of all contigs for each isolate were performed using reference sequences for each of the MLST loci. Loci identified in this way were used to search the *A. baumannii* MLST sequence type database at: http://www.pasteur.fr/cgi-bin/genopole/PF8/mlstdbnet.pl?file=acin_profiles.xml.

Ribosomal multi-locus sequence typing (rMLST) (Jolley *et al.* 2012) was applied to isolates which underwent WGS. All 53 rMLST loci were identified in each of the isolates studied and in other sequences used in phylogenetic analysis using TBLASTX searches against the loci from an appropriate reference sequence, downloaded from the Bacterial Isolate Genome Sequence database ($BIGS_{DB}$) available at http://rmlst.org/. The reference sequences used were *V. cholerae* N1961 (AE003852) for *Vibrio* spp. and *A. johnsonnii* SH046 (NZ_ACPL00000000.1) for *Acinetobacter* spp. Further sequences with annotated rMLST loci were downloaded from $BIGS_{DB}$ for use in phylogenies. Translated protein sequences for all rMLST loci for each sequence were then concatenated in Geneious and aligned as described above.

2.16 Phylogenetic analysis

For phylogenetic analysis of 16S rRNA genes, concatenated rMLST loci, and individual proteins from pNDM-116-14, protein or nucleotide alignments were first performed as described above. Final alignments were used to create maximum-likelihood (ML) phylogenetic trees using PhyML 3.1 (Guindon *et al.* 2010) in SeaView (Galtier *et al.* 1996), with 100 bootstraps on the most likely tree (other parameters default settings).

RAST and ConjScan identified genes coding for putative replicase, relaxase and T4SS proteins in pNDM-116-14. BLAST searches of the NCBI reference sequence protein database identified potential homologues, and sequences with relatively close identity and well characterised proteins were chosen to be included in phylogenetic analysis for the replicase protein, RepE, the relaxase, TraI, and the ATPase, TraC. Protein alignments were performed using MAFFT as above. Complete protein sequences of RepE and TraC were used but in the case of the TraI, BLAST searches and alignments were restricted to the first 300 bp, in which the relaxase domain is normally found, as done by Garcillán-Barcia *et al.* (Garcillán-Barcia *et al.* 2009). ML phylogenetic trees were drawn using PhyML 3.1 as above.

The phylogenetic tree of *V. cholerae* core genome SNPs was performed by Dr T. Connor. This tree includes a subset of the isolates included in a similar analysis by Mutreja et al. (Mutreja et al. 2011), with a full list given in Appendix 5.1. Reads from each of the isolates were mapped to the reference assembly of *V. cholerae* N16961 (created by concatenating the two chromosomes together; accession numbers AE003852 and AE003853 for chromosomes I and II respectively) and high quality SNPs identified as described previously (Harris *et al.* 2010). Using the resultant SNP alignment, an approximately-ML phylogeny was produced using FastTree 2.1 (Price *et al.* 2009, 2010). FastTree was run on the nucleotide SNP alignment using a general time-reversible model with gamma correction (with 4 rate categories) for among-site rate variation. Only reads mapped to the reference and present in all isolates included in the tree were used for this analysis.

2.17 Conjugation experiments

Conjugation experiments were performed using *bla*$_{NDM-1}$ positive *Acinetobacter* spp. and *V. cholerae* BRV8 as donors (see Table 2.5) and *E. coli* UAB190 or *A. pittii* AG3528 (both rifampicin resistant) as recipients. Mating experiments were initially performed as described previously in LB broth and on CBA plates at 30°C and 37°C, incubated overnight (Walsh et al. 2011). Strains were initially grown overnight in LB broth at 37°C and adjusted to give about 10^8 cfu/ml of donor and 10^7 cfu/ml of recipients in each of the mating mixtures. In order to maintain antimicrobial selection of the MGEs harbouring *bla*$_{NDM-1}$ in the donor cells, overnight LB broth cultures were supplemented with 10 µg/ml of meropenem (AstraZeneca, London, UK) for *Acinetobacter* spp. and ampicillin 100 µg/ml (Sigma-Aldrich, St. Louis, USA) for BRV8. Prior to setting up the mating mixtures cells were pelleted by centrifugation for 5 min at 3,600 rpm and re-suspended in LB broth to a final concentration of 10^8 cfu/ml. Selection was performed on Brilliance^{TM} UTI Clarity Agar or LB agar supplemented with 200 µg/ml rifampicin (Sigma-Aldrich) for recipient selection; ampicillin 100 µg/ml and rifampicin 200 µg/ml, or meropenem 0.5 µg/ml and rifampicin 200 µg/ml, for UAB190 background transconjugants, or meropenem 1 µg/ml and rifampicin 200 µg/ml for AG3528 background transconjugants. For BRV8 the experiment was also attempted with SOS induction as described by Beaber *et* al (Beaber et al. 2004). In brief 30 µl of overnight broths were added to 3ml of fresh LB and incubated for 2 hours. Ciprofloxacin was then added to the BRV8 broth culture to a final concentration of 0.5 µg/ml, and both cultures incubated for one further hour prior to setting up mating mixtures.

Five colonies with the morphology of the recipient background on selective media were subcultured with continued antimicrobial selection. Colonies from the

subculture were then checked for purity, and *bla*$_{NDM-1}$ PCR and MALDI-TOF were performed. For the successful mating experiments with *Acinetobacter* spp. described in Chapter 4, preliminary results suggested that plate mating at 30°C were the most efficient conditions tested. These conditions were therefore used for replicates to calculate the mating efficiency, defined as the number of transconjugants per recipient cell. Results given are the average of three duplicate experiments. Unsuccessful mating experiments were repeated at least 3 times by broth and plate mating methods.

2.18 Passage experiments

A passage experiment was performed on CHI-40-1 and its transconjugants UAB190$_{NDMP2}$ and AG3528$_{NDMP1}$. Cultures from selective plates were inoculated into 5 ml of LB broth with and without antibiotic selection and incubated overnight at 37°C. The following day 10 µl of the overnight cultures was re-inoculated into 5 ml of fresh broth with the same selection as the starting culture. CBA plates were inoculated daily to check purity and cultures were stored each day in LB broth with 10% glycerol at -80°C. This procedure was repeated on 14 consecutive days. Antibiotic selection was with meropenem (10 µg/mL) for CHI-40-1 and AG3528$_{NDMP1}$ and meropenem (1 µg/mL) for UAB190$_{NDMP2}$. Stored cultures were then investigated by S1 PFGE and probing for *bla*$_{NDM-1}$ and *traA*, as described, as well as qPCR (see above).

Chapter 3

Plasmid carriage of *bla*$_{NDM-1}$ in clinical *Acinetobacter baumannii* isolates from India.

3.1 Introduction

As discussed in Introduction section 1.3.2 NPGNB have been isolated around the world, with many cases linked to travel, especially to South Asia (Yong et al. 2009b; Kumarasamy et al. 2010; Nordmann *et al.* 2011a; Johnson and Woodford 2013). The group of pathogens for which there is the most clinical and public health concern are the Enterobacteriaceae. However, *A. baumannii* has also been found to harbour *bla*$_{NDM-1-like}$ genes and has been the subject of clinical concern and scientific interest for a variety of reasons. Firstly, as discussed in Introduction section 1.5.1, *A. baumannii* has in the past decades been established to be a successful nosocomial pathogen worldwide, with certain strains associated with MDR phenotypes (Diancourt *et al.* 2010; Higgins et al. 2010; Zarrilli et al. 2013). Carbapenem resistance is relatively common in some of these strains, but this is predominantly due to the presence, and increased expression of, intrinsic and acquired OXA-type β-lactamases, combined with increased expression of multi-drug efflux pumps and reduced outer membrane permeability (Vila *et al.* 2007; Higgins et al. 2010; Zarrilli et

al. 2013). Secondly, NDM-producing *A. baumannii* (NPAB) have been reported from clinical samples more frequently than other bacterial species, outside Enterobacteriaceae (Chen et al. 2011; Nordmann et al. 2011b; Bonnin et al. 2012b; Johnson and Woodford 2013; Jain et al. 2014).

Finally, genetic analysis has identified a strong likelihood that *bla*$_{\text{NDM-1-like}}$ genes arose by a fusion between an ancestral MBL and an aminoglycoside resistance gene called *aphA6* (Toleman et al. 2012). This fusion event probably occurred in bacteria of the *Acinetobacter* genus. *bla*$_{\text{NDM-1-like}}$ genes have, so far, always been found in association with IS*Aba125*, although in many Enterobacteriaceae sequences only a 3' fragment of the element is present (Poirel et al. 2011b; Poirel et al. 2012a; Toleman et al. 2012). This IS was first described in *Acinetobacter* spp. and most complete IS*Aba125* elements in the database are in *Acinetobacter* spp. This same IS has been associated with *aphA6,* which is also predominantly found in *Acinetobacter* spp. (Lambert *et al.* 1990; Nigro *et al.* 2011). Alignments of sequences from IS*Aba125* to *aphA6* and IS*Aba125* to *bla*$_{\text{NDM-1}}$ show that the sequences share 100% identity extending from the 3' end of IS*Aba125* up until the first 20 bps of *bla*$_{\text{NDM-1}}$ (see Figure 3.1) (Toleman et al. 2012).

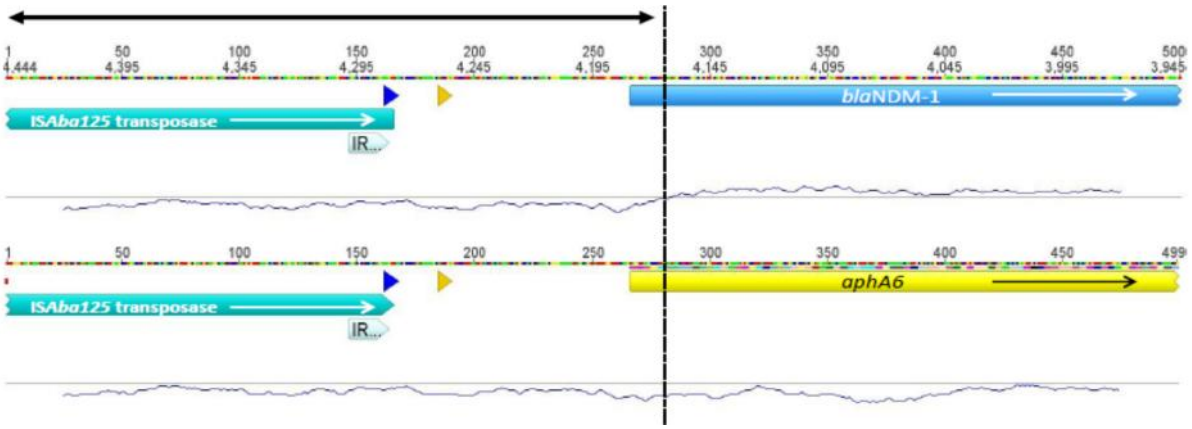


Figure 3.1 – Alignment of 500bp sections of DNA including the 5' ends of *bla*$_{NDM-1}$ and *aphA6* genes and 260bp of upstream sequence. Reproduced from Toleman et al 2012 (Toleman et al. 2012). ORFs are depicted as coloured blocks with arrows indicating the direction of transcription. The double-ended arrow indicates the region of sequence that is 100% identical. The right inverted repeat of IS*Aba125* is depicted as a thick arrow at the end of the transposase ORFs. Promoter sequences are depicted as a blue arrow at -35 and a yellow arrow at -10. The trace underneath each sequence is the %GC which is calculated using a sliding window of 45 bp and the dotted vertical line indicates the point at which the GC% changes from below 50% to above 50% within the *bla*$_{NDM-1}$ sequence.

However, it is likely that the ancestral MBL originates from some other environmental bacterial species. *Acinetobacter* spp. are organisms with a low GC percentage of around 30-40%. In *Acinetobacter* spp. *bla*$_{NDM-1}$ is usually found on a composite Tn formed by two IS*Aba125* elements, Tn*125* (See General Introduction section 1.3.4, Figure 1.7 and Figure 3.6) (Poirel et al. 2012a; Toleman et al. 2012). The contents of Tn*125* are generally highly conserved in *Acinetobacter* spp. with the occasional exceptions resulting from truncation by insertion of ISs (Espinal et al. 2011; Poirel et al. 2012a; Bonnin *et al.* 2013). Most of the contents of Tn*125*, from

bla$_{\text{NDM-1-like}}$ until just prior to the downstream IS*Aba125*, is of a high GC% of between 60-70% (Poirel et al. 2012a). This means that neither the ancestral MBL nor its surrounding context is likely to originate from *Acinetobacter*. A large part of this sequence includes gene coding regions which show synteny with sequences from *Xanthomonas* and *Pseudoxanthomonas* species (Sekizuka et al. 2011). *A. baumannii* is commonly associated with healthcare settings but other *Acinetobacter* species are mainly found at environmental sites, including soil (Diancourt et al. 2010; Visca *et al.* 2011). Therefore, they could be found in similar ecological niches to the *Xanthomonads*, which are recognised as plant pathogens, and so have the opportunity to acquire foreign DNA from them.

Tn*125* is found inserted in several different genetic locations, with direct repeats at either end indicating movement by transposition. Isolates from Europe and the Middle East, which have had the *bla*$_{\text{NDM-1}}$ contexts analysed in detail contain Tn*125* in a chromosomal location (Espinal et al. 2011; Pfeifer et al. 2011; Bonnin et al. 2012b; Bonnin et al. 2013). In China *bla*$_{\text{NDM-1}}$ is also associated with Tn*125* but the Tn is reported mostly on plasmids in both *A. baumannii* and other *Acinetobacter* spp. (Hu et al. 2012; Wang et al. 2012; Zhou et al. 2012; Zhang et al. 2013b).

At present the clinical importance and epidemiology of NPAB are poorly defined. A small number of studies have reported on NPAB, causing infections in intensive care patients in Indian hospitals (Karthikeyan et al. 2010; Bharadwaj et al. 2012). Clinical cases and colonisation with NPAB have also been reported from Pakistan and Bangladesh (Islam et al. 2012; Hasan *et al.* 2014; Sartor et al. 2014). In China *bla*$_{\text{NDM-1}}$ has been frequently identified in *Acinetobacter* spp, including *A. baumannii,* both in clinical cases and from environmental sources (Chen et al. 2011; Hu et al. 2012; Wang et al. 2012; Zhang et al. 2013b). European countries have

reported an increasing number of NPAB isolates, with many cases having epidemiological links with travel to North Africa or occasionally the Balkans (Bonnin et al. 2012b; Poirel et al. 2012a; Bonnin et al. 2013). Notably in France a clonal outbreak occurred involving 7 patients on a surgical intensive care unit, in which a patient repatriated from Algeria was the likely index case (Decousser et al. 2013). In the United Kingdom 16 isolates of NPAB were submitted to the Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) from 2008 to 2013, making it the most commonly identified non-fermentative GNB in the series (Jain et al. 2014). Studies from the Middle East (Espinal et al. 2011; Ghazawi et al. 2012; Espinal *et al.* 2013; Rafei *et al.* 2014) and North Africa (Bakour et al. 2014) have reported significant number of isolates from clinical or screening samples. Recent case reports have also identified NPAB in Honduras, Brazil and Kenya (Revathi *et al.* 2013; Waterman *et al.* 2013; Pillonetto *et al.* 2014).

Of note few of the cases which have undergone MLST profiling are within clonal complex 1-3 (CC1-3, previously global/ European clones I-III) according to the definition of Diancourt et al (Diancourt et al. 2010). Most of the isolates from France were ST85, including the recent outbreak strain, as were isolates from one Algerian hospital and isolates from injured Syrian civilians treated in Lebanon (Decousser et al. 2013; Bakour et al. 2014; Rafei et al. 2014). Most of the isolates from another site in Algeria and sporadic isolates from other parts of the world, including France, were ST25 (Decousser et al. 2013; Revathi et al. 2013; Waterman et al. 2013; Bakour et al. 2014; Pillonetto et al. 2014). *A. baumannii* isolated in Egypt and the Middle East which harboured *bla*$_{\text{NDM-2}}$ were all ST103 (note all ST numbers quoted according to the Pasteur MLST typing scheme, ST numbers differ for the PubMLST scheme and

the three locus scheme) (Espinal et al. 2011; Kaase et al. 2011; Ghazawi et al. 2012).

This chapter reports on the genetic contexts of *bla*$_{\text{NDM-1}}$ in *A. baumannii* obtained from clinical samples from Tamil Nadu, India. Part of this data has been published previously and is included as Appendix 3.1 (Jones et al. 2014a). Strain background, the presence of and mobility of plasmids carrying *bla*$_{\text{NDM-1}}$ and plasmid sequences were determined, representing the first extensive genetic characterisation of NPAB isolates from India. South Asia, as far as current data can determine, has a higher incidence of NPE than other parts of the world. As *Acinetobacter* spp. are proposed to have been the source of *bla*$_{\text{NDM-1-like}}$ genes, NPAB isolates in Indian hospitals were of potential significance. Any MGEs in these strains could act as significant vectors for the dissemination of *bla*$_{\text{NDM-1-like}}$ genes into Enterobacteriaceae.

3.2 Nucleotide sequence accession numbers

WGS assemblies have been deposited under project accession PRJEB8567 for *A. baumannii* isolates CHI-32, CHI-34 and CHI-45-1. Accession numbers for CHI-32 contigs are CVLF01000001-CVLF01000164, and for plasmid pNDM-32 LN833432. Accession numbers for CHI-34 and CHI-45-1 contigs are CVLD01000001-CVLD01000140 and CVLE01000001-CVLE01000165 respectively.

3.3 Results

3.3.1 Preliminary findings

Nine *Acinetobacter* spp. isolates from a hospital in Tamil Nadu, India, collected in 2005 were studied (see Methods Table 2.1). Most isolates were from patients on intensive care and were isolated from blood, pus and respiratory secretions (see Table 3.1). Further details with regards to patient demographics, clinical state and outcomes were not available. Initial identification by BD phoenix did not consistently identify isolates as *Acinetobacter* spp. However, all isolates were $bla_{\text{OXA-51-like}}$ positive by PCR, consistent with being *A. baumannii* (Turton et al. 2006b), except CHI-40-1. Susceptibility testing showed that all isolates were extensively-drug resistant (XDR) according to the definition of Magiorakos et al (Magiorakos et al. 2012). The *A. baumannii* isolates were resistant to all drugs tested except colistin and in two cases amikacin (see Table 3.1). Seven isolates were positive for $bla_{\text{NDM-1}}$ by PCR, with CHI-41 and CHI-44 being the only $bla_{\text{NDM-1}}$ negative isolates (see Table 3.2).

Isolate	Specimen type	Azt	Caz	Taz	Imp	Mem	Ami	Gent	Tob	Cip	Col	Rif	Tig
***A. baumannii* CHI-16**	Blood	>16	≥256	>16/4	≥32	≥32	6	≥256	32	≥32	≤1	6	2
***A. baumannii* CHI-18**	Blood	>16	≥256	>16/4	≥32	≥32	≥256	≥256	≥256	≥32	≤1	4	3
***A. baumannii* CHI-32**	Blood	>16	≥256	>16/4	≥32	≥32	≥256	≥256	≥256	≥32	≤1	6	2
***A. baumannii* CHI-34**	Sputum	>16	≥256	>16/4	≥32	≥32	6	≥256	48	≥32	≤1	4	2
***A. bereziniae* CHI-40-1**	Pus	>16	≥256	>16/4	≥32	≥32	48	≥256	12	≥32	≤1	≥256	0.75
***A. baumannii* CHI-40-2**	Pus	>16	≥256	>16/4	≥32	≥32	≥256	≥256	≥256	≥32	≤1	6	2
***A. baumannii* CHI-41**	Sputum	>16	≥256	>16/4	≥32	≥32	≥256	≥256	≥256	≥32	≤1	6	1.5
***A. baumannii* CHI-44**	Endotracheal aspirate	>16	≥256	>16/4	≥32	≥32	≥256	≥256	≥256	≥32	≤1	6	2
***A. baumannii* CHI-45-1**	Endotracheal aspirate	>16	≥256	>16/4	≥32	≥32	≥256	≥256	≥256	≥32	≤1	3	2

Table 3.1 – Study isolates, specimen type and MIC (mg/L) results for *Acinetobacter* isolates. Table cell red – resistance; green – sensitive; grey – no breakpoint. Species specific *Acinetobacter* spp. breakpoints not available for aztreonam, ceftazidime, piperacillin-tazobactam or tigecycline, for which results are based on EUCAST PK/PD non-species specific breakpoints. Ami – amikacin; Azt – aztreonam; Caz – ceftazidime; Cip – ciprofloxacin; Col – colistin; Imp – imipenem; Mem – meropenem; Taz – piperacillin-tazobactam; Tob – tobramycin.

CHI-32	CHI-34	CHI-45-1
Aminoglycoside		
	aadA1	*aadA1*
aadA2		*aadA2*
	aac(3)-Ia	*aac(3)-Ia*
aac(3)-IId	*aac(3)-IId*	*aac(3)-IId*
	aph(3')-Ia	*aph(3')-Ia*
armA		*armA*
β-lactam		
bla_{NDM-1}	bla_{NDM-1}	bla_{NDM-1}
bla_{OXA-23}	bla_{OXA-23}	bla_{OXA-23}
bla_{OXA-69}	bla_{OXA-69}	bla_{OXA-69}
bla_{ADC-25}/ bla_{ADC-30}	bla_{ADC-25}/ bla_{ADC-30}	bla_{ADC-25}/ bla_{ADC-30}
	bla_{TEM-1D}	bla_{TEM-1D}
MLS - Macrolide-Lincosamide-StreptograminB		
msr(E)		*msr(E)*
mph(E)		*mph(E)*
Phenicol		
floR	*floR*	*floR*
	catA1	*catA1*
Sulphonamide		
sul1	*sul1*	*sul1*
Tetracycline		
	tet(A)	*tet(A)*
Trimethoprim		
dfrA12		*dfrA12*

Table 3.2 – Resistance genes detected by Resfinder search of WGS contigs from *A. baumannii* CHI-32, CHI-34 and CHI-45-1. Colour of table cells indicates approximate % ID with reference genes used for Resfinder searches: Dark green – 100% ID; Light green – 99% ID; Grey – 98% ID.

All the *A. baumannii* isolates carried *bla*$_{\text{OXA-23-like}}$ genes, confirmed to be associated with IS*Aba1* in CHI-32, CHI-34 and CHI-45-1, and so with a strong upstream promoter (Turton et al. 2006a; Corvec et al. 2007), by PCR (see Figure 3.2). Sequencing of the *bla*$_{\text{OXA-51-like}}$ gene PCR amplicons showed that all *A. baumannii* isolates contained *bla*$_{\text{OXA-69}}$, except for CHI-41 and CHI-44 (both also *bla*$_{\text{NDM-1}}$ negative), which contained *bla*$_{\text{OXA-66}}$ (see Table 3.2). PCR with primers designed to see if *bla*$_{\text{OXA-69}}$ was also linked to an IS*Aba1* element failed to produce a PCR product for CHI-32, CHI-34 and CHI-45-1. Later analysis of WGS contigs for these isolates showed that *bla*$_{\text{OXA-69}}$ was not linked to any IS. The *bla*$_{\text{OXA-69}}$ gene was present on large contigs (~160 kb for CHI-32 and ~220 kb for CHI-34 and CHI-45-1) with close identity to part of the chromosomal sequences of several *A. baumannii* isolates, including *A. baumannii* AYE. The β-lactamase is flanked by ORFs proposed to code for a phosphinotricin acetyltransferase and a suppressor of F exclusion of phage T7 (see Figure 3.3). However, the chromosomal AmpC type β-lactamase of *A. baumannii* has IS*Aba1* and its promoter sequence upstream of it, exactly as found in, for example, *A. baumannii* NCGM 237 (see Figure 3.4). IS*Aba1* has also been shown to be associated with increased expression of *bla*$_{\text{ADC}}$ genes and cephalosporin resistance (Heritier *et al.* 2006; Lopes and Amyes 2012). The AmpC β-lactamase in *A. baumannii* CHI-32 differs by 1 AA from ADC-30 (as in *A. baumannii* NCGM 237) and ADC-25.

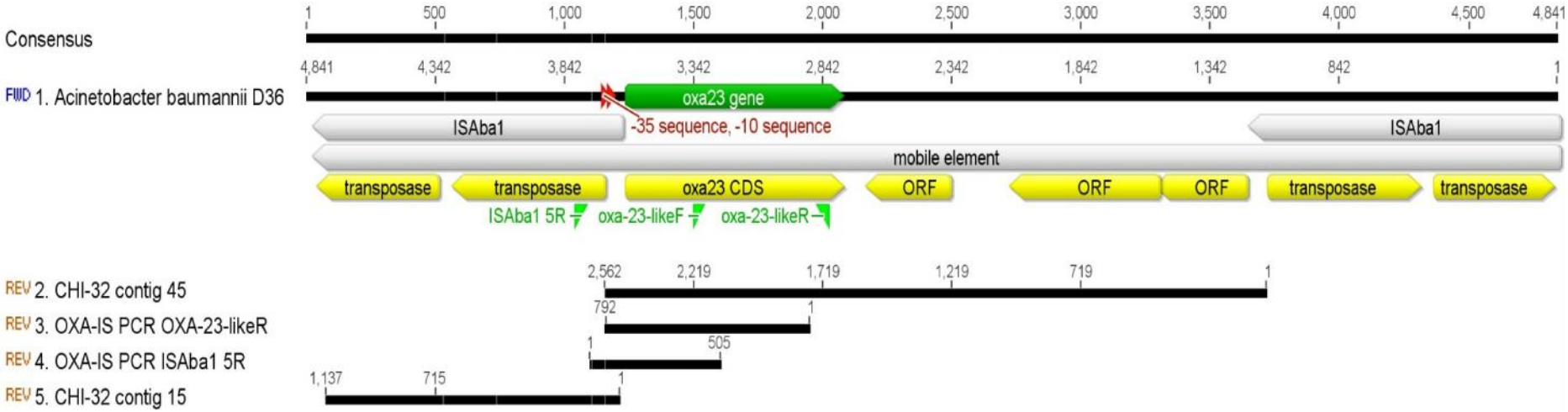


Figure 3.2 – Context of *bla*$_{OXA-23}$ in *A. baumannii* CHI-32 compared to *A. baumannii* D36 as a reference. *A. baumannii* CHI-32 results are shown as an example but identical contigs were present in *A. baumannii* CHI-34 and CHI-45-1. Image shows an assembly of two *A. baumannii* CHI-32 contigs and sequence products of PCR with the primers oxa-23-likeR and ISAba1 5R against a reference *bla*$_{OXA-23}$ context from *A. baumannii* D36 (Accession number JN107991). ORFs shown as yellow arrowed boxes. MGEs shown as grey arrowed boxes. The *bla*$_{OXA-23}$ gene shown as green arrowed box. The -35 and -10 sequence of the *bla*$_{OXA-23}$ promoter sequence are shown as red triangles. Primers used in PCR reactions and sequencing marked as light green triangles. Black lines show sequence with 100% identity across assembly. Bases with less than 100% identity appears as grey lines in the sequence. Image drawn in Geneious version 5.6.

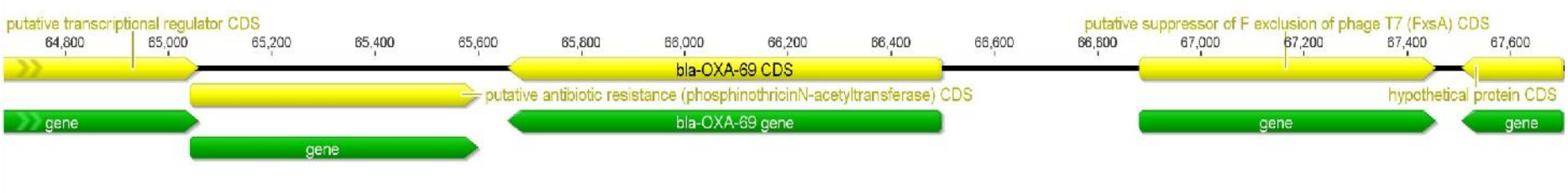


Figure 3.3 – Gene map of *bla*$_{OXA-69}$ context from *A. baumannii* CHI-32. Annotation transferred in Geneious from the identical sequence in *A. baumannii* AYE. Sequence also identical in *A. baumannii* CHI-34 and CHI-45-1. CDS yellow arrowed boxes, genes – green arrowed boxes. Numbers above sequence line denote the position in the complete contig 254 of WGS assembly of CHI-32.

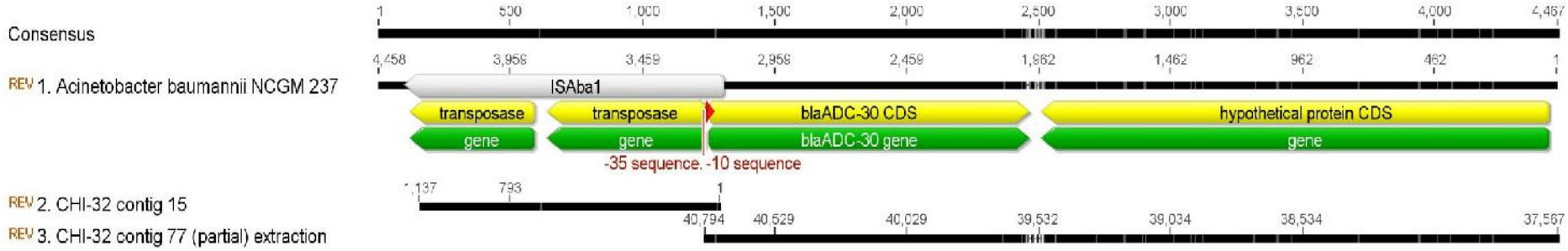


Figure 3.4 – Context of chromosomal *bla*$_{ADC-30-like}$ gene in *A. baumannii* CHI-32 compared to *A. baumannii* NCGM 237 as a reference. *A. baumannii* CHI-32 results are shown as an example but identical contigs were present in *A. baumannii* CHI-34 and CHI-45-1. Image shows an assembly of *A. baumannii* CHI-32 contigs against a reference AmpC context from *A. baumannii* NCGM 237 (Accession number AP013357). Labelling as for Figure 3.2 and 3.3.

Further AMR genes were detected in the WGS assemblies of CHI-32, CHI-34 and CHI-45-1. A summary of the results is shown in Table 3.2. As will be seen later in this chapter, most of the genes present in CHI-32 were present on the plasmid pNDM-32, which harboured *bla*$_{NDM-1}$. In CHI-34 and CHI-45-1 the genes are probably found predominantly on a plasmid similar to pNDM-32 and on a chromosomal resistance island which is truncated in CHI-32 (see later and Figure 3.19). Other than the β-lactamases already discussed the only exception to this in all of the isolates was *floR*. This is found on relatively small contigs, ranging in size from 2,534 bp in CHI-32 to 4,092 bp in CHI-45-1. In all cases *floR* is probably flanked by IS*1008* and an IS most closely related to IS*Aba19*. Although the same or related ISs were also present in the assembly of pNDM-32, the contig harbouring *floR* from CHI-32 was not demonstrated to be a part of this plasmid by PCR analysis. The location of these contigs has not been established for any of the isolates.

The only documented difference in the susceptibility profiles of CHI-32, CHI-34 and CHI-45-1 was in the aminoglycoside results. CHI-32 and 45-1 showed high-level resistance to all of the agents tested, while CHI-34 is susceptible to amikacin and has an MIC to tobramycin of 32 mg/L, which is well above the clinical resistance breakpoint of >4 mg/L, but significantly lower than the MICs in CHI-32 and CHI-45-1 of ≥ 256 mg/L. CHI-34 only contains one fairly broad spectrum enzyme, *aac(3) IId*, which has activity conferring resistance to tobramycin in addition to gentamicin. CHI-32 and CHI-45-1 also contains the 16S rRNA methyltransferase gene, *armA*, which would be expected to confer high-level resistance to amikacin as well as gentamicin and tobramycin.

3.3.2 MLST and PFGE typing

A. baumannii isolates were typed by the MLST method described by Turton et al. (Turton et al. 2007) and by pulsed field gel electrophoresis (PFGE) of *Apa*I digested genomic DNA. A summary of typing results is given in Table 3.3. All *A. baumannii* producing NDM-1 were within group II by MLST, which corresponds with CC1 (Turton et al. 2007; Diancourt et al. 2010). Later the full 7 loci MLST typing scheme for *A. baumannii* (Diancourt et al. 2010) was applied to the isolates CHI-32, CHI-34 and CHI-45-1 which had undergone WGS. This was consistent with the earlier results, with all three isolates being ST1 (part of CC1). CHI-32, CHI-34 and CHI-45-1 also had identical rMLST profiles to several strains in the rMLST database including the multi-drug resistant ST1 strain AYE. Phylogenetic analysis of the rMLST profile of CHI-40-1 later confirmed that this was an *Acinetobacter bereziniae* isolate (see Chapter 4). The two *bla*$_{\text{NDM-1}}$ negative isolates were within group I by the Turton MLST scheme, corresponding to CC2 (formerly global clone II) (Diancourt et al. 2010). *Apa*I profiles were similar but not identical for all group II *A. baumannii* isolates and differed substantially from group I isolates and *A. bereziniae* CHI-40-1 (see Figure 3.5).

Isolate	*bla*$_{NDM-1-like}$ PCR	*bla*$_{OXA-51-like}$ PCR	3 Locus MLST Type	7 Locus MLST type	rMLST	PFGE group
***A. baumannii* CHI-16**	*bla*$_{NDM-1}$	*bla*$_{OXA-69}$	group II	ND	ND	A
***A. baumannii* CHI-18**	*bla*$_{NDM-1}$	*bla*$_{OXA-69}$	group II	ND	ND	A
***A. baumannii* CHI-32**	*bla*$_{NDM-1}$	*bla*$_{OXA-69}$	group II	ST1	100% ID *A. baumannii* AYE	A
***A. baumannii* CHI-34**	*bla*$_{NDM-1}$	*bla*$_{OXA-69}$	group II	ST1	100% ID *A. baumannii* AYE	A
***A. bereziniae* 40-1**	*bla*$_{NDM-1}$	NEG	NA	NA	Groups with *A. bereziniae**	C
***A. baumannii* CHI-40-2**	*bla*$_{NDM-1}$	*bla*$_{OXA-69}$	group II	ND	ND	A
***A. baumannii* CHI-41**	NEG	*bla*$_{OXA-66}$	group I	ND	ND	B
***A. baumannii* CHI-44**	NEG	*bla*$_{OXA-66}$	group I	ND	ND	B
***A. baumannii* CHI-45-1**	*bla*$_{NDM-1}$	*bla*$_{OXA-69}$	group II	ST1	100% ID *A. baumannii* AYE	A

Table 3.3 – Results of *bla*$_{NDM-1}$ and *bla*$_{OXA-51-like}$ PCR and results of typing methods applied to study *Acinetobacter* spp. isolates. Subtypes of *bla*$_{NDM-1-like}$ and *bla*$_{OXA-51-like}$ genes were determined by sequencing of PCR products. See methods for details of different typing schemes. *See ML phylogenetic tree based on rMLST profiles of different *Acinetobacter* spp. presented in Chapter 4. Table cell colours represent: Green – *bla*$_{NDM-1}$ PCR positive; Red – PCR negative for *bla*$_{NDM-1}$ or *bla*$_{OXA-51-like}$; Blue – all typing results for PFGE group A isolates; Yellow – all typing results for PFGE group B isolates; Orange – all typing results for PFGE group C isolates.

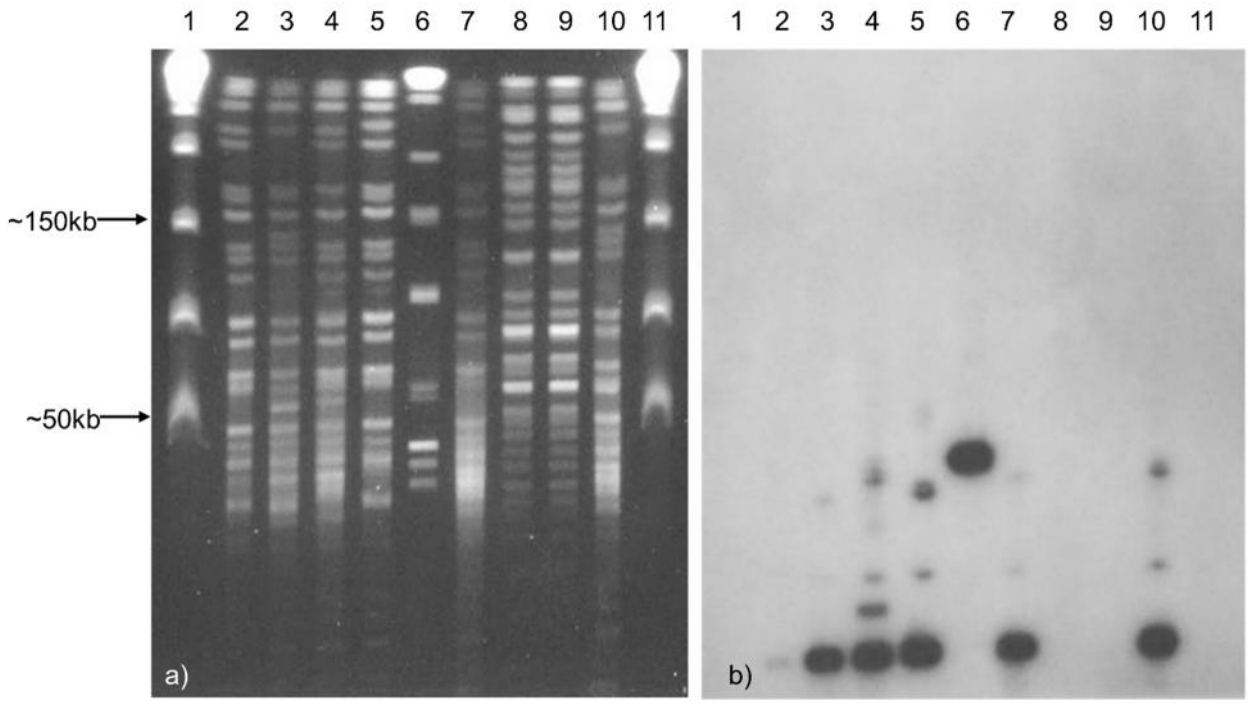


Figure 3.5 a) *Apa*I profile of *Acinetobacter* spp. isolates; b) Autoradiograph of a) directly labelled with *bla*$_{NDM-1}$. 1 λ ladder (~50-1000 kb); 2 CHI-16; 3 CHI-18; 4 CHI-32; 5 CHI-34; 6 CHI-40-1; 7 CHI-40-2; 8 CHI-41; 9 CHI-44; 10 CHI-45-1; 11 λ ladder

3.3.3 Immediate *bla*$_{NDM-1}$ contexts

Although the wider *bla*$_{NDM-1}$ contexts were later defined using WGS data, initially the local contexts were characterised using a primer walking strategy (see Appendix 3.3). As primer walking PCR results were consistent for all three isolates studied, PCR amplicons were only fully sequenced for CHI-45-1. The full Tn*125* structure normally associated with *bla*$_{NDM-1}$ was present; however, the IS*CR27* transposase gene contains an IS*15-Δ* insertion (99% nt sequence identity with IS*26*, see figure 3.6). Other than this insertion the context showed 99.9% identity with the sequence from *A. baumannii* 161/07 (Pfeifer et al. 2011), the only difference being 4

SNPs in the upstream IS*Aba125* transposase gene (nucleotide co-ordinates from HQ857107: 1,245G>A; 1,264C>T; 1,267C>T; 1,453A>C). Sequence analysis of PCR amplicons revealed that the fragment of IS*CR27* downstream of the IS*15-Δ* insertion is present both in its normal position and upstream of *bla*$_{NDM-1}$ (see figure 3.6).

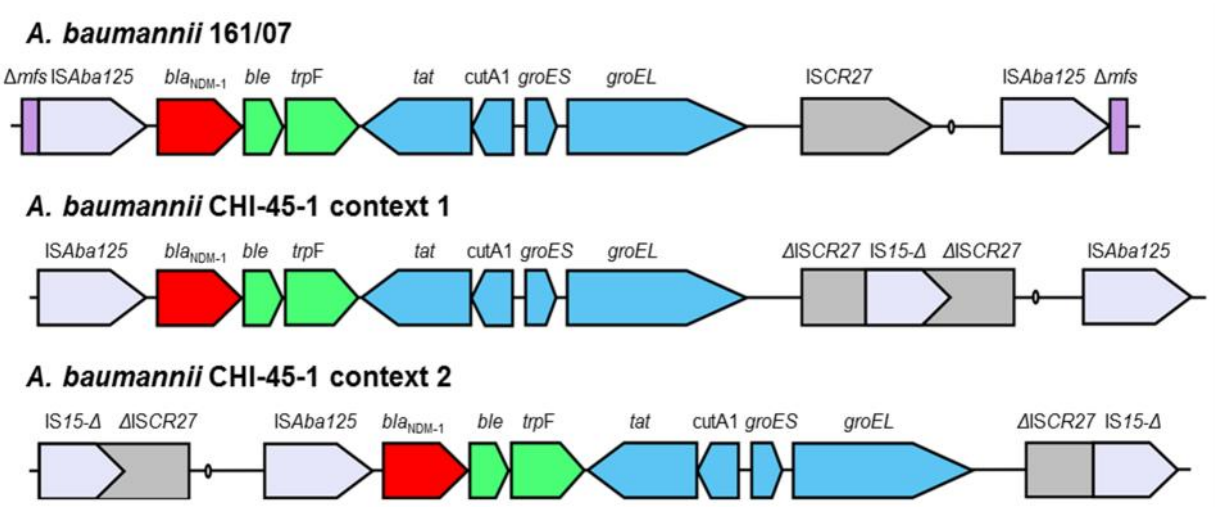


Figure 3.6 – Gene maps of the genetic context of *bla*$_{NDM-1}$ in *A. baumannii* CHI-45-1 compared to *A. baumannii* 161/07. ORFs are colour coded with the direction of transcription indicated by arrow heads, truncated remnants of ORFs are shown as rectangles. Red – *bla*$_{NDM-1}$; lime green – usually immediately downstream of *bla*$_{NDM-1}$; blue – from a common context in *Xanthomonas* and *Pseudoxanthomonas*; dark grey – IS*CR27* transposase; light grey – IS transposases, note transposases are labelled with IS name; *ble* – bleomycin resistance gene; *trpF* – phosphoribosylanthranilate isomerase gene; *tat* – twin-arginine translocation pathway signal sequence domain gene; *cutA1* – periplasmic divalent cation tolerance gene; *groES* – co-chaperonin gene; *groEL* – chaperonin gene; IS*CR27* – IS*CR27* transposase gene; *oriIS* – origin of insertion of IS*CR27*; *Δmfs* – major facilitator superfamily (MFS) metabolite/H+ symporter gene.

3.3.4 S1 plasmid analysis, restriction analysis and gene probing

PFGE gels of S1 digested genomic DNA from all isolates revealed that all of the *Acinetobacter* spp. isolates studied contained multiple plasmids. Probing of the S1 gel showed that *bla*$_{NDM-1}$ was on bands, ranging in size from ~45kb to ~500kb. For *A. baumannii* isolates, bands of increasing size, in intervals of approximately the size of the smallest band for each isolate, and reducing intensity were noted (see Figure 3.7). There are some exceptions to this pattern. For example, CHI-32 has a band of ~85 kb, a fainter band of ~170 kb and then a band of intermediate intensity at ~310 kb. Results of probing with IS*CR27*, IS*15- Δ* and IS*Aba125* showed that each sequence was also present on bands of similar size to that which *bla*$_{NDM-1}$ was located in *A. baumannii* isolates (see Figures 3.8-3.10). The size of the smallest *bla*$_{NDM-1}$ positive band, and thus the size of the interval between bands of larger size, differed between isolates. For example, in CHI-34 the smallest band was ~50 kb whilst in CHI-32 and CHI-45-1 it was ~85 kb. In addition in many cases the gene probes also hybridised with chromosomal bands. CHI-40-1 contained *bla*$_{NDM-1}$, IS*Aba125* and IS*CR27*, but not IS*15-Δ*, on bands of ~50 kb and ~250 kb. Results from this isolate are discussed in more detail in Chapter 4.

Gene probing of *Apa*I gels showed that *bla*$_{NDM-1}$ was present on bands of various sizes and intensities. The brightest bands were all of the same size and considerably smaller than the smallest unit of λ ladder size marker used. Results of probing *Not*I gels with *bla*$_{NDM-1}$ and IS*15-Δ* for CHI-32 and CHI-45-1 showed that these genes were both present on bands which had migrated slightly further than the smallest λ molecular weight marker (See Figure 3.11).

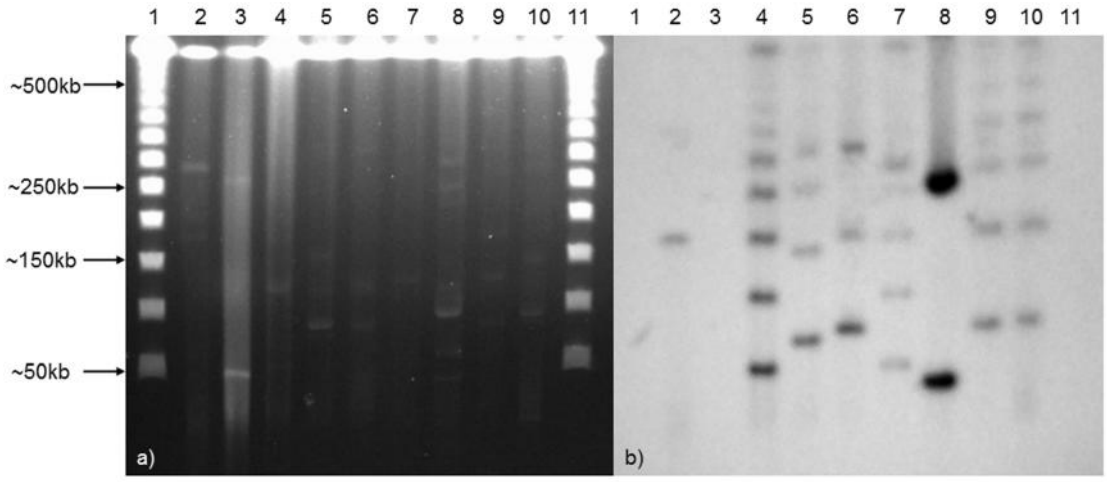


Figure 3.7 – a) S1 endonuclease digests of *Acinetobacter* spp. isolates; b) Autoradiograph directly labelled with *bla*$_{NDM-1}$. 1 – λ (~50-1000kb); 2 – *K. pneumoniae* 05-506 (NDM positive control); 3 – *E. coli* UAB190 (NDM negative control); 4 – CHI-16; 5 – CHI-18; 6 – CHI- 32; 7 CHI-34; 8 – CHI-40-1; 9 – CHI-40-2; 10 – CHI-45-1; 11 – λ

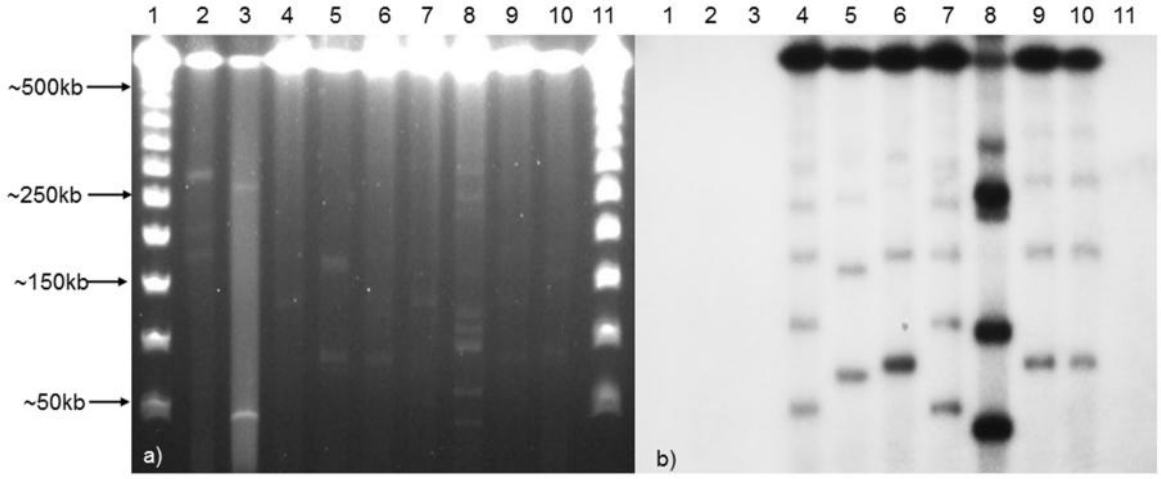


Figure 3.8 – a) S1 endonuclease digests of *Acinetobacter* spp. isolates; b) Autoradiograph of S1 gel directly labelled with IS*Aba125*. 1 – λ (~50-1000kb); 2 – *K. pneumoniae* 05-506; 3 – *E. coli* UAB190; 4 – CHI-16; 5 – CHI-18; 6 – CHI- 32; 7 CHI-34; 8 – CHI-40-1; 9 – CHI-40-2; 10 – CHI-45-1; 11 – λ

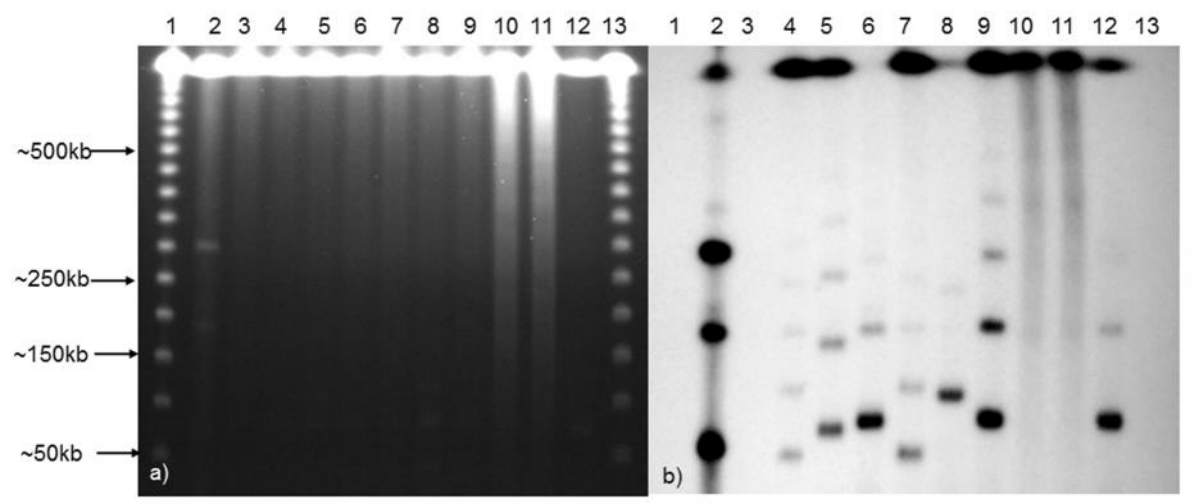


Figure 3.9 – a) S1 endonuclease digests of *Acinetobacter* spp. isolates; b) Autoradiograph of S1 gel directly labelled with IS*15-Δ*. 1 – λ (~50-1000kb); 2 – *K. pneumoniae* 05-506; 3 – *E. coli* UAB190; 4 CHI-16; 5 CHI-18; 6 CHI-32; 7 CHI-34; 8 CHI-40-1; 9 CHI-40-2; 10 CHI-41; 11 CHI-44; 12 CHI-45-1; 13 λ ladder.

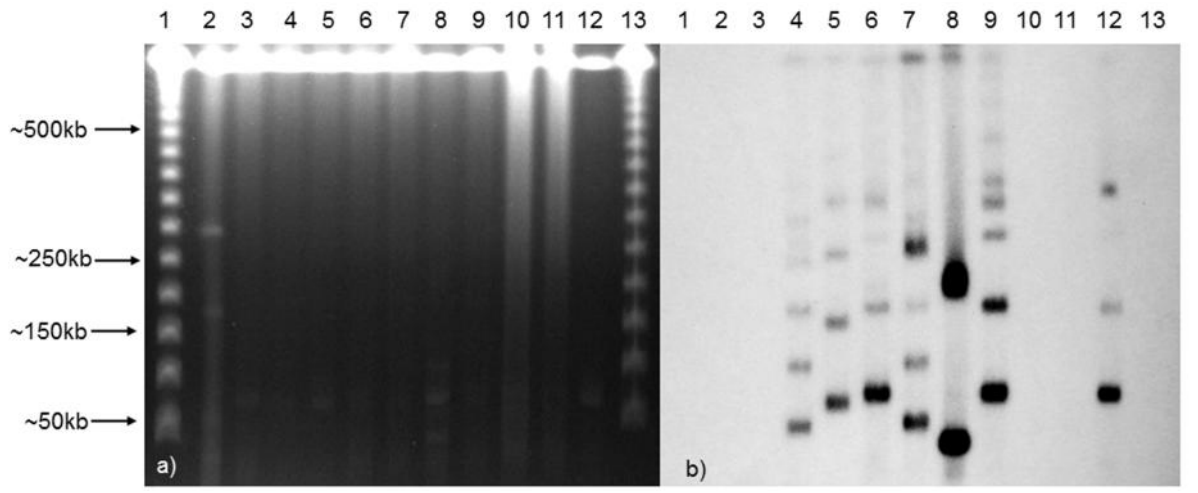


Figure 3.10 – a) S1 endonuclease digests of *Acinetobacter* spp. isolates; b) Autoradiograph of S1 gel directly labelled with IS*CR27*. 1 – λ (~50-1000kb); 2 – *K. pneumoniae* 05-506; 3 – *E. coli* UAB190; 4 CHI-16; 5 CHI-18; 6 CHI-32; 7 CHI-34; 8 CHI-40-1; 9 CHI-40-2; 10 CHI-41; 11 CHI-44; 12 CHI-45-1; 13 λ ladder.

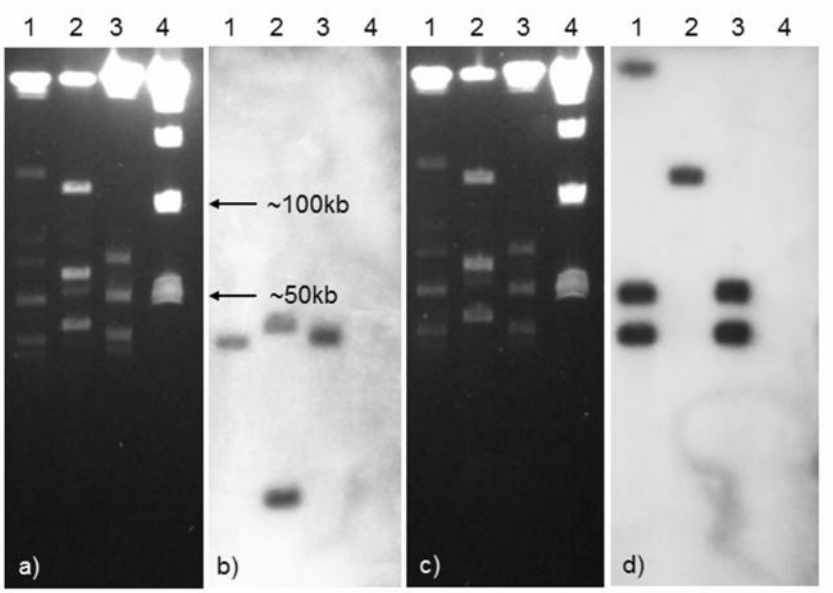


Figure 3.11 – a) and c) *Not*I restriction digests of NDM producing *Acinetobacter* spp. and controls; b) In gel hybridisation of a) with *bla*$_{NDM-1}$; d) in gel hybridisation of c) with IS*15-Δ*. 1 – CHI-45-1; 2 – CHI-40-1; 3 – CHI-32; 4 – λ (~50-1000kb).

3.3.5 Conjugation experiments

Mating experiments with *A. baumannii* CHI-32, CHI-34 and CHI-45-1, and *A. bereziniae* CHI-40-1 as donors and *E. coli* UAB190 and *Acinetobacter pittii* AG3528 as recipients were performed multiple times in broth and on solid media. No transconjugants were obtained using *A. baumannii* donors but were obtained using the CHI-40-1 donor in both recipient backgrounds. Results of these experiments are presented in more detail in Chapter 4.

3.3.6 Sequence of pNDM-32 from CHI-32 and comparison with related genetic contexts

Further analysis of the *bla*$_{NDM-1}$ positive *A. baumannii* isolates was performed on WGS assemblies of CHI-32, CHI-34 and CHI-45-1. Final assembly of the *bla*$_{NDM-1}$ plasmid with closure of contig gaps was finally only attempted for CHI-32 plasmid, designated pNDM-32 (LN833432). This isolate was chosen as the whole genome sequence of CHI-32 did not contain as many contigs which appeared to be linked to IS*15-Δ*, which was an immediate part of the *bla*$_{NDM-1}$ context, thus reducing the number of permutations that needed to be investigated by PCR and sequencing .

The final assembly of the plasmid pNDM-32 was 84,623 bp long, with a total GC% of 44.8 %, containing 108 ORFs (see Figure 3.12 and Appendix 3.4). These included 22 coding for IS transposases (a further 4 ORFs shown in 3.12 probably represent truncated and inactivated transposase derivatives), 8 coding for AMR determinants and 44 for hypothetical proteins of unknown function. The plasmid contained a sequence coding for a replicase protein identical to that from *A. baumannii* plasmid pWA3 (Accession number JQ241791, see Figure 3.13). Fu et al. designated the replicase of pWA3 *repAci10* and also detected the same replicase gene in a number of different *Acinetobacter* spp. harbouring *bla*$_{OXA-58}$ and their transformants (Fu *et al.* 2014). As in pWA3 next to the replicase is a gene coding for a partition protein, *parA*. However, no type IV secretion system or relaxase could be identified in the plasmid sequence.

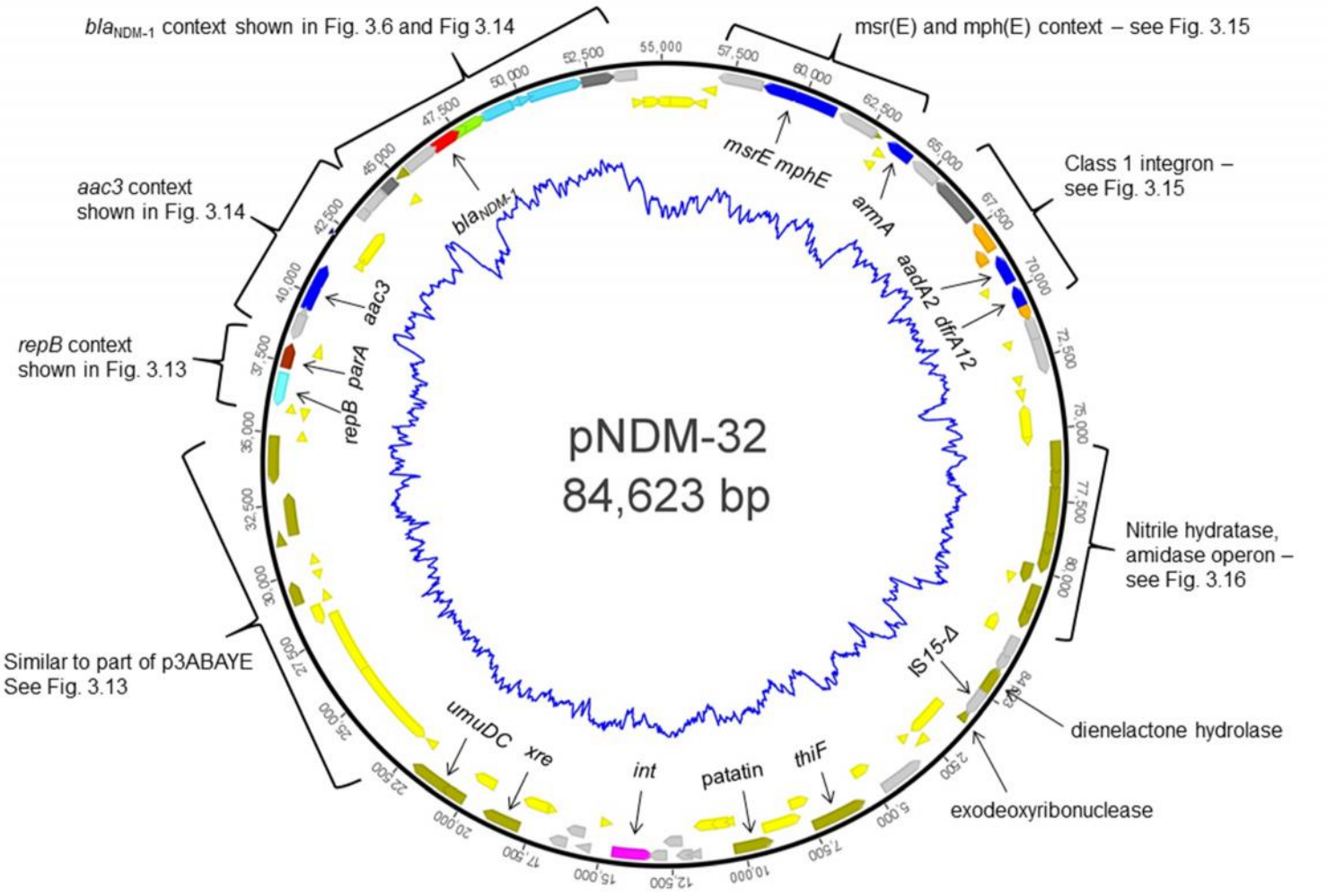


Figure 3.12 – Gene map of complete *A. baumannii* CHI-32 plasmid, pNDM-32 (LN833432). ORFs are colour coded as in Figure 3.6, with the direction of transcription indicated by arrowheads. In addition: blue – other AMR genes; light orange – genes from

conserved sequences of class 1 integrons; dark grey – IS*CR* elements; turquoise – plasmid replicase, *repB* dark brown – plasmid partition gene, *parA;* pink – phage like integrase, *int*; light brown – ORF proposed to code for proteins of known function; yellow – ORF proposed to code for hypothetical protein of unknown function. Gene names used where ORF sequence previously designated with a gene name. Where no known gene name, labels given are with an abbreviation of the proposed product of the ORF. As previously ISs are labelled with the name of the element but the figure shows the transposase ORF. In addition to annotations already given in Figure 3.6: *repB* – plasmid replicase gene; *parA* – plasmid partition gene; *aac3* (IId) – aminoglycoside resistance gene coding for aminoglycoside 3-N-acetyltransferase; *msrE* and *mphE* – macrolide resistance genes; *armA* – aminoglycoside resistance gene, coding for a 16S rRNA methyltransferase; *aadA2* – aminoglycoside resistance gene, coding for aminoglycoside *O*-nucleotydyltransferase; *dfrA12* – trimethoprim resistance gene, coding for dihydrofolate reductase; exodexoyribonuclease – putative exodexoyribonuclease VII large subunit, degrades single stranded DNA; dienelactone hydrolase – putative dienelactone hydrolase coding sequence; *thiF* – sulfur carrier adenylyltransferase gene; patatin – putative patatin like phospholipase; *int* – phage like integrase gene; *xre* – XRE-family transcriptional regulator; *umuD* – error prone DNA repair gene; *umuC* – error-prone, lesion bypass DNA polymerase V gene. Blue line on the inside of the sequence indicates GC content averaged over a sliding window of 180 residues.

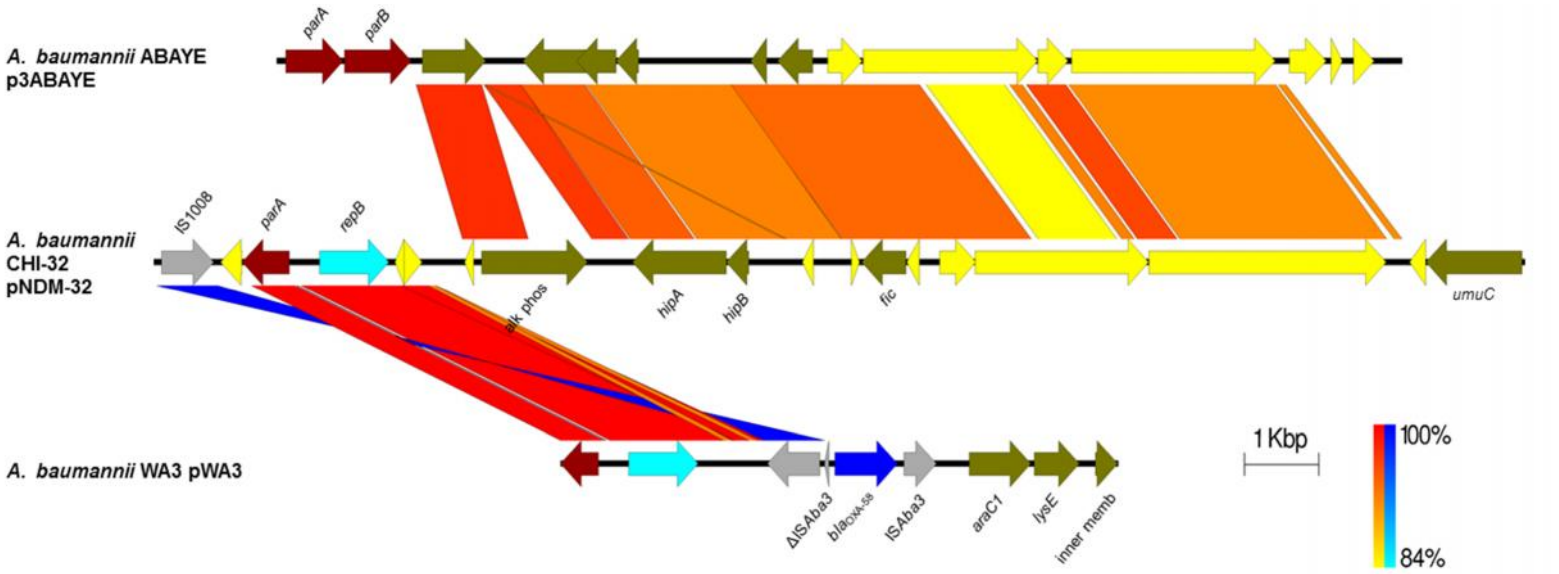


Figure 3.13 – Comparison of pNDM-32 *repB* and *hipA* contexts to pWA3 and p3ABYE, respectively. BLAST comparison run using WebAct. Figure drawn using Easyfig 2.1. Areas with BLAST hits in same orientation shown by bars between sequences ranging from red to yellow. BLAST hits for sequences in reverse orientation ranging from dark to light blue, key shows % ID of BLAST hit matching to bar colours. ORFs are colour coded and annotated as for Figure 3.6 and 3.12. In addition to annotations already given in Figure 3.13: *parB* – plasmid partition gene; alk phos – putative alkaline phosphatase gene; *hipA* – gene for HipA toxin; *hipB* – gene for transcriptional regulator/ antitoxin of HipA; *fic* – putative cell filamentation gene; *bla*$_{\text{OXA-58}}$ – OXA-58 β-lactamase gene; *araC1* – transcriptional regulator gene; *lysE* – threonine efflux gene; inner memb – putative inner membrane protein coding sequence.

Immediately upstream of this is an approximately 11.5 kb region with significant identity to part of plasmid p3ABAYE from *A. baumannii* AYE (Accession number CU459140, See Figure 3.13). Most of the ORFs in this region are proposed to code for hypothetical proteins of no known function but it also-contains an ORF proposed to code for the protein HipA. HipA forms part of a toxin-antitoxin system, with transcription controlled by HipB in models studied in *E. coli* and *Shewanella* spp. (Germain *et al.* 2013; Wen *et al.* 2014). A BLASTP search with HipA from pNDM-32 detected identity with three conserved domains of HipA; namely, couple_hipA, hipA_C, and hipA_N. A MAFFT alignment of the protein sequences of the putative HipA of pNDM-32 showed 19.3% identity with HipA from *E. coli* K12. Upstream of HipA in pNDM-32 is a sequence coding for a putative transcriptional regulator according to the RAST annotation. However, BLASTP identified a hipB type transcriptional regulator domain and the protein shared 20.8% ID with HipB from *E. coli* K12. This region also contains sequences coding for a putative protein involved in cell division, Fic, and an alkaline phosphatase.

The immediate *bla*$_{\text{NDM-1}}$ context is identical to that described for CHI-45-1, as predicted by the results of the earlier PCR analysis of CHI-32. Assembly of pNDM-32 was only possible with the CHI-45-1 context B from Figure 3.4, in which a fragment of IS*CR27* has been moved upstream of *bla*$_{\text{NDM-1}}$. The location of the alternative context (CHI-45-1 context A in Figure 3.4) was not formally confirmed. With the exception of the contigs harbouring *bla*$_{\text{NDM-1}}$ and the 3' fragment of IS*CR27*, the only contigs which were identified by BLAST analysis to be linked to IS*Aba125* probably represented part of the *A. baumannii* chromosome, in that they had lower coverage than the plasmid contigs and close identity to chromosomal contexts from other *A. baumannii* isolates.

In pNDM-32 the IS*15-Δ* element upstream of *bla*$_{NDM-1}$ has interrupted an IS*Cfr1* insertion sequence. Upstream of this are several coding sequences for hypothetical proteins, a putative tunicamycin resistance protein and an *aac3* gene coding for an aminoglycoside N(3')-acetyltransferase III. An identical context is present in several publicly available plasmid sequences from Enterobacteriaceae and *Acinetobacter* spp, some of which also contain *bla*$_{NDM-1}$. An example is given in Figure 3.14 of one of these plasmid sequences from *E. coli*, pNDM102337 (Accession number JF714412), which is most similar to the context in pNDM-32.

A second region containing AMR genes also has close identity to sequences from several plasmids in Enterobacteriaceae, including some harbouring *bla*$_{NDM-1}$, and from both plasmid and chromosomal *A. baumannii* sequences. A comparison with one of the most closely related contexts, from the *C. freundii* plasmid pCTX-M3 (Accession number AF550415) is shown in Figure 3.15. This region contains a class 1 integron, although in pNDM-32 the integrase has become truncated, with an *aadA2* gene, coding for an aminoglycoside O-adenylyltransferase, as a gene cassette. The 3' conserved sequence contains the usual *qacEΔ1* and *sul1* genes, and an IS*CR1*, as seen in complex class 1 integrons. Downstream of the integron there are two ISs, an *armA* gene, coding for a ribosomal RNA methyltransferase responsible for broad spectrum aminoglycoside resistance, and the genes *mphE* and *msrE* which are responsible for macrolide resistance.

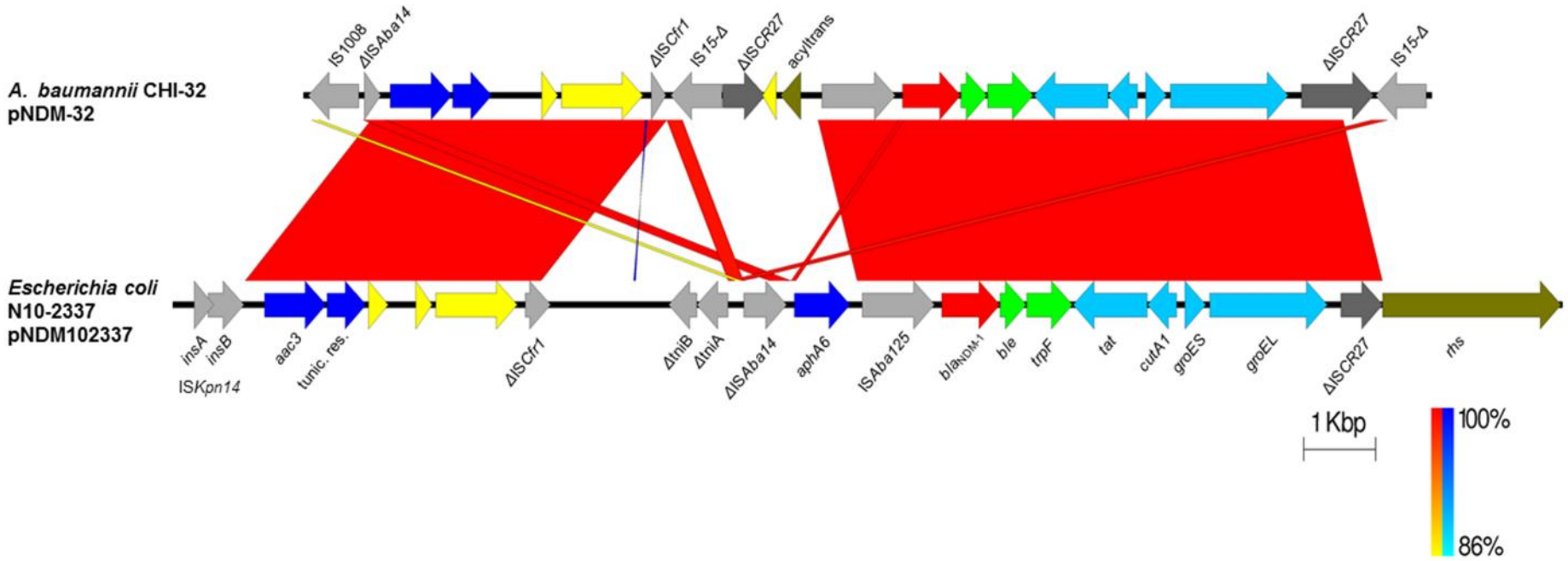


Figure 3.14 – Comparison of *aac3* and *bla*$_{NDM-1}$ contexts from pNDM-32 and pNDM102337. BLAST comparison run using WebAct and shown as in Figure 3.13. Figure drawn using Easyfig 2.1. ORFs are colour coded and annotated as for Figure 3.6 and 3.13. For ISs with multiple ORFs both gene names associated with the ORF and full IS name are given. In addition to annotations already given in Figures 3.6 and 3.13: acyltrans – putative acyltransferase gene; tunic. res. – putative tunicamycin resistance gene; *aphA6* – aminoglycoside resistance gene coding for aminoglycoside O-phosphotransferase; *rhs* – rearrangement hotspot gene (widely distributed GNB but of uncertain function).

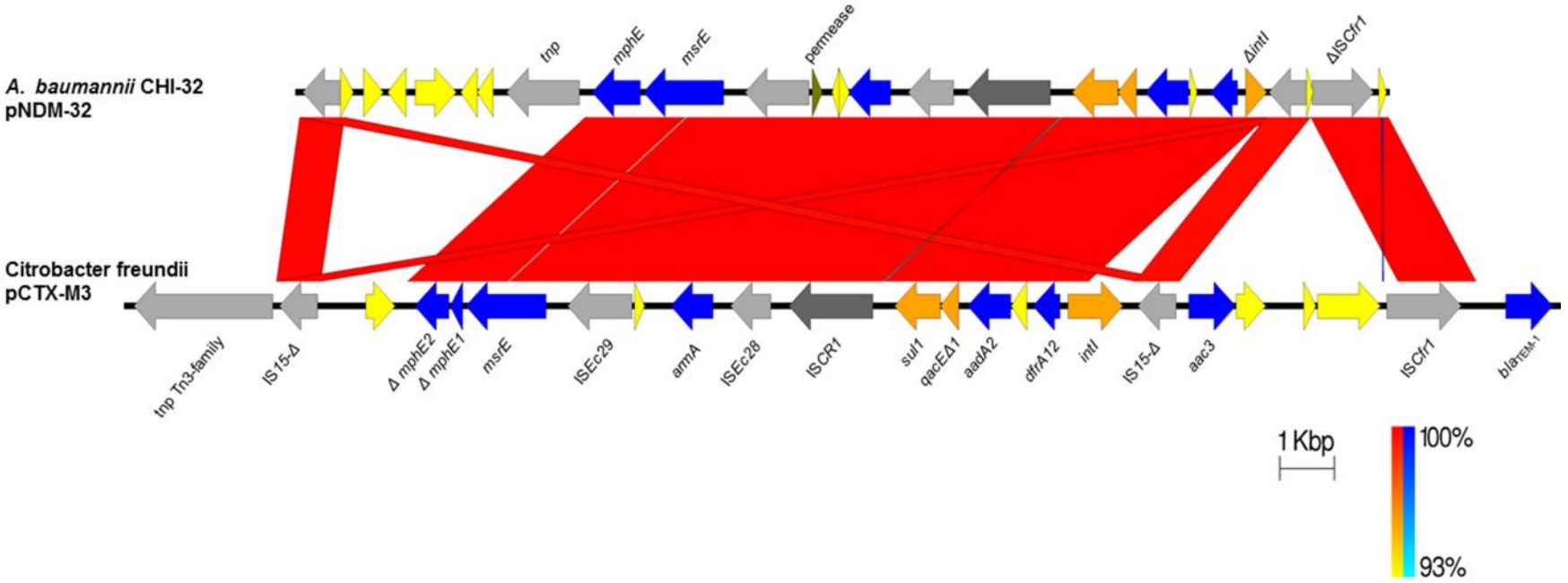


Figure 3.15 – Comparison of class 1 integron, *armA* and macrolide resistance context from pNDM-32 and pCTX-M3. BLAST comparison run using WebAct and shown as in Figure 3.13. Figure drawn using Easyfig 2.1. ORFs are colour coded and annotated as for Figure 3.6 and 3.12. In addition to annotations already given in Figures 3.6 and 3.12: *tnp* – putative transposase, permease – putative permease gene; *Δint* – truncated class 1 integron integrase gene; ΔIS*Cfr1* – truncated IS*Cfr* transposase; *tnp* Tn*3*-family – Tn3-family transposase; Δ*mphE1*/ *E2* – truncated derivatives of macrolide phosphotransferase gene; *sul1* – dihydropteroate synthase gene mediating sulphonamide resistance; *qacEΔ1* – multidrug resistance exporter gene; *bla*$_{TEM-1}$ – TEM-1 β-lactamase gene.

In addition pNDM-32 harbours a cluster of genes, probably containing a cobalt-type nitrile hydratase operon and a gene coding for an amidase. This 10 gene cluster spans 7,792 bp and has close sequence identity with several similar gene clusters found in *K. oxytoca* (e.g. 98.8% ID with the sequence *from K. oxytoca* E718, accession CP003683, see Figure 3.16). Although similar sequences can be identified in many species of bacteria the sequence in pNDM-32 has much closer identity with that in *K. oxytoca* and the gene cluster has a GC content of 59.8%, considerably higher than the average GC content of pNDM-32 and closer to that expected in *K. oxytoca* (the GC% of the chromosome of *K. oxytoca* KCTC 1686 is 56.0%, while that of the full *A. baumannii* CHI-32 assembly is 39%). BLAST searches based on the protein sequences of the α and β subunits of the nitrile hydratase targeted at *Acinetobacter* genus refseq proteins revealed homologues in several species but not *A. baumannii*. BLASTX searches of *A. baumannii* CHI-32 contigs failed to detect any other homologues of the nitrile hydratase α or β subunits.

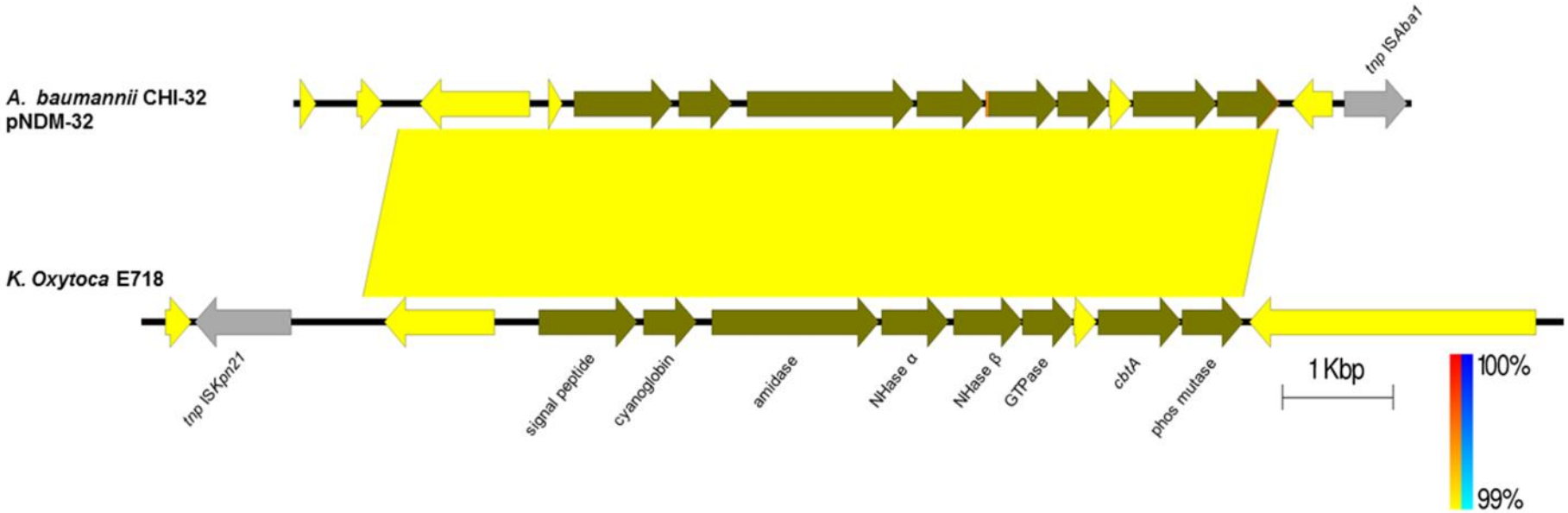


Figure 3.16 – Comparison of Nitrile hydratase/ amidase operon from pNDM-32 and the chromosome of *K. oxytoca* E718. BLAST comparison run using WebAct and shown as in Figure 3.13. Figure drawn using Easyfig 2.1. ORFs are colour coded and annotated as for Figure 3.6 and 3.12. In addition to annotations already given in Figures 3.6 and 3.12: *tnp* – transposase coding sequences, cyanoglobin – putative haemoglobin-like gene, possibly involved in non-haem co-factor binding; amidase – see main text; NHase α and β – cobalt containing nitrile hydratase genes, α and β subunits (see main text); GTPase – putative metal chaperone involved in NHase activation; *cbtA* – putative cobalt transporter gene; phos mutase – putative phosphoglycerate mutase gene.

A region of pNDM-32 of ~23 kb is not shown in any of the comparison figures, although large sections showed substantial identity with *Acinetobacter* spp. whole genome sequence contigs, for example *A. baumannii* BZICU-2 scaffold25_3 (Accession NZ_ALOH01000133.1) and *A. baumannii* Naval-57 contigs 7180000008033 and 7180000008032 (Accession numbers NZ_AMFP01000069.1 and NZ_AMFP01000016.1). This region contains an ORF coding for a putative P4-type phage like integrase with substantial identity to proteins conserved in *Acinetobacter* spp. As well as ORFs coding for several transposases and hypothetical proteins, it also contained ORFs proposed to code for a dienelactone hydrolase, an exodexoyribonuclease VII large subunit, a sulfur carrier adenylyltransferase, a patatin like phospholipase, an XRE-family transcriptional regulator, an error prone DNA repair protein and an error-prone, lesion bypass DNA polymerase V.

3.3.7 Contigs with identity to pNDM-32 in CHI-34 and CHI-45-1

The complete sequences of the plasmids in CHI-34 and CHI-45-1 were not confirmed as for pNDM-32. Equivalent contigs to those found in CHI-32 were found in both CHI-34 and CHI-45-1 (See Figure 3.17). However, CHI-34 lacked a large region found in pNDM-32 containing the class-1-integron harbouring *aadA2* and *dfrA12*, the *mphE-msrE* context and the sequences coding for the putative NHase and amidase proteins. The concatenated sequences of the pNDM-32 related contigs in CHI-34 came to a combined length of 53,827 bp and those in CHI-45-1 came to 83,054 bp.

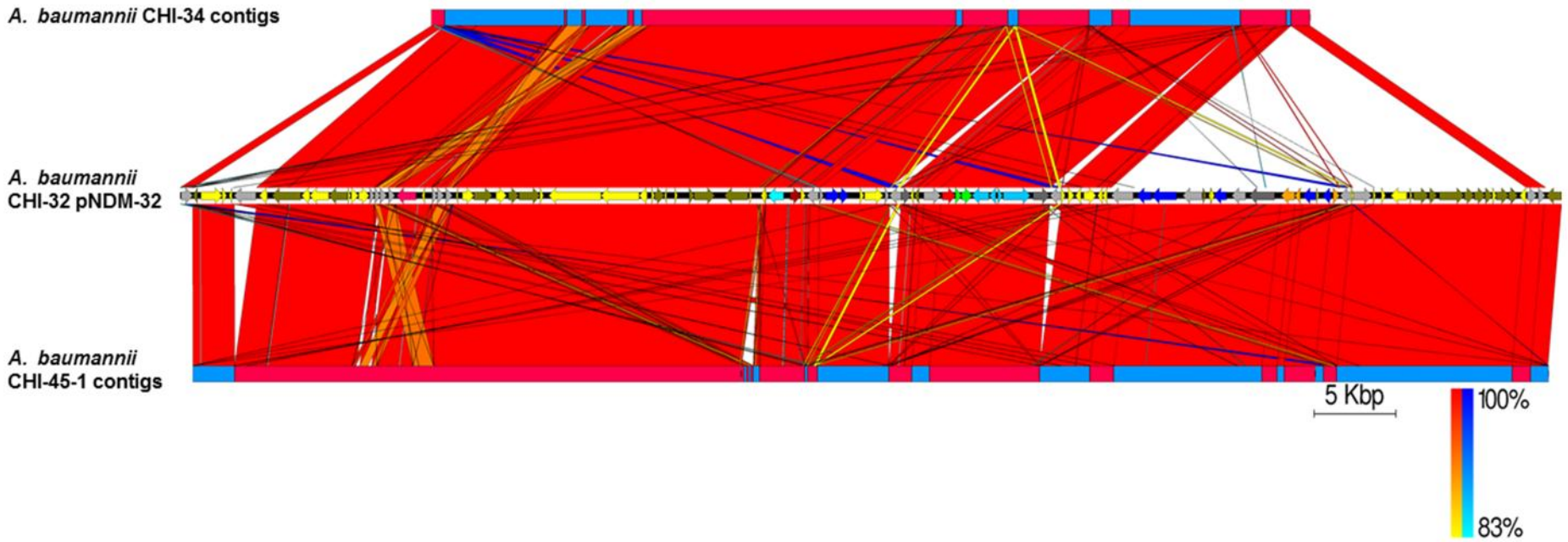


Figure 3.17 – ACT comparison of contigs from *A. baumannii* CHI-34 and CHI-32 which have substantial regions with close identity to pNDM-32 from *A. baumannii* CHI-32. BLAST comparison run using WebAct and shown as in Figure 3.13. Contigs with identity initially identified by BLAST searches. Contigs larger than the size of pNDM-32, small areas of identity < 100 bp and/or < 90% identity were not included in the ACT comparison. Figure drawn in EasyFig 2.1. Contigs shown as alternate red or blue rectangles on sequence line in ACT comparison.

3.3.8 Contigs with identity to AbaR3 in CHI-34 and CHI-45-1

The resistance genes identified in the WGS assemblies of CHI-34 and CHI-45-1 but absent in CHI-32 were found to be clustered together on a small number of contigs. These contigs were found to share 99-100% identity to regions of an *A. baumannii* resistance island, Aba3R, present in *A. baumannii* A85. In these two isolates there were contigs with close identity to the entire *A. baumannii* A85 AbaR3. Figure 3.18 shows the contigs from *A* CHI-34, with closest identity to AbaR3, mapped to the *A. baumannii* A85 reference sequence (Accession KC118540).

AbaR3 is a ~63 kb mobile element found interrupting the *comM* gene, which is thought to code for a bacterial competence protein. The AMR genes are located within a ~26 kb region. At the 5' end of this are the tetracycline resistance gene and its regulator gene, *tetM* and *tetR*. Immediately downstream are a gene coding for a possible multidrug transporter superfamily protein and the chloramphenicol resistance gene, *catA1*. The $bla_{\text{TEM-1}}$ β-lactamase gene is found as part of a truncated Tn*1* and the aminoglycoside resistance gene, *aphA1*, is flanked by two copies of IS*26*/ IS*15-Δ*. Two other aminoglycoside resistance genes, *aac(3)-Ia* and *aadA1*, are present as gene cassettes within a class-1-integron. In addition AbaR3 contains arsenic and mercury resistance operons and numerous other ISs and Tns. Tn*6108*, carries a copper/ zinc/ cobalt exporter gene and its regulator. Duplicates of this Tn bound the multiple antibiotic resistance region. Analysis of *A. baumannii* CHI-32 showed that contigs are present with identity to the AbaR3 sequence, excluding the region between the two copies of Tn*6108* (see Figure 3.19).

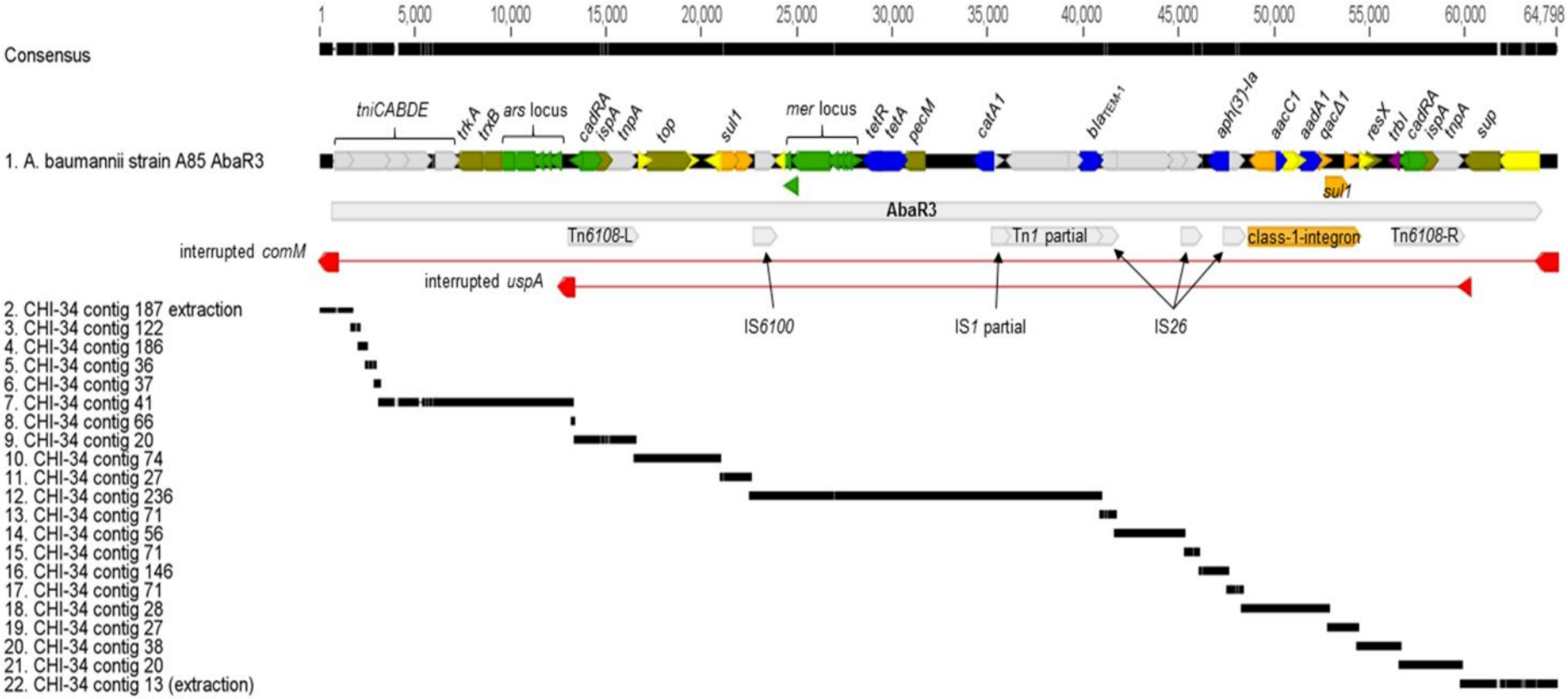


Figure 3.18 – *A. baumannii* CHI-34 contigs mapped to the multi-drug resistance genomic island AbaR3 from *A. baumannii* A85 (Accession KC118540). Annotation is as for Figure 3.6 and Figure 3.12, except that genes interrupted by the insertion AbaR3 and the multi-drug resistance region are shown in red. Note that contigs 27 and 71 appear multiple times in the assembly. In addition to ORF annotated in earlier figures: *comM* – bacterial competence gene; *usp* – universal stress protein gene; *trkA* –

putative flavoprotein involved in K+ transport gene; *tniCADBE* – genes predicted to code for transposition proteins; *trxB* – putative thioredoxin reductase gene; *ars* locus – arsenical resistance gene locus; *cadRA* – transcriptional regulator and copper/ zinc/ cadmium efflux system genes; *ispA* – lipoprotein signal peptidase gene; *top* – DNA topoisomerase I gene; *mer* locus – mercury resistance gene locus; *tetRA* – transcriptional regulator and tetracycline resistance genes; *pecM* – multidrug transporter superfamily gene; *catA1* – chloramphenicol resistance gene; *aph(3')-Ia* – aminoglycoside resistance gene, coding for aminoglycoside *O*-phosphotransferase; *resX* – resolvase/ invertase gene; *trbI* – conjugal transfer gene; *sup* – sulphate permease gene.

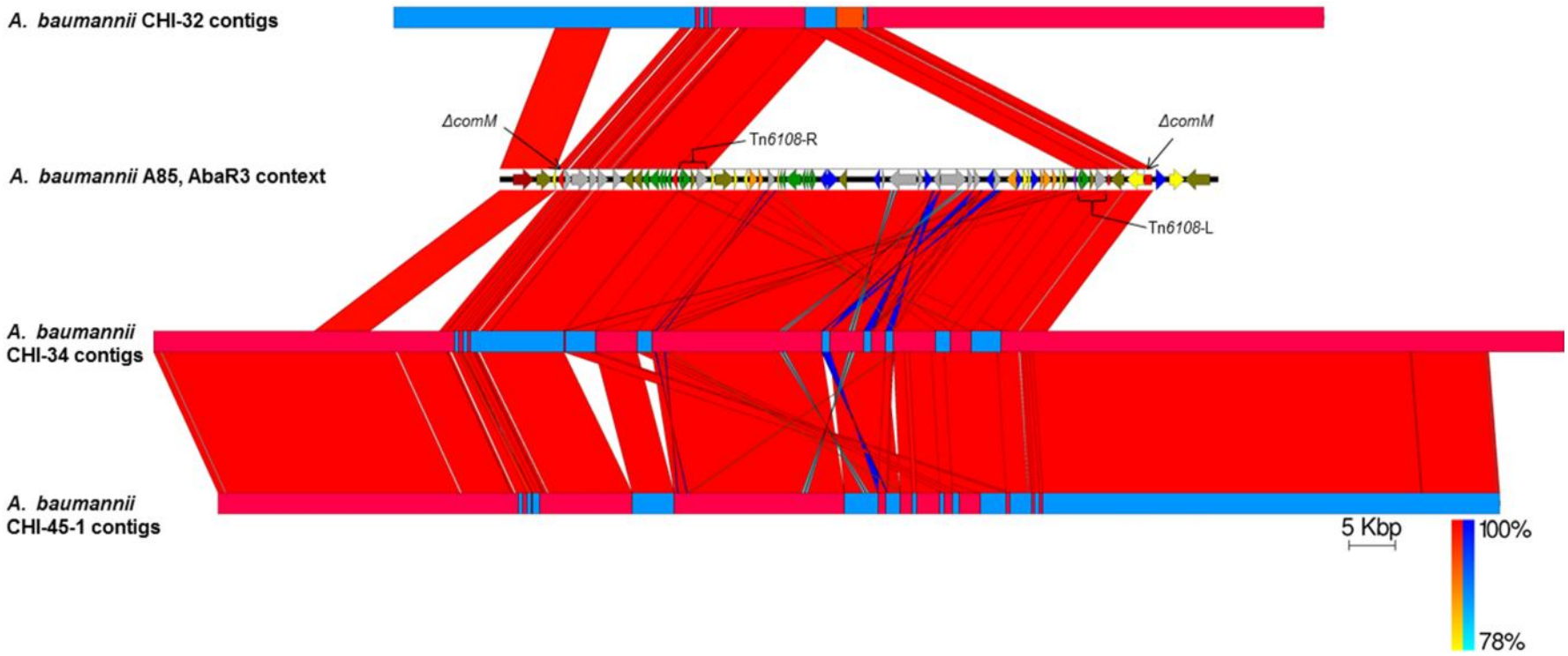


Figure 3.19 – ACT comparison of contigs from *A. baumannii* CHI-32, CHI-34 and CHI-45-1 with AbaR3 from *A. baumannii* A85. BLAST comparison run using WebAct and shown as in Figure 3.13. Contigs with identity initially identified by BLAST searches. Contigs with closest identity to *A. baumannii* A85 AbaR3 context shown. For some regions similar contigs with lower identity also identified. Figure drawn in EasyFig 2.1. Contigs shown as alternate red or blue rectangles on sequence line in ACT comparison. *A. baumannii* A85 annotations as shown in Figure 3.18. Note that the region absent in *A. baumannii* CHI-32 flanked by the two copies of Tn*6108*.

3.4 Discussion

The *A. baumannii* isolates analysed in this study are the earliest found to produce NDM-1, having been initially identified in 2005 (Berrazeg et al. 2014; Jones et al. 2014a). This is the first time that genetic contexts from *A. baumannii* isolates from the Indian subcontinent have been analysed in detail. That these clinical isolates of NPAB were clonally related demonstrates the potential for $bla_{\text{NDM-1}}$ establishing itself in successful strain backgrounds capable of disseminating in the hospital environment and further compromising therapeutic options in the treatment of significant bacterial pathogens. Furthermore with the full genome sequence it was shown that the strain involved was not only of the globally successful ST1 strain but that it also had an identical rMLST profile to other MDR ST1 isolates like *A. baumannii* AYE. Although $bla_{\text{NDM-1}}$ producing ST1 strains have been previously described in Switzerland (with a proposed Balkan origin) and Algeria, the majority of isolates, for which ST types have been determined, internationally are ST25 and ST85 for NDM-1 producers and ST103 for NDM-2 producers (Espinal et al. 2011; Bonnin et al. 2012b; Decousser et al. 2013; Bakour et al. 2014). This may reflect differences in epidemiology of NDM producers locally, regionally or a temporal shift, since the isolates described in this chapter were first identified several years prior to most of the other isolates studied.

In the present study no comparison of SNPs in the core genomes of these isolates was undertaken, which would have offered the highest level of discrimination between strains. Such an analysis, however, would have been most informative if more isolates had been sequenced and if further information were available with regards to when and where these isolates were obtained, in order to potentially track transmission events. Other WGS studies have shown that core genomes, excluding

regions of high recombination, from common STs or CCs are very closely related in *A. baumannii*, despite the inclusion of some isolates acquired over several years and from different geographical locations (Adams *et al.* 2008; Adams et al. 2010; Wright et al. 2014). However, even closely phylogenetically related *A. baumannii* isolates can exhibit significant genetic and phenotypic differences between one another, as a result of regions of apparently high recombination and differences in gene content. The latter is probably largely as a result of differences in MGEs present and IS mediated deletion events (Snitkin et al. 2011; Wright et al. 2014). The finding that the isolates which underwent WGS in this study had identical rMLST profile to one another, and to other ST1 isolates but had marked differences in the number of AMR genes present and the size of their MGEs is consistent with the more in depth analysis conducted in these other studies.

In the present study clinical data on the patients infected with NDM producing *Acinetobacter* spp. are lacking, so it is not possible to assess their clinical significance. However, it is of note that three of the isolates were from blood samples, suggesting that they were most likely to be causing significant infections (although contamination or transient bacteraemia cannot be excluded) and that the acquisition of *bla*$_{\text{NDM-1}}$ has probably not significantly attenuated the pathogenic potential of these organisms.

In these NPAB isolates several factors suggest that there is a degree of redundancy in the AMR genes present. Therefore the phenotypic and clinical significance of the acquisition of *bla*$_{\text{NDM-1}}$ and the MGEs associated with it in *A. baumannii* may be limited. MDR, including carbapenem resistance, is well recognised in *A. baumannii*, without the need for *bla*$_{\text{NDM-1}}$ (Higgins et al. 2010; Zarrilli et al. 2013). The *bla*$_{\text{NDM-1}}$ negative isolates obtained in this study had susceptibility

profiles similar to the NPAB isolates. All of the isolates analysed in detail had *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{ADC-30-like}}$ genes associated with IS*Aba1*. These genes, associated with a strong promoter from IS*Aba1*, are likely to have been sufficient to result in clinically relevant resistance to all available β-lactams in *A. baumannii* (Turton et al. 2006a; Lopes and Amyes 2012; Villalon *et al.* 2013). However, factors effecting membrane permeability or drug efflux which may also be important to the β-lactam resistance profile (Vila et al. 2007) have not been investigated. These isolates all also had multiple aminoglycoside resistance genes, many of which had relatively limited, and similar, predicted spectra of activity (Ramirez and Tolmasky 2010). The presence of *armA* would be expected to be of phenotypic significance, since it has far broader activity (Wachino and Arakawa 2012). In its absence CHI-34 was susceptible to amikacin whereas CHI-32 and CHI-45-1 were resistant.

The findings with regards to the local *bla*$_{\text{NDM-1}}$ context, and the year of isolation of these organisms, are compatible with the hypothesis that *bla*$_{\text{NDM-1}}$ could have disseminated from *Acinetobacter* to Enterobacteriaceae in South Asia. However, they do not provide strong evidence for this having occurred. In these isolates Tn*125* has been disrupted by IS*15-Δ* (similar to IS*26*) and subsequent re-arrangement, possibly resulting from homologous recombination based around IS*Aba125*, has resulted in *bla*$_{\text{NDM-1}}$ being within an IS*15-Δ* composite Tn, which could potentially mobilise *bla*$_{\text{NDM-1}}$. Otherwise the genes usually found on Tn*125* are conserved and so the genetic context is compatible with being the progenitor of *bla*$_{\text{NDM-1}}$ contexts in many of the Enterobacteriaceae for which sequences are available (Partridge and Iredell 2012).

The appearance of the S1 gels hybridised with *bla*$_{\text{NDM-1}}$, IS*CR27*, IS*Aba125* and IS*15-Δ* were unusual since all were present on multiple bands in the NPAB

isolates, with the bands spaced at regular intervals in most cases. It was hypothesised that this is likely to represent concatemers of the plasmids containing *bla*$_{NDM-1}$. Concatemer formation can occur for a number of reasons and is part of the transposition process for IS*6* family elements like IS*15-Δ* (Mahillon and Chandler 1998), of which there are 4 copies in the assembly of pNDM-32, including the 2 which flank the immediate context of *bla*$_{NDM-1}$. It is possible that the *A. baumannii* strains studied were recombination deficient resulting in failure to resolve concatemers (Lambert *et al.* 1994; Mahillon and Chandler 1998). However, various studies suggest that a significant amount of recombination often occurs in *A. baumannii*.

Alternatively there could have been a failure to resolve replicative concatemers because of some feature of the plasmid, the bacterial strain or the growth conditions (Summers 1998; Sengupta and Austin 2011; Pinto *et al.* 2012). For some plasmids it is reported that in certain genetic backgrounds or during bacteriophage infection plasmid replication can proceed with linear concatemers of the plasmid replicon (Viret *et al.* 1991). This can result in failure of plasmid partition leading to the plasmid loss in some daughter cells (Viret et al. 1991; Sengupta and Austin 2011). Stability was not formally analysed for these isolates and for general subculture of all of the *bla*$_{NDM-1}$ positive isolates investigated in this thesis antimicrobial selection pressure was maintained to avoid plasmid loss. Thus, it is possible that without this expedient stability would have been poor. It should also be noted that the submitting laboratory in India had found CHI-41 and CHI-44, the two ST2 isolates, to be *bla*$_{NDM-1}$ positive, whilst in multiple PCR and probing experiments performed for this thesis, these isolates were consistently negative.

As *bla*$_{NDM-1}$ was located on plasmids in these isolates it was initially thought that these might have mobile potential. However, mating experiments failed to demonstrate conjugation, at least into an *E. coli* or *A. pittii* background. Furthermore the assembly of pNDM-32 did not demonstrate any known plasmid relaxase or transfer genes, suggesting that the plasmids harbouring *bla*$_{NDM-1}$ are non-mobilizable. However, in this regard several points need to be considered. Firstly it is possible that amongst the ORFs present in the assembly of pNDM-32 there are sequences coding for previously uncharacterised relaxase and conjugative transfer proteins. Despite the increasing number of sequences of *Acinetobacter* plasmids published (Bertini et al. 2010; Fondi *et al.* 2010; Carattoli 2013) relatively little research has focused on plasmid function in *Acinetobacter* spp. The plasmid pNDM-40-1 from CHI-40-1 (which is discussed in detail in Chapter 4) contains no identifiable replicase sequence, despite the fact that a replicase is a prerequisite for a functional plasmid and that there is quite good evidence that pNDM-40-1 and related plasmids are autonomously replicating plasmids with conjugative potential.

Secondly, it must be considered that the assembly could be in error. The final assembly of pNDM-32 was particularly challenging because of the large number of ISs present and the fact that results of in gel hybridisation with *bla*$_{NDM-1}$ of S1 and *Apa*I gels suggest that the *bla*$_{NDM-1}$ context and the plasmids may exist in several forms. These results and the results of the probed *Not*I gels are; however, consistent with the assembly representing the predominant form of the *bla*$_{NDM-1}$ context and the PCRs and sequencing experiments (confirming all proposed contig links to be present) suggest that the assembly is accurate. However, a substantial amount of additional experimental work would be required to exclude all other potential

permutations of the plasmid sequence and other potential *bla*$_{NDM-1}$ contexts on the chromosome.

Finally, even if pNDM-32 is non-mobilizable it is possible that the plasmid could still be spread to new hosts by transformation or transduction, although this is predicted to be more likely for smaller plasmids (Viret et al. 1991; Smillie et al. 2010). Alternatively the IS*15-Δ* composite Tn could transfer *bla*$_{NDM-1}$ to new MGE by transposition (Lambert et al. 1994) or part of the plasmid sequence could be introduced to a different replicon by homologous recombination events (Darmon and Leach 2014). It has been proposed that *A. baumannii* may be naturally competent, as one means to explain the species apparent ability to readily acquire resistance genes and the large number of ORFs which show evidence of recombination (Traglia et al. 2014; Wright et al. 2014). As discussed in Introduction section 1.5.1 there is, however, limited *in vitro* evidence that *A. baumannii* are naturally transformable, other than rare strains (Traglia et al. 2014), although recent studies suggest that transformation may be more readily demonstrable under appropriate experimental conditions (Rumbo et al. 2011; Wilharm et al. 2013).

The appearance of the probed *Apa*I PFGE gel, with *bla*$_{NDM-1}$ on fragments of multiple sizes, could be as a result of the plasmid concatemer formation discussed above, as well as being compatible with the PCR studies suggesting that two forms of the immediate *bla*$_{NDM-1}$ context exist. Further alternative contexts not defined by the experiments done here could also exist. Alternatively it is conceivable that not all of the genomic DNA had been successfully digested by the *Apa*I enzyme, although this seems less likely in view of the other findings already cited.

As expected with resistance plasmids, including those associated with *bla*$_{NDM-1}$, the plasmids from the *A. baumannii* strain harboured multiple AMR determinants and MGEs, including Tns, ISs including IS*CR*s and a class 1 integron (Carattoli 2013). Much of the sequence containing resistance determinants showed high degrees of identity with those identified in other resistance plasmids, both with and without the *bla*$_{NDM-1}$ gene, in Enterobacteriaceae and *Acinetobacter* spp. These various contexts however, were arranged in a novel fashion, in a plasmid type not previously associated with these resistance determinants (as far as could be determined by the results of the BLAST searches carried out). There was also evidence of ongoing plasticity, in view of the fact that different isolates contained different forms of the plasmid, the different versions of the immediate *bla*$_{NDM-1}$ context discussed above and the loss of most of AbaR3 in CHI-32. This novel plasmid is likely to have resulted from a combination of transposition events mediated by Tns and ISs, and homologous recombination events, based around the large number of repetitive sequences present.

An unusual feature of pNDM-32 was the large gene cluster that included sequences coding for a nitrile hydratase operon and an amidase. A BLASTN search showed high levels of identity only with chromosomal sequences from *K. oxytoca*, with the entire 7.8 kb sequence in perfect synteny. The GC % of the region was also compatible with an acquisition from *K. oxytoca* or a related species. Some microorganisms are known to be able to utilise nitrile compounds for growth, as a carbon and/ or nitrogen source (Kobayashi and Shimizu 1998; Brandão *et al.* 2003). Nitrile hydratases and amidases form one pathway that has been shown to allow some bacteria to do this.

These processes have been studied extensively in some bacterial species, such as *Rhodococcus* spp., because of their practical applications in the industrial production of useful amide compounds and bioremediation of nitrile contaminated environments (Kobayashi and Shimizu 1998; Brandão et al. 2003). Despite this, and the fact that nitrile metabolism has been described for a large number of bacteria isolated from various sites, including environments contaminated with nitrile compounds from industry and agriculture, relatively little is known about how widespread these genes are in bacteria and their primary purpose (Kato *et al.* 2000). Pubmed searches for papers on nitrile hydratases in *Acinetobacter* and *Klebsiella* produced no references. The results of the BLAST searches based on the protein sequences of the α and β subunits of the nitrile hydratase suggested that closely related proteins are probably uncommon in *A. baumannii* and that there were no homologues in *A. baumannii* CHI-32.

Further work would be required on *A. baumannii* CHI-32 to establish whether the genes in this operon are significantly expressed and whether the strain has the ability to utilise nitrile compounds. However, given that contamination of the environment with synthetic nitrile compounds by waste water from industry and agriculture is known to occur, and that there are many naturally occurring nitrile compounds (Kato et al. 2000; Brandão et al. 2003), it is conceivable that the sharing of such sequences on MGE could offer a further selection advantage to bacteria in the environment. This could create an additional selection pressure for the maintenance of this resistance plasmid and the survival of strains harbouring it, including for *A. baumannii* survival in environmental niches not usually associated with this species.

A further region of interest in pNDM-32 was the putative *hipAB* toxin-antitoxin locus. HipA is associated with a bacterial persistence phenotype, resulting in a decreased rate of growth but continuing survival in the face of bactericidal antimicrobials (Germain et al. 2013). Thus this locus in pNDM-32 may allow the induction of a persistence phenotype under appropriate environmental conditions. The putative HipA was only distantly related (19.3% AA identity) to HipA in *E. coli* K12, in which the *hipAB* operon has been extensively studied. However, it is of note that in *Shewanella oneidensis* MR-1 the *hipAB* operon has been shown to have similar effects, despite the fact that the HipA protein studied shared only 28% identity with that of *E. coli* K12 (Wen et al. 2014). If this region in pNDM-32 represents a functional *hipAB* locus it could provide a further selection advantage for plasmid acquisition and maintenance. However, persistence has been observed in many bacterial pathogens, and it is also possible that the *A. baumannii* isolates studied here could have multiple other mechanisms mediating persistence, including other homologues of HipA. This has not been studied further at the present time.

The decision not to attempt full assembly of the plasmids in CHI-34 and CHI-45-1 was partly determined by the fact that early analysis indicated that the plasmids in these other isolates were very similar to pNDM-32. On the basis of the BLAST and ACT comparisons between pNDM-32 and the WGS contigs in CHI-34 and CHI-45-1 it is predicted that CHI-34 contains a related plasmid which has either not acquired, or lost, a large region containing several of the AMR genes and the NHase/ amidase functions. CHI-45-1 contains a plasmid which is mostly identical to pNDM-32. The combined length of the contigs in CHI-34 with close identity to pNDM-32 is also consistent with the predicted size of the plasmid in this isolate on the basis of the S1 in gel hybridisation results.

A feature of MDR *A. baumannii* isolates that have undergone extensive molecular investigation has been the presence of several MDR islands (Adams et al. 2010; Hamidian and Hall 2011). Although the main focus of the current work was the MGEs associated with *bla*$_{NDM-1}$ it had been the intention, once the decision was made to analyse WGS data, to look to see whether these isolates also contained MDR GEIs. Of the three isolates which underwent WGS only *A. baumannii* CHI-32 did not contain a complete element with close identity to AbaR3. That the isolate chosen for most detailed analysis of the wider *bla*$_{NDM-1}$ context was the only isolate which did not contain this island was not by chance. As stated above *A. baumannii* CHI-32 was chosen because the complexity of the assembly options was reduced because there were less contigs linked to IS*15-Δ*. As can be seen from Figure 3.19a the reference AbaR3, and in all likelihood the related element in *A. baumannii* CHI-34 and CHI-45-1, contains 3 copies of IS*26*/ IS*15-Δ*. Thus the additional assembly options were linking the plasmid contigs to the AbaR3-like element. As these links were not investigated and the assembly links between AbaR3-like contigs in *A. baumannii* CHI-34 and CHI-45-1 it cannot be excluded that these elements contain a further copy of *bla*$_{NDM-}$1. As *bla*$_{NDM-1}$, IS*CR27*, IS*Aba125* and IS*15-Δ* are all present on the chromosome by the S1 hybridisation studies it is reasonable to hypothesise that *bla*$_{NDM-1}$ could have been transferred to this region by IS*15-Δ* mediated transposition/ co-integration. However, PCR results suggest it is likely that one chromosomal copy is flanked by IS*Aba125* at both ends and so is in a different chromosomal location.

Results for *A. bereziniae* CHI-40-1 have been discussed relatively little in the current Chapter. Some results are presented here for convenience, as initial experiments were done on all isolates simultaneously. However, as the genetic

context and strain background were so different, detailed genetic analysis is presented separately. As will be discussed at the beginning of Chapter 4, although *A. baumannii* is of greater clinical interest than other *Acinetobacter* spp., there is no particular reason to think that *A. baumannii* is more likely to have been an intermediate step in the spread of $bla_{\text{NDM-1-like}}$ genes to Enterobacteriaceae than other *Acinetobacter* spp. As the only strain containing a plasmid harbouring $bla_{\text{NDM-1}}$ which could be conjugatively transferred into other bacterial species, *A. bereziniae* CHI-40-1 seemed more likely to have potential to contribute to the dissemination of $bla_{\text{NDM-1}}$ and its immediate genetic context. This was therefore the focus of subsequent study, rather than seeking to resolve some of the questions raised in the foregoing discussion with regards to the *A. baumannii* isolates which were the main subject of the current chapter.

Chapter 4

Characterisation of plasmids harbouring *bla*$_{NDM-1}$ in extensively drug-resistant (XDR) *Acinetobacter* species from India and Pakistan

4.1 Introduction

As discussed in the General Introduction section 1.5.1 and Chapter 3, *A. baumannii* is the primary *Acinetobacter* species of medical importance, but many other *Acinetobacter* spp. are commonly found in the environment and occasionally cause opportunistic infections in humans (Higgins et al. 2010; Visca et al. 2011). Evidence was presented in Chapter 3, suggesting that *bla*$_{NDM-1}$ was formed in an *Acinetobacter* background (Toleman et al. 2012). Although it has been proposed that this occurred in *A. baumannii* there is no good evidence to suggest that these events occurred in one species of *Acinetobacter* over another. Tn*125* harbouring *bla*$_{NDM-1-like}$ genes are found in many species of *Acinetobacter*, sequence data deposited at NCBI indicate that both IS*Aba125* and *aphA6* are found in many other *Acinetobacter* spp. and all *Acinetobacter* have fairly similar GC contents.

There are only a few published examples of transfer of *bla*$_{NDM-1}$ from *A. baumannii* to other bacteria (see General Introduction section 1.4.1 and Chapter 3). The analysis of the plasmids harbouring *bla*$_{NDM-1}$ in *A. baumannii*, reported in Chapter 3, did not suggest that they were conjugative plasmids. However, if *bla*$_{NDM-1}$ and its immediate context was initially established in *Acinetobacter* spp. then

significant HGT must have occurred to result in the rapid appearance of *bla*$_{NDM-1}$ genes in so many species of GNB.

The published examples of conjugative transfer of *bla*$_{NDM-1}$ from *A. baumannii* are from studies conducted on isolates from China. NPE are apparently uncommon in China, despite quite extensive efforts to detect them (Chen et al. 2011; Ho et al. 2011; Lai *et al.* 2011; Fu et al. 2012; Ho et al. 2012; Hu et al. 2012; Wang et al. 2012; Yang et al. 2012; Zhou et al. 2012; Hu et al. 2013; Wang et al. 2013). However, *bla*$_{NDM-1}$ has been observed more often in *Acinetobacter* spp., including *A. baumannii*, in strains identified from clinical, environmental and farm animal samples (Chen et al. 2011; Hu et al. 2012; Yang et al. 2012; Zhou et al. 2012; Zhang et al. 2013a; Zhang et al. 2013b). The plasmids harbouring *bla*$_{NDM-1}$ that have been sequenced so far from China are very closely related to one another, despite being reported in many different species including *A. baumannii*, *A. pittii*, *A. junii* and *A. lwoffii* (Chen et al. 2011; Chen *et al.* 2012; Fu et al. 2012; Hu et al. 2012; Yang et al. 2012; Zhou et al. 2012; Sun et al. 2013b; Zhang et al. 2013b). Here after these plasmids will be designated as pNDM-BJ01-like, after the first such plasmid sequenced, from *A. lwoffii* BJ01 (rMLST analysis below suggests this isolate is in reality an *Acinterobacter johnsonnii* strain). As pNDM-BJ01-like plasmids have been shown repeatedly to be transferable to recipient bacteria, including *Escherichia coli*, *in vitro*, they represent a potential vector for the spread of *bla*$_{NDM-1}$ between species of bacteria. As these plasmids have most often been described in “environmental” *Acinetobacter* spp. this could suggest that *A. baumannii* should not be regarded as being the species from which *bla*$_{NDM-1}$ is most likely to have disseminated.

However, *bla*$_{NDM-1}$ appears to be much less frequent in Enterobacteriaceae in China, which argues against a major dissemination of NDM-1 from *Acinetobacter* to

Enterobacteriaceae having taken place there. It was hypothesized that pNDM-BJ01-like plasmids, or other conjugative plasmids, could be detectable in clinical and/or environmental *Acinetobacter* spp. isolates in countries reporting a higher prevalence of NPE, including India and Pakistan. In Chapter 4 the further characterisation of CHI-40-1, first reported in Chapter 3, is described. By analysis of WGS data this isolate was found to contain a conjugative plasmid harbouring *bla*$_{\text{NDM-1}}$ that is very similar to pNDM-BJ01. Further phenotypic analysis of this plasmid is presented and it is shown that closely related plasmids carrying *bla*$_{\text{NDM-1}}$, are present in *Acinetobacter* spp. isolated from faecal samples from patients in Karachi, Pakistan.

4.2 Nucleotide sequence accession numbers

Accession numbers for pNDM-40-1, from *A. bereziniae* CHI-40-1, and the partial sequence from pNDM-69122, from *A. haemolyticus* 69122-EW, are KF702385 and LN611576 respectively. *A. bereziniae* CHI-40-1 assembly contigs are deposited under study accession PRJEB7120, contig accession numbers CDEL01000001-CDEL01000324.

4.3 Results

4.3.1 Preliminary results on all isolates

Most of the data from this Chapter has been published previously (Jones et al. 2015) and is included in Appendix 4.1. The Indian clinical isolate CHI-40-1 and the five isolates from the Pakistani faecal samples included four different *Acinetobacter* spp. that were all *bla*$_{\text{NDM-1}}$ positive. MIC results are shown in Table 4.1. All isolates were resistant to most drugs tested including all β-lactam antibiotics, with the exception that the *A. haemolyticus* isolates were susceptible to aztreonam. Notably

all strains were sensitive to colistin and resistant to tigecycline and the *A. haemolyticus* and *A. schindleri* isolates were sensitive to co-trimoxazole. There are no *Acinetobacter* or PK/PD breakpoints defined by EUCAST for fosfomycin, but the MICS of all isolates were below breakpoints used for Enterobacteriaceae causing urinary tract infections. Species identification by MALDI-TOF and phylogenetic analysis of 16S rRNA gene sequences were concordant for all but CHI-40-1. CHI-40-1 was confirmed as an *A. bereziniae* by analysis of rMLST loci. The 16S rRNA gene and rMLST phylogenetic trees are shown in Figure 4.1. Sequences for two other *bla*$_{\text{NDM-1}}$ positive *Acinetobacter* spp. for which WGS were available, are included in both trees. *A. lwoffii* WJ10621 (Accession AFQY00000000.1) contains plasmid pNDM-BJ01 harbouring *bla*$_{\text{NDM-1}}$ and *A. pittii* D499 (NZ_AGFH00000000.1) contains the similar plasmid pAB_D499. Both strains were isolated from clinical samples in China (Hu *et al.* 2011; Yang et al. 2012). The phylogenetic trees suggest that strain WJ10621 is in fact an *A. johnsonnii* isolate. The rMLST ML phylogenetic tree clearly shows that CHI-40-1 comes from a genetic background distinct from the other *bla*$_{\text{NDM-1}}$ positive *Acinetobacter* spp included. This reflects levels of nucleotide identity of 91.3% and 91.7% across all rMLST loci with *A. pittii* D499 and *A. johnsonnii* WJ10621, respectively.

	AZT	CAZ	IMP	MEM	PTZ	AMI	GENT	TOB	CIP	COL	FOS	RIF	SXT	TIGE
CHI-40-1	48	≥256	≥32	≥32	96	48	≥256	12	≥32	0.75	8	≥256	≥32	0.75
69122-EW	3	≥256	≥32	≥32	24	32	2	8	≥32	0.75	32	4	0.25	1
73261-EC	4	≥256	≥32	≥32	32	32	2	12	≥32	1	24	6	0.25	0.75
70114-EC	4	≥256	≥32	≥32	32	32	2	8	≥32	1	24	6	0.19	0.75
73668-ECT	16	≥256	≥32	≥32	48	6	192	≥256	≥32	0.5	4	≥256	0.25	0.5
74312-EC	48	≥256	≥32	≥32	≥256	48	96	12	≥32	0.5	16	≥256	≥32	0.75
AG3528	16	3	0.75	0.75	2	2	0.75	0.75	4	1	16	-	0.125	-
UAB190	0.125	0.25	0.38	0.047	2	2	8	1.5	0.006	0.5	3	-	0.094	-
AG3528$_{NDMP1}$	16	≥256	≥32	≥32	96	3	0.75	0.75	3	1	16	-	0.125	-
UAB190$_{NDMP2}$	0.064	≥256	24	4	256	2	8	1.5	0.006	0.5	3	-	0.094	-

Table 4.1 – MIC (mg/L) for all NDM-1 producing *Acinetobacter* isolates, mating experiment recipients and representative transconjugants.

Red - Resistant; Orange - Intermediate resistance; Green – Sensitive; Grey – no breakpoint; White – not done. Species specific *Acinetobacter* spp. breakpoints not available for aztreonam, ceftazidime, piperacillin-tazobactam or tigecycline, for which results are based on EUCAST PK/PD non-species specific breakpoints. AZT – aztreonam; CAZ – ceftazidime; IMP – imipenem; MEM – meropenem; PTZ – piperacillin-tazobactam; AMI – amikacin; GENT – gentamicin; TOB – tobramycin; CIP – ciprofloxacin; COL – colistin sulphate; FOS – fosfomycin; RIF – rifampicin; SXT – co-trimoxazole; TIGE – tigecycline.

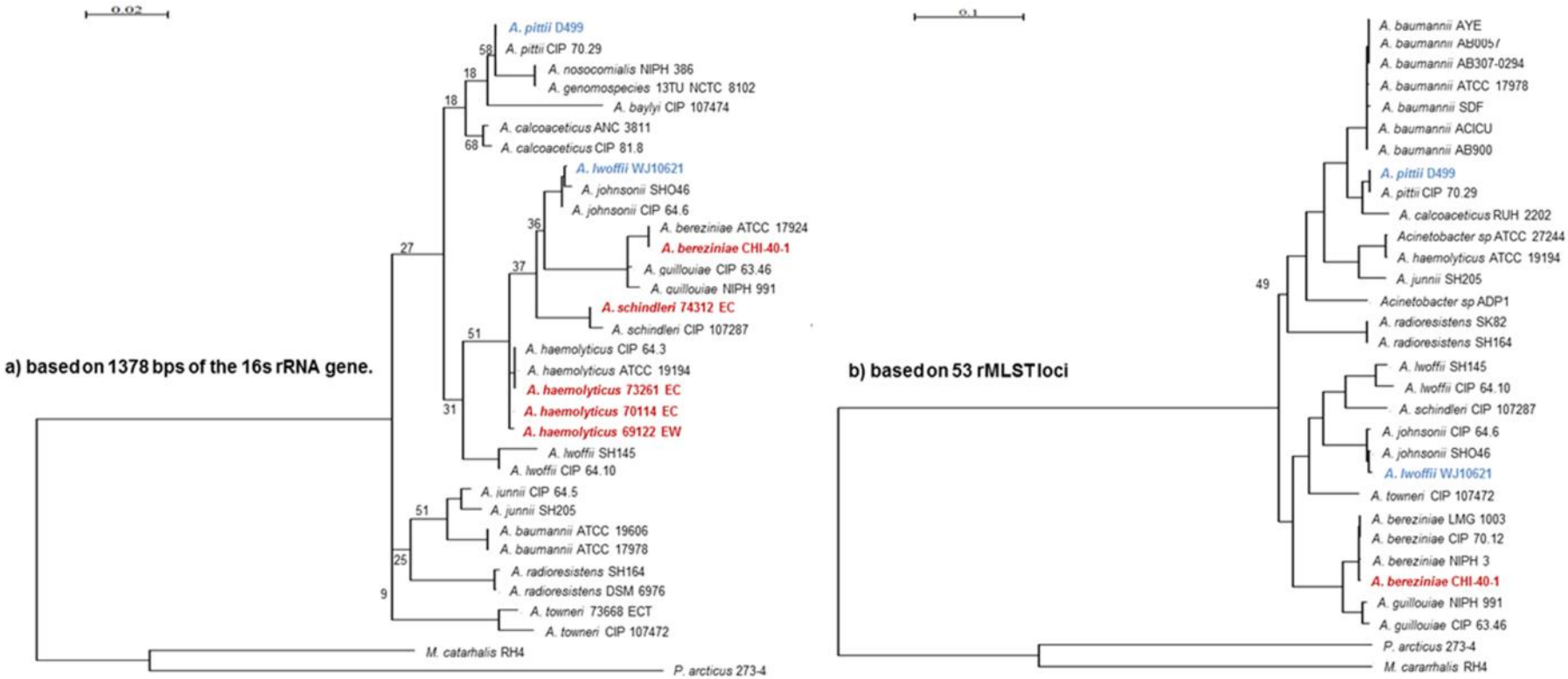


Figure 4.1 – Phylogenetic trees of *Acinetobacter* spp. a) Based on 1378 bps of the 16s rRNA gene; b) Based on 53 rMLST loci. Performed with 100 bootstraps. Only bootstrap values of < 70 are shown. Isolate names shown in red are *bla*$_{NDM-1}$ *Acinetobacter* spp. characterised for this study. Isolate names shown in blue are *bla*$_{NDM-1}$ *Acinetobacter* spp. for which WGS are publically available from the NCBI database. All other sequences are from *Acinetobacter* spp. isolates that do not harbour *bla*$_{NDM-1}$ available from NCBI nucleotide, draft genome or complete genome databases or in the rMLST database.

S1 PFGE and *bla*$_{NDM-1}$ probing showed that CHI-40-1 harboured multiple plasmids, with *bla*$_{NDM-1}$ present on bands of ~45kb and ~250kb (Figures 3.7 in Chapter 3 and Figure 4.2). IS*CR27* and IS*Aba125* were also present on bands of the same size, although in the case of IS*Aba125* this was also present on 3 other plasmid bands and the chromosome (Figures 3.8 and 3.10). IS*15-Δ*; however, was only present on bands of ~100 kb and faintly visible on a band of ~230 kb (i.e. not associated with *bla*$_{NDM-1}$, Figure 3.9) Probing for the conjugative relaxase, *traA*, of pNDM-BJ01-like plasmids showed that the gene was present on bands of the same size (Figure 4.3). PFGE performed on CHI-40-1 DNA subjected to an S1 concentration gradient showed that the higher the S1 concentration the fainter the 250kb band appeared (Figure 4.4). The *bla*$_{NDM-1}$ gene was present on a single *Apa*I restriction fragment of ~45 kb (Figure 3.5).

PCR analysis showed that sequences with significant identity to the pNDM-BJ01 plasmid backbone were present in CHI-40-1 and that *bla*$_{NDM-1}$ was found within a Tn*125* element (see Appendix 4.3). To allow detailed analysis of the *bla*$_{NDM-1}$ context and the strain background CHI-40-1 was submitted for WGS.

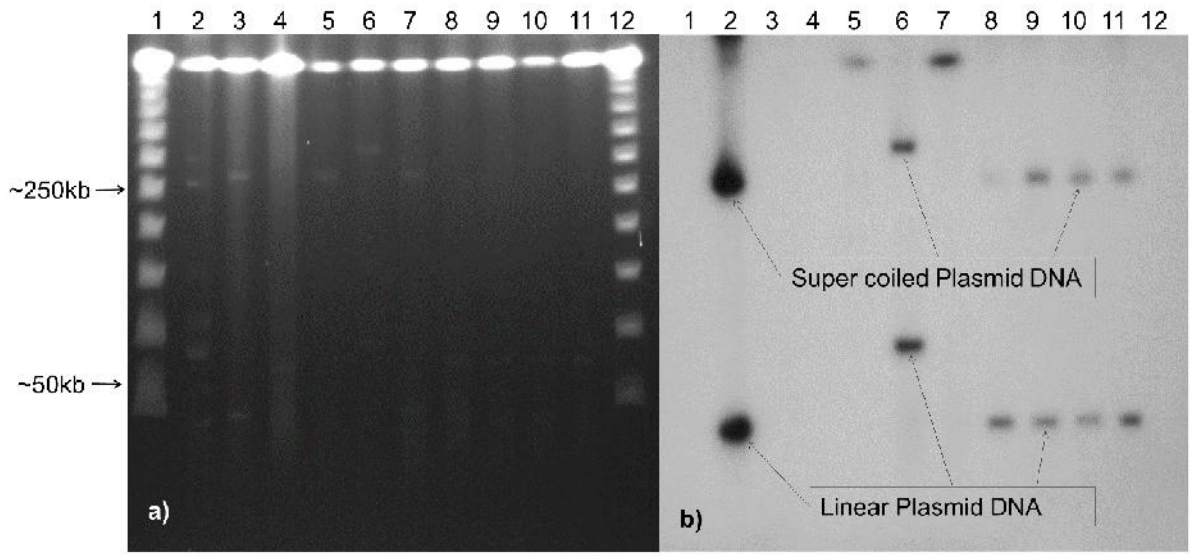


Figure 4.2 – a) Pulsed field gel of S1 digested genomic DNA from CHI-40-1, recipients and transconjugants; b) in gel hybridisation with *bla*$_{NDM-1}$ gene probe. 1 – λ concatemer (~50-1000kb); 2 – CHI-40-1; 3 – UAB190; 4 – AG3528; 5 – UAB190$_{NDMP1}$; 6 – UAB190$_{NDMP2}$; 7 – UAB190$_{NDMP3}$; 8 – AG3528$_{NDMP1}$; 9 – AG3528$_{NDMP2}$; 10 – AG3528$_{NDMB1}$; 11 – AG3528$_{NDMB2}$ 12 - λ .

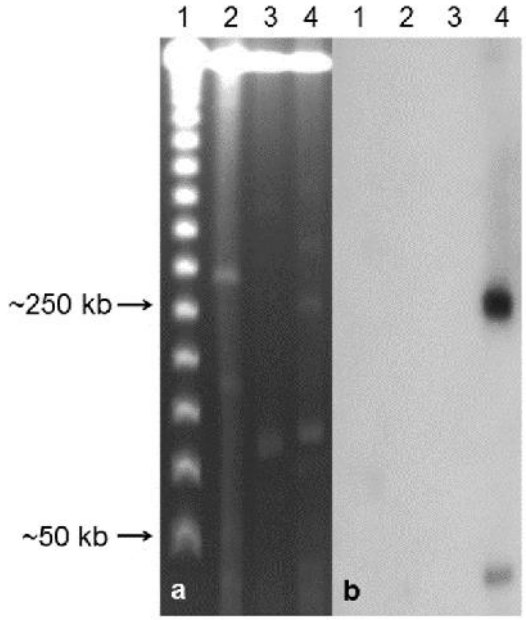


Figure 4.3 – a) Pulsed field gel of S1 digested genomic DNA from *A. bereziniae* CHI-40-1 and control strains; b) in gel hybridisation with *traA* gene probe. 1 – λ concatemer (~50-1000kb); 2 – *K. pneumoniae* 05-506, 3 – CHI-45-1 , 4 – CHI-40-1

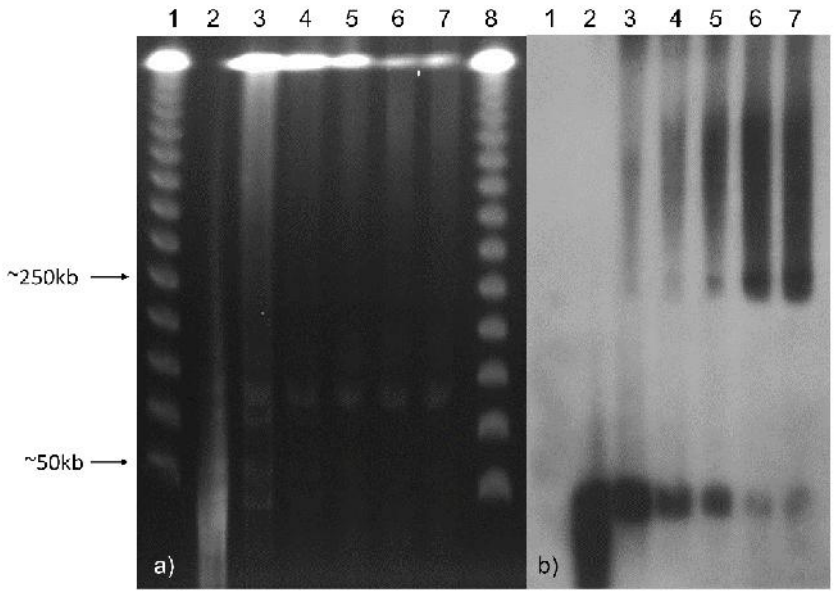


Figure 4.4 – a) Pulsed field gel showing genomic DNA from *A. bereziniae* CHI-40-1 digested with different concentrations of S1 nuclease; b) Direct gene probe of pulsed field gel in a) with *bla*$_{NDM-1}$. 1 – λ concatemer (~50-1000kb); 2-7 – CHI-40-1 digested S1 nuclease: 2 – neat; 3 – 1 in 10 dilution; 4 – 1 in 50 dilution; 5 – 1 in 100 dilution; 6 – 1 in 500; 7 – 1 in 1000 dilution; 8 – λ concatemer.

4.3.2 Sequence of pNDM-40-1 from CHI-40-1 and comparison with related genetic contexts

Following *de novo* assembly of CHI-40-1 WGS, 324 contigs were obtained, with a mean GC content of 38% and a combined size of 4.78Mb. BLAST searches identified 23 contigs with 99-100% nucleotide identity to pNDM-BJ01 (Accession number JQ001791). The complete plasmid sequence was closed by PCR and sequencing of amplicons (See Figure 4.5 and Appendix 4.4). The plasmid, pNDM-40-1 (Accession number KF702385), is 45,827bp and has substantial similarity to NDM plasmids sequenced from several *Acinetobacter* spp. backgrounds. The GC content of the entire plasmid is 40.1%. However, the GC content of the plasmid backbone is 36.2% and that of the variable region (from IS*Aba14* to the end of Tn*125*, nts 5,427-16,280) is 52.5%.

At the time of writing, complete sequences of 9 pNDM-BJ01-like plasmids were available in GenBank. The backbone of pNDM-40-1 is 100% identical at the nucleotide level to that of pNDM-BJ01 (bases 1-5,684 and 17,987-47,274, Accession number JQ001791), pNDM-BJ02 (JQ060896), pAbNDM-1 (JN377410) and pXM1 (AMXH01000087). pNDM-AB (KC503911), pM131_NDM-1 (JX072963), pNDM-Iz4b (KJ547696) exhibit minor differences to the backbone of pNDM-BJ01 and to one another. pNDM-AB differs the most, because of a 3.5 kb insertion containing the genes *traD*, *insB* and a putative Type I restriction-modification system methyltransferase (Figure 4.5a and Table 4.2). The gene *traD* as annotated in pNDM-AB is 454bp longer than in pNDM-40-1, although the gene as annotated in pNDM-40-1 retains the complete *traD* functional domain. These plasmids were all identified in isolates from China, and were found in 5 different *Acinetobacter* spp. (see Table 4.2).

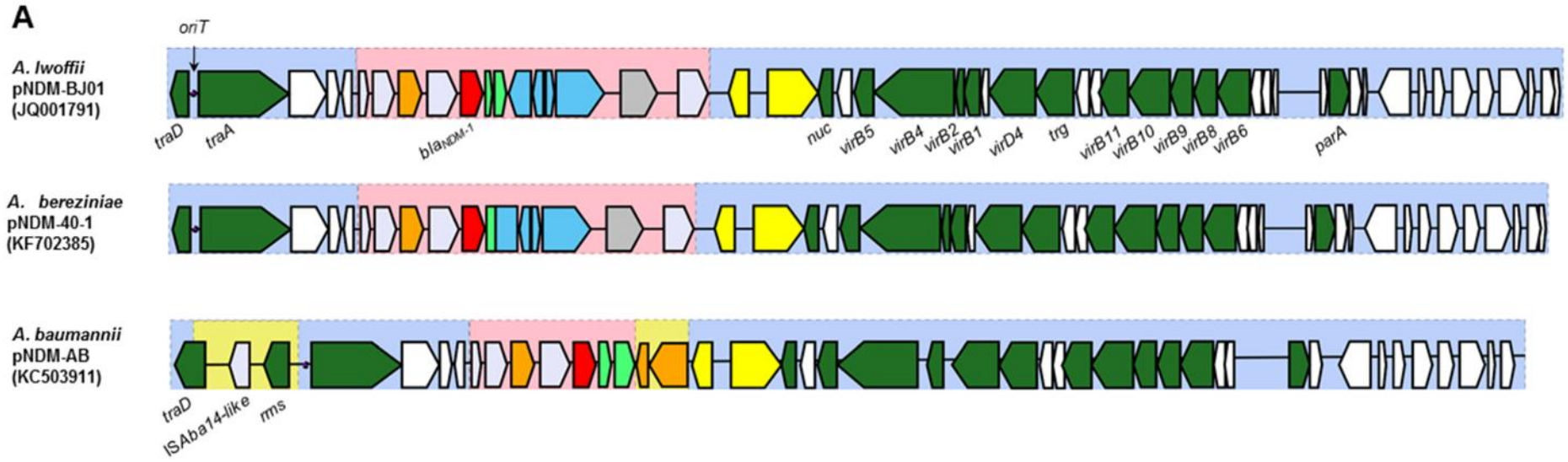
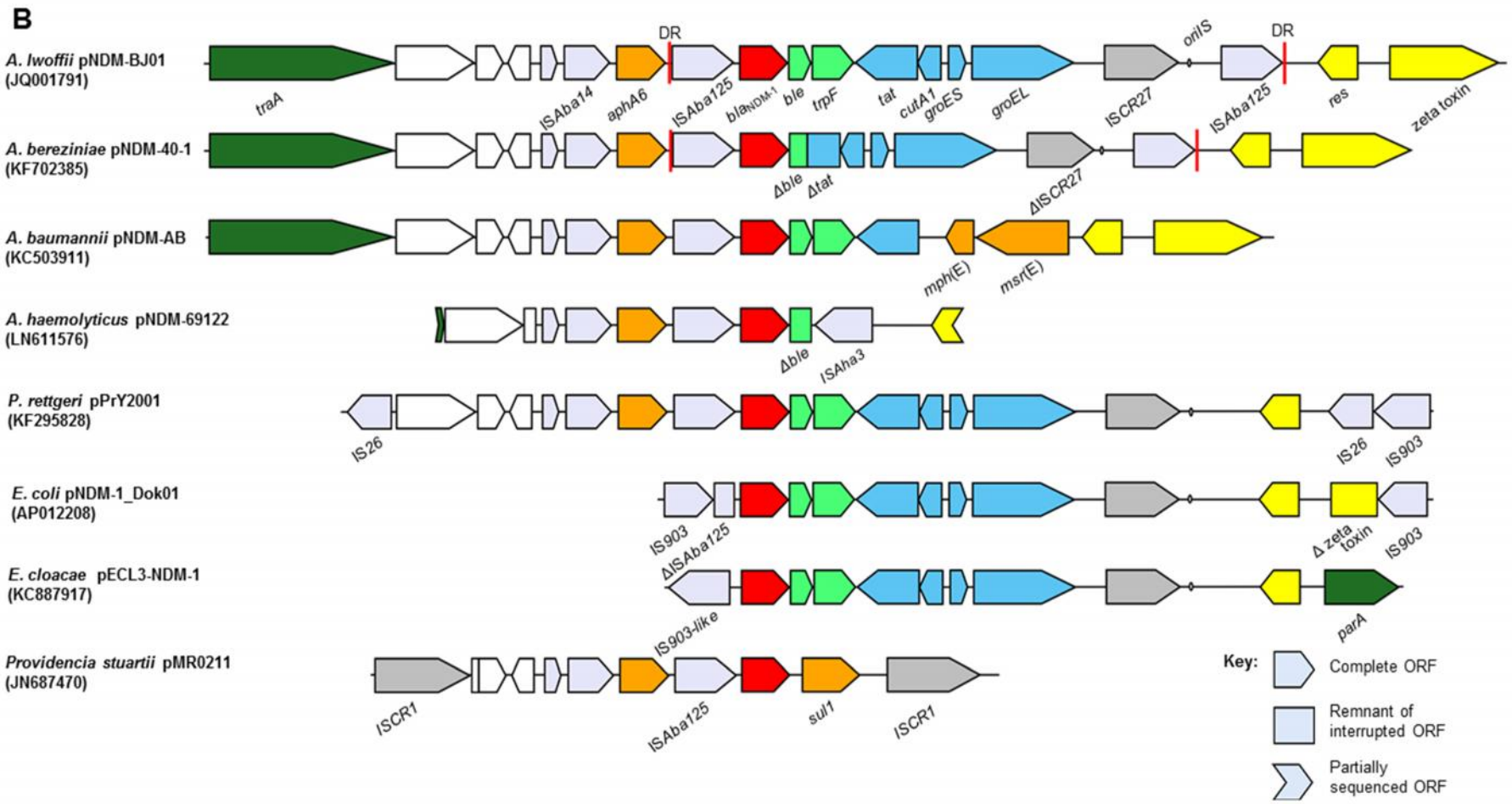


Figure 4.5 – a) Maps of plasmids pNDM-BJ01, pNDM-40-1 and pNDM-AB. b) Immediate *bla*$_{NDM-1}$ context from pNDM-40-1, *A. haemolyticus* 69122-EW and related sequences in *Acinetobacter* and Enterobacteriaceae. ORFs are colour coded with the direction of transcription indicated by arrow heads, truncated remnants of ORFs are shown as rectangles. Red – *bla*$_{NDM-1}$; orange – other antibiotic resistance; lime green – usually immediately downstream of *bla*$_{NDM-1}$; blue – from a common context in *Xanthomonas* and *Pseudoxanthomonas*; dark grey – IS*CR27* transposase; light grey – IS transposases, note transposases are labelled with IS name, and as *tnp* for uncharacterised elements; yellow – resolvase and zeta-toxin from pNDM-BJ01-like plasmids; dark green – named plasmid backbone genes; white – hypothetical proteins. Regions with a light blue shaded background contain plasmid backbone with close identity amongst pNDM-BJ01-like plasmids. The pink shaded regions represent genes normally found

in the *bla*$_{NDM-1}$ context in *Acinetobacter* spp. Regions from pNDM-AB with a yellow background represent genes with no significant identity to those in pNDM-40-1. *oriT* –origin of transfer; *traD* – conjugal transfer gene; *traA* – MobA/L type relaxase gene; *res* – resolvase gene; *nuc* – nuclease homologue; *virB1-B11 and VirD4* – putative T4SS genes; *trg* – putative lytic transglycosylase gene; *parA* – putative plasmid partition gene; *insB* – putative transposase, IS*Aba14*-like; *rms* – putative type I restriction-modification system methyltransferase subunit gene; *aphA6* – aminoglycoside resistance gene; *ble* – bleomycin resistance gene; *trpF* – phosphoribosylanthranilate isomerase gene; *tat* – twin-arginine translocation pathway signal sequence domain gene; *cutA1* – periplasmic divalent cation tolerance gene; *groES* – co-chaperonin gene; *groEL* – chaperonin gene; IS*CR27* – insertion sequence common repeat 27 transposase gene; *oriIS* – origin of insertion of IS*CR27*; *res* – putative resolvase gene; *msr*(E) and *mph*(E) – macrolide resistance genes.


B
A. lwoffii pNDM-BJ01 (JQ001791)
traA
ISAba14
aphA6
DR
ISAba125
blaNDM-1
ble
trpF
tat
cutA1
groES
groEL
ISCR27
orfIS
DR
res
zeta toxin
A. bereziniae pNDM-40-1 (KF702385)
Δble
Δtat
ΔISCR27
ISAba125
A. baumannii pNDM-AB (KC503911)
mph(E)
msr(E)
A. haemolyticus pNDM-69122 (LN611576)
Δble
ISAha3
P. rettgeri pPrY2001 (KF295828)
IS26
IS26
IS903
E. coli pNDM-1_Dok01 (AP012208)
IS903
ΔISAba125
Δ zeta toxin
IS903
E. cloacae pECL3-NDM-1 (KC887917)
IS903-like
parA
Providencia stuartii pMR0211 (JN687470)
ISCR1
ISAba125
sul1
ISCR1
Key:
Complete ORF
Remnant of interrupted ORF
Partially sequenced ORF

Species/ strain	Plasmid	Accession No.	Backbone compared to pNDM-BJ01	Resistance region compared to pNDM-BJ01	Country of isolation	Travel History	Reference
A.lwoffii WJ10621	pNDM-BJ01	JQ001791	NA	NA	China		(Hu et al. 2012)
A. bereziniae CHI-40-1	pNDM-40-1	KF702385	Identical	17,688C>T, 17,760T>C in 3' IS*Aba125*, 10,121_11,420del including 3' end of *ble* to 5' end of *tat*, 15,761_15,912del within IS*CR27*.	India		This work
A. calcoaceticus XM1570	pXM1	AMXH01000087	Identical	17,688C>T, 17,760T>C in 3' IS*Aba125*.	China		(Sun et al. 2013b)
A.lwoffii WJ10659	pNDM-BJ02	JQ060896	Identical	16,859_17,969del including most of 3' IS*Aba125*, excluding only 3' 18bp.	China		(Hu et al. 2012)
A. baumannii GF216	pNDM-AB	KC503911	47,274_1ins – 3,530bp long containing part of *traD*, *insB*, methyltransferase	12,036-18,237 from *cutA* to 3' IS*Aba125* replaced by sequence including *msr*(E) and *mph*(E).	China		(Zhang et al. 2013b)
A. pittii D499	pAB_D499	AGFH01000030	32,541T>A in *virB10*, 46,541_46,712del.	8,364A>G in 5' IS*Aba125,* 10,531C>G in *trpF*, 16,866-18,101 containing 3' IS*Aba125*, IS*Aba11*-like insertion 3' end, 18,123T>A.	China		(Yang et al. 2012)
A. baumannii ZW85-1	pAbNDM-1	JN377410	Identical	17,132A>G, 17,151T>C, 17,154T>C, 17,340C>A, 17,688C>T, 17,760T>C and 17,984_17,985insCC in 3' IS*Aba125*.	China		-
Acinetobacter sp. M131	pM131_NDM-1	JX072963	47,200T>C in hypothetical protein coding sequence.	16,866-18,101 containing 3' IS*Aba125*, IS*Aba11*-like insertion 3' end.			-
A. lwoffii Iz4b	pNDM-Iz4b	KJ547696	504G>A in *traA*, 31,694T>G in virB11, 40,341_40,342insC, 43,781_44,487del	8,328A>C in 5' IS*Aba125*, 17,688C>T and 17,760T>C in 3' IS*Aba125*.			-
A. soli TCM341	Unnamed (contig 5)	JAPY01000005	20,767G>T and 20,977_21,019del in putative zeta-toxin coding sequence.	8,174A>G in 5' IS*Aba125*, 16,859_17,969del including most of 3' IS*Aba125*, excluding only 3' 18bp.			-
A. schindleri MRSN 10319	Unnamed	Not applicable	> 99.9% identity	Unclear from report	USA	Afghanistan	(McGann *et al.* 2013)
A. pittii 2012276	Unnamed	Not applicable	Similar based on partial sequencing	Full Tn*125* as in JQ001791	Belgium	India and Egypt	(Bogaerts et al. 2011)
A. baumannii MRSN 12227	Unnamed	Not applicable	>99% identity	Full Tn*125* as in JQ001791	Honduras		(Waterman et al. 2013)

Table 4.2 – Strain details and sequence differences for fully sequenced or published reports of pNDM-BJ01-like plasmids.

The *bla*$_{NDM-1}$ context in pNDM-40-1 is similar to that found in pNDM-BJ01 and many other *Acinetobacter bla*$_{NDM-1/2}$ contexts (Espinal et al. 2011; Pfeifer et al. 2011; Bonnin et al. 2012b; Hu et al. 2012). The structure of Tn*125* containing *bla*$_{NDM-1-like}$ genes is described in detail in Chapter 3. Tn*125* in pNDM-40-1 contains two deletions relative to this: 150bp are missing within IS*CR27* and a 1,298bp deletion from the 3' end of *ble* to *tat* (Figure 4.5b). Otherwise, Tn*125* in pNDM-40-1 is identical to that in pNDM-BJ01 with the exception of the downstream IS*Aba125* in which there are two SNPs. The context is largely identical in other pNDM-BJ01-like plasmids. However, pNDM-BJ02 and contig 5 from *Acinetobacter soli* TCM341 lack the 3' IS*Aba125*, whilst in pAB-D499 and pM131_NDM-1 there is an IS*Aba11* inserted at the 3' end of the element. In pNDM-AB a large part of the context from *cutA1* to the 3' IS*Aba125* is replaced by the macrolide resistance genes *msr*(E) and *mph*(E) (Figure 4.6b and Table 4.2) (Zhang et al. 2013b). In all pNDM-BJ01-like plasmids the aminoglycoside resistance gene *aphA6* is found immediately upstream of Tn*125*.

GenBank searches show that *bla*$_{NDM-1}$ contexts in Enterobacteriaceae have high degrees of identity with the *bla*$_{NDM-1}$ context from pNDM-BJ01-like plasmids. In most cases this is restricted to genes that make up part of the full Tn*125* element harbouring *bla*$_{NDM-1}$, with at least a fragment of the IS*Aba125* upstream of *bla*$_{NDM-1}$ and the *ble* and *trpF* genes being present in almost all cases. Four sequences from Enterobacteriaceae with regions of close identity to the *bla*$_{NDM-1}$ context in pNDM-BJ01, which included part of the plasmid backbone, were available at the time of writing (Figure 4.6b) (Partridge and Iredell 2012). These were in plasmids found in *P. rettgeri* (KF295828), *Providencia stuartii* (JN687470), *E. coli* (AP012208), and *E. cloacae* (KC887917). Plasmid pPrY2001 from *P. rettgeri* contains the most extensive

region of identity. The sequence is nearly identical to that found in pNDM-BJ01 from the far 3' end of *traA* to the resolvase gene, with the main difference being the absence of the 3' IS*Aba125*.

All pNDM-BJ01-like plasmids share a common region with putative genes coding for a type IV secretion system (T4SS). These are involved in constructing the conjugation machinery and mediating conjugative transfer of plasmid DNA to recipient bacteria (Juhas *et al.* 2008). *traA* codes for a putative MobA/L type relaxase, which nicks the plasmid at the origin of transfer leading to unwinding of the supercoiled plasmid prior to conjugative transfer. A putative *oriT* was identified upstream of *traA*. This contained a *nic* site with 67% nucleotide identity to the consensus sequence of the *nic* sites of several Mob_Q type plasmids adjacent to imperfect inverted repeats characteristic of this family of *oriT*s (Figure 4.6) (Francia *et al.* 2004).

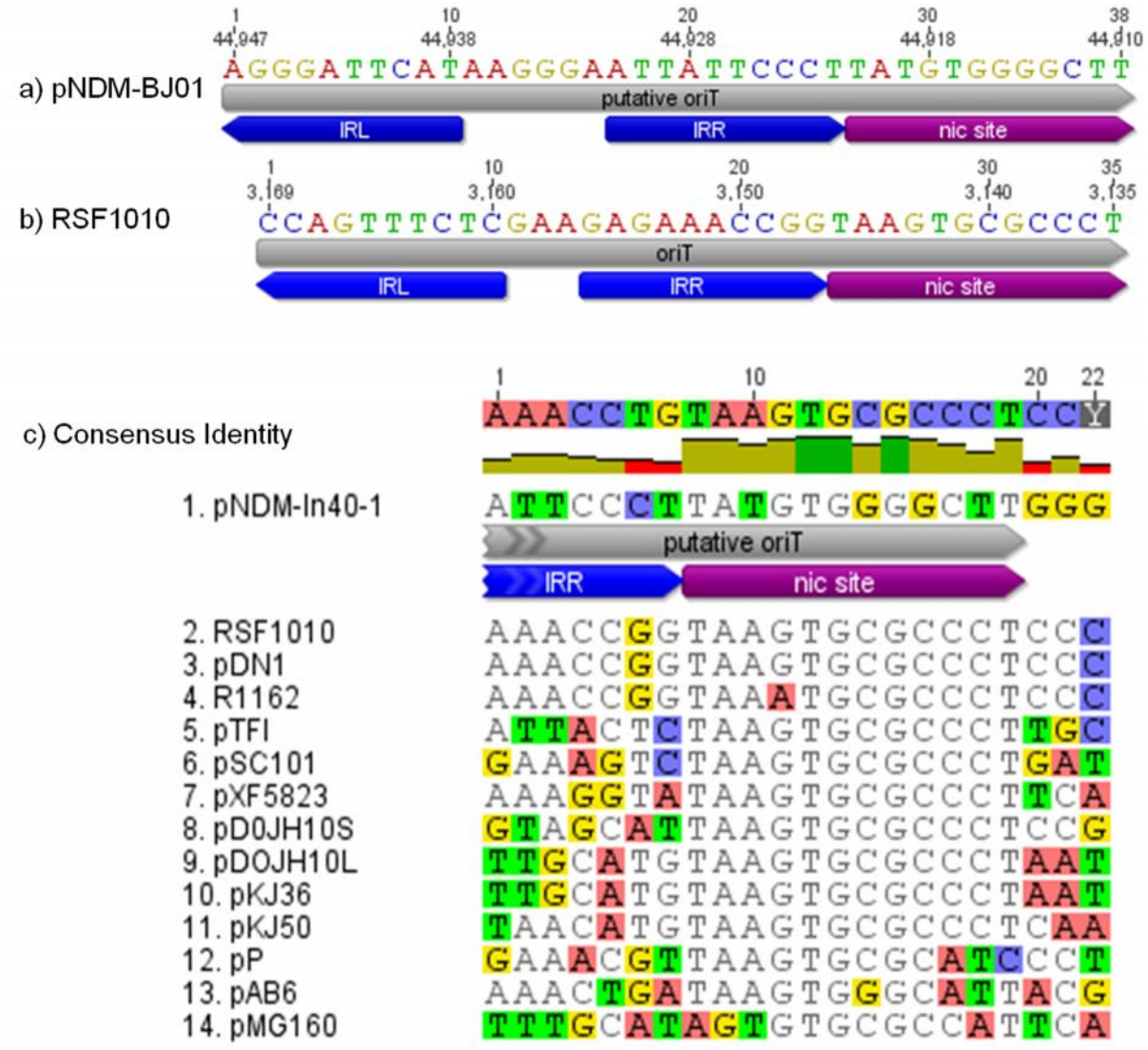


Figure 4.6 – Putative *oriT* of pNDM-BJ01 and comparison with other Mob_Q family *nic* sites. a) Sequence of putative *oriT* of pNDM-40-1; b) Sequence of *oriT* in plasmid RSF1010; c) MUSCLE alignment of Mob_Q family *nic* sites against the putative site in pNDM-40-1. Images drawn in Geneious 5.6.

pNDM-BJ01-like plasmids all contain *parA* genes proposed to code for a plasmid partition system and a putative zeta-toxin, which may contribute to plasmid stability by a toxin-antitoxin addiction system. However, it has so far not been possible to identify the replicase or the origin of replication of these plasmids. Replicon typing schemes for Enterobacteriaceae and *Acinetobacter,* have previously

been applied *in silico* to pNDM-BJ01-like plasmids (Hu et al. 2012; Zhang et al. 2013b). None of the putative genes from the glimmer gene model had significant identity with known replicase genes.

Several sequences in *bla*$_{NDM-1}$ negative *Acinetobacter* spp. contain regions with significant identity to the backbone of pNDM-BJ01-like plasmids (Figure 4.7 and Table 4.3). Most closely related are multiple contigs from *A. ursingii* NIPH 706 that contain 37 ORFs in synteny with the genes in pNDM-40-1, coding for proteins with amino-acid sequence identities between 88-100%. One of these contigs contains the resolvase gene adjacent to IS*Aba14*. The rest of the genes in the *bla*$_{NDM-1}$ context are absent. Less closely related but complete plasmid sequences are present in *Acinetobacter* spp. NIPH 2168 and *A. radioresistans* WC-A-157. These contain genes with substantial identity to the T4SS genes, *parA* and *traA*, from pNDM-BJ01-like plasmids. The NIPH 2168 plasmid does not contain any identifiable regions coding for antibiotic resistance or other known phenotypically important genes. pWC-A-157; however, codes for genes, including *copA* and *arsB2*, proposed to be associated with resistance to copper, arsenic and other transition or heavy metals (Rosen 2002; Teixeira *et al.* 2008; Davolos and Pietrangeli 2013). *A. radioresistans* SK82 probably contains a plasmid very similar to pWC-A-157, with the same T4SS and heavy metal resistance regions present on separate contigs. The *A. radioresistens* sequences both contain an identifiable replicase gene downstream of *parA* which is absent in the plasmids harbouring *bla*$_{NDM-1}$.

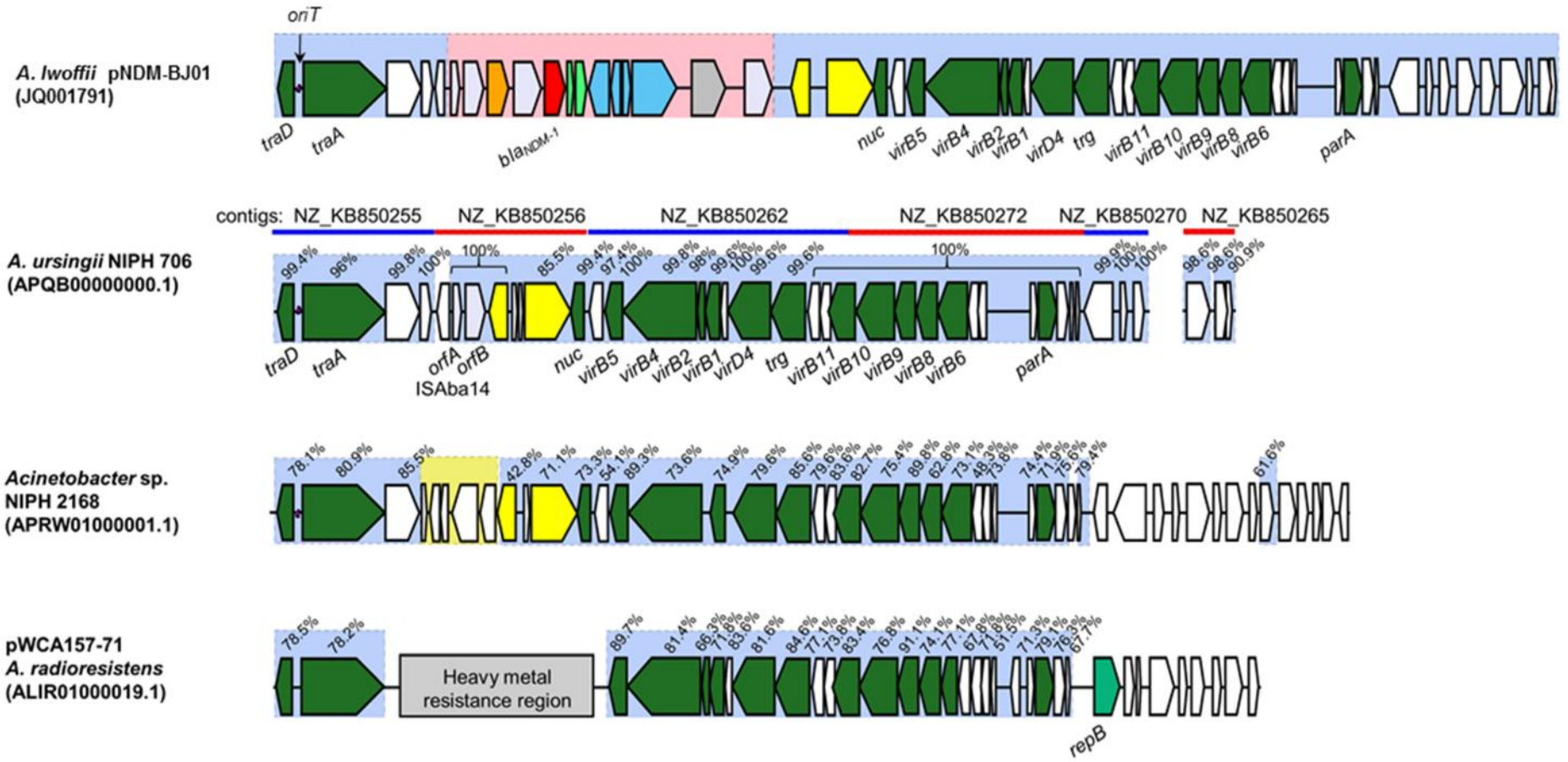


Figure 4.7 – Gene maps of complete sequence of pNDM-BJ01 and related sequences not harbouring *bla*$_{NDM-1}$. ORFs in the immediate context of *bla*$_{NDM-1}$ are colour coded as in figure 4.5. All other ORFs are coded dark green for named genes, coding for putative proteins with function based on conserved functional domains within the translated product or close identity with a protein

of known function. ORFs coding for hypothetical proteins of unknown function are white. *repB* in pWCA157-71 is lime green. Regions with a light blue shaded background contain related ORFs found in the plasmid backbones. The pink shaded regions represent ORFs normally found in the *bla*$_{NDM-1}$ context in *Acinetobacter* spp. Regions with a shaded yellow background represent ORFs with no significant homology to pNDM-BJ01 but which are inserted within regions with otherwise conserved gene order. Dark blue and red lines mark the boundaries between *A. ursingii* NIPH 706 contigs. Percentages above ORFs represent degree of amino-acid sequence identity of translated protein sequences in pNDM-40-1, based on MUSCLE alignments. For ease of comparison, where alternative names are used for ORFs in different sequences, the gene names used to annotate pNDM-40-1 are used for all sequences. *oriT* – putative origin of transfer; *traD* – putative conjugal transfer gene; *traA* – putative MobA/L type relaxase gene; *orfA, orfB* – transposase genes of *ISAba14*; *res* – resolvase; *ztx* – putative zeta toxin gene; *nuc* – nuclease homologue gene; *virB1-B11 and VirD4* – group of putative T4SS genes necessary to mediate conjugative transfer; *trg* – putative lytic transglycosylase gene; *parA* – putative plasmid partition gene; *insB* – putative integrase gene *rms* – putative type I restriction-modification system methyltransferase subunit gene; *repB* – putative plasmid replicase gene.

Strain	Plasmid	Accession	Backbone compared to pNDM-BJ01	Resistance region compared to JQ001791	Country of isolation
A. ursingii NIPH 706		APQB00000000.1	6 contigs contain 36 genes with 85.5-100% AA identity.	*bla*$_{NDM-1}$ and Tn*125* absent.	Czech Republic
Acinetobacter sp. NIPH 2168		APRW01000001.1	Contains 26 genes with 42.8-89.3% AA identity.	*bla*$_{NDM-1}$ and Tn*125* absent.	Netherlands
A. radioresistens WC-A-157	pWCA157-71	ALIR01000019.1	Contains 24 genes with 51.5-91.1% AA identity. 50kb of the 70kb plasmid has little identity.	*bla*$_{NDM-1}$ and Tn*125* absent. Contains copper and arsenic resistance regions.	Not specified
A. radioresistens SK82		ACVR00000000.1	1 contig contains 24 genes with 51.5-91.1% AA identity, in synteny.	*bla*$_{NDM-1}$ and Tn*125* absent. Contains copper and arsenic resistance regions as in pWCA157-71 present on another contig.	Not specified

Table 4.3 - Strain details for *Acinetobacter* spp. isolates containing sequences related to pNDM-BJ01-like plasmids but lacking the *bla*$_{NDM-1}$ gene.

Several other antibiotic resistance genes were identified in the CHI-40-1 contigs. A *bla*$_{OXA-58}$ gene, coding for a carbapenem hydrolysing OXA-type β-lactamase, was present with fragments of IS*Aba3*-like elements at either end of the contig. *bla*$_{OXA-58}$ is usually flanked by interrupted IS*Aba3*-like elements. The 5' IS*Aba3*-like fragment usually provides a strong promoter signal to the gene (Poirel and Nordmann 2006b) and this was also present on the CHI-40-1 contig (CDEL01000322). *arr-3*, *dfrA1* and *sul1*, coding for genes associated with resistance to rifampicin, trimethoprim and sulphonamides, respectively, were all identified as part of a class-1-integron (CDEL01000320). Sequences coding for aminoglycoside resistance genes *strA*, *strB* (CDEL01000323) and *aacC2* (CDEL01000324), the sulphonamide resistance gene, *sul2* (CDEL01000319), and macrolide resistance genes, *msr*(E) and *mph*(E) (CDEL01000321) were also identified. There appear to be two copies of *aphA6*. One of the contigs making up part of the *aphA6* sequence from pNDM-40-1 has high coverage relative to other contigs that make up the plasmid sequence (CDEL01000117). There are also two contigs with very close identity to contigs that make up the sequence of *aphA6* in pNDM-40-1 (CDEL01000157 and CDEL01000138). It is further likely that the second copy has IS*Aba125* upstream of the gene, since contig 151 (CDEL01000151) could be potentially linked to *aphA6* as well as *bla*$_{NDM-1}$, and also has high coverage. CHI-40-1 also contains a mercury resistance island, most similar to that found in *Acinetobacter* spp. LS56-7 but with substantial identity to sequences present in many *Acinetobacter* spp. and Enterobacteriaceae (CDEL01000139).

4.3.3 Conjugation experiments

Donor	UAB190	AG3528
CHI-40-1	$7.267x10^{-4} \pm 1.206x10^{-3}$	$1.598x10^{-5} \pm 1.291x10^{-5}$
69122 EW	$1.251x10^{-5} \pm 2.945x10^{-5}$	$2.279x10^{-4} \pm 1.575x10^{-4}$
74312 EC	$<1x10^{-9}$	$5.060x10^{-4} \pm 5.382x10^{-6}$
73668 ECT	$<1x10^{-9}$	$<1x10^{-9}$

Table 4.4 – Conjugation rates obtained from plate mating experiments. Numbers shown are means of 3 separate experiments with 2 standard deviations, calculated as transconjugant cfu/ recipient cfu.

From the two donor strains containing pNDM-BJ01-like plasmids, CHI-40-1 and 69122 EW, transconjugants were obtained in both UAB190 and AG3528 recipients. Transconjugants were also obtained from the 74312 EC donor in AG3528 but not UAB190. Mating rates are shown in Table 4.4. Antimicrobial susceptibility results for CHI-40-1 transconjugants and their recipient strains are shown in Table 4.1. MICs to all β-lactams except aztreonam increased but UAB190$_{NDMP2}$ MICs to meropenem were in the intermediate range only.

S1 PFGE and in gel hybridisation with *bla*$_{NDM-1}$ for transconjugants obtained with CHI-40-1 as a donor are shown in Figure 4.2. In *A. pittii* transconjugants *bla*$_{NDM-1}$ positive bands were ~45kb as expected for pNDM-40-1. However, UAB190 transconjugants were positive for *bla*$_{NDM-1}$ either on the chromosome or on a ~90kb plasmid.

4.3.4 Passaging Experiment

Probing of PFGE gels of S1 digested genomic DNA from CHI-40-1, UAB190$_{NDMP2}$ and AG3528$_{NDMP1}$ over the course of the 14 day passage showed that in all cases *bla*$_{NDM-1}$ bands did not alter in size (see Figure 4.8). Intensity of the *bla*$_{NDM-1}$ bands in CHI-40-1 was relatively similar over the course of the experiment with and without meropenem selection. The intensity of the *bla*$_{NDM-1}$ bands for both transconjugant strains was stable with antibiotic selection but decreased significantly over the course of the passage without meropenem selection. S1 digests of passaged transconjugant strains probed with *traA* demonstrated that this gene was on fragments of the same size as *bla*$_{NDM-1}$ (see Figure 4.9). The size of the bands did not alter over the course of the passage but the intensity of the *traA* bands increased over the course of the passage.

A more accurate assessment of the loss of *bla*$_{NDM-1}$ and *traA* gene copies over the course of the experiment with meropenem selection versus without selection was obtained by qPCR. Regression analysis of ΔΔCt values (Figures 4.10 and 4.11) showed that there were statistically significant falls in the quantity of both *bla*$_{NDM-1}$ and *traA* template present over the course of the passage experiment for both transconjugant strains without meropenem selection. For the donor strain *A. bereziniae* CHI-40-1 the opposite trend was seen. Although the trend was statistically significant for *bla*$_{NDM-1}$ ($p=0.038$), the size of the effect was small. *bla*$_{NDM-1}$ and *traA* both remained detectable throughout the 14 day passage experiment, even in the absence of antibiotic selection.

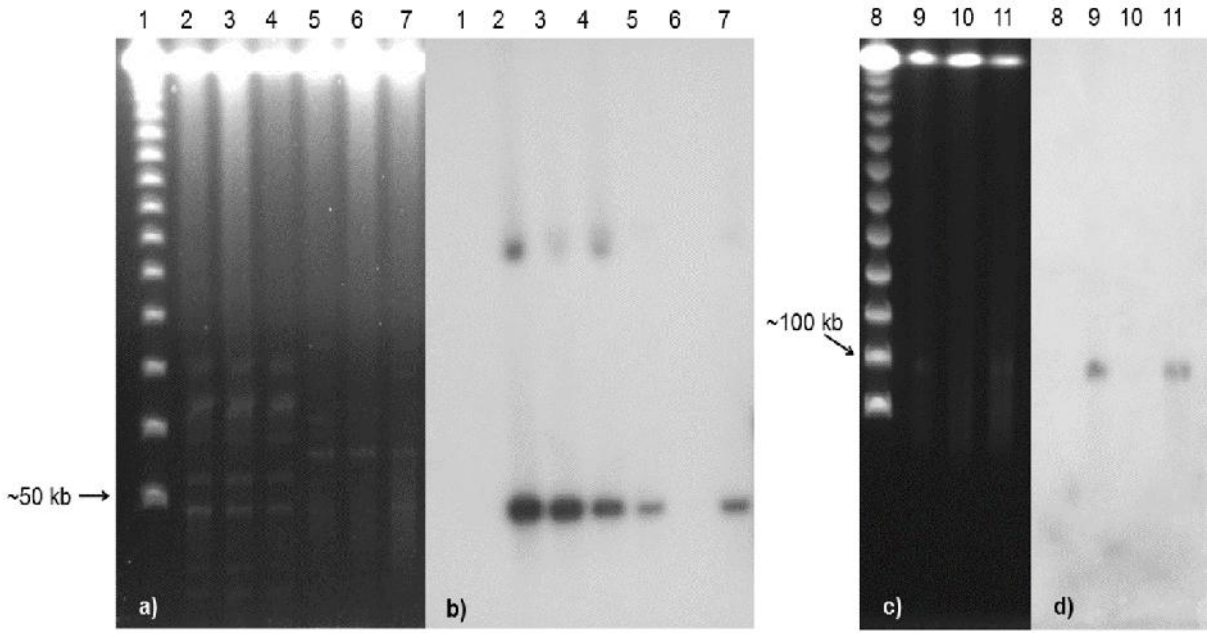


Figure 4.8 – Pulsed field gels of S1 digested genomic DNA from passaged isolates and in gel hybridisation with *bla*$_{NDM-1}$ gene probe. a) Pulsed field gel of CHI-40-1 and AG3528$_{NDMP1}$ at start of passage (D0) and after 14 day passage without antibiotics (D14N) and with meropenem (D14M); b) in gel hybridisation of a); c) Pulsed field gel of UAB190$_{NDMP2}$ at D0, D14N and D14M; d) in gel hybridisation of b). 1 - λ concatemer (~50-1000kb); 2 – CHI-40-1 D0; 3 – CHI-40-1 D14N; 4 – CHI-40-1 D14M; 5 – AG3528$_{NDMP1}$ D0; 6 - AG3528$_{NDMP1}$ D14N; 7 - AG3528$_{NDMP1}$ D14M; 8 – λ; 9 – UAB190$_{NDMP2}$ D0; 10 – UAB190$_{NDMP2}$ D14N; 11 – UAB190$_{DMP2}$ D14M

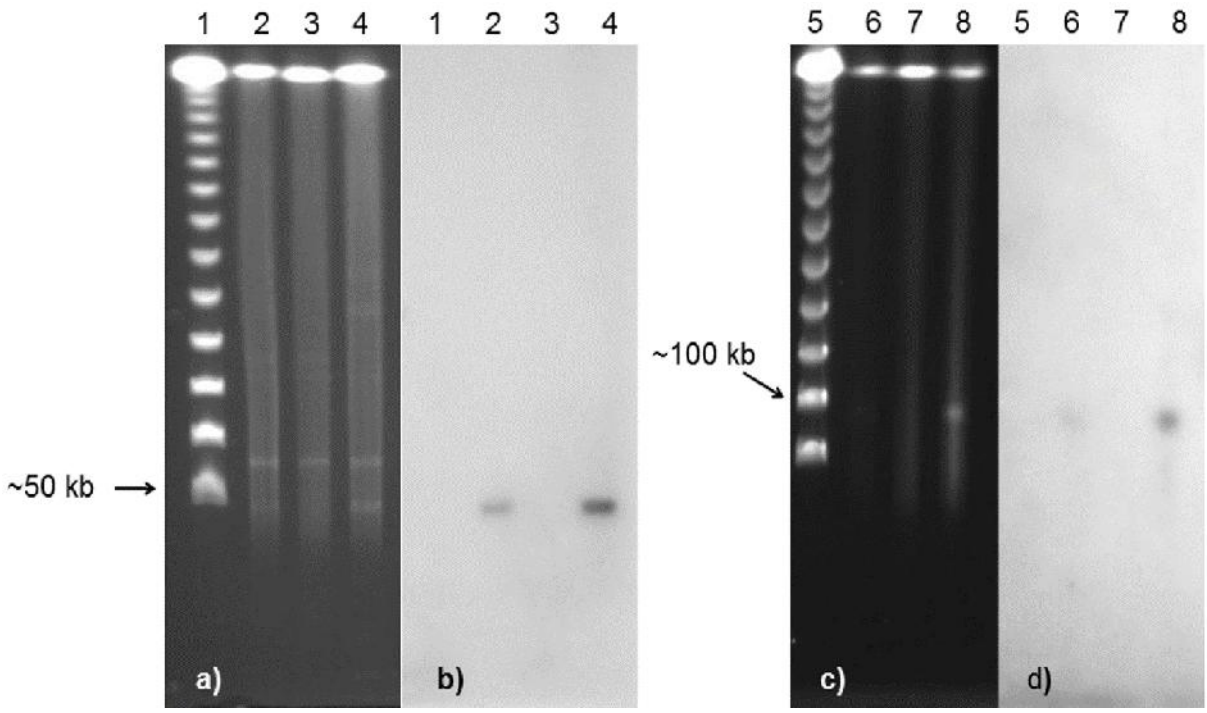


Figure 4.9 – Pulsed field gels of S1 digested genomic DNA from passaged isolates and in gel hybridisation with *traA* gene probe. a) Pulsed field gel of AG3528$_{NDMP1}$ at start of passage (D0) and after 14 day passage without antibiotics (D14N) and with meropenem (D14M); b) in gel hybridisation of a); c) Pulsed field gel of UAB190$_{NDMP2}$ at D0, D14N and D14M; d) in gel hybridisation of b). 1 - λ concatemer (~50-1000kb); 2 – AG3528$_{NDMP1}$ D0; 3 – AG3528$_{NDMP1}$ D14N; 4 - AG3528$_{NDMP1}$ D14M; 5 – λ; 6 – UAB190$_{NDMP2}$ D0; 7 – UAB190$_{NDMP2}$ D14N; 8 – UAB190$_{DMP2}$ D14M

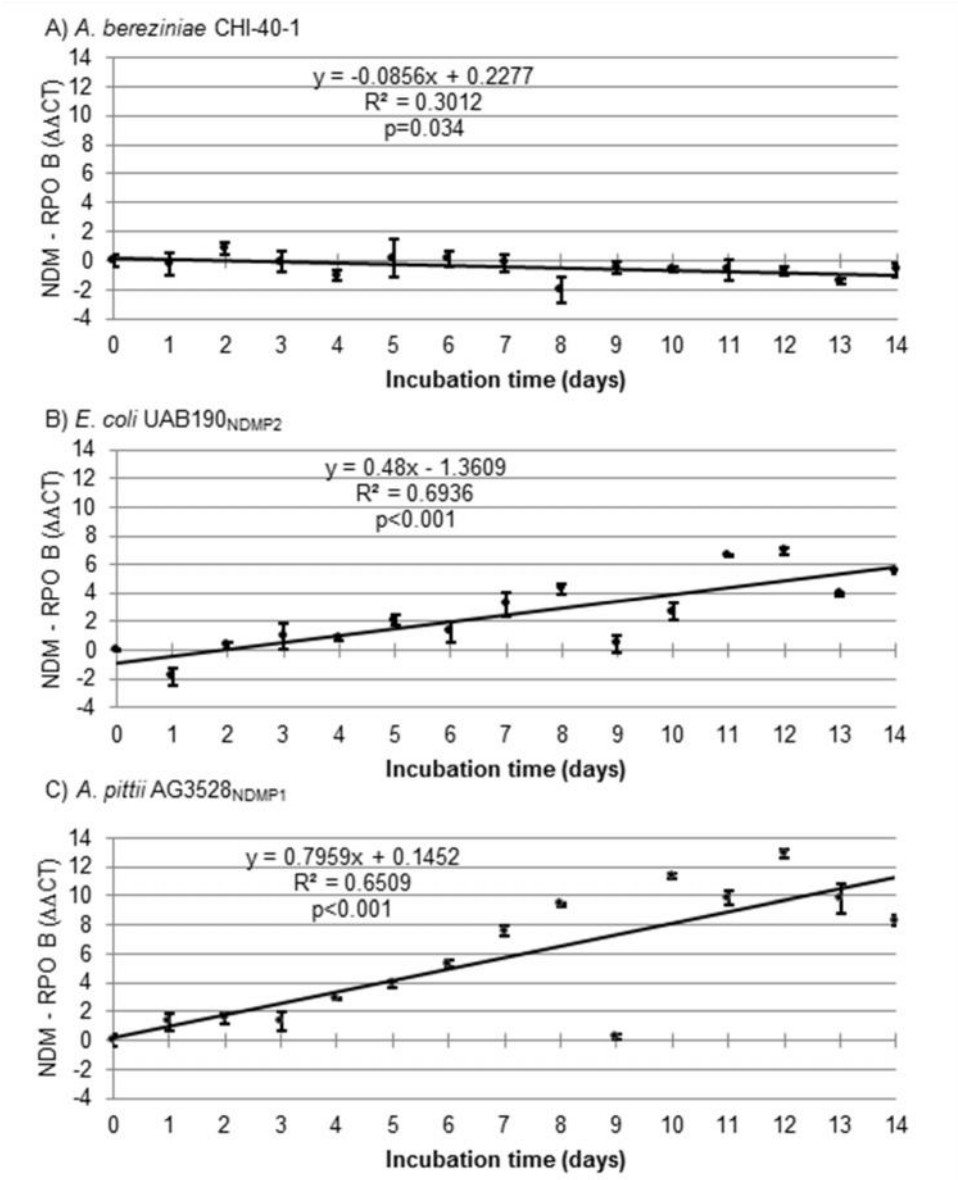


Figure 4.10 – Estimated quantity of *bla*$_{NDM-1}$ gene present relative to *rpoB* gene over the course of the passage experiment with meropenem selection versus no antibiotic selection by ΔΔCT method. Results are shown for a) the *bla*$_{NDM-1}$ positive donor strain CHI-40-1 and transconjugants b) UAB190$_{NDMP2}$ and c) AG3528$_{NDMP1}$. Note that a positive slope indicates a fall in quantity of *bla*$_{NDM-1}$ gene detected relative to reference in the absence of antibiotic selection. Results based on means of three replicate real time PCRs, error bars show 2 standard deviations.

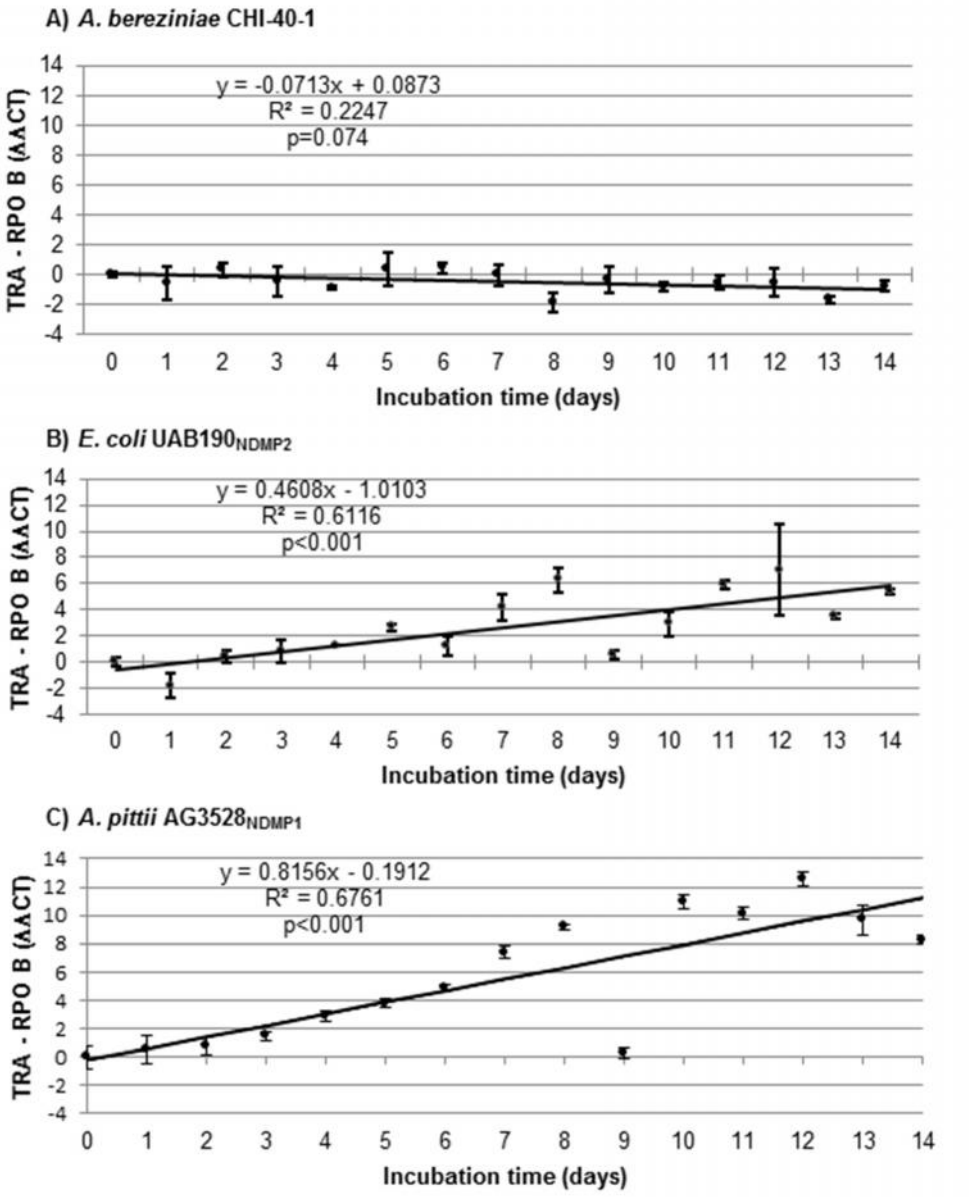


Figure 4.11 – Estimated quantity of *traA* gene present relative to *rpoB* gene over the course of the passage experiment with meropenem selection versus no antibiotic selection by ΔΔCT method. Results are shown for a) the *bla*$_{NDM-1}$ positive donor strain CHI-40-1 and transconjugants b) UAB190$_{NDMP2}$ and c) AG3528$_{NDMP1}$. Note that a positive slope indicates a fall in quantity of *traA* gene detected relative to reference in the absence of antibiotic selection. Results based on means of three replicate real time PCRs, error bars show 2 standard deviations.

4.3.5 Genetic contexts of *bla*$_{NDM-1}$ in *Acinetobacter* spp. isolates from Karachi

Analysis of *bla*$_{NDM-1}$ positive *Acinetobacter* spp. isolates from Pakistan was undertaken to see if a similar context to that found in CHI-40-1 was present. Probing of S1 PFGE gels showed that *bla*$_{NDM-1}$ was present on ~45kb plasmids in *A. haemolyticus* isolates but was on a plasmid ~50kb in *A. towneri* 73668-ECT and possibly a ~300kb in *A. schindleri* 74312-EC (see Figure 4.12). *Apa*I profiles showed that the three *A. haemolyticus* strains were clonally related, with the other strains having unique profiles (See Figure 4.13).

PCR analysis revealed that the *A. haemolyticus* strain contained sequences similar to several sections of the pNDM-BJ01 backbone and that the *bla*$_{NDM-1}$ context could be linked to *traA* and *res* genes found in these plasmids. The immediate context differed from that described in pNDM-BJ01 in that most of Tn*125* was missing. PCR analysis revealed that the *A. haemolyticus* strain, but not the other two *Acinetobacter* spp. isolates from Karachi, contained several regions of a pNDM-BJ01-like plasmid backbone. The immediate *bla*$_{NDM-1}$ context in 69122-EW was linked to pNDM-BJ01-like backbone genes *traA* upstream and the resolvase gene downstream (Figure 4.5b and Appendix 4.5). The immediate genetic context differed from that described in pNDM-BJ01 in that most of Tn*125* was missing. A previously uncharacterised insertion sequence, IS*Aha3*, most similar to IS*Alw1* (95% AA identity between transposases), was inserted between *ble* and the putative resolvase gene. No direct repeats (DRs) were observed, but this was not uncommon with other closely related ISs deposited in ISFinder. It is possible that transposition of IS*Aha3* resulted in deletion of the sequence often found between *ble* and *res* and also resulted in the loss of one of the DRs (Weinert *et al.* 1983). S1 PFGE and in-gel

hybridisation showed that *bla*$_{NDM-1}$ was present on ~45kb plasmids in 69122-EW, pNDM-69122 (see Figure 4.12).

For 74312-EC and 73668-ECT no sequence from pNDM-BJ01-like plasmids was detected. In both cases the genes *aphA6* and IS*Aba125* were detected upstream of *bla*$_{NDM-1}$ and the *ble* gene was downstream. In 74312-EC *trpF* and *tat* were also detected downstream of *ble*. However, the rest of the genes usually associated with Tn*125* harbouring *bla*$_{NDM-1/2}$ in *Acinetobacter* spp. appear to be missing in these strains.

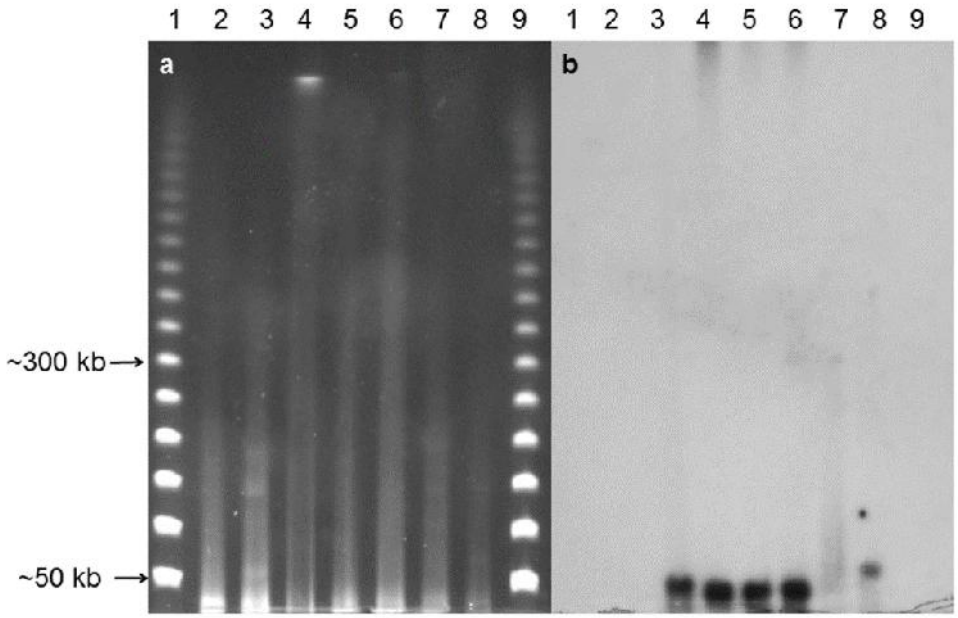


Figure 4.12 – a) Pulsed field gel of S1 digested genomic DNA from *Acinetobacter* spp. Isolates from Karachi; b) in gel hybridisation with *bla*$_{NDM-1}$ gene probe. 1 – λ, 2 AG3528, 3 CHI-40-1, 4 – 70114-EC, 5 – 73261-EC, 6 – 69122-EW, 7 – 74312-EC, 8 – 73668-ECT, 9 – λ

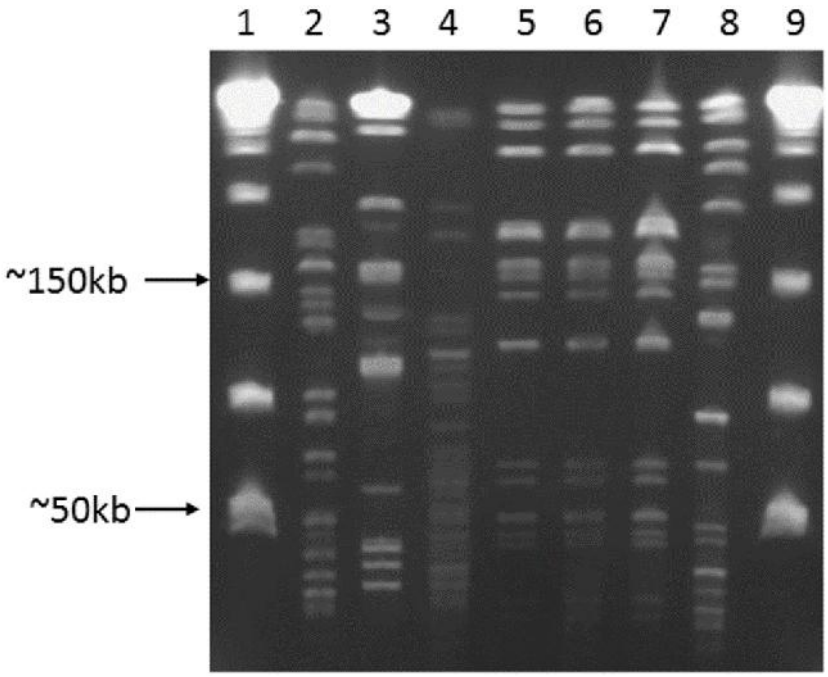


Figure 4.13 – Pulsed field gel of *Apa*I digested genomic DNA from *Acinetobacter* spp. Isolates from Karachi. 1 – λ concatemer (~50-1000kb), 2 CHI-45-1, 3 CHI-40-1, 4 – 74312-EC, 5 – 70114-EC, 6 – 73261-EC, 7 – 69122-EW, 8 – 73668-ECT, 9 – λ.

4.4 Discussion

Many of the complete NDM plasmid sequences from *Acinetobacter* spp. in the NCBI database are from Chinese isolates; are ~45-50kb and are very similar to pNDM-BJ01. pNDM-BJ01 contains a single *Apa*I restriction site, in keeping with the ~45kb bands present in both the S1 and *Apa*I gels. PFGE performed on *A. bereziniae* CHI-40-1 DNA subjected to an S1 concentration gradient showed that the higher the S1 concentration the fainter the 250kb band appeared (Figure 4.3). It was concluded that the strain harboured just one NDM-1 plasmid, which was likely to be similar to pNDM-BJ01, and that the 250kb band represented residual supercoiled plasmid DNA that had not been nicked by the enzyme (See original description of S1

method for comparison of S1 PFGE gels of digested and undigested genomic DNA) (Barton *et al.* 1995).

The complete sequence of pNDM-40-1 shares close sequence identity with the other fully sequenced pNDM-BJ01-like plasmids harbouring *bla*$_{NDM-1}$, suggesting that these plasmids share a close common ancestor. The pNDM-BJ01-like plasmids have a GC content similar to that of most *Acinetobacter* spp. WGS deposited in GenBank (~40%). The small number of related sequences not associated with *bla*$_{NDM-1}$, are more distantly related to pNDM-BJ01-like plasmids, but are also exclusively found in *Acinetobacter* spp. It is proposed that these findings are compatible with this plasmid lineage having evolved within the *Acinetobacter* genus and with the acquisition of *bla*$_{NDM-1}$ being a relatively recent event. As previously reported for pNDM-BJ01-like plasmids, the GC proportion of the region within Tn*125*, which includes *bla*$_{NDM-1}$, is much higher than that of the rest of the plasmid, strongly suggesting that, with the exception of the gene fusion at the 5' end of *bla*$_{NDM-1}$, this entire sequence is derived from an unrelated species of bacteria (Hu et al. 2012; Toleman et al. 2012).

Recent reports suggest that NPE may be more common than originally demonstrated in China (Qin et al. 2014; Zhou *et al.* 2014) and it is possible that the spread of *bla*$_{NDM-1}$ into Enterobacteriaceae could have occurred in China. However, the high prevalence in some regions of the Indian subcontinent of NPE has been well documented and most reports of travel-associated colonisation with NPGNB involved the subcontinent (Kumarasamy et al. 2010; Johnson and Woodford 2013). The characterisation of pNDM-40-1 in *A. bereziniae* from India and the identification of pNDM-BJ01-like plasmids in clonal *A. haemolyticus* isolates from Pakistan is therefore potentially significant.

bla$_{NDM-1}$ was also recently identified on pNDM-BJ01-like plasmids from clinical isolates of *A. schindleri* recovered from a soldier returning from Afghanistan (McGann et al. 2013), of *A. pittii* from a patient who had travelled to both Egypt and India (Bogaerts *et al.* 2013) and from a patient with peritonitis with *A. baumannii* in Honduras (Waterman et al. 2013). A plasmid with many pNDM-BJ01-like features (~45 kb, readily transferred by conjugation, sequence of the immediate *bla*$_{NDM-1}$ context similar) was also identified in an *A. pittii* isolate from a patient from Turkey, with no significant travel history, in 2006 (Roca *et al.* 2014). Insufficient data is available to establish whether these cases could be linked to recent spread from China or whether these plasmids are prevalent in a wider geographical region including the Indian Subcontinent, and further systematic studies would be required to clarify this point.

Including the results reported here, pNDM-BJ01-like plasmids have been reported in nine different species of *Acinetobacter*. The three strains harbouring pNDM-BJ01-like plasmids for which whole genome sequences are available appear on distinct branches of the rMLST phylogentic tree, reflecting significant differences in the nucleotide identity across the concatenated rMLST loci. However, the methods used to define the species of *Acinetobacter* harbouring these plasmids in some publications are questionable, as demonstrated by the fact that the strain WJ10621, in which pNDM-BJ01 was identified, was reported on the basis of phenotypic tests to be an *A. lwoffii* (Hu et al. 2012), while in this study it is shown that the strain clusters with *A. johnsonnii* strains by both 16S rRNA gene and rMLST phylogenetic analysis. The rMLST method provides robust speciation, with results similar to core *Acinetobacter* genus genome analysis (Chan *et al.* 2012; Jolley et al. 2012). In contrast, speciation by the phenotypic tests used by the majority of microbiology

laboratories is unreliable (Visca et al. 2011). In the absence of WGS results for Pakistani isolates, phylogenetic analysis of 16S rRNA genes and MALDI-TOF results were concordant in all cases, providing a reasonably robust identification. However, identity of 16S rRNA genes, even using very stringent similarity scores to define common species, can be inaccurate for several genera, including *Acinetobacter* (Chan et al. 2012). Accordingly, 16S rRNA gene should not be used for phylogenetic inferences where WGS are available.

Of the species identified in this study, *A. bereziniae*, *A. schindleri* and *A. haemolyticus* have all been isolated from clinical samples, including normally sterile sites, despite the first two species only being fully described in recent years (Castellanos Martinez *et al.* 1995; Nemec et al. 2001; Dortet *et al.* 2006; Quinteira *et al.* 2007; Gundi *et al.* 2009; Nemec et al. 2010; Figueiredo *et al.* 2012). They have all also been previously associated with XDR phenotypes and production of carbapenem hydrolysing enzymes (Quinteira et al. 2007; Bonnin *et al.* 2012a; Figueiredo et al. 2012; Park *et al.* 2012; McGann et al. 2013; Yamamoto *et al.* 2013). There is very little information published on *A. towneri* and to my knowledge no publications reporting clinical isolation of this organism (Carr et al. 2003). The clinical significance of these organisms is therefore uncertain, although *bla*$_{\text{NDM-1}}$ is also associated with *A. baumannii* and *A. pittii* which are better recognised as opportunistic nosocomial pathogens (Chen et al. 2011; Visca et al. 2011; Yang et al. 2012; Bogaerts et al. 2013; Zhang et al. 2013b).

Most descriptions of conjugative transfer of *bla*$_{\text{NDM-1}}$ from *Acinetobacter* spp. *in vitro* are from isolates with pNDM-BJ01-like plasmids (Chen et al. 2011; Hu et al. 2012; Yang et al. 2012; Zhang et al. 2013b). Conjugation rates were similar for the pNDM-BJ01-like plasmids, pNDM-40-1 and pNDM-69122, into *E. coli* and *A. pittii*

recipients. In the transconjugant strains *E. coli* UAB190$_{NDMP2}$ and *A. pittii* AG3528$_{NDMP1}$, containing pNDM-40-1, both *bla*$_{NDM-1}$ and *traA* copy number were stable in the presence of meropenem but decreased in its absence. Despite this, both genes were still detectable by qPCR at the end of the antibiotic free passage. These results are similar to those described by Huang *et al* (Huang et al. 2013) for the plasmid pKPX-1 in *K. pneumoniae* KPX, although in that study the increase in *bla*$_{NDM-1}$ copy number with increasing imipenem exposure was mediated by tandem repeats of an 8.6kb cassette rather than simply influenced by plasmid copy number and stability. In contrast Chen *et al* (Chen et al. 2011) reported that *E. coli* transconjugants obtained by mating with NPAB lost *bla*$_{NDM-1}$ following a single passage. However, the plasmids described were not sequenced, so may be very different to the pNDM-BJ01 plasmids, a different recipient strain was used and the method employed to test for *bla*$_{NDM-1}$ was not fully explained and may have been less sensitive than that employed in this study. To our knowledge, no other groups have specifically tested the stability of known pNDM-BJ01-like plasmids.

Representative *E. coli* and *A. pittii* transconjugants had higher MICs to all β-lactams, with the exception of aztreonam, compatible with the acquisition of *bla*$_{NDM-1}$ alone being responsible for the change in susceptibility profile. There was no change in the susceptibility of other antimicrobials in transconjugants, compared to their recipient backgrounds, including in susceptibility profile to aminoglycosides. Although pNDM-40-1 contains *aphA6*, it has already been reported that identical sequences from pNDM-BJ01-like plasmids are not associated with amikacin resistance in other isolates, and that there is not an identifiable promoter sequence upstream of *aphA6* in these plasmids (Hu et al. 2012; Waterman et al. 2013). In contrast, when IS*Aba125* and its promoter are upstream of *aphA6*, it is associated with amikacin

resistance (Nigro et al. 2011). Although CHI-40-1 contains other aminoglycoside modifying enzymes these do not explain the high-level of resistance to amikacin in this isolate. However, analysis of the contigs in CHI-40-1 suggests that there is a second copy of the gene which does have IS*Aba125* upstream of it. PCR analysis to confirm the assembly of this alternative context has not; however, been undertaken. The location of this second copy of *aphA6* has also not been analysed.

It was not initially anticipated that the stability of *bla*$_{NDM-1}$ and *traA* would be similar in UAB190$_{NDMP2}$ to that in AG3528$_{NDMP1}$. As well as being much more closely related to the donor strain, *A. pittii* is a species in which pNDM-BJ01-like plasmids have been isolated on several occasions (Yang et al. 2012; Bogaerts et al. 2013). However, in all *E. coli* UAB190 transconjugants subjected to S1 analysis, *bla*$_{NDM-1}$ and the plasmid relaxase from pNDM-40-1, *traA*, were found on the chromosome or larger plasmids. It may be that the only way that pNDM-40-1 can replicate in this host is by integration in the chromosome or another replicating plasmid. This may in turn explain the relative stability of *bla*$_{NDM-1}$ and *traA* in UAB190$_{NDMP2}$. Stability was not studied in other transconjugants, in which chromosomal integration was observed, and so may have been very different. WGS of the *E. coli* transconjugants would have also been useful, to allow analysis of whether the full pNDM-40-1 was integrated into new replicons, where the sequence was integrated and to generate hypotheses as to the mechanism of integration. The small decrease in *bla*$_{NDM-1}$ and *traA* copy number in the presence of meropenem in CHI-40-1 was also unexpected. This may be related to the presence in CHI-40-1 of a second carbapenemase, *bla*$_{OXA-58}$. Hence, even with meropenem exposure the *bla*$_{NDM-1}$ gene might not offer any significant survival advantage at the concentrations used in the current experiment.

No complete pNDM-BJ01-like plasmid has yet been described in Enterobacteriaceae. However, sequence data now strongly suggests that at least some *bla*$_{NDM-1}$ contexts in Enterobacteriaceae are derived from pNDM-BJ01-like plasmids, since several examples demonstrate high levels of identity with the plasmid backbone sequences that flank IS*Aba14*, *aphA6* and Tn*125* containing *bla*$_{NDM-1}$. The results of the mating and passage experiment make it feasible that these plasmids could have been transferred into Enterobacteriaceae and maintained for long enough for other MGE to move *bla*$_{NDM-1}$ and its immediate context to a new genetic location, for example Tn*125* or Tns formed by other ISs, as with the IS*903* elements in the *E. coli* plasmid pNDM-Dok-01 (Sekizuka et al. 2011).

Although pNDM-40-1 was maintained in UAB190$_{NDMP2}$ in the passage experiment, if it was not able to replicate autonomously, this element would be unlikely to have the same evolutionary success in Enterobacteriaceae as would result from *bla*$_{NDM-1}$, and its immediate context, being transferred onto MGEs that were fully functional in these backgrounds. For example, pNDM-Dok-01, is an incA/C plasmid (Sekizuka et al. 2011), a lineage that has a broad host range and as well as being one of the incompatibility types most often associated with *bla*$_{NDM-1,}$ has previously been involved in the dissemination of *bla*$_{CMY-2}$ amongst Enterobacteriaceae (Carattoli et al. 2012). The diversity of MGEs associated with *bla*$_{NDM-1}$ in Enterobacteriaceae, in addition to the diversity of species and strains carrying them, would suggest that *bla*$_{NDM-1}$ contexts in Enterobacteriaceae are highly plastic and mobile (Nordmann et al. 2011b; Walsh et al. 2011; Carattoli 2013). Why this is the case is not clear. However, even if movement of the *bla*$_{NDM-1}$ context from pNDM-BJ01-like plasmids to a new mobile genetic element were to be quite a rare event, the subsequent dissemination and diversification could occur rapidly.

The lack of an identifiable replicase or origin of replication in any of the pNDM-BJ01-like plasmids indicates that there is a considerable amount of work that needs to be done to understand these MGEs. The ability to autonomously replicate is a prerequisite for a plasmid (Francia et al. 2004). However, the wide range of replication strategies already described for plasmids in different bacterial species and the fact that very little work has been done on *Acinetobacter* plasmids other than for *A. baumannii* mean that the lack of an identifiable replicase is not entirely surprising (Francia et al. 2004; Carattoli et al. 2005; Bertini et al. 2010). One possibility is that *traA* could serve the functions of a relaxase and a replicase gene. This has been shown to be the case for RSF1010, the prototypical Mob_Q type plasmid from *E. coli*, which has been well characterised (Frey *et al.* 1992). Although RSF1010 is a mobilizable rather than conjugative plasmid it is noteworthy for having a very broad host range, in common with pNDM-40-1. It has been proposed that mobilizable and conjugative plasmids could all be classified with reference to their relaxase gene sequences instead of replication region (Francia et al. 2004). In accordance with this I propose that, in the absence of a known incompatibility or Rep type, pNDM-BJ01-like plasmids be classified as Mob_Q type plasmids.

Chapter 5

Vibrio cholerae: an environmental reservoir for mobile elements and antimicrobial resistance genes including *bla*$_{NDM-1}$?

5.1 Introduction

The focus of study in terms of NPGNB has been on the clinical and molecular epidemiology of the Enterobacteriaceae and *A. baumannii* (Gaillard *et al.* 2010; Poirel et al. 2012a). This is understandable, given the emergence of XDR phenotypes in important pathogens, like *E. coli* and *K. pneumoniae*, is of considerable public health concern (Kumarasamy et al. 2010; Johnson and Woodford 2013), and the proven potential of *A. baumannii* as a nosocomial pathogen associated with MDR and XDR phenotypes (Higgins et al. 2010; Visca et al. 2011). However, in Chapter 4 it was argued that the plasmids responsible for the dissemination of *bla*$_{NDM-1}$ genes from the genus *Acinetobacter* to the Enterobacteriaceae were likely to have arisen in "environmental" species with less pathogenic potential. In Chapter 3 it was noted that there is some evidence that the contents of Tn*125*, the Tn commonly harbouring *bla*$_{NDM-1}$ in *Acinetobacter* spp., was probably assembled in some other environmental species (Poirel et al. 2012a; Toleman et al. 2012).

In 2011 a study of seepage and tap water samples from around central New Delhi showed that the *bla*$_{NDM-1}$ gene could be detected by PCR at several sites.

NPGNB were cultured from many of these samples. Most of these were not Enterobacteriaceae but “environmental” organisms, such as *Pseudomonas* spp., *Stenotrophomonas maltophilia* and the aquatic organisms *Aeromonas caviae* and *V. cholerae* (Walsh et al. 2011). Since this study “environmental” bacteria have been found to harbour *bla*$_{\text{NDM-1}}$ on many occasions, as summarised in the General Introduction section 1.3.2 and Table 1.2. These have included bacteria isolated from clinical specimens as both causes of opportunistic infections and colonising flora, faecal screening samples and further environmental studies (Jovcic et al. 2011; Perry et al. 2011; Bharadwaj et al. 2012; Darley et al. 2012; Liu *et al.* 2012; Wang et al. 2012; McGann et al. 2013; Wang et al. 2013). These include many of the “environmental” *Acinetobacter* spp. as discussed in Chapter 4, whilst *Pseudomonas* spp. have also been prominent amongst carriage isolates (Jovcic et al. 2011; Bharadwaj et al. 2012; Hu et al. 2012; Wang et al. 2012; Yang et al. 2012).

Our laboratory is currently involved in the largest clinical prevalence and risk factor study ever undertaken of carriage and infection with NPGNB, carried out on patients admitted to a public hospital in Karachi, Pakistan. In that study only a small proportion of *bla*$_{\text{NDM-1}}$ positive bacteria isolated are NPE, with many “environmental” organisms including *Acinetobacter* spp. and *Pseudomonas* spp. representing the bulk of the organisms identified. Other bacteria identified include *Aeromonas* spp., *Comomonas aquatica* and *Achromobacter* spp., all of which are associated with aquatic environments (T.R. Walsh personnel communication).

Amongst the “environmental” organisms associated with significant clinical infections caused by NPGNB two cases have involved *V. cholerae*. A case of clinical cholera in a two-year-old boy in Puducherry, India, was reported in 2012 (Mandal et al. 2012). An O1 El Tor Ogawa *V. cholerae* strain harbouring *bla*$_{\text{NDM-1}}$ was isolated

from the boy's faeces. Although the strain was resistant to several antimicrobials, including cefotaxime, it was not resistant to carbapenems and the boy was treated successfully with fluid and electrolyte replacement and received ciprofloxacin, to which the organism was sensitive (Mandal et al. 2012). In 2011 a *V. cholerae* strain was isolated from the blood of a burns victim, who having sustained his injuries in Bihar, India, travelled back to the UK and was treated at a burns unit in Bristol (Darley et al. 2012). Very little else has been learned about *bla*$_{NDM-1}$ in *V. cholerae*. It was felt that the molecular epidemiology of *bla*$_{NDM-1}$ in *V. cholerae* was worthy of further investigation for a variety of reasons, relating to the clinical importance of some strains of *V. cholerae*, the ecological niches which the organism occupies and its potential for genetic plasticity.

There are several reasons to conclude that the role of HGT in the environment is important to the evolution of *V. cholerae*. These are reviewed in more detail in section 1.5.2 of the introduction. In summary *V. cholerae* is; naturally transformable in the presence of chitin (Lo Scrudato and Blokesch 2012; Sun et al. 2013a); there is evidence for closely related strains switching serotype by acquisition of GEIs with the genetic determinants of the o-somatic antigens (Ramamurthy et al. 2003; Blokesch and Schoolnik 2007; Chun et al. 2009); broad host range MGEs associated with AMR genes are common in *V. cholerae*, especially SXT/ R391 ICEs but with IncA/C plasmids also being identified (Burrus et al. 2006; Pan et al. 2008; Pugliese et al. 2009; Wozniak et al. 2009; Ceccarelli et al. 2013); all contain super integrons which probably contribute to their genetic plasticity (Rowe-Magnus et al. 1999; Baharoglu et al. 2012); and several elements associated with *V. cholerae*, including SXT/ R391 ICEs and super integrons, are influenced by SOS induced stress responses, potentially increasing plasticity in extreme environmental

conditions, such as with antimicrobial exposure (Beaber et al. 2004; Guerin et al. 2009; Baharoglu et al. 2012). Given that *V. cholerae* are naturally present in marine environments, and can also be found in sewage and the human gut, these organisms have the potential to share genetic information with both marine environmental organisms and human commensals and pathogens (Harris et al. 2012).

Our laboratory's culture collection includes all of the *bla*$_{NDM-1}$ positive isolates of *V. cholerae* so far identified, with the exception of the O1 El Tor isolate (Mandal et al. 2012) from the clinical cholera case. I set out to better characterise these isolates in terms of their strain background, the genetic contexts of *bla*$_{NDM-1}$ and the presence of other antimicrobial and heavy metal resistance determinants. In so doing the aim was to better understand how these strains had acquired *bla*$_{NDM-1}$ and assess the impact that the dissemination of *bla*$_{NDM-1}$ to *V. cholerae* could have on its further spread amongst environmental and pathogenic strains of bacteria.

5.2 Nucleotide sequence accession numbers

WGS assemblies have been deposited under project accession PRJEB8515 for *V. cholerae* isolates 116-17a, 116-17b, 116-14 and BRV8. Accession numbers are: CGII01000001-CGII01000203 for 116-17a contigs and LN831185 for plasmid pNDM-116-17; CGHE01000001-CGHE01000186 for 116-17b contigs; CGIJ01000001-CGIJ01000203 for 116-14 contigs and LN831184 for plasmid pNDM-116-14; CTBD01000001-CTBD01000152 for BRV8 contigs (SGI-NDM-1 is located on contig 38, accession CTBD01000091).

5.3 Results

5.3.1 Origin of study isolates

Two environmental *V. cholerae* isolates, 116-14 and 116-17, were identified from a single seepage water sample obtained from Gole Market, New Delhi, as part of a study of NPGNB in the city environment (Walsh et al. 2011). In this study seepage water sites, that is rivulets or pools of water in the streets, and tap water samples were sampled within a 12 km radius of central New Delhi. 51/171 seepage water samples and 2/51 tap water samples were positive for *bla*$_{\text{NDM-1}}$ by PCR and/ or in gel hybridisation. The sites positive for the *bla*$_{\text{NDM-1}}$ gene within central New Delhi are shown in Figure 5.1.

20 NPGNB were isolated on selective media, and identified biochemically by BD phoenix or API 20E. For the presumptive *V. cholerae* isolates speciation was supported by the use of the species specific *trkA* gene PCR and isolates were also tested with strain specific anti-sera for 01 and 0139 serotypes. Of note only 5 of the isolates identified in this study were Enterobacteriaceae but the method of isolation used conditions that were designed to optimise the identification of CRE. Although not reported in the original article, putative *V. cholerae* were isolated on TCBS agar from several other sites around the city, although these isolates were not *bla*$_{\text{NDM-1}}$ positive (T.R. Walsh, personal communication). Other organisms which are associated with aquatic environments were found to be *bla*$_{\text{NDM-1}}$ positive, including *A. caviae*, *Achromobacter* spp., *Pseudomonas* spp. and *S. maltophilia*.

The *bla*$_{\text{NDM-1}}$ positive *V. cholerae* isolates were not extensively characterised for this study. However, S1 PFGE and in gel hybridisation with a *bla*$_{\text{NDM-1}}$ gene probe show that these isolates harboured *bla*$_{\text{NDM-1}}$ on plasmids of approximately 170 kb in 116-17 and 400 kb in 116-14. In gel hybridisation with probes specific for different,

well characterised, plasmid incompatibility types showed hybridisation of the 170 kb plasmid with an IncA/C plasmid probe. However, none of the set of incompatibility typing probes hybridised with the 400 kb plasmid. Conjugation experiments were also carried out by broth and plate mating assays at room temperature, 30ºC and 37ºC overnight. From both *V. cholerae* isolates *bla*$_{NDM-1}$ could be transferred into *E. coli* J53 and *Salmonella enteritidis* R08/R10 at room temperature and 30 ºC but not at 37 ºC.

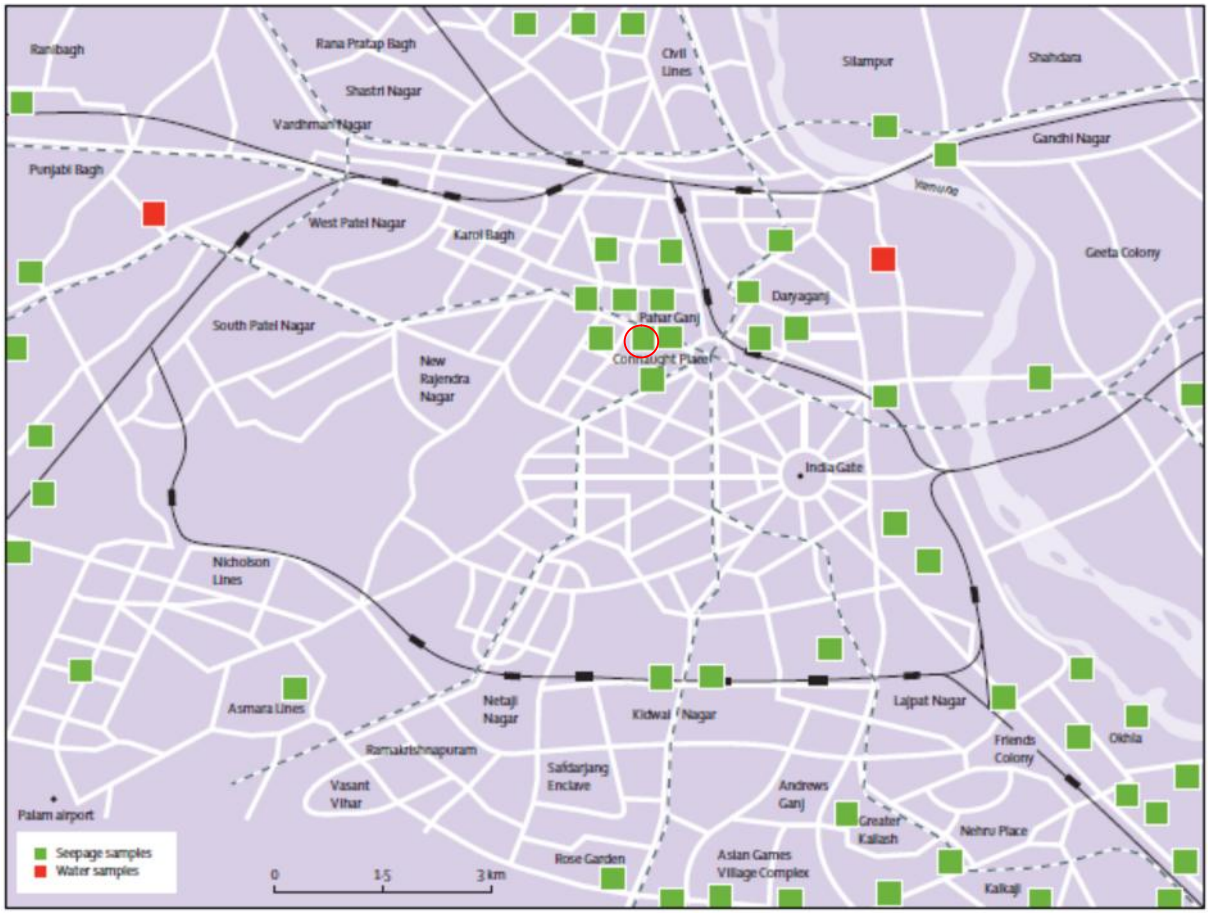


Figure 5.1 – Map of *bla*$_{NDM-1}$ positive samples from New Delhi centre and surrounding areas. The site that the *V. cholerae* strains were isolated from is highlighted on the map. Reproduced with modification from Walsh et al with permission (Walsh et al. 2011).

BRV8 originated from the blood of a burns patient. This 49-year-old British man was admitted to Frenchay burns unit in April 2011 (Darley et al. 2012). He had travelled to Bihar, in northern India. An accident in a faulty shower had resulted in him sustaining a severe electric shock. The immediate history following the electric shock is unclear but he had subsequently spent time in two hospitals in India and received unspecified antibiotics. He presented at Bristol 4 days following the electrocution, having flown back to the United Kingdom. At admission he was found to have very extensive burns of his face, neck, chest, left arm, left leg and the soles of his feet. He was hypotensive, had developed acute renal failure and required emergency management, with resuscitation and intensive care treatment. His wounds required extensive debridement and grafting. At admission he was found to have a high temperature and raised inflammatory markers. Multiple wound swabs and blood cultures were taken and on infection control and microbiology advice he was kept in single room isolation, with contact precautions, and started on empirical meropenem. The reason for the immediate isolation was the travel history, which was identified as a risk factor for MDR infections, including with NPGNB.

This infection control approach was vindicated by the isolation of multiple NPGNB. The blood cultures drawn soon after admission grew a possible *Vibrio* spp., which was identified at the Bristol microbiology laboratory as being resistant to all the first line agents it was tested against, including meropenem, ciprofloxacin and the aminoglycosides. It was subsequently identified as a *V. cholerae* and found not to agglutinate with anti-sera for O1 or O139.

5.3.2 Preliminary findings

The environmental isolates of *V. cholerae* 116-14, 116-17 and BRV8 had been stored at -80 °C for further study. These were subcultured on to LB agar with 0.5 µg/mL of meropenem (LBMer0.5), TCBS and CBA. On these initial plates two colonial morphotypes were seen for 116-14 and 3 for 116-17. Appearances on TCBS and LB were similar except for colony size, but on CBA there was variation in colony size, degree of haemolysis and colony colour. On subculture to LBMer0.5 agar and CBA of each morphotype, only a single colonial type was identified. 116-14a and 116-14b were indistinguishable, as were 116-17a and 116-17c. However, colonies of 116-17b were smaller than other 116-17 subcultures and associated with less haemolytic, small grey colonies on CBA. All isolates including each of the "colonial variants" were re-identified by BD phoenix as *V. cholerae*, except for 116-17b which was identified as a *V. mimicus*, and confirmed to be $bla_{\text{NDM-1}}$ positive by PCR.

5.3.3 PFGE and in gel hybridisation

The relatedness of the *V. cholerae* isolates was further explored by PFGE of *Not*I digested genomic DNA for each *V. cholerae* isolate. Results are shown in Figure 5.2a. Profiles for the "colonial variants" of 116-14 were identical, as were profiles for 116-17a and 116-17c. 116-17b, however, although being very similar to 116-17a differed by several bands. 116-17, 116-14 and BRV8 each presented distinct profiles. S1 PFGE was also performed and in gel hybridisation performed with $bla_{\text{NDM-1}}$ gene probes (Figure 5.2 b and c). This confirmed previous findings for all isolates with the exception of 116-17b, in which $bla_{\text{NDM-1}}$ was located on the chromosome, rather than on a ~170kb plasmid as for 116-17a and as originally reported for 116-17 (Walsh et

al. 2011). Subsequent analysis was performed on 116-17a, 116-17b, 116-14a and BRV8.

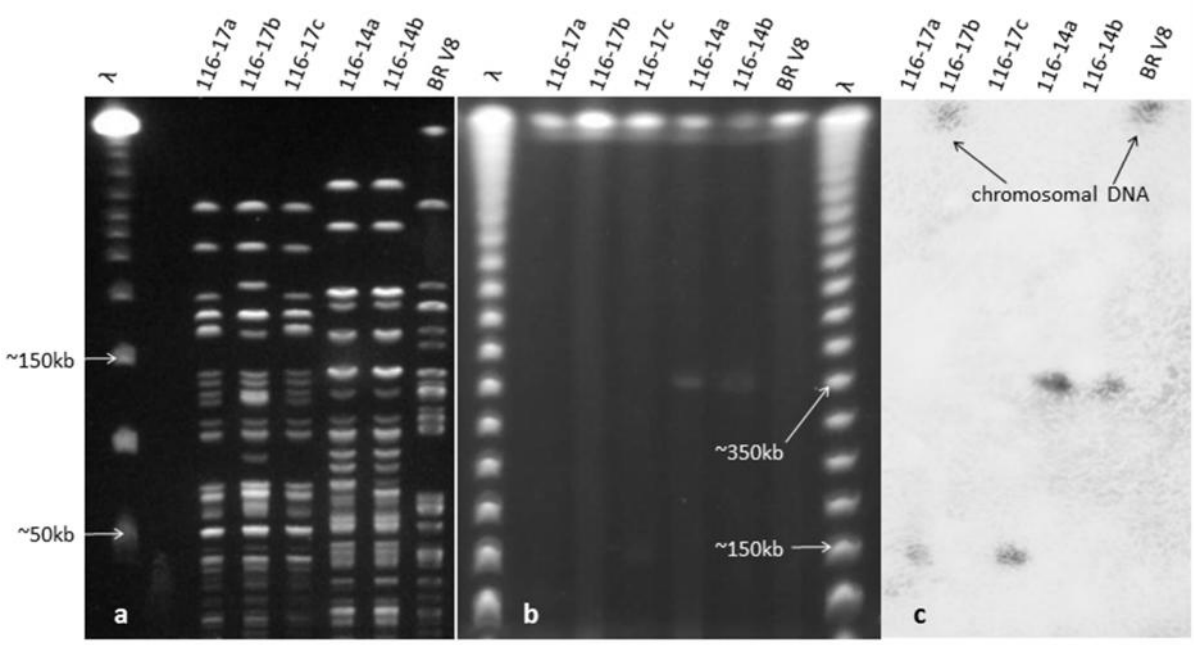


Figure 5.2 PFGE profiles and in gel hybridisation of *V. cholerae* isolates: a) *Not*I restriction digest PFGE profile; b) S1 digest PFGE profile; c) In gel hybridisation with *bla*$_{NDM-1}$ gene probe of S1 PFGE gel.

5.3.4 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing results for unique isolates are shown in Table 5.1. All exhibit high level resistance to most β-lactams and increased MICs to the carbapenems, although MICs remained just below sensitive-intermediate breakpoints to meropenem in 116-14 and imipenem in 116-17b. The strains are universally resistant to azithromycin but all sensitive to tetracycline. BRV8 and 116-17b remain susceptible to trimethoprim-sulfamethoxazole and both 116-17a and b are susceptible to chloramphenicol. Of note 116-17b had considerably lower MICs to trimethoprim-sulfamethoxazole and slightly lower MICs to chloramphenicol than 116-17a, despite being of a similar strain background. All of the isolates were sensitive to

at least two classes of antimicrobials which have breakpoints for use in *Vibrio* spp. according to CLSI guidelines (Clinical and Laboratory Standards Institute 2006). MICs to gentamicin, tobramycin and amikacin were substantially higher for BRV8 than for the other isolates. The correlation with the genotype of each strain is discussed below.

	116-17a	116-17b	116-14a	BR V8
Amoxicillin	≥256	≥256	≥256	≥256
Ceftazidime	≥256	≥256	≥256	≥256
Cefotaxime	≥32	≥32	≥32	≥32
Imipenem	6	4	8	12
Meropenem	6	6	2	6
Aztreonam	1	0.75	1.5	1.5
Tetracycline	0.75	0.75	1	0.125
Tigecycline	0.125	0125	0.064	0.094
Co-trimoxazole*	≥32	0.047	≥32	0.75
Ciprofloxacin	3	1	2	4
Azithromycin	8	8	8	3
Chloramphenicol	6	1	12	16
Gentamicin	0.25	0.25	0.5	>256
Tobramycin	1	1	1	>256
Amikacin	2	2	3	>256
Colistin	2	1	0.75	6

Table 5.1 – MICs (mg/L) as determined by MIC test strip for *V. cholerae* isolates. Cells shaded red for resistant, amber for intermediate resistance, green for sensitive, grey if no genus specific breakpoints available. Interpretation according to CLSI criteria (Clinical and Laboratory Standards Institute 2006). *****Trimethoprim-sulfamethoxazole.

5.3.5 De novo assembly results

WGS was performed on the four unique *V. Cholera* isolates. A summary of the outputs of the de novo assemblies of the WGS of these isolates is shown in Table 5.2.

Strain	No. of Contigs	Genome size	GC content (%)
116-17a	203	4,147,683bp	47.8
116-17b	186	4,125,773bp	47.8
116-14a	308	4,268,020bp	47.4
BRV8	158	4,105,007bp	47.6

Table 5.2 – summary statistics of de novo genome assemblies performed using Velvet assembly tool. GC – guanine cytosine. Genome size and GC% both based on a concatenation of all contigs, generated in Geneious.

5.3.6 Speciation by rMLST

Following WGS of the *V. cholerae* isolates, rMLST was used to provide a robust speciation. The ML phylogenetic tree shown in Figure 5.3 shows that the rMLST profiles of all the study isolates cluster with the *V. cholerae* strains and are distinct from *V. mimicus* (the closest relative to *V. cholerae*) and other clinically relevant *Vibrio* spp. 116-17b is not shown in the tree since its rMLST profile was identical to that of 116-17a, except for a SNP in locus 1. Note that this is contrary to the result obtained by automated biochemical testing performed by BD phoenix for 116-17b, which identified this isolate as a *V. mimicus* isolate.

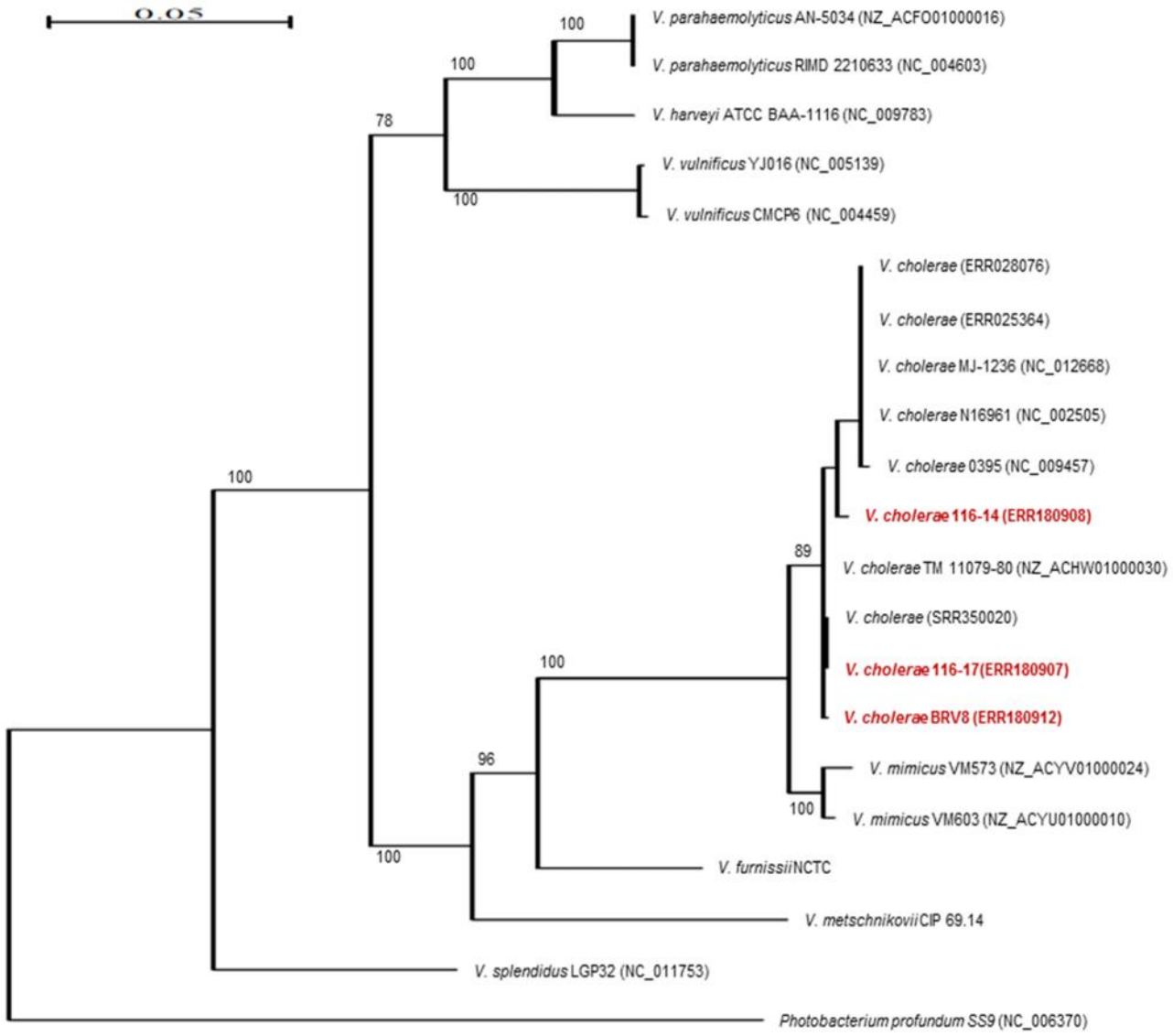


Figure 5.3 – ML phylogenetic tree of rMLST profiles of NPVC isolates compared with related species. Study isolates are shown in red. Bootstrap values for main branches (100 replicates) are shown.

5.3.7 Core genome phylogeny

To investigate the phylogenetic relationships between the study isolates and other *V. cholerae* isolates an approximately-ML phylogenetic tree of core genome SNPs was constructed (see Figure 5.4, full list of included isolates given in Appendix 5.1). This confirmed that 116-17a and 116-17b are representatives of the same strain, with almost identical core genomes. The *bla*$_{\text{NDM-1}}$ positive isolates cluster together; however, there is significant phylogenetic diversity between strains. They show substantial phylogenetic separation from the other *V. cholerae* sequences included in the tree, including classic and 7th pandemic isolates. They are closest to the L4 group which is comprised of the non-conventional, 01 serotype, clinical isolates A215, isolated in California in 1985, and A325, isolated in Argentina in 1993 (Mutreja et al. 2011).

5.3.8 Sequence analysis of pNDM-116-17 from 116-17a

The contig containing the *bla*$_{\text{NDM-1}}$ gene and linked contigs were identified as outlined in the Methods (see Chapter 2, section 2.11). 10 contigs were assembled to give the complete sequence of pNDM-116-17, an IncA/C plasmid of 167,382 bp with a GC content of 51.6%, coding for 194 putative ORFs (See Figure 5.5 and Appendices 5.2 and 5.3).

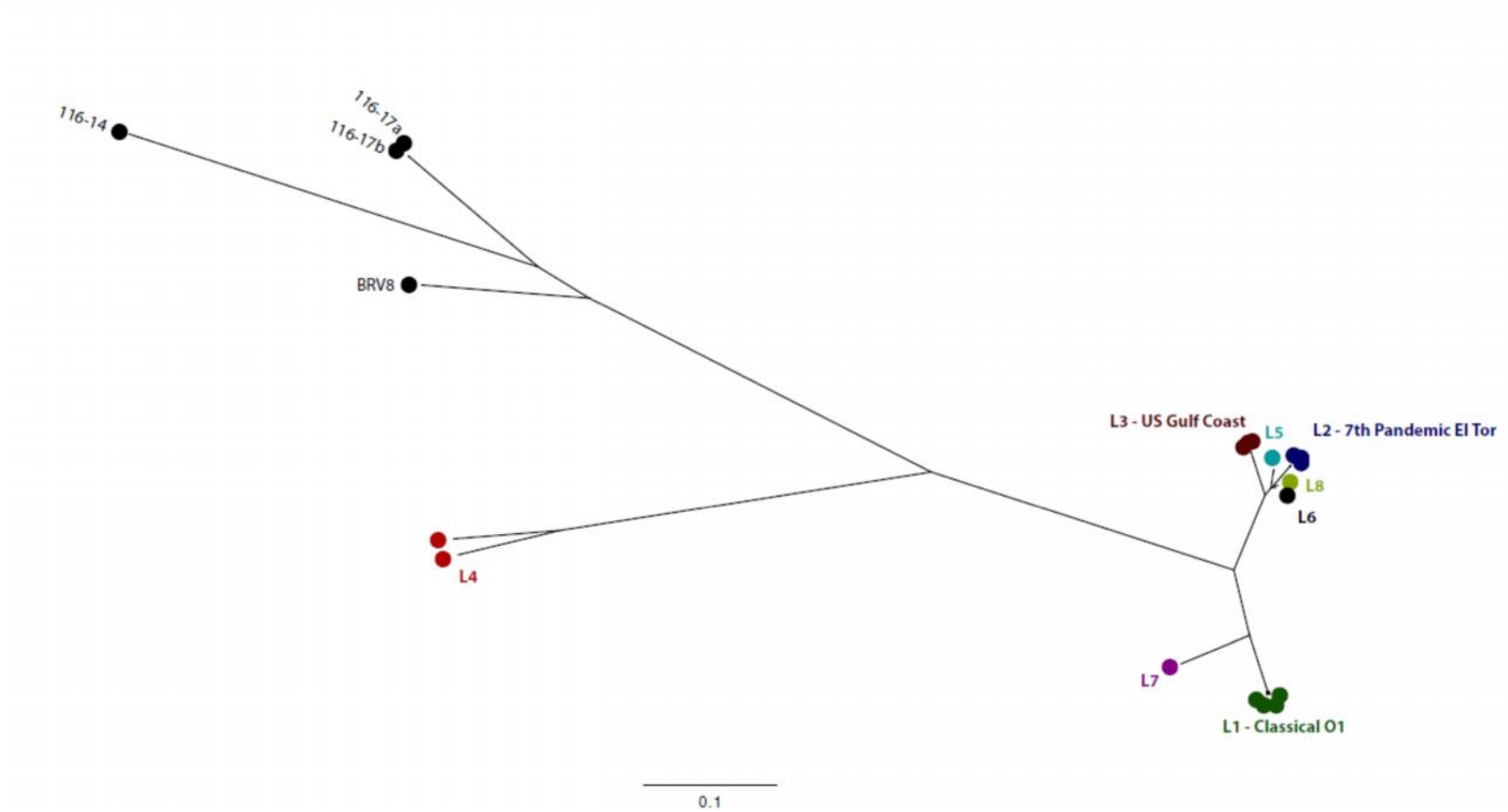


Figure 5.4 – Approximately-ML phylogenetic tree based on core genome SNPs: comparison of NPVC isolates with an international collection of *V. cholerae* of environmental and clinical origin. A full list of isolates included in the tree is shown in Appendix 5.1.

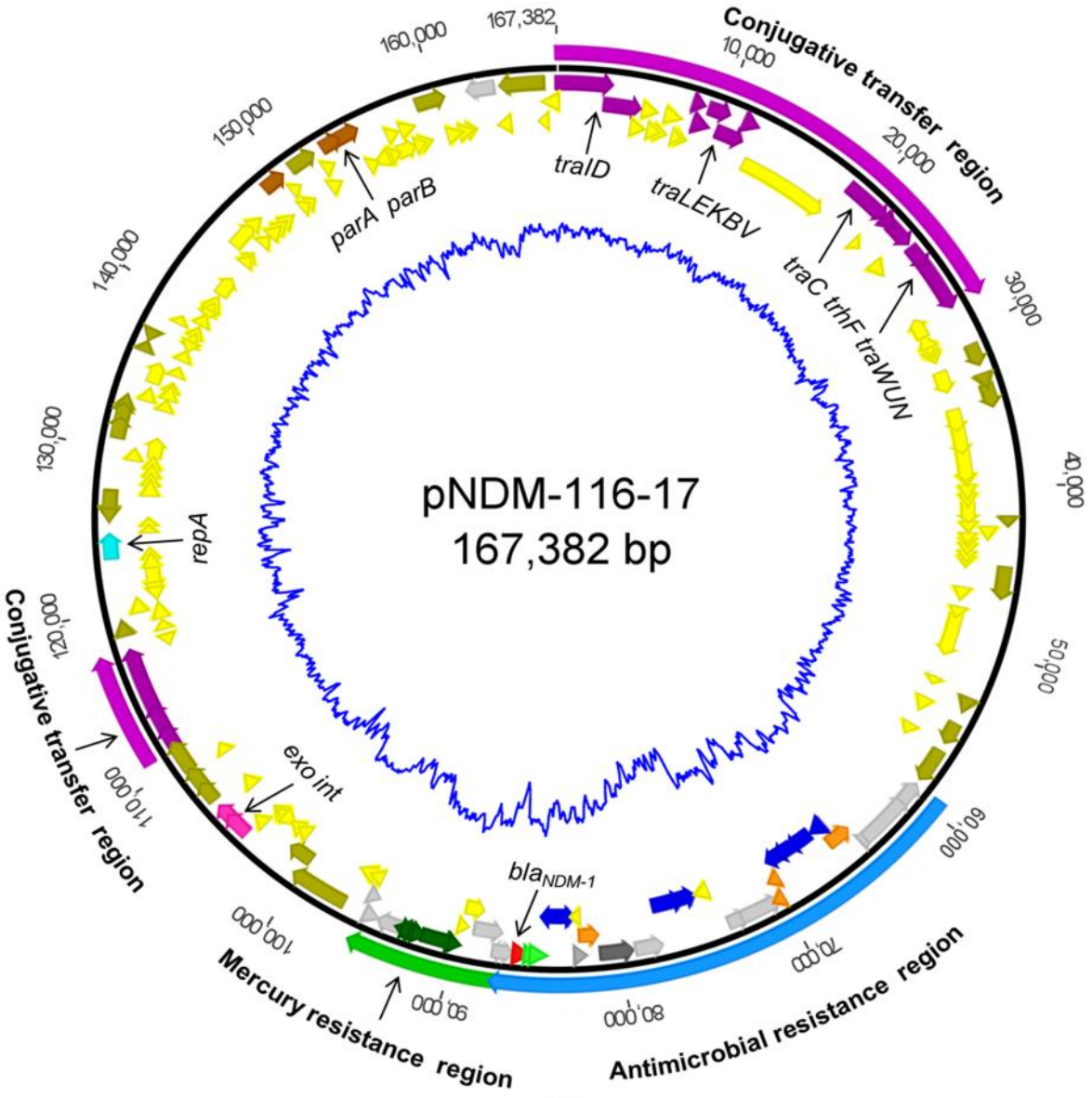

pNDM-116-17
167,382 bp
Conjugative transfer region
Antimicrobial resistance region
Mercury resistance region
Conjugative transfer region
traID
traLEKBV
traC trhF traWUN
parA parB
repA
exo int
bla NDM-1
10,000
20,000
30,000
40,000
50,000
60,000
70,000
80,000
90,000
100,000
110,000
120,000
130,000
140,000
150,000
160,000
167,382

Figure 5.5 – Gene map of IncA/C plasmid pNDM-116-17 (LN831185) from 116-17. Arrows indicate direction of gene transcription. ORFs are colour coded: red – *bla*$_{NDM-1}$; blue – other AMR genes; light orange – genes from conserved sequences of class 1 integrons; dark grey – IS*CR1*; light green – *trpF* and *ble* genes usually present downstream of *bla*$_{NDM-1}$; dark green – mercury resistance genes; light grey – IS (note ORFs are shown rather than the complete IS); turquoise – plasmid replicase, *repA*; purple – relaxase and T4SS genes, *tra*; dark brown – plasmid partition genes, *parA* and *parB;* pink – phage like integrase and excisionase genes, *int* and *xis*; light brown – other genes coding for proteins with proposed functions; yellow – genes coding for hypothetical proteins. Coloured bars on the outside of the map indicate regions of functional importance: purple – conjugative transfer; blue – AMR genes; green – mercury resistance. Base pairs numbered around outside of the map. Blue line on the inside of the sequence indicates GC content averaged over a sliding window of 300 residues. Identity of genes are given in the main text.

The *bla*$_{NDM-1}$ gene is contained within a large variable region containing many AMR genes and a group of genes associated with resistance to the heavy metal mercury. The sequence of pNDM-116-17 contains sequences with close identity to all of the genes proposed to be essential for IncA/C plasmid replication, maintenance and conjugative transfer. In addition to the IncA/C plasmid replicase, *repA*, these include the conjugative relaxase, *traI*, the components of the Type IV secretion system, *traBCDEFGHKLNUVW* and the plasmid partition genes *parA* and *parB* (Johnson and Lang 2012). pNDM-116-17 also contains the genes *int* and *xis*, coding for putative phage integrase and excisionase, respectively. These are present in many IncA/C plasmids, although their function is not known. IncA/C plasmids normally replicate autonomously, although chromosomal integration of IncA/C plasmids has been described previously (Johnson and Lang 2012).

Most sequenced IncA/C plasmids have close backbone identity with each other, with most variability occurring within regions which appear to be hotspots for the integration of foreign DNA (Carattoli et al. 2012; Johnson and Lang 2012). The core regions of pNDM-116-17 display >99% identity with many IncA/C plasmids identified from NCBI databases, including 8 harbouring *bla*$_{NDM-1}$. These plasmids are *E. coli* NDM-1 Dok01 plasmid pNDM-1_Dok01 (AP012208); *E. coli* N10-0505 plasmid pNDM10505 (JF503991); *E. coli* N10-2337 plasmid pNDM102337 (JF714412); *K. pneumoniae* ATCC BAA-2146 plasmid pNDM-US (CP006661); *K. pneumoniae* KP1 plasmid pKP1-NDM-1 (KF992018); *K. pneumoniae* Kp7 plasmid pNDM-KN (JN157804); *K. pneumoniae* N10-0469 plasmid pNDM10469 (JN861072); and *P. stuartii* plasmid pMR0211 (JN687470).

The immediate *bla*$_{NDM-1}$ context within pNDM-116-17 varies from that previously described in other IncA/C plasmids harbouring the gene and is instead

most similar to that of IncL/M plasmids pNDM-OM (JX988621, from *K. pneumoniae* 601) and pNDM-HK (HQ451074, from *E. coli* strain HK-01) (see Figure 5.6 and 5.7). A small part of the context around *bla*$_{NDM-1}$ is shared with several examples sequenced from Enterobacteriaceae, including the two plasmids just mentioned. Only a small fragment of the IS*Aba125* element upstream of *bla*$_{NDM-1}$ remains, having been interrupted by an IS*26* element. The promoter sequence at the 3' end of the element, upstream of *bla*$_{NDM-1}$ is, however, intact. Downstream of *bla*$_{NDM-1}$ the genes *ble* and *trpF* are followed by a fragment of the AmpC-type β-lactamase gene, *bla*$_{DHA-1}$, and its regulator gene, *ampR*. The genes *qacΔE1*, *sul1* and IS*CR1*, exactly as found in the 3' conserved sequence of some class 1 integrons, are found after *ampR*. The rest of the class 1 integron is missing.

The pNDM-116-17 context then differs from other closely related sequences in that IS*Ec29* and the macrolide resistance genes, *msr*(E) and *mph*(E), are immediately downstream of IS*CR1*, rather than being preceded by IS*Ec29* and the 16s RNA methylase gene, *armA*, as is the case in pNDM-HK. *mph*(E) is followed by a common arrangement, with a gene coding for a hypothetical protein, a gene proposed to code for a replicase from an *Acinetobacter* spp. plasmid, an IS*26* element and then an interrupted transposase gene. pNDM-116-17 again diverges from related sequences in that a class 1 integron containing genes coding for aminoglycoside (*aadA1*), β-lactam (*bla*$_{OXA-10}$), chloramphenicol (*cmlA7*) and rifampicin (*arr3*) are found next, although class 1 integrons are a common feature of many *bla*$_{NDM-1}$ contexts in Enterobacteriaceae. The immediate *bla*$_{NDM-1}$ contexts identified in all the *V. cholerae* isolates sequenced in this study and other sequences available from public databases are discussed further in a later section of this chapter.

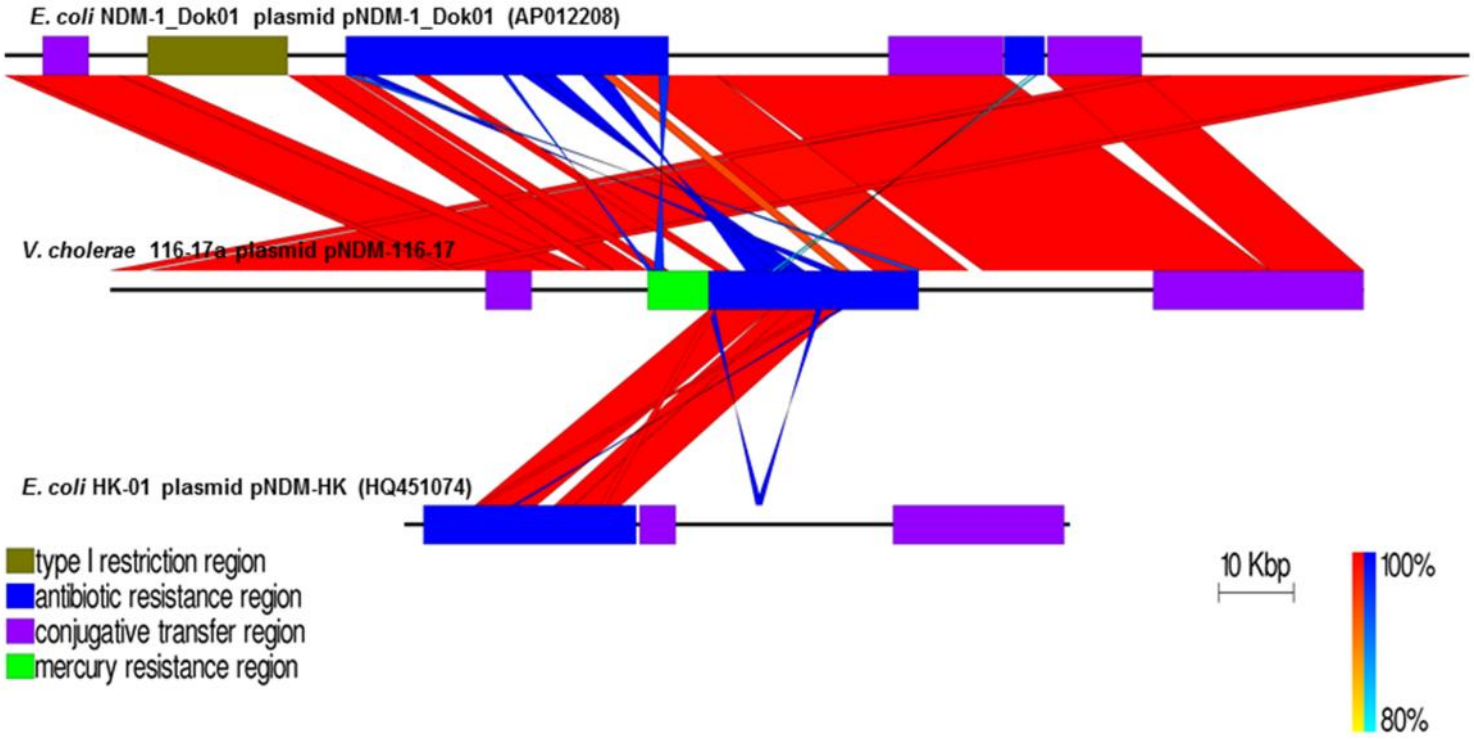


Figure 5.6 – ACT comparison of complete sequence of pNDM-116-17 with the IncA/C plasmid pNDM-Dok01 and the IncL/M plasmid pNDM-HK. BLAST comparison run using WebAct. Figure drawn using Easyfig 2.1. Areas with BLAST hits in same orientation shown by bars between sequences ranging from red to yellow. BLAST hits for sequences in reverse orientation ranging from dark to light blue, key shows % ID of BLAST hit matching to bar colours. Coloured boxes indicate gene regions as shown in the key on the left side of the figure.

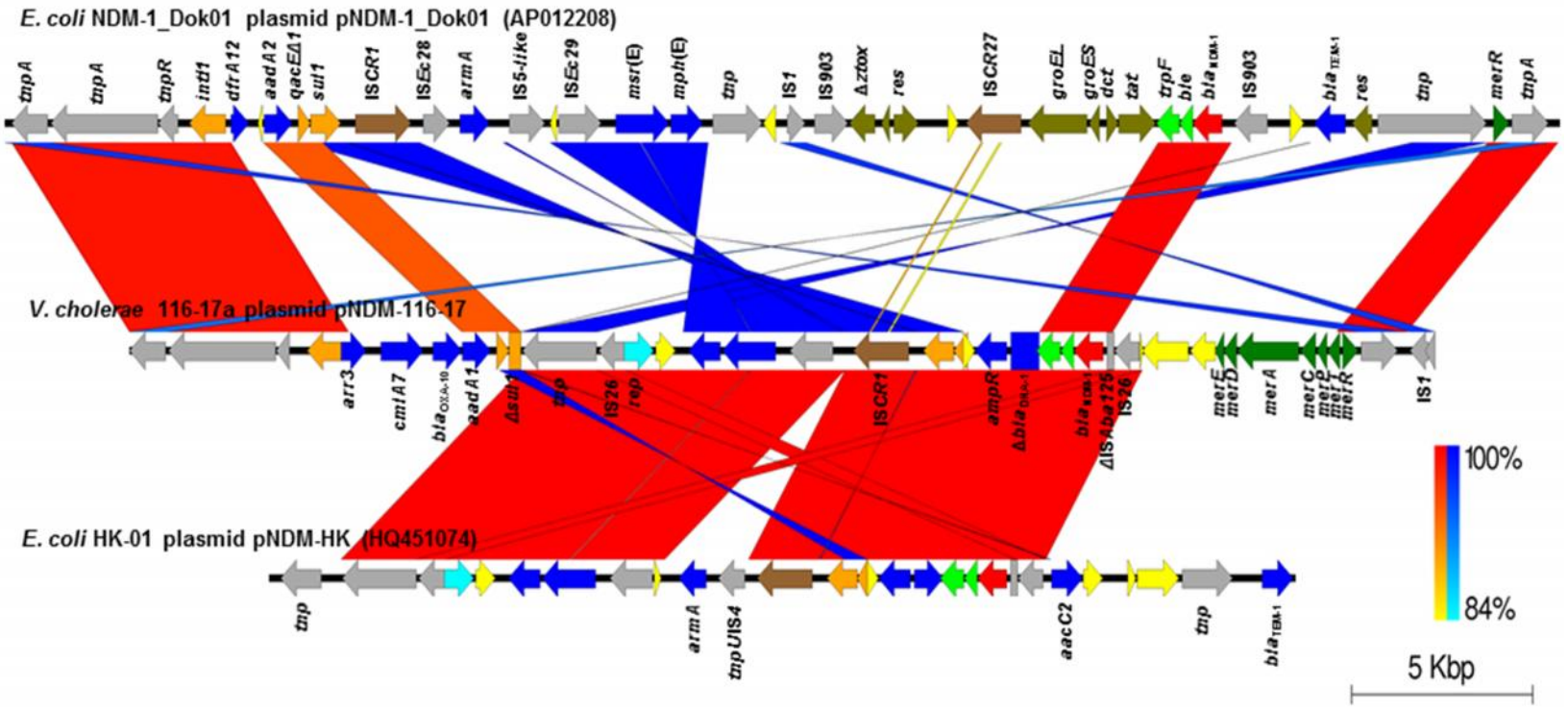


Figure 5.7 – ACT comparison of antibiotic and mercury resistance region of pNDM-116-17 with the resistance regions from pNDM-Dok01 and pNDM-HK. ORFs are colour coded as in Figure 5.5. The direction of transcription is indicated by arrow heads, truncated remnants of ORFs are shown as rectangles. BLAST comparison run using WebAct. Figure drawn using Easyfig 2.1. BLAST hits shown as in Figure 5.6. Annotations are given in the main text except: *dfrA12* – trimethoprim resistance gene, coding for dihydrofolate reductase; Δ*ztox* – truncated sequence coding for putative zeta-toxin; *res* – resolvase gene; *aacC2* – aminoglycoside resistance gene coding for aminoglycoside 3-N-acetyltransferase; *bla*$_{TEM-1}$ – TEM type β-lactamase gene.

The 6 other IncA/C plasmids with similar backbones containing *bla*$_{NDM-1}$ all contain an insertion which includes the *bla*$_{CMY}$ gene at a different integration hotspot to that harbouring *bla*$_{NDM-1}$, located between some of the conjugation apparatus genes. None of these plasmids possess the mercury resistance operon which is found immediately upstream of *bla*$_{NDM-1}$ in pNDM-116-17; although they do contain a fragment of the *merR* gene (see Figure 5.7). The genes usually found within this operon are; *merA,* which codes for a mercuric reductase; *merC*, *merP* and *merT*, which all code for mercury transporter proteins; *merR*, which codes for the main regulatory protein; and *merD*, which codes for a co-regulator (Mathema *et al.* 2011). Mercury resistance operons with close identity to that found in pNDM-116-17 are present in several other IncA/C plasmids (Johnson and Lang 2012).

5.3.9 Evidence of chromosomal integration of pNDM-116-17 in 116-17b

The assembly of 116-17b included 9 contigs with 99.8 to 100% nucleotide identity over their entire length to sections of the pNDM-116-17 sequence, encompassing almost the complete plasmid. However, no plasmid harbouring *bla*$_{NDM-1}$ was seen on the S1 PFGE gels. The 74 and 167 kb contigs which contain most of the IncA/C plasmid backbone genes could be linked by PCR to large contigs containing *V. cholerae* chromosomal genes and sequences with close identity to SXT/R391 family ICEs. The position of the contigs with identity to pNDM-116-17 suggest that the entire plasmid had integrated at a hotspot within the ICE which commonly contains AMR genes (Wozniak et al. 2009). Loss of resistance genes and changes in the resistance profile of 116-17b relative to 116-17a also support this (see discussion below). However, the insertion event seems likely to have been mediated by IS*10* elements, which are found at either end of the integrated IncA/C plasmid,

linking it to the ICE context, rather than to the IncA/C plasmid having integrated in a manner analogous to an ICE (see Figures 5.8 and Appendices 5.4 and 5.5).

5.3.10 Sequence analysis of pNDM-116-14 from 116-14

61 contigs from the 116-14 sequence were linked to assemble plasmid pNDM-116-14 (LN831184), which is 354,308bp long and has a GC content of 44.4%. pNDM-116-14 codes for 400 putative coding sequences (see Figure 5.9, Appendices 5.6 and 5.7). Of these 244 code for hypothetical proteins of unknown function. The genes coding for proteins of known function, or proposed function based on homology with known proteins, include 7 intact AMR genes, in addition to *bla*$_{\text{NDM-1,}}$ 7 metal resistance genes, 39 genes from ISs, 1 intact replicase gene, 2 plasmid partition genes and 11 genes involved in conjugative transfer.

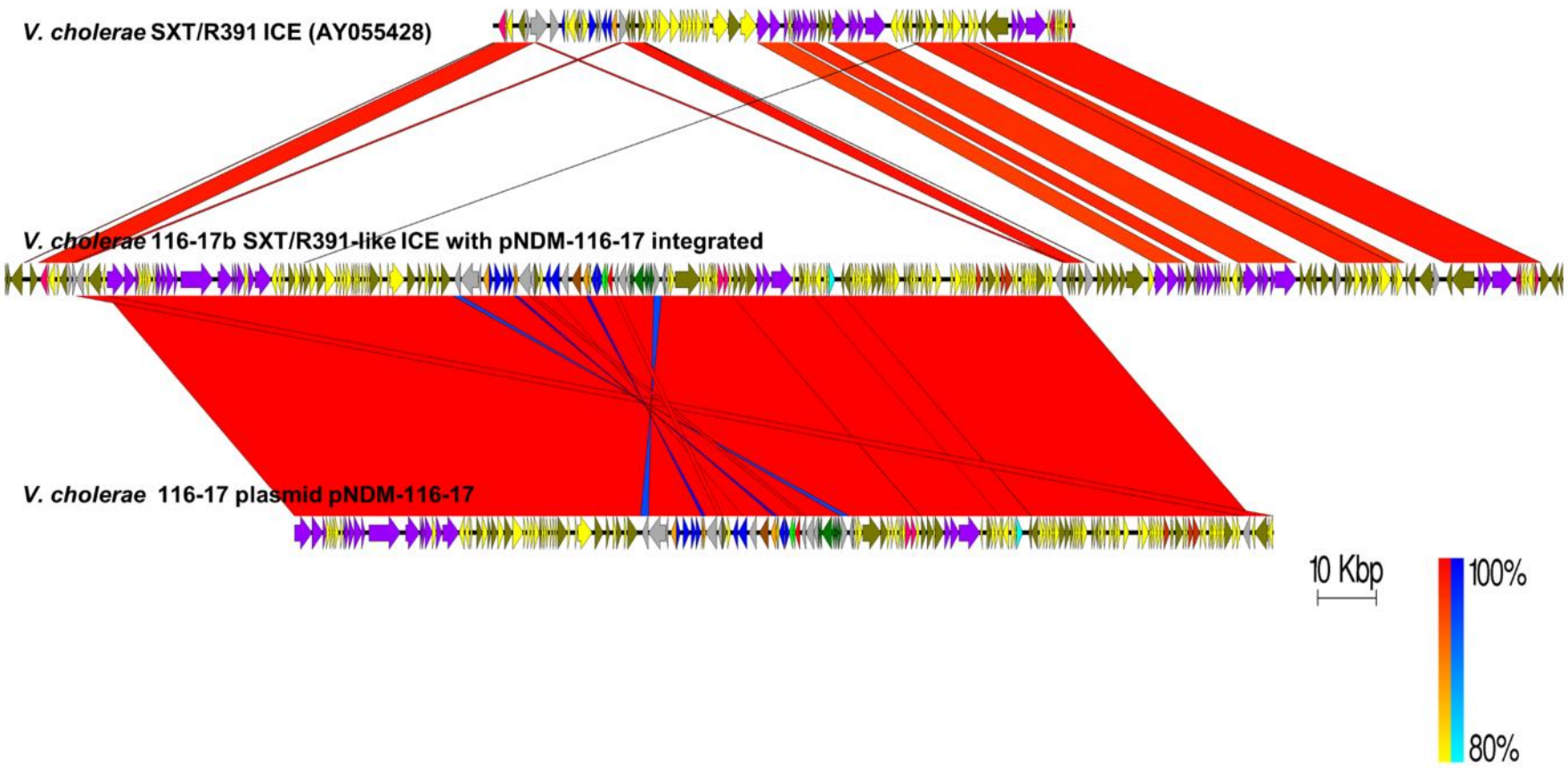


Figure 5.8 – ACT Comparison of contigs linking SXT/R391-like ICE to pNDM-116-17 in 116-17b to pNDM-116-17 and reference SXT/R391 ICE. Genes are colour coded as in Figure 5.5. BLAST comparison run using WebAct. Figure drawn using Easyfig 2.1. BLAST hits shown as in Figure 5.6.

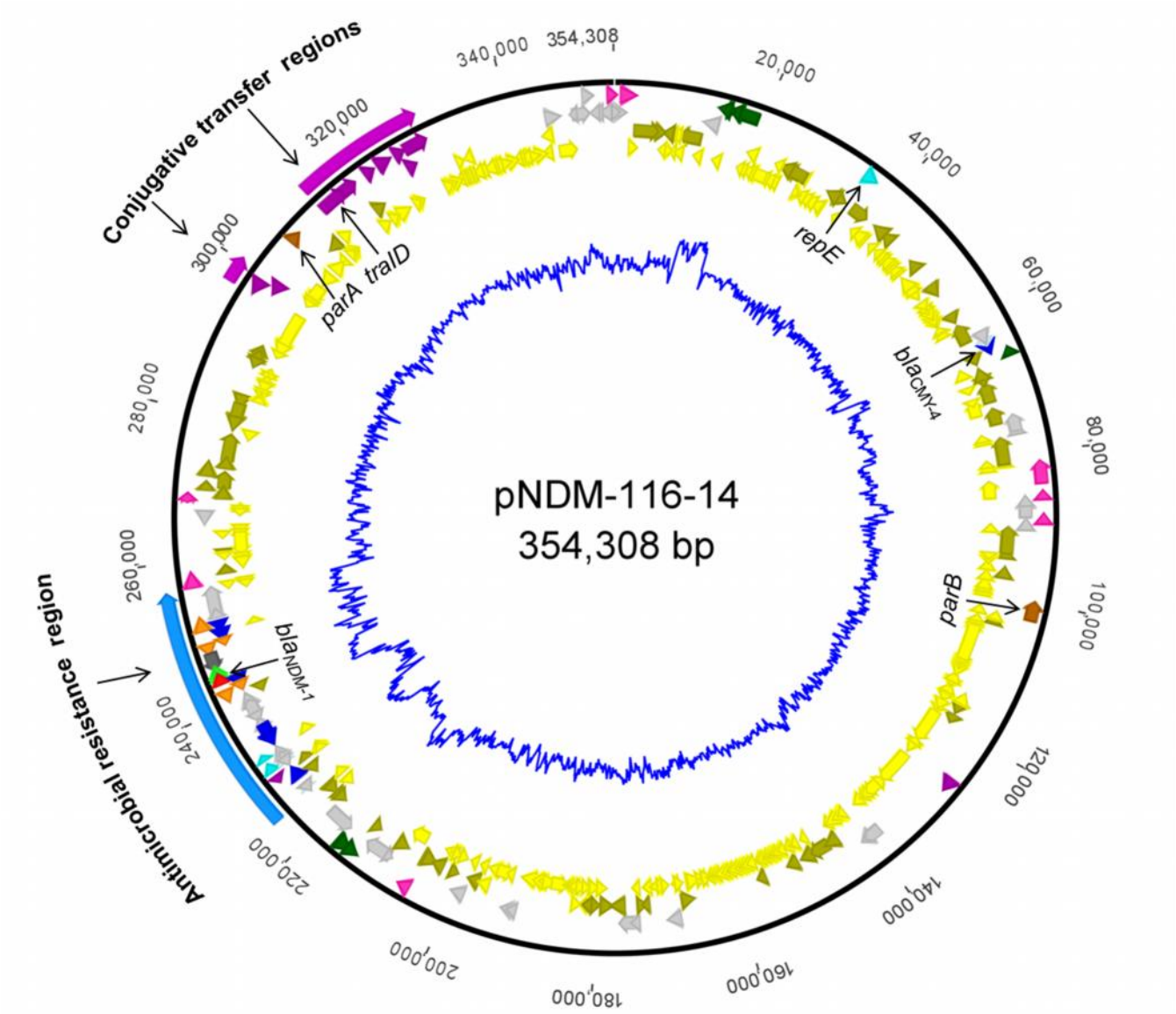


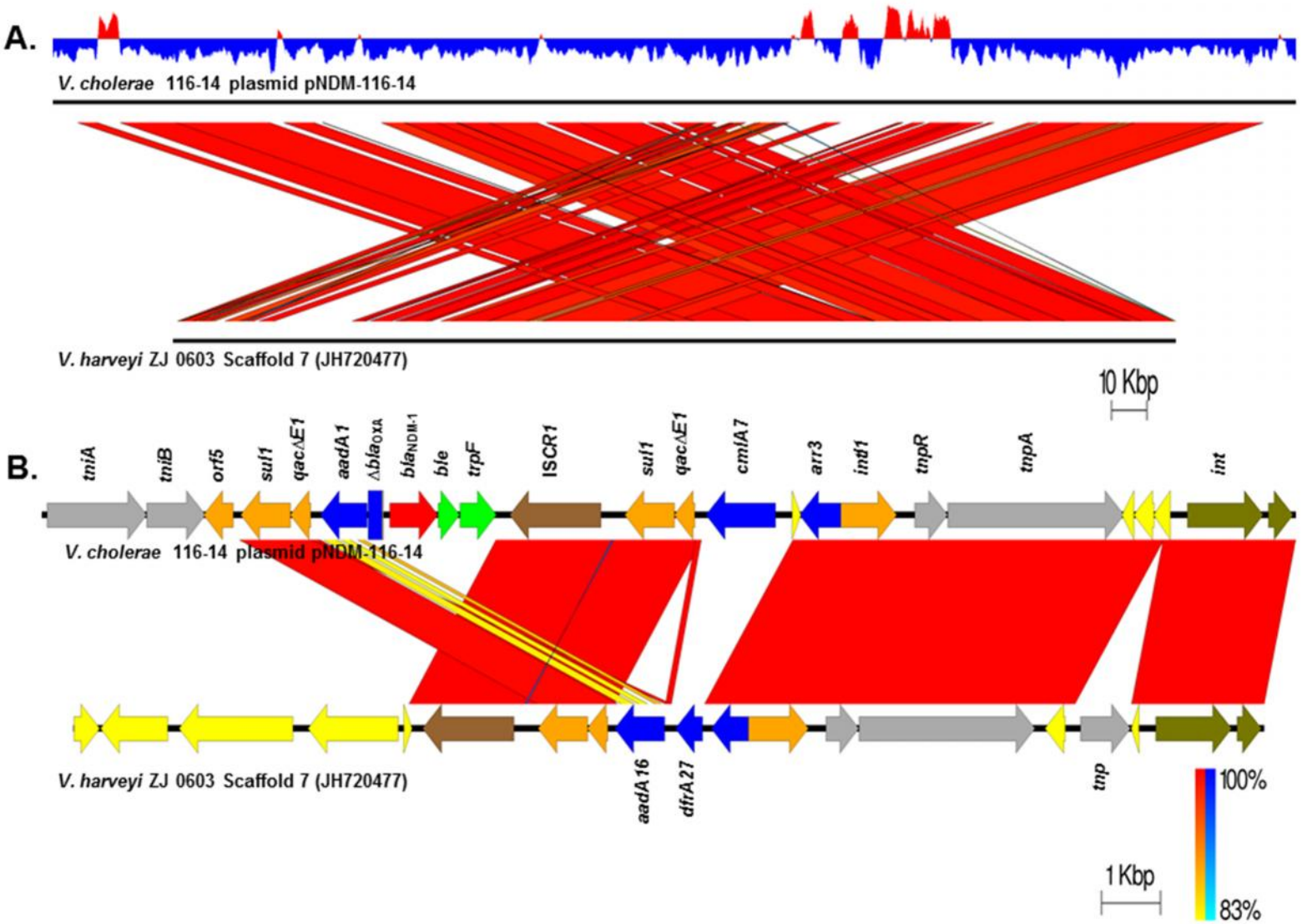
Figure 5.9 – Gene map of plasmid pNDM-116-14 (LN831184) from 116-14. All labels and features are as indicated for Figure 5.5. Annotations are as given in the main text.

A large AMR region of more than 30 kb surrounds the *bla*$_{NDM-1}$ gene. Downstream of *bla*$_{NDM-1}$ are the common *ble* and *trpF* genes. Downstream of *trpF* is an IS*CR1* element at the 3' end of a complex class 1 integron. This class 1 integron contains the chloramphenicol and rifampicin resistance genes, *cmlA7* and *arr3*. Downstream of the integrase gene, *int*, of the class 1 integron are the resolvase, *tnpR*, and transposase, *tnpA*, genes of a Tn*3* family IS. Immediately upstream of *bla*$_{NDM-1}$ is a fragment of IS*Aba125* which, as usual, retains the promoter sequence for *bla*$_{NDM-1}$. Unusually this fragment is present adjacent to the 3' remnant of a *bla*$_{OXA}$ beta-lactamase gene which had probably been inserted as a gene cassette within a class 1 integron. Upstream of this gene fragment are an *aadA1* gene cassette, coding for an aminoglycoside 3' adenyltransferase, followed by the 3' conserved sequence of a classic class 1 integron. The remaining 15kb of this region upstream of *bla*$_{NDM-1}$ includes several ISs, including 3 copies of IS*26*, and the macrolide resistance genes, *msr*(E) and *mph*(E), and the tetracycline efflux gene, *tetA*, with its transcriptional regulator, *tetR*. Comparison of the immediate context of *bla*$_{NDM-1}$ in pNDM-116-14 with related contexts is discussed further later in this chapter.

pNDM-116-14 shows extensive identity with scaffold 7 from the whole genome sequence of *V. harveyi* ZJ0603 (see Figure 5.10). The majority of this 286kb scaffold is present on pNDM-116-14 with nucleotide identities of between 98-100% between the homologous regions. Most of the antibiotic resistance genes, including *bla*$_{NDM-1}$, present in pNDM-116-14 are absent in *V. harveyi* ZJ0603 but it does contain a class 1 integron in a similar location to that of the integron downstream of *bla*$_{NDM-1}$ in pNDM-116-14 (see Figure 5.10b). Despite containing different gene cassettes these integrons have several features in common; IS*CR1* is present in the 3'CS; similar rifampicin resistance genes are upstream of the integrase and Tn*3*-like resolvase

and transposase genes are downstream. Amongst the other regions absent from the *V. harveyi* ZJ0603 scaffold are all of the heavy metal resistance genes and a region containing several restriction modification genes with significant identity to genes found in SXT/R391-like ICEs (see Figure 5.10c).

The *repE* gene coding for a putative replicase protein is present in both pNDM-116-14 and the *V. harveyi* ZJ0603 scaffold 7. However, the next closest homologue of this protein has only 49.4% AA identity across a 237aa conserved region. A phylogenetic analysis (See Figure 5.11a) of the closest BLASTP hits to this protein shows that the most closely related replicase homologues, for which the incompatibility type has been experimentally determined, are IncF replicases, but there is also some identity with IncN replicase genes. The sequence immediately upstream of *repE* in pNDM-116-14 does not show significant identity with IncF or IncN origins of replication. However, this region did contain five 21bp imperfect repeats adjacent to an AT rich region. This could represent an iteron controlled origin of replication similar to that found in several incompatibility types including IncF replication regions (del Solar *et al.* 1998), but would require experimental confirmation.


A.
V. cholerae 116-14 plasmid pNDM-116-14
V. harveyi ZJ 0603 Scaffold 7 (JH720477)
10 Kbp
B.
tniA
tniB
orf5
sul1
qacΔE1
aadA1
ΔblaOXA
blaNDM-1
ble
trpF
ISCR1
sul1
qacΔE1
cmlA7
arr3
intI1
tnpR
tnpA
int
V. cholerae 116-14 plasmid pNDM-116-14
V. harveyi ZJ 0603 Scaffold 7 (JH720477)
aadA16
dfrA27
tnp
100%
83%
1 Kbp

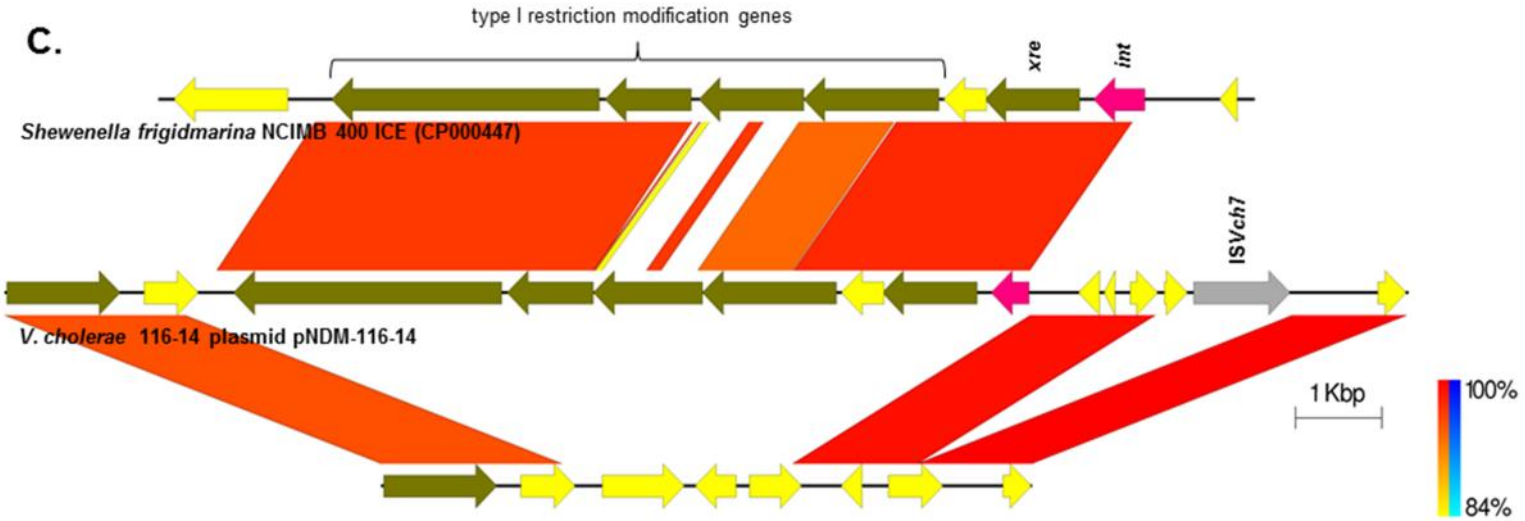
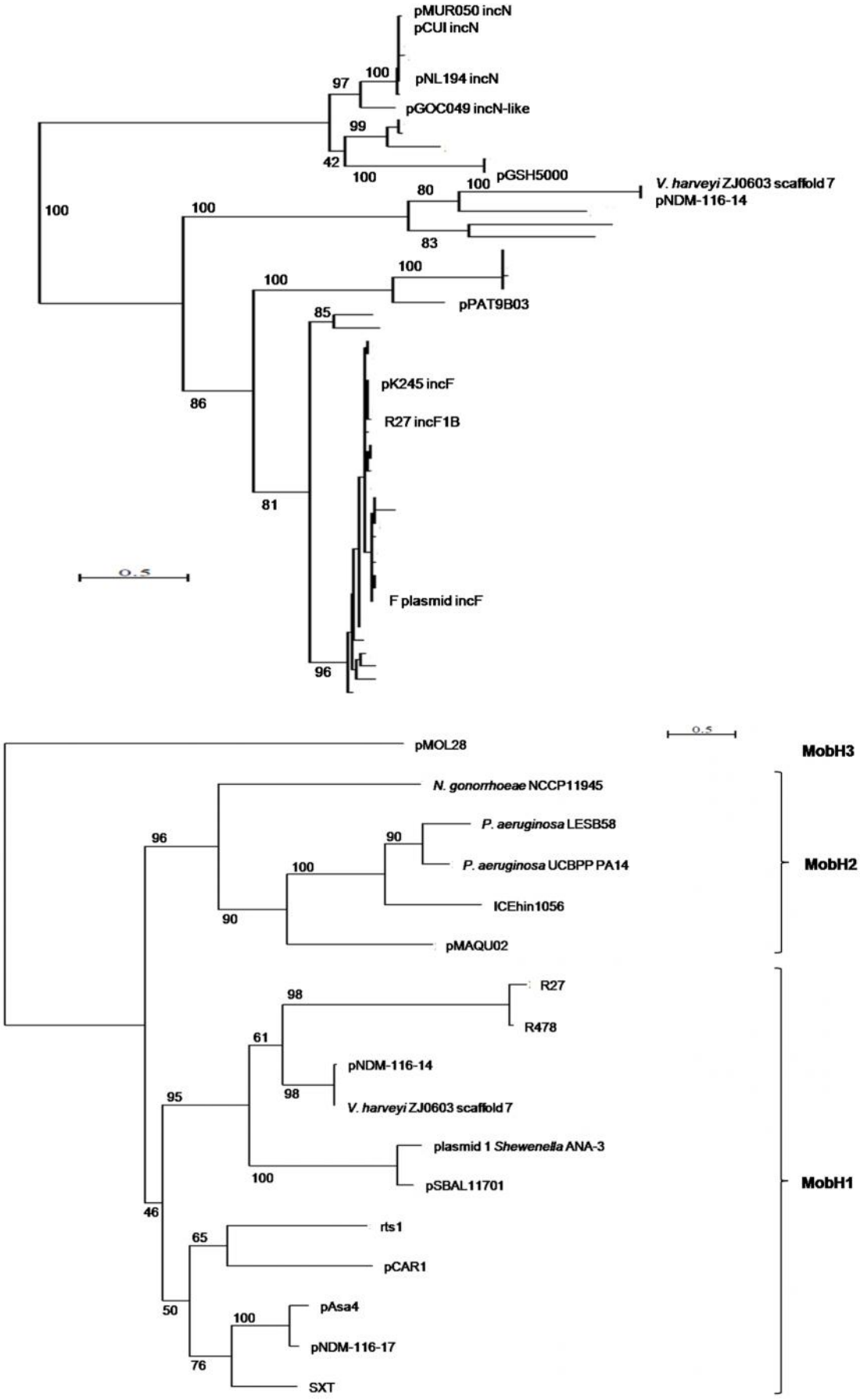


Figure 5.10 – ACT comparison of: a) complete pNDM-116-14 with Scaffold 7 from *V. harveyi* ZJ0603; b) 3' end of AMR region in pNDM-116-14 with class 1 integron region of *V. harveyi* ZJ0603 scaffold 7; c) type 1 restriction modification gene cluster in pNDM-116-14 and *Shewenella frigidmarina* NCIMB 400 ICE and its absence from *V. harveyi* ZJ0603 scaffold 7. ORFs are colour coded as in Figure 5.5. The direction of transcription is indicated by arrow heads, truncated remnants of ORFs are shown as rectangles. Top histogram represents GC% content of pNDM-116-14. BLAST comparison run using WebAct. Figure drawn using Easyfig 2.1. BLAST hits shown as in Figure 5.6. Annotations as stated in the main text except: *tniA* and *tniB* – transposase and transposition associated gene*;* orf5 – gene of unknown function associated with 3' conserved sequence of class 1 integron; *aadA16* – aminoglycoside *O*-nucleotydyltransferases, aminoglycoside resistance genes; *int* – phage integrase like genes; *dfrA27* – trimethoprim resistance, dihydrofolate reductase type XXVII gene; *xre* – transcriptional regulator gene.


pMUR050 incN
pCUI incN
pNL194 incN
pGOC049 incN-like
pGSH5000
V. harveyi ZJ0603 scaffold 7
pNDM-116-14
pPAT9B03
pK245 incF
R27 incF1B
F plasmid incF
0.5
pMOL28
N. gonorrhoeae NCCP11945
P. aeruginosa LESB58
P. aeruginosa UCBPP PA14
ICEhin1056
pMAQU02
R27
R478
pNDM-116-14
V. harveyi ZJ0603 scaffold 7
plasmid 1 Shewenella ANA-3
pSBAL11701
rts1
pCAR1
pAsa4
pNDM-116-17
SXT
MobH3
MobH2
MobH1

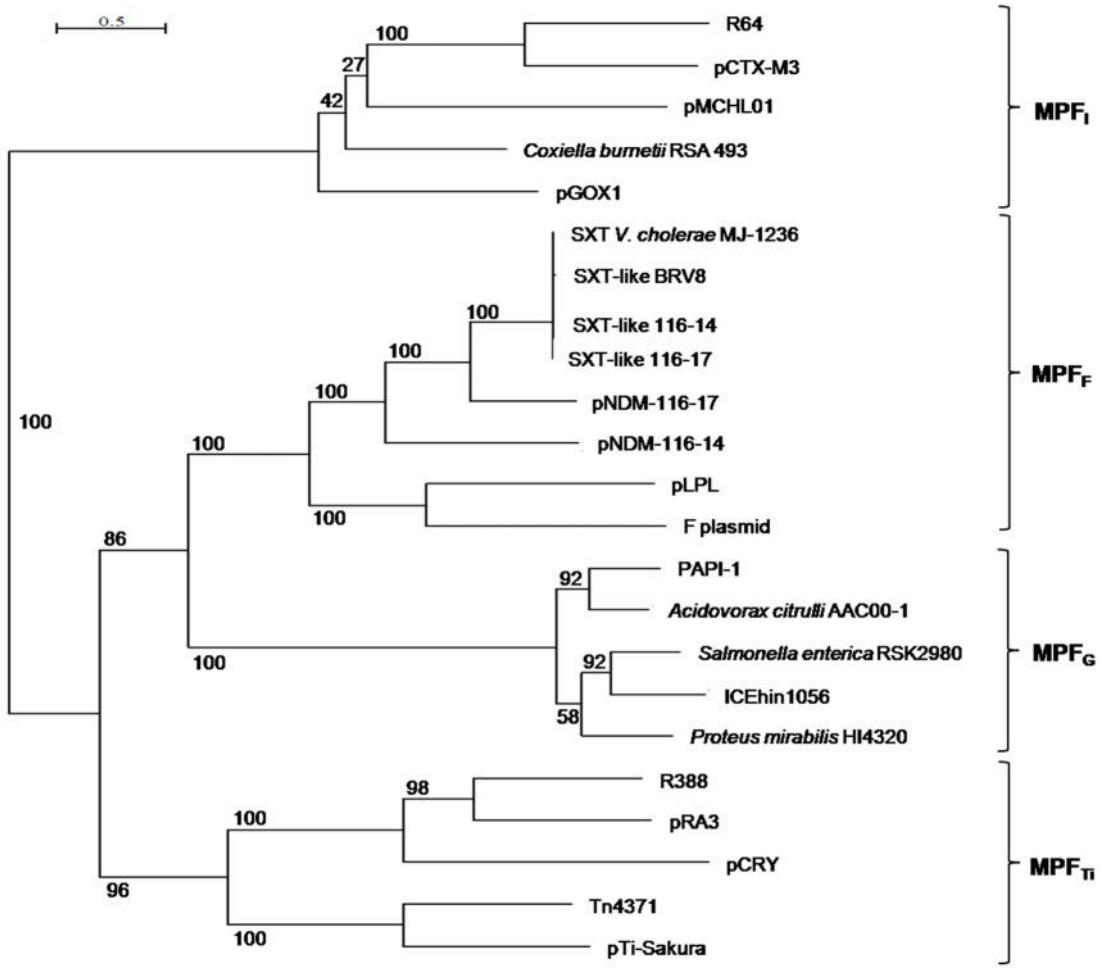


Figure 5.11 a) Phylogenetic tree of replicase proteins with identity to RepE of pNDM-116-14; b) Phylogenetic tree of MobH relaxases with identity to TraI of pNDM-116-14; c) Phylogenetic tree of VirB4/ TraC like ATPases from T4SS related to TraC of pNDM-116-14. Bootstrap values shown on main branches.

Plasmid pNDM-116-14 also contains genes coding for a putative MobH group relaxase (*traI*) and a full set of F plasmid-like type IV secretion system (T4SS) proteins. Phylogenies were constructed for the TraI and the ATPase protein of the T4SS, TraC. No substantial identity is found between the MobH relaxases and other relaxase families (Garcillán-Barcia et al. 2009) so the tree was built using close BLAST matches of TraI from pNDM-116-14 including known MobH type relaxases, mainly from the MobH1 and MobH2 subgroups, with a MobH3 protein as an outgroup. This demonstrates that TraI from p116-14 is located within the MobH1 phylogroup, which includes plasmid R27, SXT/ R391 ICEs and IncA/C plasmids (See Figure 5.11b). Furthermore, *traI* and the genes of the T4SS preserved broad synteny with these genes in other elements with MobH1 phylogroup relaxases.

The TraC phylogeny was used as being representative of the T4SS. Previous phylogenetic studies have shown relaxases, homologues of the type IV coupling protein, VirD4, and homologues of the ATPase protein VirB4/TraC seem to have evolved independently. However, VirB4/TraC phylogenies cluster consistently in clades which are representative of the 4 T4SS families (F, G, I and Ti) which have thus far been identified in proteobacteria (Guglielmini *et al.* 2013). Examples of VirB4/ TraC homologues for each of these 4 families were included. The tree shows the monophyly of each group, with TraC of pNDM-116-14 firmly rooted within the F-family and more closely related to proteins from IncA/C plasmids and SXT/ R391-like ICEs than it is to the F-plasmid (See Figure 5.11c).

5.3.11 Sequence analysis of *Salmonella* Genomic Island-1-like element, SGI-NDM-1, from BRV8

The wider chromosomal context of *bla*$_{NDM-1}$ in BRV8 was defined by PCR linking of 7 contigs to give a super-contig with a full length of 144,419bp (CTBD01000091, See Appendices 5.8 and 5.9). Much of this contig contains genes found commonly on chromosome 1 of *V. cholerae* but *bla*$_{NDM-1}$ was contained within a GEI similar to *Salmonella* Genomic Island-1 (SGI-1, see Figure 5.12). This element, which was named SGI-NDM-1, is 51,342bp long and has a GC content of 47.1%. SGI-1-like elements include genes coding for a phage like integrase and excisionase as well as three conjugative transfer proteins of the F plasmid family, TraGHN and a replicase with similarity to that of IncW plasmids (Mulvey *et al.* 2006). SGI-NDM-1, in common with other SGI-1-like elements was inserted with the direct repeat which defines its 5' end present within the 3' end of the *trmE* gene (coding for a tRNA GTPase), which is not interrupted by the insertion. Downstream of the SGI-NDM-1 was the gene *mioC* (coding for a flavodoxin protein).

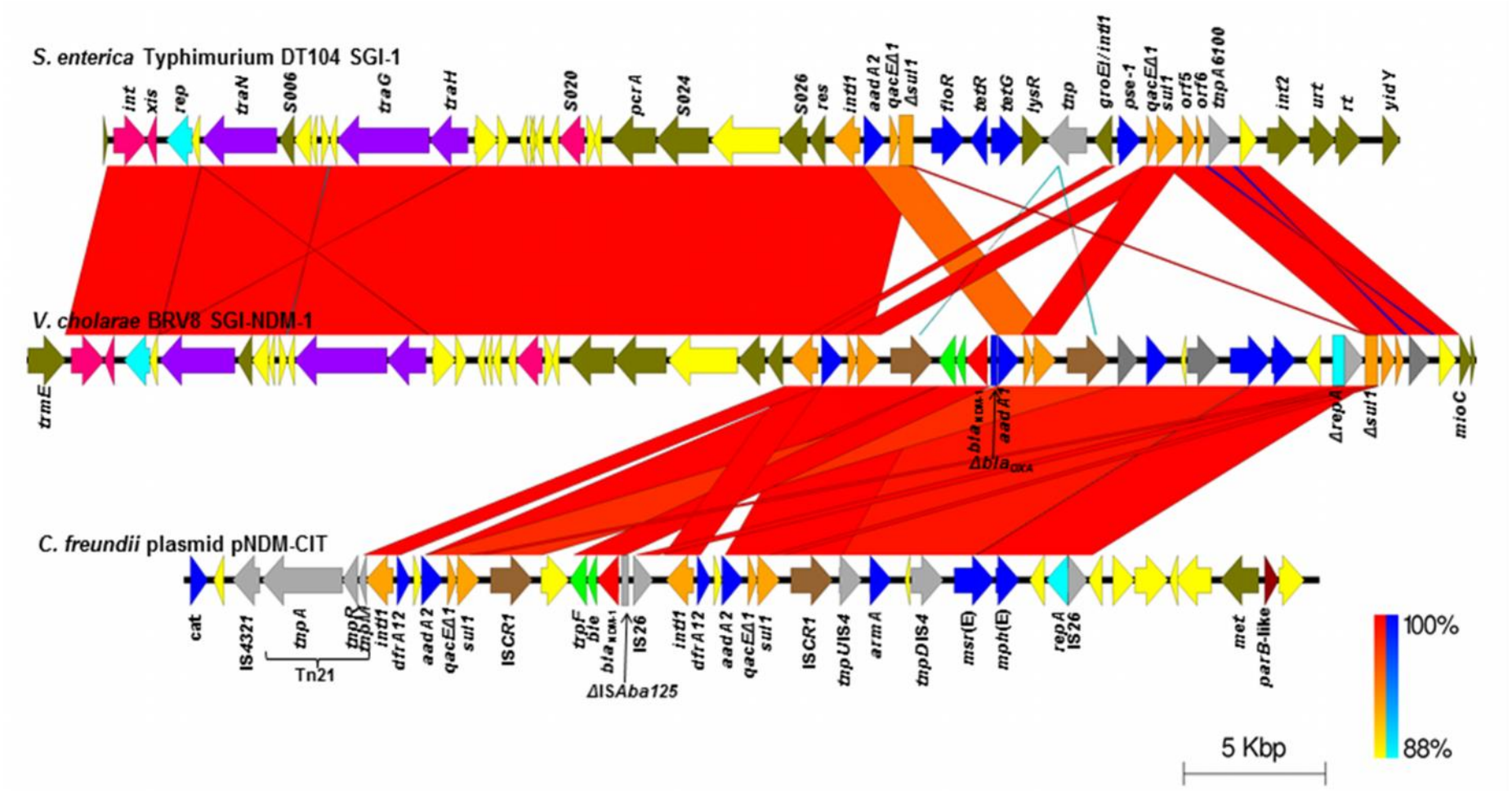

S. enterica Typhimurium DT104 SGI-1
int
xis
rep
traN
S006
traG
traH
S020
pcrA
S024
S026
res
intI1
aadA2
qacEΔ1
Δsul1
floR
tetR
tetG
lysR
tnp
groEl/intI1
pse-1
qacEΔ1
sul1
orf5
orf6
tnpA6100
int2
urt
rt
yidY
V. cholarae BRV8 SGI-NDM-1
trmE
ΔrepA
Δsul1
mioC
C. freundii plasmid pNDM-CIT
cat
IS4321
tnpA
Tn21
tnpR
tnpM
intI1
dfrA12
aadA2
qacEΔ1
sul1
ISCR1
trpF
ble
IS26
ΔISAba125
tnpUIS4
armA
tnpDIS4
msr(E)
mph(E)
repA
met
parB-like
100%
88%
5 Kbp

Figure 5.12 – ACT comparison of SGI-NDM-1 (CTBD01000091) from BRV8 with SGI-1 from *S. enterica* Typhimurium DT104 and the plasmid pNDM-CIT from *C. freundii*. Genes are colour coded as in Figure 5.5. BLAST comparison run using WebAct. Figure drawn using Easyfig 2.1. BLAST hits shown as in Figure 5.6. Annotations as described in main text except: *yidC* – inner membrane translocase gene; *trmE* – tRNA GTPase gene; *rep* – *incW* like replicase gene; *traN* – putative mating pair stabilisation gene; S006 – putative regulator gene; *traG* and *traH* – putative pilus assembly gene; S020 – putative integrase gene; *pcrA* – DNA helicase gene; S024 – putative exonuclease gene; *ftsH* – putative cell division gene; *res* – resolvase gene; *gidA* – tRNA uridine 5-carboxymethylaminomethyl modification; *int2* – phage integrase pseudogene; *urt* – gene coding for hypothetical protein; *rt* – reverse transcriptase gene; *yidY* – putative drug translocase gene; *cat* – chloramphenicol acetyltransferase gene; *met* – modification methylase gene; *parB*-like – putative partition protein.

The first 26,414kb of SGI-NDM-1, from the imperfect repeat marking the 5' boundary of the element to the start of the class 1 integron, shares >99% nucleotide identity to SGI-1 in *S. enterica* Typhimurium DT104 and to several other examples of SGI-1-like elements in *S. enterica*, *Proteus mirabilis* and *V. cholerae*. Variation between these SGI-1-like elements occurs predominantly in a 3' region which differs greatly in length and gene content but is characterised by the presence of 1 or more class 1 integrons, AMR genes and ISs. Some similarity is seen between the "variable region" in SGI-NDM-1 with most other SGI-1-like elements because of the class 1 integrons, sometimes with common gene cassettes. However, the *bla*$_{NDM-1}$ context found in SGI-NDM-1 shows far greater identity with the contexts found in the plasmid pNDM-CIT (JX182975, from *C. freundii*) and the plasmid pNDM-116-14 described above (see Figures 5.12 and discussion below) than with the variable region of any other SGI-1-like elements.

The *bla*$_{NDM-1}$ gene has been identified on one other occasion in a related genomic island, PGI1-PmPEL from *P. mirabilis* PEL. This element is similar to SGI-W from *Salmonella* Heidelberg, but differs significantly from SGI-NDM-1 both in its backbone and in the variable region, as shown in Figure 5.13. The conserved region of PGI1-PmPEL is clearly related to SGI-1-like elements, with many of the genes in synteny to their homologues in SGI-1-like IMEs, and some amino-acid sequence identity of the coding sequences. For example, the integrase and putative mating pair stabilisation proteins of PGI1-PmPEL share 84.6% and 53.9% AA identity, respectively, to those in SGI-NDM-1. The sequences with high levels of identity in the variable regions of these elements are based on the conserved regions of class 1 integrons and the *bla*$_{NDM-1}$, *ble*, *trpF* gene cluster. The other AMR genes and ISs

differ significantly between the two sequences, while PGI1-PmPEL also contains a mercury resistance island.

At the 5’ end of the variable region of SGI-NDM-1 is a class 1 integron with a single *aadA2* gene cassette, coding for an aminoglycoside 3’ adenyltransferase and an IS*CR1* element at its far 3’ end. This is followed by the *trpF* gene, *ble* and *bla*$_{\text{NDM-1}}$. There is then a structure identical to that described in pNDM-116-14, with remnants of a class 1 integron, with an intact *aadA1* gene cassette, and a truncated *bla*$_{\text{OXA}}$ gene cassette adjacent to the fragment of IS*Aba125* upstream of *bla*$_{\text{NDM-1}}$. This class 1 integron in SGI-NDM-1, however, terminates at its 3’ end with IS*CR1*, rather than orf5 as in pNDM-116-14. The aminoglycoside resistance methylase gene, *armA*, follows, flanked by IS*Ec29* and IS*Ec28* elements, followed by the *msr*(E) and *mph*(E) genes, and then IS*26*. The far 3’ end of SGI-NDM-1, beyond IS*26*, is conserved in most SGI-1-like elements and contains the 3’ end of a classic class 1 integron, truncated at *sul1*, with an intact *orf5* and *orf6*; IS*6100*; a gene coding for a hypothetical protein of unknown function; and the right hand direct repeat.

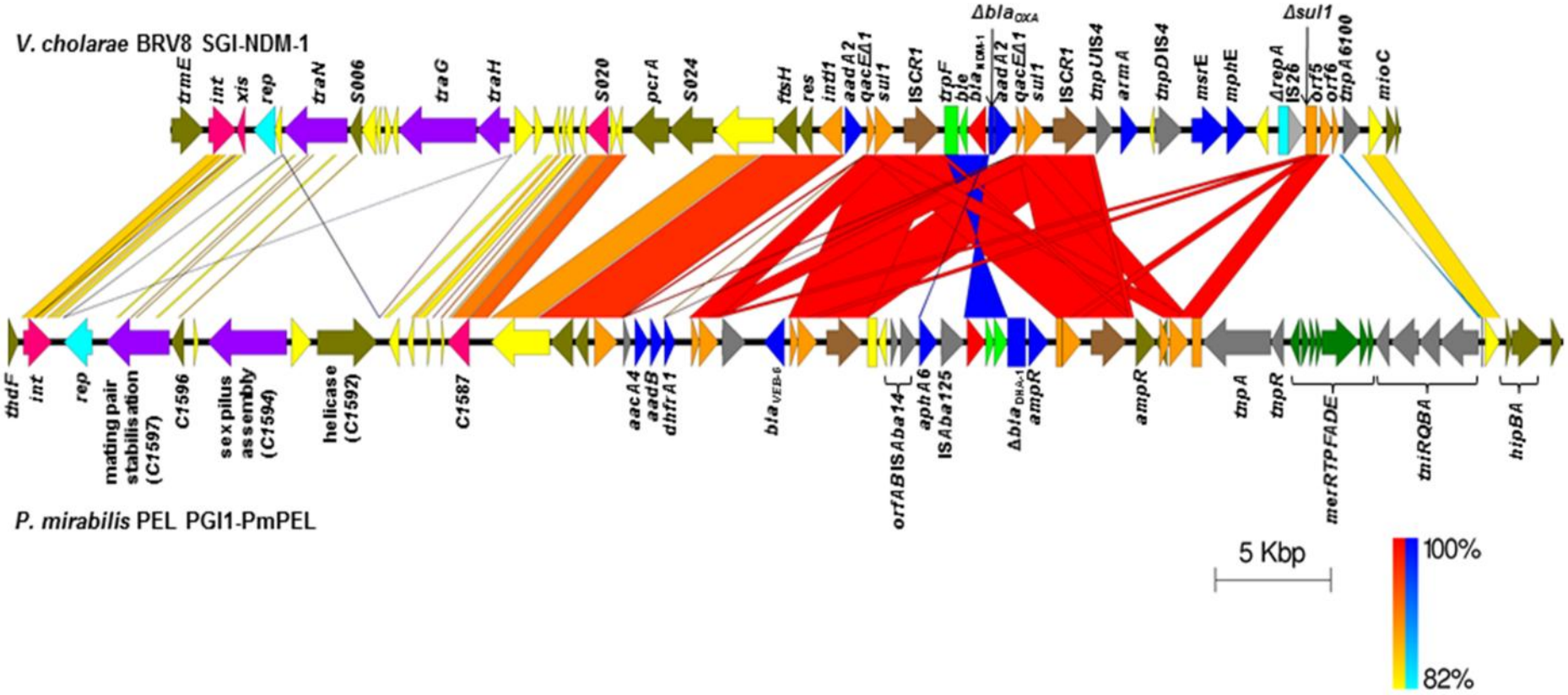

V. cholarae BRV8 SGI-NDM-1
tmE
int
xis
rep
traN
S006
traG
traH
S020
pcrA
S024
ftsH
res
intI1
aadA2
qacEΔ1
sul1
ISCR1
trpF
ble
blaOXA-1
ΔblaOXA
aadA2
qacEΔ1
sul1
ISCR1
tnpUIS4
armA
tnpDIS4
msrE
mphE
ΔrepA
IS26
orf5
orf6
tnpA6100
Δsul1
mioC
thdF
int
rep
mating pair stabilisation (C1597)
C1596
sex pilus assembly (C1594)
helicase (C1592)
C1587
aacA4
aadB
dhfrA1
blaVEB-6
orfAB
ISAba14
aphA6
ISAba125
ΔblaOXA-1
ampR
ampR
tnpA
tnpR
merRTPFADE
tniRQBA
hipBA
P. mirabilis PEL PGI1-PmPEL
5 Kbp
100%
82%

Figure 5.13 – ACT comparison of SGI-NDM-1 from BRV8 with PGI1-PmPEL from *P. mirabilis* PEL. Genes are colour coded as in Figure 5.5. BLAST comparison run using WebAct. Figure drawn using Easyfig 2.1. BLAST hits shown as in Figure 5.6. Annotations as described in main text and Figure 5.12 except: *thdF* – tRNA modification GTPase; *C1592* – putative regulator; *C1587* – integrase family protein; *aacA4* – aminoglycoside *N*-acetyltransferase, aminoglycoside resistance genes; *aadB* – aminoglycoside 2“ adenyltransferase, aminoglycoside resistance genes; *dhfrA1* – trimethoprim-resistant dihydrofolate reductase type I gene; bla_{VEB-1} – extended spectrum β-lactamase gene; *aphA6* – aminoglycoside *O*-phosphotransferase, aminoglycoside resistance gene; Δbla_{DHA-1} – fragment of AmpC type β-lactamase gene; *ampR* – lysR family bla_{DHA-1} regulator gene; *merRTPFADE* – genes making up a mercury resistance locus; *tniRQBA* – Tn genes; *hipA* – serine/threonine protein kinase gene; *hipB* – transcriptional regulator gene.

SGI-1-like elements do not possess a functional conjugative transfer system but they can excise and exist in an extrachromosomal form in the same way as ICEs. They can also be mobilised in trans by IncA/C plasmids but not by plasmids of several other incompatibility groups (Wozniak et al. 2009; Douard et al. 2010). No IncA/C plasmid sequence was detected in BRV8 and no large plasmids were seen on S1 gels for this strain. The strain did, however, contain an SXT/R391-like ICE, the conjugative transfer genes of which are similar to those found in incA/C plasmids (Johnson and Lang 2012). Several experiments to see if *bla*$_{\text{NDM-1}}$ positive transconjugants could be obtained in an *E. coli* recipient were unsuccessful, both with and without pre-treatment with ciprofloxacin to stimulate ICE excision and transfer by SOS induction. Further analysis of the contigs containing the transfer genes of the SXT/R391-like ICE revealed that the genes *traFHG* were missing. These have previously been identified as being necessary for SXT/R391-like ICE transfer (Wozniak et al. 2009).

5.3.12 Comparison of immediate genetic contexts of *bla*$_{\text{NDM-1}}$

Figure 5.14 shows the immediate genetic context of *bla*$_{\text{NDM-1}}$ in 116-17a, 116-14 and BRV8 and examples of closely related *bla*$_{\text{NDM-1}}$ contexts from other species. The immediate context in 116-17b is not shown as the assemblies suggested that it was identical to that in 116-17a. The *V. cholerae* contexts all contain class 1 integrons or fragments of class 1 integrons and IS*CR1* elements, which form part of the 3'CS of complex class 1 integrons (Toleman et al. 2006). All these contexts contain several antibiotic resistance genes, some of which are present in all the *V. cholerae* contexts. Most of these resistance genes are present either as gene cassettes (e.g. *aadA1*) within class 1 integrons or are flanked by IS (e.g. *msr*(E),

mph(E)). All these contexts contain one or more IS*26* elements. They also contain a fragment of IS*Aba125*, with its associated promoter sequence, upstream of *bla*$_{NDM-1}$ and the genes *ble* and *trpF* downstream. This short sequence is the only part of the *V. cholerae bla*$_{NDM-1}$ context which is retained from the Tn*125* sequence frequently seen in *Acinetobacter* spp. (represented by pNDM-BJ01 in Figure 5.14) and thought to be the precursor of *bla*$_{NDM-1}$ contexts which have disseminated and diversified amongst GNB. The genetic structure in the *E. coli* plasmid pNDM-1_Dok01 is noteworthy since it may represent an evolutionary intermediate between the context seen in pNDM-BJ01 and those most closely related to the *V. cholerae* contexts. In pNDM-1_Dok01 most of the structure immediately downstream of *bla*$_{NDM-1}$ is identical to that in pNDM-BJ01 but further downstream are found a class-1-integron, IS*CR1* and the macrolide resistance genes *msr*(E) and *mph*(E).

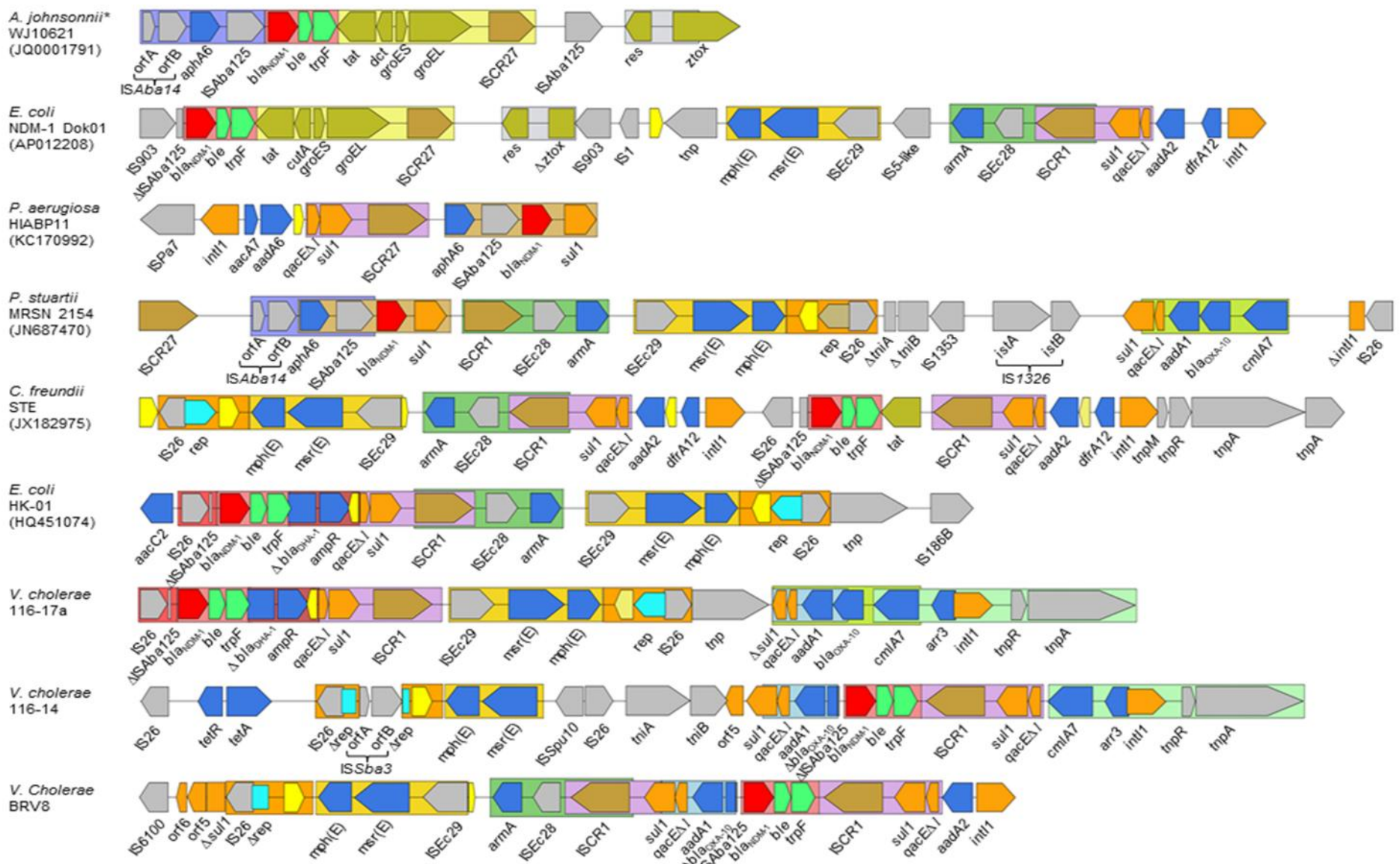

A. johnsonnii*
WJ10621
(JQ0001791)
orfA
orfB
ISAba14
aphA6
ISAba125
blaNDM-1
ble
trpF
tat
dct
groES
groEL
ISCR27
ISAba125
res
ztox
E. coli
NDM-1 Dok01
(AP012208)
IS903
ΔISAba125
blaNDM-1
ble
trpF
tat
cutA
groES
groEL
ISCR27
res
Δztox
IS903
IS1
tnp
mph(E)
msr(E)
ISEc29
IS5-like
armA
ISEc28
ISCR1
sul1
qacEΔ1
aadA2
dfrA12
intI1
P. aerugiosa
HIABP11
(KC170992)
ISPa7
intI1
aacA7
aadA6
qacEΔ1
sul1
ISCR27
aphA6
ISAba125
blaNDM-1
sul1
P. stuartii
MRSN 2154
(JN687470)
ISCR27
orfA
orfB
ISAba14
aphA6
ISAba125
blaNDM-1
sul1
ISCR1
ISEc28
armA
ISEc29
msr(E)
mph(E)
rep
IS26
ΔtniA
Δ tniB
IS1353
istA
istB
IS1326
sul1
qacEΔ1
aadA1
blaOXA-10
cmlA7
ΔintI1
IS26
C. freundii
STE
(JX182975)
IS26
rep
mph(E)
msr(E)
ISEc29
armA
ISEc28
ISCR1
sul1
qacEΔ1
aadA2
dfrA12
intI1
IS26
ΔISAba125
blaNDM-1
ble
trpF
tat
ISCR1
sul1
qacEΔ1
aadA2
dfrA12
intI1
tnpM
tnpR
tnpA
tnpA
E. coli
HK-01
(HQ451074)
aacC2
IS26
ΔISAba125
blaNDM-1
ble
trpF
Δ blaOHA-1
ampR
qacEΔ1
sul1
ISCR1
ISEc28
armA
ISEc29
msr(E)
mph(E)
rep
IS26
tnp
IS186B
V. cholerae
116-17a
IS26
ΔISAba125
blaNDM-1
ble
trpF
Δ blaOHA-1
ampR
qacEΔ1
sul1
ISCR1
ISEc29
msr(E)
mph(E)
rep
IS26
tnp
Δsul1
qacEΔ1
aadA1
blaOXA-10
cmlA7
arr3
intI1
tnpR
tnpA
V. cholerae
116-14
IS26
tetR
tetA
IS26
Δrep
orfA
orfB
Δrep
ISSba3
mph(E)
msr(E)
ISSpu10
IS26
tniA
tniB
orf5
sul1
qacEΔ1
aadA1
Δ blaOXA-10
ΔISAba125
blaNDM-1
ble
trpF
ISCR1
sul1
qacEΔ1
cmlA7
arr3
intI1
tnpR
tnpA
V. Cholerae
BRV8
IS6100
orf6
orf5
Δsul1
IS26
Δrep
mph(E)
msr(E)
ISEc29
armA
ISEc28
ISCR1
sul1
qacEΔ1
aadA1
Δ blaOXA-10
ΔISAba125
blaNDM-1
ble
trpF
ISCR1
sul1
qacEΔ1
aadA2
intI1

Figure 5.14 – Gene maps of immediate *bla*$_{NDM-1}$ contexts for *V. cholerae* strains 116-17, 116-14 and BRV8, compared to related contexts in Enterobacteriaceae, *Acinetobacter* spp. and *P. aeruginosa*. Genes are colour coded as in Figure 5.5. Gene fragments are shown without directional arrows. Coloured boxes around the gene maps highlight regions with at least 99% nucleotide identity present in multiple contexts, with each colour indicating a different homologous region. Gene annotations not in main text or previous Chapter 5 figures: *aphA6* – aminoglycoside *O*-phosphotransferase, aminoglycoside resistance gene; *tat* – twin-arginine translocation pathway signal sequence gene; *cutA* – periplasmic divalent cation tolerance gene; *groES* – co-chaperonin gene; *groEL* – chaperonin gene; *res* – putative resolvase gene; *ztox* – putative zeta-toxin gene; *dfrA12* – dihydrofolate reductase type XII; *aacA7* – aminoglycoside *N*-acetyltransferase, aminoglycoside resistance genes.

Comparing the immediate *bla*$_{NDM-1}$contexts in the *V. cholerae* strains with one another and related contexts from other species there was evidence of substantial genetic re-arrangements, gene acquisition and loss between related contexts. Many of these changes can be hypothesised to result from the action of the IS, particularly IS*CR1*, and homologous recombination events. The IS*CR1* elements are thought to mobilise in a fashion similar to the genetically related IS*911* family of ISs (see introduction section 1.4.5). This mechanism can result in transposition of the sequence upstream of the element (Toleman et al. 2006), which would explain the unusual genetic structure in which *bla*$_{NDM-1}$ is found in both 116-14 and BRV8, inserted within a remnant of a *bla*$_{OXA}$ gene cassette within a class 1 integron. This mechanism could also explain the formation of the immediate context downstream of *bla*$_{NDM-1}$ in the *P. stuartii* plasmid pMR0211, in which *ble* and *trpF* have been replaced with a *sul* gene, which is immediately upstream of IS*CR1*, as seen in the 3'CS of class 1 integrons, but with the rest of the integron missing.

It is also likely that homologous recombination based on the 3'CS of class 1 integrons is responsible for some of the differences seen. For example, an extensive region upstream of *bla*$_{NDM-1}$ is identical in pNDM-116-14 and SGI-NDM-1 sequences. However, upstream of *bla*$_{NDM-1}$ the 3'CS contains *orf5* in pNDM-116-14, as in common class 1 integrons, but it contains IS*CR1* in SGI-NDM-1, as is found in complex class 1 integrons (Toleman et al. 2006). The most likely explanation for these variants is homologous recombination based on the 3'CS, which has been experimentally shown to occur. As the resistance regions of SGI-1-like elements usually contain one or more class 1 integrons and the *V. harveyi* context related to pNDM-116-14 also contains a class 1 integron it is possible that homologous

recombination events could have introduced the *bla*$_{NDM-1}$ contexts into these elements.

5.3.13 Relationship between AMR phenotype and genotype

Much of the resistance profile can be explained by the acquired resistance genes present in these strains, most of which are found within the MGEs described above. Some of these resistance genes, however, may have a limited impact on resistance phenotype. 116-14 contains the tetracycline efflux gene, *tetA,* and its regulator, *tetR*, but MICs to tetracycline are still within the sensitive range in 116-14 and not much higher than they are in the rest of the *V. cholerae* isolates. The sulphonamide resistance gene, *sul1*, is present in all isolates, and the chloramphenicol resistance gene, *cmlA7*, is present in all isolates except BRV8. However, in the absence of the sulphonamide, trimethoprim and chloramphenicol resistances genes *sul2*, *dhfR18* and *floR* in 116-17b, this isolate has considerably lower MICs than 116-17 to chloramphenicol and co-trimoxazole. These genes, along with the aminoglycoside resistance genes *strA* and *strB*, are commonly found within the same insertion hotspot of SXT/R391 family ICEs as the IncA/C plasmid has inserted in, and so this event probably also resulted in their deletion in 116-17b.

116-17a, 116-17b and 116-14 all harbour the AmpC type β-lactamase *bla*$_{CMY-4}$. In the case of 116-17a the gene is probably chromosomal in location, having inserted upstream of the *rtxA* gene encoding the RTX toxin. In 116-14 there are two copies of *bla*$_{CMY-4}$, one found within pNDM-116-14 at a site distant from *bla*$_{NDM-1}$, and another which is probably found on a second plasmid given the high coverage of the contigs involved. In addition 116-17a and 116-17b harbour an intact *bla*$_{OXA-10}$ gene. BRV8

lacks these other β-lactamases but has similar MICs to the other *V. cholerae* isolates to all of the β-lactam antimicrobials tested.

116-17a	116-17b	116-14	BRV8
Aminoglycoside			
aadA1	*aadA1*	*aadA1*	*aadA1*
strA		*strA*	*strA*
strB		*strB*	*strB*
			aadA2
			armA
β-lactam			
bla_{NDM-1}	bla_{NDM-1}	bla_{NDM-1}	bla_{NDM-1}
bla_{CMY-4}	bla_{CMY-4}	bla_{CMY-4}	
bla_{OXA-10}	bla_{OXA-10}		
MLS - Macrolide-Lincosamide-StreptograminB			
mph(E)	*mph*(E)	*mph*(E)	*mph*(E)
msr(E)	*msr*(E)	*msr*(E)	*msr*(E)
Phenicol			
cmlA1	*cmlA1*	*cmlA1*	
floR		*floR*	*floR*
Rifampicin			
arr-3	*arr-3*	*arr-2*	
Sulphonamide			
sul1	*sul1*	*sul1*	*sul1*
sul2		*sul2*	
Trimethoprim			
*dhfR18**			
Tetracycline			
		tet(C)	

Table 5.3 – Resistance genes detected in WGS contigs from *V. cholerae* isolates. Colour of table cells indicates approximate % ID with reference genes used for Resfinder searches: Dark green – 100% ID; Light green – 99% ID; Grey – 98% ID.

* Not identified by Resfinder.

All of the *V. cholerae* isolates studied have multiple aminoglycoside resistance genes except for 116-17b. BRV8 has high MICs to all of the aminoglycosides tested, which is in keeping with the presence of the RNA methylase gene, *armA,* in this isolate, which is known to be associated with resistance against most clinically available aminoglycosides. All other isolates have much lower MICs to the aminoglycosides tested, in keeping with the narrow spectrum of the aminoglycoside modifying enzymes coded for by the genes *strA* (*aph(3")-Ib*), *strB* (*aph(6)-Id*) and *aadA1*.

5.3.14 Other MGEs present in NPVC isolates

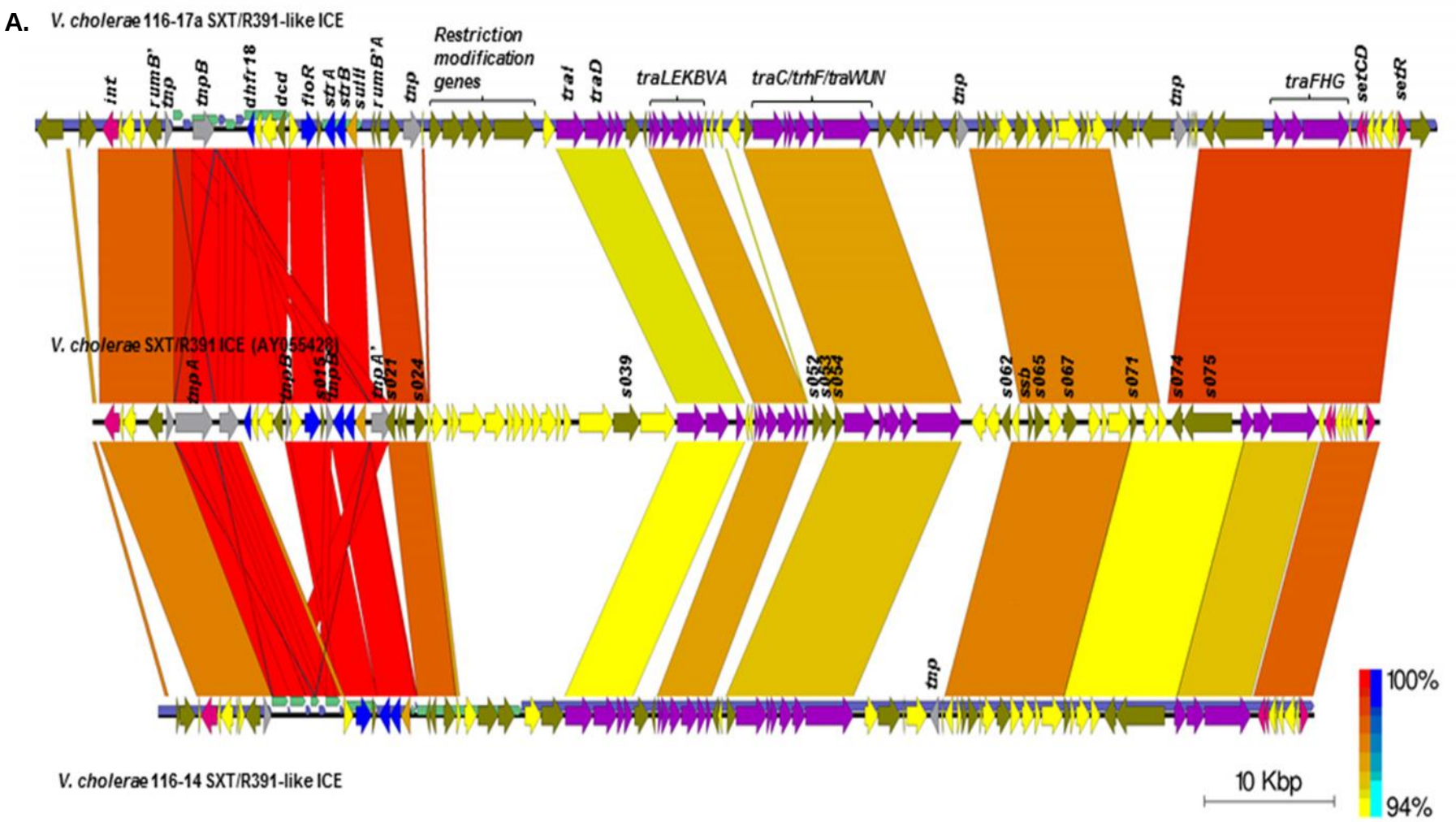
De novo assembly contigs were compared to the full genome sequence of *V. cholerae* N16961 using ACT. Comparison with this serotype 01, biovar El Tor strain, the sequence of which has been fully closed and annotated, demonstrated that in all cases both the VPI-1 island and the CTXΦ prophage were absent in their entirety from the study isolates.

All of the genome assemblies contained contigs with high levels of identity to SXT/R391 ICEs. The SXT/R391-like ICE sequence have not been closed but comparison of contigs with regions of significant identity to a SXT/R391 reference sequence (Accession AY055428) was performed using ACT, and are shown in Figure 5.15. The SXT/R391-like ICEs present in 116-17 and 116-14 are very similar. The 5' end of these elements probably contain the AMR genes mentioned in the preceding section, inserted in variable region III (see Figure 5.16) and in the same gene order as in the reference sequence. The core SXT/ R391-like genes are all present in both elements, but there are substantial differences from the reference in other variable regions. The ICE in BRV8 also contains most of the core SXT/R391-

like genes, with the exception of the *traFHG* genes already mentioned. However, in this ICE there is no insertion within variable region III (Figure 5.16), with the *rumB* gene remaining intact. Instead the genes *strA*, *strB* and *floR* are found at the 3' end of the ICE, close to the location where *traFHG* would usually be found.

5.4 Discussion

All the strains studied in this work lack the VPI-1 and CTXφ which are understood to be crucial to the pathogenesis of cholera and found in all strains which have been linked with epidemic and pandemic disease (Waldor and Mekalanos 1996; Karaolis et al. 1999; Muanprasat and Chatsudthipong 2013). This is in keeping with these strains being non-O1, non-O139 serotypes and with the fact that they are environmental and blood culture isolates. As discussed in the general introduction non-O1, non-O139 strains have been associated with other clinical infections ranging from mild gastroenteritis to sepsis associated with significant mortality (Ko et al. 1998; Morris 2003). The pathogenic potential of all the study strains is therefore uncertain.


A.
V. cholerae 116-17a SXT/R391-like ICE
int
rumB'
tnp
tnpB
dhfr18
dcd
floR
strA
strB
sulII
rumB'A
tnp
Restriction modification genes
traI
traD
traLEKBVA
traC/trhF/traWUN
tnp
tnp
traFHG
setCD
setR
V. cholerae SXT/R391 ICE (AY055428)
tnpA
tnpB
s015
tnpB'
tnpA'
s021
s024
s039
s052
s053
s054
s062
ssb
s065
s067
s071
s074
s075
tnp
V. cholerae 116-14 SXT/R391-like ICE
100%
94%
10 Kbp

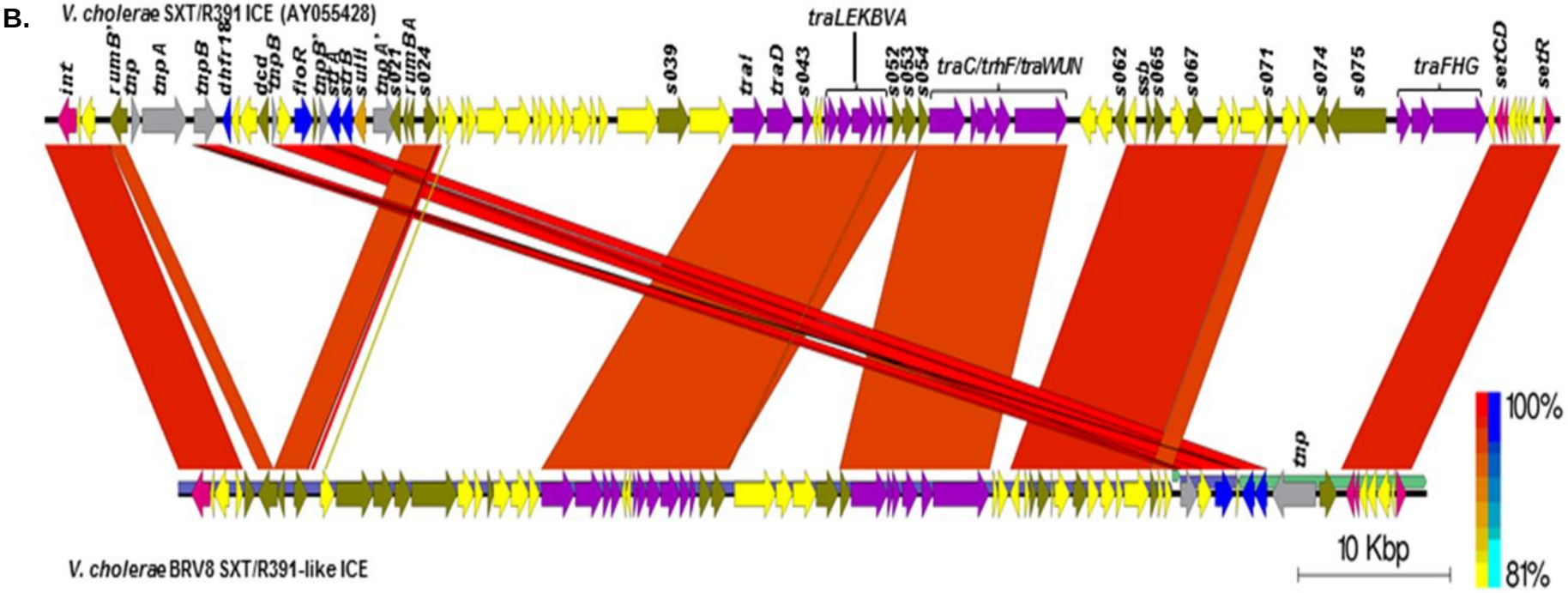


Figure 5.15 ACT comparison of contigs from NPVC containing sequences with identity to SXT/R391-like ICEs with reference SXT sequence (AY055428): a) 116-17a and 116-14 versus reference; b) BRV8 versus reference. Genes are colour coded as in Figure 5.5. Green and blue arrows above the sequence line denote contigs from the NPVC isolates. BLAST comparison run using WebAct. Figure drawn using Easyfig 2.1. BLAST hits shown as in Figure 5.6. Annotations as described in main text or earlier figures in Chapter 5 except: *int* - SXT/ R391 integrase gene; *rumB'* – gene for UV repair DNA polymerase, truncated at 3' end; 'rumB – gene for UV repair DNA polymerase, truncated at 5' end; *tnp* – genes coding for putative transposases

of IS; *dct* - deoxycytidine triphosphate deaminase gene; *sulII* - dihydropteroate synthase type II sulphonamide resistance gene; *s021* – gene with putative product similar to *V. cholerae* MutL involved in methyl directed DNA repair; *rumA* – UV repair gene; *s024* – gene with product similar to polymerase epsilon subunit from R391; *s039* – gene with product similar to lon protease from *Thermus thermophiles*; *traI* – conjugative relaxase gene; traD – conjugative coupling factor gene; s043 – putative conjugative coupling factor gene; *traLEKBVACWUNFHG/ trhF* – conjugative transfer genes; *s052* – gene with product similar to *ynd* from pTi in *Agrobacterium tumefaciens*; *s053* – gene similar to *ync* from pTi in *Agrobacterium tumefaciens*; *s054* – gene with putative disulfide bond isomerase product; *s062* – gene similar to *nucM* from *Pectobacterium chrysanthemi*; *ssb* – single-stranded DNA binding protein gene; *s065* – putative DNA recombination protein gene similar to *bet* from Bacteriophage 933W; *s067* – gene similar to *cobS* from pMT1 in Yersinia pestis; *s071* –putative DNA repair *radC* gene; *s074* – putative response regulator gene; *s075* – putative histidine kinase gene; *setCD* – transcriptional activator genes; *setR* – transcriptional repressor similar to cI from Bacteriophage 434.

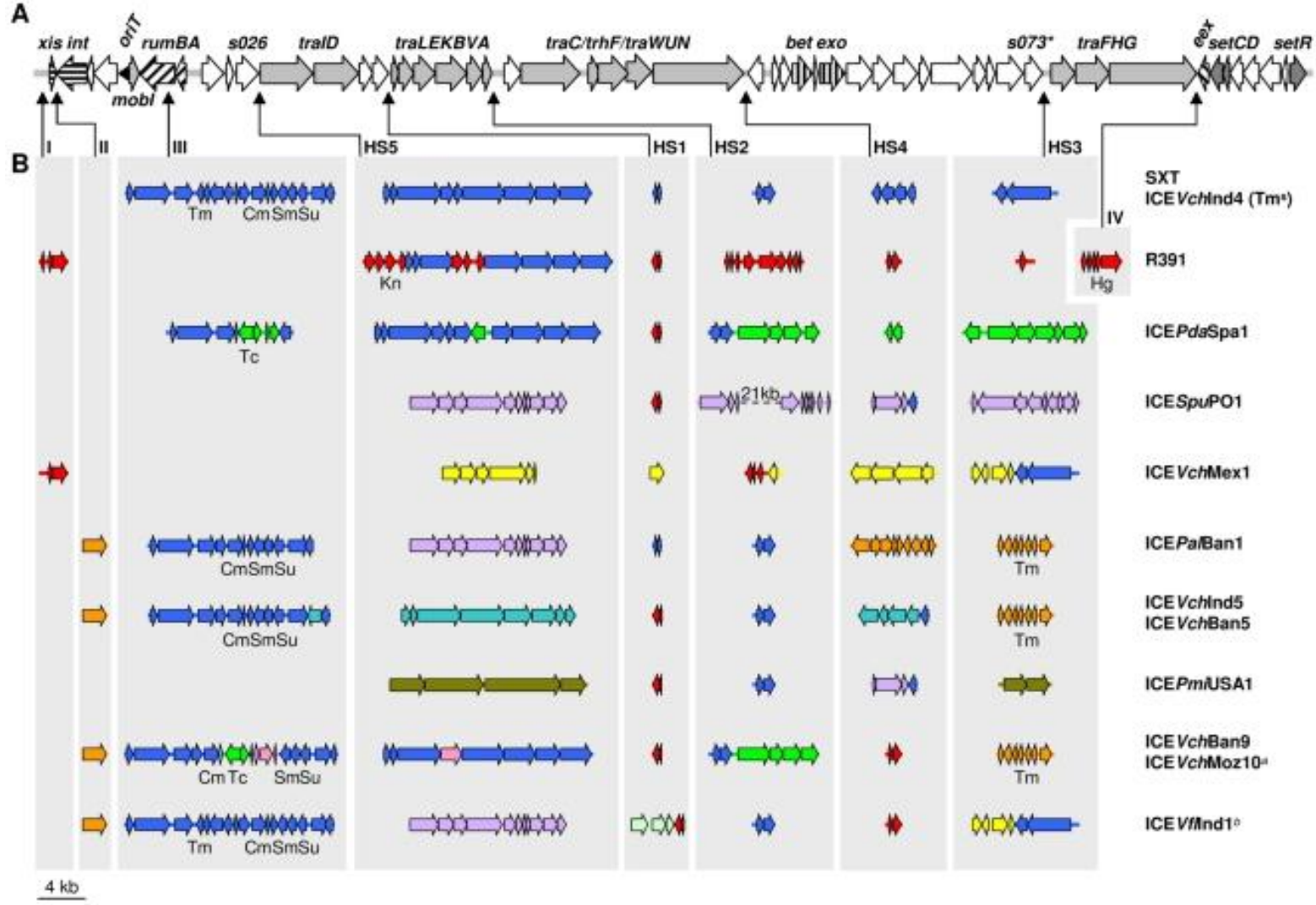

A
xis int
orfT
rumBA
mobI
s026
traID
traLEKBVA
traC/trhF/traWUN
bet exo
s073*
traFHG
setCD
setR
I
II
III
HS5
HS1
HS2
HS4
HS3
IV
B
Tm
CmSmSu
Kn
Hg
Tc
21kb
CmTc
SmSu
SXT
ICE Vchlnd4 (Tm^s)
R391
ICE PdaSpa1
ICE SpuPO1
ICE VchMex1
ICE PalBan1
ICE Vchlnd5
ICE VchBan5
ICE PmiUSA1
ICE VchBan9
ICE VchMoz10^d
ICE Vflnd1^b
4 kb

Figure 5.16 – Structure of the genomes of 13 SXT/R391 ICEs. (A) The upper line represents the set of core genes (thick arrows) and sequences common to all 13 SXT/R391 genomes analyzed. Hatched ORFs indicate genes involved in site-specific excision and integration (*xis* and *int*), error-prone DNA repair (*rumAB*), DNA recombination (*bet* and *exo*) or entry exclusion (*eex*). Dark gray ORFs correspond to genes involved in regulation (*setCDR*). Light gray ORFs represent genes encoding the conjugative transfer machinery, and white ORFs represent genes of unknown function. (B) Variable ICE regions are shown with colors according to the elements in which they were originally described SXT (blue), R391 (red), ICE*Pda*Spa1 (green), ICE*Spu*PO1 (purple), ICE*Vch*Mex1 (yellow), ICE*Pal*Ban1 (orange), ICEVchInd5 (turquoise), ICE*Pmi*USA1 (olive), ICE*Vch*Ban9 (pink), ICE*Vfl*Ind1 (light green). Thin arrows indicate the sites of insertion for each variable region and HS1–HS5 represent hotspots 1–5. Roman numerals indicate variable regions not considered true hotspots. Cm, chloramphenicol; Hg, mercury; Kn, kanamycin; Sm, streptomycin; Su, sulfamethoxazole; Tc, tetracycline; Tm, trimethoprim. * indicates that s073 is absent from ICE*Pda*Spa1. a ICE*Vch*Moz10, which lacks *dfrA1* in the integron structure, does not confer resistance to Tm. b The purple gene content of ICE*Vfl*Ind1 was deduced from partial sequencing, PCR analysis and comparison with ICESpuPO1. Reproduced with permission from Wozniack RAF *et al.*, PLOS Genetics 2009;5 (12):1-13 (Wozniak et al. 2009).

The clinical strain, BRV8, was isolated from a patient who was, at the time, profoundly immunocompromised by the burn injuries he had sustained (Darley et al. 2012). Major burns constitute an immunosuppressed state because of the lack of barrier function of the skin and also because of the effects of the major inflammatory response. Furthermore, the clinical findings which suggested the presence of systemic infection at the time the organism was isolated could also have been partially, or completely, explained by the ongoing inflammatory response secondary to his injuries (Greenhalgh *et al.* 2007). Therefore, even for this isolate it is difficult to conclude with much confidence that the strain would have significant pathogenic potential in a less compromised host. It is, however, noteworthy that despite having wounds colonised with multiple XDR, NPE that the only organism isolated from blood early in the clinical course was BRV8 (Darley et al. 2012).

Although some virulence determinants have been proposed for non-O1, non-O139 *V. cholerae*, these are poorly characterised in these organisms (Morris 2003). For this reason these genetic determinants have not been sought in the study isolates, as no meaningful conclusions could be drawn about the strains pathogenic potential from a genotypic analysis. Never the less, the resistance genes, and the MGEs associated with them, identified in these organisms have significant clinical and public health ramifications. Firstly, there is a high likelihood of these elements being shared with other *Vibrio* spp. in aquatic environments because of the proven potential for gene exchange within the species and the genus. *bla*$_{\text{NDM-1}}$ has on at least one occasion been acquired by a strain with epidemic/ pandemic potential (Mandal et al. 2012). This has concerning implications for the potential of these organisms to develop XDR phenotypes, even though β-lactam antibiotics are not normally recommended for cholera treatment (Kitaoka et al. 2011; Harris et al. 2012).

As seen in this study, and the wider literature, *bla*$_{NDM-1}$ is commonly associated with MGEs carrying a multitude of other resistance genes (Kumarasamy et al. 2010; Carattoli 2013; Johnson and Woodford 2013) and so finding *bla*$_{NDM-1}$ can be seen as a marker for the sharing of such elements in populations of these bacteria.

Of the MGEs found to be associated with *bla*$_{NDM-1}$ in this study perhaps the most worrisome are the IncA/C plasmids. They are now observed as one of the incompatibility types most frequently associated with *bla*$_{NDM-1}$ and have previously contributed to the dissemination of other β-lactamases, notably *bla*$_{CMY-2}$ (Carattoli et al. 2012; Johnson and Lang 2012; Carattoli 2013). The many differences described here between the variable regions of pNDM-116-17 and other IncA/C plasmids which harbour *bla*$_{NDM-1}$ suggest that the acquisition of *bla*$_{NDM-1}$ by pNDM-116-17 is likely to be a separate evolutionary event to that which resulted in the *bla*$_{NDM-1}$ context seen in most other IncA/C plasmids that have been sequenced. IncA/C plasmids have a broad host range, having first been identified in *Aeromonas* spp. and *Vibrio* spp. they have subsequently been found in many Enterobacteriaceae (including *S. enterica* and *Yersinia pestis*) and *Xenorhabdus nematophila* and can be transferred to recipients of different species *in vitro* (Johnson and Lang 2012). It has previously been reported that pNDM-116-17 could be transferred by conjugation in to an *E. coli* recipient (Walsh et al. 2011). It has been hypothesised that IncA/C plasmids may be unstable without selection pressure in some Enterobacteriaceae and that aquatic organisms like *Vibrio* spp. are the source of IncA/C plasmids. *Vibrio* spp. may, therefore, act as a reservoir from which other GNB could acquire these plasmids (Johnson and Lang 2012).

Although *bla*$_{NDM-1}$ has been identified in a chromosomal location on several occasions the literature and sequence databases do not yet contain many examples

of *bla*$_{NDM-1}$ incorporated into GEIs. As noted above, an IME similar to SGI-NDM-1 described here, PGI1-PmPEL from *P. mirabilis*, has recently been described (Girlich et al. 2014). However, these IMEs are only distantly related, with significant sequence divergence between even their conserved regions, as well as having major differences in their variable regions. Thus, these represent separate acquisitions of *bla*$_{NDM-1}$ by GEIs, and there is as yet no evidence of dissemination of *bla*$_{NDM-1}$ in any bacterial species by GEIs with mobile potential.

Theoretically, the chromosomal location of these elements would be expected to result in greater genetic stability of the resistance gene contexts containing *bla*$_{NDM-1,}$ than if found on a plasmid. Thus SGI-NDM-1 in *V. cholerae* could represent a further stable reservoir of AMR genes, with mobile potential. Although it was not demonstrated that SGI-NDM-1 has mobile potential in this study, it is possible that, with the appropriate plasmid vector to transfer the element in trans, it would be so. However, attempts to do this with PGI1-PmPEL were unsuccessful, although the presence of the excised extrachromosomal form of the genetic element was demonstrated (Girlich et al. 2014).

The rapid emergence of SGI-1-like elements associated with antibiotic resistance in *S. enterica* serovars and later in *P. mirabilis* demonstrates their potential to disseminate and acquire resistance genes in significant bacterial pathogens (Mulvey et al. 2006; Boyd *et al.* 2008; Hall 2010; Siebor and Neuwirth 2013). Although there are 4 SGI-1-like sequences from *V. cholerae* available in the NCBI database as of January 2015, a search of Pubmed did not identify any publications relating to SGI-1-like elements in *V. cholerae*. SGI-1-like elements may represent a group of elements which have contributed to the genomic plasticity of *V. cholerae* strains, and warrant further study. Given the history of the rapid expansion

of SXT (Burrus et al. 2006; Mutreja et al. 2011) in epidemic strains of cholera and of SGI-1 in *S. enterica* Typhimurium the acquisition of *bla*$_{NDM-1}$ by SGI-1-like elements could threaten a similar event in pathogenic strains of bacteria, including in *V. cholerae*.

The significance of the finding of *bla*$_{NDM-1}$ on the mega-plasmid pNDM-116-14 is less easy to predict, since very little is known about plasmids of this type. Previous work suggests the plasmid can be transferred to *E. coli* recipients (Walsh et al. 2011) and pNDM-116-14 does contain genes which are homologues to all the genes known to be required for conjugative transfer. That a sequence with large regions in synteny with pNDM-116-14 has also been found in a *V. harveyi* isolate suggests that in nature these elements have some mobile potential.

These findings with regards to the broader context of *bla*$_{NDM-1}$ demonstrate once again the variety of genetic contexts associated with *bla*$_{NDM-1}$. There is also evidence of significant genetic plasticity within the local genetic contexts, although these are sufficiently closely related to have arisen from a recent, common source. More data would be required in *Vibrio* spp. and other environmental organisms to draw firm conclusions about the extent to which *bla*$_{NDM-1}$ genetic contexts are being shared between different species and whether the diversification of the contexts observed here has occurred within *V. cholerae*. However, the nature of the MGEs identified, which all have GC percentages similar to those in *Vibrio* spp. and have all been identified previously in *V. cholerae* and/or other *Vibrio* spp., is compatible with the broader genetic contexts having diversified within *V. cholerae* or related species. The degree of variation observed in this small data set suggests that environmental organisms could have an important influence on the shaping of genetic contexts and

their subsequent dissemination and that *V. cholerae* could be a useful model organism for studying this.

Another important un-answered question is whether *bla*$_{\text{NDM-1}}$, or other AMR genes of concern, like *bla*$_{\text{CMY-4}}$ and *armA*, can be found relatively frequently in *Vibrio* spp. Thus far we have not made any systematic attempt to look for resistant *Vibrio* spp. in environmental or clinical samples. The NDM-1 producing strains identified so far were found either by chance in clinical isolates or from environmental samples using techniques optimised to identify CRE (Walsh et al. 2011; Darley et al. 2012; Mandal et al. 2012). The diversity of the context observed in this small data-set, and the means used to acquire these isolates, would seem to suggest that our findings are the tip of the iceberg.

Recent work suggests that environmental bacteria could be a significant reservoir of *bla*$_{\text{NDM-1}}$ genes. Sharing of *bla*$_{\text{NDM-1}}$ genes with organisms like *V. cholerae* could contribute to the genetic diversity of *bla*$_{\text{NDM-1}}$ contexts and increase the chances of dissemination by successful MGEs to pathogenic strains of bacteria. It also suggests that the environment could be an ongoing reservoir for MGEs and AMR genes, including *bla*$_{\text{NDM-1}}$, despite efforts targeted within clinical settings to control the spread of MDR and XDR pathogens. More study is warranted into *V. cholerae*, other aquatic organisms and organisms from different environmental reservoirs on the prevalence of clinically important AMR determinants. Furthermore, understanding MGEs associated with AMR determinants and the dynamics of HGT in environmental settings could provide useful information to guide strategies to contain the spread of AMR.

Chapter 6

General Discussion

6.1 Methods to define molecular epidemiology and their limitations

It is hoped that by studying the genetic contexts of AMR genes like *bla*$_{NDM-1}$ that greater understanding of both the epidemiology and the means of dissemination of these resistance genes may be gained. However, the sequence data available from *bla*$_{NDM-1}$ contexts, including the data produced for this thesis, only provides a small part of the story and the approach has several clear limitations.

Firstly, since *bla*$_{NDM-1}$ has spread so widely amongst many bacterial species (Kumarasamy et al. 2010; Nordmann et al. 2011b; Walsh et al. 2011; Berrazeg et al. 2014), the sequence can only provide a snap shot of existing genetic contexts. Furthermore, although it has become increasingly straightforward and cheap to sequence large numbers of bacterial WGS using next generation sequencing technologies such as Illumina, sequence assembly can be challenging where large numbers of repetitive sequences are present (Baker 2012; Miyamoto *et al.* 2014; Wajid and Serpedin 2014). Thus, providing full assemblies of multiple, and sometimes quite large (e.g. the mega-plasmid pNDM-116-14 was 354,308 bp in length), is not a trivial undertaking and occupied a large amount of the time spent analysing data for the current work. This means that the number of reliable

assemblies of wider genetic contexts of resistance genes that can be produced with currently accessible technology is much more limited than might be imagined.

This may change fairly rapidly if longer read sequencing platforms like PACBIO become more widely applied and available because of falling monetary costs, reduced sequencing error rates and increased throughput on sequencing and assembly pipelines (Miyamoto et al. 2014). In the short term, a more realistic strategy may be the selective application of sequencing methods like PACBIO on highly selected isolates to provide fully assembled reference sequences, including reliable, fully assembled MGEs (Miyamoto et al. 2014; Stoesser et al. 2014). These can then be supplemented with the results of other sequencing studies using Illumina or 454 sequencing, without necessarily attempting full assembly of MGEs, to facilitate high throughput of samples and keep costs down.

In terms of optimising the utility of sequence data, ideally isolates need to be sequenced which have been carefully selected to address specific questions, collected in a systematic fashion and with as much provenance data as possible. Given that the countries in which NPGNB are prevalent have resource poor healthcare systems and the barriers to international collaboration on researching this topic that exist in India now (Walsh and Toleman 2011a), this is challenging.

Evidently the sequence data is also a tiny part of the overall picture. Understanding of the sequence data is made possible by annotating potential coding sequences. Increasingly, annotation is provided by bioinformatic methods used to define sequence similarities with known protein sequences, obtained by translation of potential coding sequences (Aziz et al. 2008; Consortium 2014). However, with novel sequences, including some of the sequences obtained for this thesis, the quality of the annotation that can be produced in this way may be fairly poor. Many of

the MGEs sequenced during this work have been annotated with large numbers of potential coding sequences which are not known to produce any protein of known function. Thus, sequencing technology is overtaking our knowledge of protein structure and function, which in turn limits the interpretation of the sequence data.

Sequence data alone can give clues to how genetic structures have been formed, but further experimental data is needed to support hypotheses generated in this way. The majority of the information provided in this thesis is based on the analysis of sequence data alone. Some attempt was made in each results chapter to provide some functional information about MGEs and some phenotypic information about the organisms studied. However, it proved challenging to take this work to its logical conclusions within the constraints of the time available.

6.2 Overview of genetic contexts associated *bla*$_{\text{NDM-1}}$

The striking thing about the epidemiology of NDM producers has been that the gene is found in a large number of strains and species of GNB (Kumarasamy et al. 2010; Walsh et al. 2011; Carattoli 2013; Berrazeg et al. 2014). A comparison of the number of species in which *bla*$_{\text{KPC-2-like}}$ and *bla*$_{\text{NDM-1-like}}$ genes have been reported in the published literature as of 2013 is shown in Figure 6.1. NPGNB include organisms usually found in the environment, of probably limited pathogenic potential, and strains of bacteria which are not strongly associated with human disease. Furthermore, although the gene has been associated with some MGEs more frequently, and these are likely to have played a more significant role in its dissemination, there are a large number of different plasmids, Tns and some GEIs which have been found to harbour *bla*$_{\text{NDM-1}}$ (Nordmann et al. 2011b; Carattoli 2013; Johnson and Woodford 2013; Girlich et al. 2014).

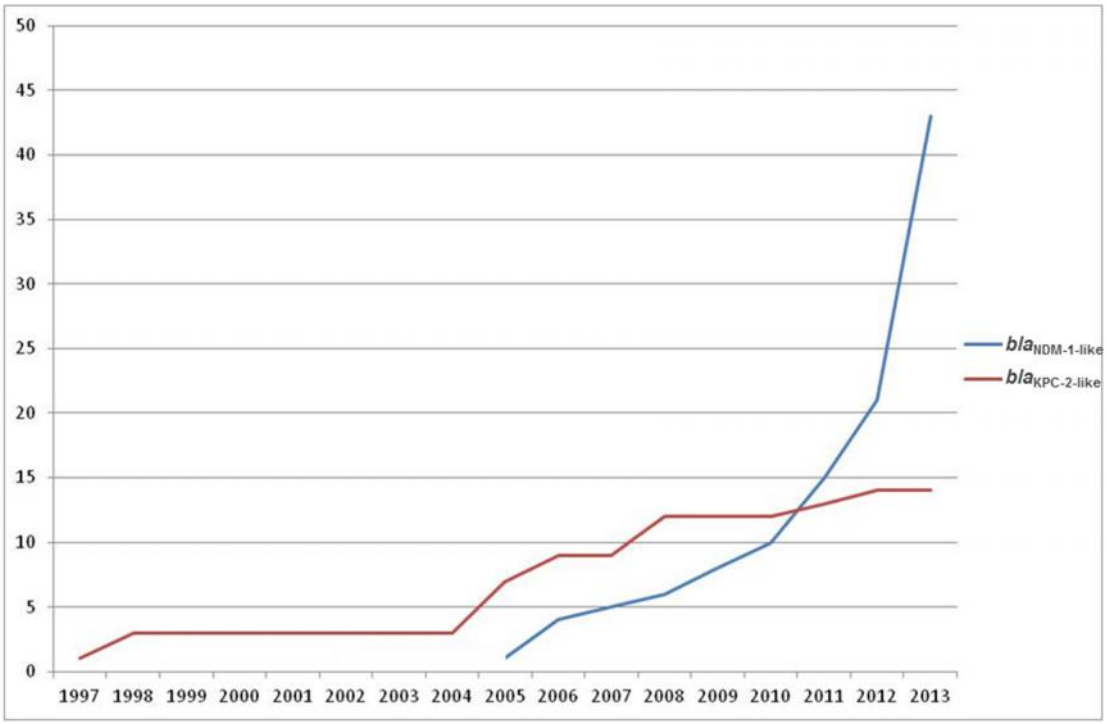


Figure 6.1 – Graph showing the number of species in the published literature which harbour *bla*$_{\text{NDM-1-like}}$ versus *bla*$_{\text{KPC-2-like}}$ genes producers. Graph courtesy of Tim R Walsh.

Although representing a small proportion of the available sequence data, and a highly selected sample, the results presented in this thesis are consistent with the above statements. The *A. baumannii* clinical isolates described in Chapter 3 probably carried *bla*$_{\text{NDM-1}}$ on very similar plasmids, but this was a reflection of clonal spread of a successful strain within a single clinical setting (Jones et al. 2015), and the plasmid was of a type not previously associated with *bla*$_{\text{NDM-1}}$. Even in the case of these *Acinetobacter* isolates there was evidence of genetic plasticity, with *bla*$_{\text{NDM-1}}$ present in multiple genetic locations and examples of variants of the plasmid which had lost large sections of the DNA sequence. This thesis also reports *bla*$_{\text{NDM-1}}$ in four other *Acinetobacter* spp. strains, with the gene present on different plasmids, albeit that in two the plasmid backbone was almost certainly similar and shared close

identity with plasmids that have been detected in *Acinetobacter* spp. in various geographic locations, but predominantly China (Jones et al. 2015). Plasmids in the other species seemed likely to be of a novel background, as far as association with *bla*$_{NDM-1}$, but have not been extensively characterised at present. *bla*$_{NDM-1}$ was further described in *V. cholerae*, in 3 strain backgrounds and associated with 2 different plasmid types and a GEI. One of these plasmids was of a type which had been poorly characterised previously and never associated with *bla*$_{NDM-1}$. The other plasmid was an IncA/C plasmid. These plasmids have been associated with *bla*$_{NDM-1}$ on many occasions, but the variable region was different to those characterised previously. The GEI showed some backbone similarity with a GEI which has been associated with *bla*$_{NDM-1}$ before, but in a different species background and with a substantially different variable region (Girlich et al. 2014).

There are differences between the reported genetic diversity and epidemiology of bacteria harbouring *bla*$_{NDM-1}$ compared to that of other globally successful β-lactamase genes like *bla*$_{KPC-2}$, *bla*$_{VIM-1}$, *bla*$_{OXA-48}$ and *bla*$_{CTX-M-15}$. Most of these other genes have fairly conserved sequences immediately adjacent to them (*bla*$_{CTX-M-15}$ being an exception although it is usually associated with IS*Ecp1*) and many have been associated with a predominant species and/ or strains of bacteria (Woodford et al. 2011; Tzouvelekis et al. 2012; D'Andrea et al. 2013; Munoz-Price et al. 2013). For example *bla*$_{KPC}$ is predominantly identified in *K. pneumoniae* ST258 (Munoz-Price et al. 2013). These other β-lactamase genes are found in different species of Enterobacteriaceae and occurrence in species of other genera, like *P. aeruginosa*, does occur (Tzouvelekis et al. 2012; Munoz-Price et al. 2013). This variability in host background is likely to be as result of dissemination by plasmids. Some genes, like *bla*$_{VIM-1}$, *bla*$_{OXA-48}$ and *bla*$_{CTXM-15}$ have so far been mainly associated

with a limited range of plasmid Inc types, whilst others like *bla*$_{KPC-2}$ are, like *bla*$_{NDM-1}$, associated with several different Inc types (Carattoli 2013). *bla*$_{NDM-1-like}$ genes have been described in many diverse species of GNB, and amongst the predominant species, like *E. coli* and *K. pneumoniae*, are associated with many different strain types (Kumarasamy et al. 2010; Nordmann et al. 2011b; Walsh et al. 2011; Johnson and Woodford 2013). This raises the question, if there is a difference in the genetic diversity of *bla*$_{NDM-1-like}$ contexts compared to similar resistance genes in GNB, is there some feature of these genes, their local genetic contexts, wider genetic contexts, or the environment in which these genes are being spread, favouring broad dissemination?

6.3 Immediate context and early evolution of *bla*$_{NDM-1-like}$ genes

As with all carbapenemases the level of carbapenem resistance of the host bacteria associated with NDM-1-like enzymes are somewhat variable (Daikos *et al.* 2009; Nordmann et al. 2011b). Studies of the enzyme kinetics of NDM-1-like enzymes do not suggest that the hydrolysis of carbapenems is greater than for other clinically prevalent carbapenemases. The k_{cat}/K_m values reported for NDM-1 against meropenem and imipenem are not substantially different to those reported for VIM-2 and KPC-2, and slightly higher than for OXA-48 (Yigit *et al.* 2003; Poirel *et al.* 2004; Yong et al. 2009b; Tzouvelekis et al. 2012; Makena et al. 2014). So, although *bla*$_{NDM-1-like}$ genes are more consistently identified in Enterobacteriaceae with significant levels of carbapenem resistance than is the case with other genes, especially *bla*$_{OXA-48}$, this does not seem to be an intrinsic feature of the enzymes themselves (Daikos et al. 2009). Instead this is more likely to be related to the level of expression of the

enzyme and other features of the host strains favouring higher carbapenem MICs, such as relative membrane impermeability or efflux mechanisms.

An almost universal feature of the genetic contexts of *bla*$_{IMP}$ and *bla*$_{VIM}$ genes is that they are found as gene cassettes of class 1 integrons (Daikos et al. 2009; Walsh 2010). This may reflect the fact that the evolutionary success of these genes is associated with promotion of expression of gene cassettes near to the integrase gene, combined with the genetic plasticity associated with these elements and the selective advantage associated with other gene cassettes (Walsh *et al.* 2005; Gillings 2014). *bla*$_{NDM\text{-}1\text{-}like}$ genes have not been described so far as gene cassettes. However, IS*Aba125* has been shown to provide a strong promoter to *bla*$_{NDM\text{-}1\text{-}like}$ genes (Hornsey *et al.* 2011; Poirel et al. 2011a; Dortet et al. 2012). IS*Aba125* has also previously been associated with increasing the promotion of other resistance genes including *bla*$_{ADC}$ genes and probably *aphA6* (Nigro et al. 2011; Lopes and Amyes 2012). Indeed, in pNDM-BJ01 the *aphA6* gene does not result in amikacin resistance, which is proposed to be because the gene does not possess IS*Aba125* and its associated promoter upstream (Hu et al. 2012). *A. bereziniae* CHI-40-1, described in Chapters 3 and 4, is amikacin resistant despite containing an identical *aphA6* context but its transconjugants have the same aminoglycoside susceptibility profiles as their recipient backgrounds (Jones et al. 2015). *A. bereziniae* CHI-40-1 appears to contain a sequence variant of *aphA6* which is probably associated with a different promoter sequence in a second genetic context, explaining the apparent inconsistency.

Close analysis of the IS*Aba125*-*bla*$_{NDM\text{-}1}$ sequence and related sequences lead to the suggestion that *bla*$_{NDM\text{-}1}$ is in fact a chimeric gene, that has incorporated a small part of the 5’ sequence of *aphA6* (Toleman et al. 2012), as discussed in

Chapter 3. This same event probably resulted in the formation of the context that placed the promoter sequence upstream of *bla*$_{NDM-1}$. This has previously been identified as a key evolutionary event in the formation of *bla*$_{NDM-1-like}$ genes and their genetic contexts (Partridge and Iredell 2012; Poirel et al. 2012a; Toleman et al. 2012). As can be seen in Figure 3.1 in Chapter 3, there is a marked change in the GC% after the small 5' remnant of *aphA6*, which makes up part of *bla*$_{NDM-1}$. This is likely to reflect the fact that *aphA6* and IS*Aba125* are from an *Acinetobacter* background (most *Acinetobacter* spp. have total GC% in the region of 40%) while the rest of the *bla*$_{NDM-1}$ sequence has a much higher GC%, suggesting an alternative original background (Poirel et al. 2012a; Toleman et al. 2012).

It is not known for sure what the original source of the ancestral metallo-β-lactamase of *bla*$_{NDM-1-like}$ genes was (Sekizuka et al. 2011; Poirel et al. 2012a; Toleman et al. 2012). NDM-1-like enzymes do not show substantial similarity to any other known metallo-β-lactam (Yong et al. 2009b), with NDM-1 sharing 34.2% AA identity with its closest relative VIM-1 (based on MAAFT alignment). On the basis of the GC% of most of the *bla*$_{NDM-1}$ context within Tn*125* and phylogenetic analysis of some of the coding sequences within Tn*125*, it has been proposed that this context could have been acquired from an organism related to plant pathogens of the *Xanthomonas* or *Pseudoxanthomonas* spp (Sekizuka et al. 2011). The genes *tat*, *cutA1*, *groES* and *groEL* are found in examples of these organisms in perfect synteny.

An environmental reservoir for *A. baumannii* has not been clearly established, notwithstanding its ability to persist in the hospital environment (Visca et al. 2011). Other species of *Acinetobacter*; however, are found in various environmental and animal reservoirs. Therefore, it can be hypothesised that a context containing the

progenitor of *bla*$_{\text{NDM-1}}$, *ble*, *trpF*, the genes from the context similar to that in *Xanthomonas* spp. and IS*CR27* could have been taken up by *Acinetobacter* from a *Xanthomonas* spp., or similar species, in the environment. Two possible mechanisms have been proposed for the formation of the *bla*$_{\text{NDM-1-like}}$ context with IS*Aba125* upstream; either a deletion event or an IS*CR27* rolling circle transposition mechanism (Toleman et al. 2012). The latter mechanism would result in a fragment of *aphA6* being downstream of the *oriIS* of IS*CR27*, a sequence that has not ever been observed in sequenced isolates in *Acinetobacter* or Enterobacteriaceae, although it is feasible that the context could have evolved further prior to widespread dissemination of *bla*$_{\text{NDM-1-like}}$ genes. If IS*CR27* is functional it is possible that it could have moved the entire context on a second occasion, so that *aphA6* was no longer present at the 3' end of the element.

What factors could have influenced the genetic plasticity of the immediate *bla*$_{\text{NDM-1-like}}$ context in *Acinetobacter* spp. and Enterobacteriaceae?

It is noteworthy that despite the fact that IS*Aba125* elements upstream of *bla*$_{\text{NDM-1-like}}$ genes are frequently interrupted by the insertion of other ISs in Enterobacteriaceae, and were truncated in all of the *V. cholerae* isolates described in Chapter 5, no examples have yet been identified in which the promoter is interrupted (Poirel et al. 2011b; Toleman et al. 2012; Dortet et al. 2014). It is likely that this reflects selection bias, in that, in the absence of the promoter, carbapenem MICs would fall significantly and most studies look for the presence of the gene in isolates selected because they have reduced susceptibility to carbapenems. Alternatively, there may be some feature of the sequence upstream of the promoter that preferentially encourages insertion of transposable elements.

One hypothesis suggests that this particularly marked change in GC% encourages the insertion of ISs in some genetic backgrounds, given the large number of examples in which an IS has inserted within IS*Aba125*. If this were the case, the sequences in the *V. cholerae* strains 116-14 and BRV8 would suggest that this is also relevant to the insertion of IS*CR* elements, since it is proposed that IS*CR1* is likely to be responsible for the unusual genetic structure around *bla*$_{\text{NDM-1}}$ in these strains (see Chapter 5). In contrast, in all *Acinetobacter* contexts sequenced so far, including all of the *Acinetobacter* spp. sequences described in Chapters 3 and 4, the IS*Aba125* elements upstream of *bla*$_{\text{NDM-1-like}}$ genes are intact (Decousser et al. 2013; Jones et al. 2014a; Jones et al. 2015). Thus in these strain backgrounds the same sequence does not appear to be a hot spot for transposition events. A variation of the earlier hypothesis could suggest that it is simply the low GC region of IS*Aba125* relative to that of certain strain backgrounds, like Enterobacteriaceae and *V. cholerae*, which acts as the transposition hotspot. Although some ISs/ Tns/ IS*CR*s have specific target sites, for most, the factors dictating insertion targets are poorly defined, and changes in GC% and DNA structural features are proposed as influencing choice of insertion site (Meyer *et al.* 1980; Sengstag *et al.* 1986; Craig 1997). As it is a common finding that there are hotspots within MGEs and chromosomal sites for the insertion of foreign DNA (Darmon and Leach 2014), it does make sense that one potential way of targeting variation at a site containing accessory, rather than housekeeping genes, would be regions with a different GC%. As such, in an *Acinetobacter* background, IS*Aba125* would not be an obvious target site because its GC content is similar to that of the background.

In this scenario, instead *bla*$_{\text{NDM-1-like}}$ genes, and most of the rest of the contents of Tn*125*, would be potential hotspots for insertion. As with disruptions of the

IS*Aba125* promoter it makes sense that if there are a large number of inactivated *bla*$_{\text{NDM-1-like}}$ derivatives in *Acinetobacter* spp. we would not identify them because the techniques used to detect *bla*$_{\text{NDM-1-like}}$ genes probably would not identify these derivatives. Also, *bla*$_{\text{NDM-1-like}}$ genes may offer an evolutionary advantage to *Acinetobacter* isolates containing it. Nevertheless, according to this hypothesis we would expect that truncated versions of Tn*125* would be more frequent, rather than the full Tn*125* element that is most often reported in *Acinetobacter* spp. containing *bla*$_{\text{NDM-1-like}}$ genes (Poirel et al. 2012a; Decousser et al. 2013; Jones et al. 2015). However, although the contents of Tn*125* are rather better preserved in *Acinetobacter* contexts than in Enterobacteriaceae many examples do vary from the classic Tn*125*, to some extent.

None of the genetic contexts described in this thesis from *Acinetobacter* spp. contain Tn*125* without deletions or insertions (Jones et al. 2014a; Jones et al. 2015). Tn*125* in pNDM-40-1 from *A. bereziniae* is close to the original sequence described from *A. baumannii* 161/07 (Pfeifer et al. 2011) but contains a couple of deleted sections. In the *A. baumannii* isolates IS*CR27* is interrupted by IS*15-Δ* and the context seems to then be present in two forms, either a classic Tn*125*, with the IS*15-Δ* insertion, or in a re-arranged form as found in the assembly of plasmid pNDM-32, in which a copy of IS*15-Δ* and the 3' fragment of IS*CR27* are moved upstream of *bla*$_{\text{NDM-1}}$ (Jones et al. 2014a). In pNDM-69122 from *A. haemolyticus* an IS*Aha3* element is found between *ble* and pNDM-BJ01-like resolvase, possibly having been responsible for deletion of the rest of the genes usually found within Tn*125*. The *bla*$_{\text{NDM-1}}$ contexts from *A. schindleri* 74312-EC and *A. towneri* 73668-ECT were not well characterised, because for these isolates WGS was not undertaken and PCR analysis was only able to determine a short sequence flanking *bla*$_{\text{NDM-1}}$. However,

this probably reflected the fact that the Tn*125* context was significantly truncated in these strains also (Jones et al. 2015).

That most other reports suggest that Tn*125* remains intact, could suggest that the contents of Tn*125* offer an additional evolutionary advantage. If this was a major factor this would not explain why most of this context is not preserved in Enterobacteriaceae. This, however, would presuppose that the genes were functional in both backgrounds and/ or conveyed their selective advantage in both backgrounds. As IS*Aba125* is apparently an *Acinetobacter* spp. associated IS (Nigro et al. 2011; Poirel et al. 2012a; Toleman et al. 2012), it may be Tn*125* is not functional in an Enterobacteriaceae background. Thus the diversification seen in Enterobacteriaceae could only have occurred by *bla*$_{\text{NDM-1-like}}$ genes becoming associated with different MGEs. In other words, in Enterobacteriaceae there would be an evolutionary advantage of disruption by other ISs, while the opposite situation would exist in *Acinetobacter* if, as seems to be the case, Tn*125* can readily move the *bla*$_{\text{NDM-1-like}}$ context to new genetic locations in this genus (Poirel et al. 2012a).

6.4 What role have different plasmid backgrounds played in the dissemination of *bla*$_{\text{NDM-1}}$ between *Acinetobacter* spp. and other GNB?

The plasmids containing *bla*$_{\text{NDM-1}}$ in the *A. baumannii* isolates from India were likely to be non-mobilizable, and could not be transferred in the conjugation experiments (Jones et al. 2014a). The plasmids in two of the *Acinetobacter* spp. from Karachi were not extensively characterised but one of these could also not be transferred via conjugation, whilst the other could be transferred to an *A. pittii* but not an *E. coli* recipient (Jones et al. 2015). Thus pNDM-BJ01 plasmids, which could be transferred efficiently and can clearly be maintained in a fairly broad host range (at

least within the genus *Acinetobacter*), would probably have an evolutionary advantage, and so this may explain their apparent predominance amongst sequenced plasmids from *Acinetobacter* spp.

In Chapter 4 a similar argument to that proposed above for the infrequency of an intact Tn*125* in Enterobacteriaceae, was proposed for the lack of any complete pNDM-BJ01 plasmids in Enterobacteriaceae. It was demonstrated that both of the pNDM-BJ01-like plasmids described here, pNDM-40-1 and pNDM-69122, could be transferred by conjugation to *Acinetobacter pittii* and *E. coli* transconjugants (Jones et al. 2015). This was in keeping with several other reports which have found that pNDM-BJ01-like plasmids could be transferred to Enterobacteriaceae (Hu et al. 2012; McGann et al. 2013; Zhang et al. 2013b). It was also observed that there are a number of Enterobacteriaceae sequences which contain fragments of the backbone of these plasmids (Partridge and Iredell 2012; Jones et al. 2015). Thus it is a reasonable hypothesis that these plasmids, which are relatively frequent in *Acinetobacter* spp., have been responsible for some of the dissemination from *Acinetobacter* to Enterobacteriaceae. Despite this no complete pNDM-BJ01-like plasmids have yet been described in Enterobacteriaceae. The finding that three transconjugants which underwent further investigation contained both *bla*$_{\text{NDM-1}}$ and *traA* on a larger plasmid or the chromosome, suggested that in UAB190 integration into another replicon was required to maintain the plasmid. So, although it was efficiently transferred and could be maintained, at least with antimicrobial selection, pNDM-40-1 was not replicating autonomously in these backgrounds (Jones et al. 2015).

To some extent these findings pose more questions than they answer. To start with only three transconjugant colonies were investigated, and it would have been

interesting to investigate a larger number, to see if they all contained *bla*$_{NDM-1}$ on replicons larger than pNDM-40-1. Secondly, it would have been useful to investigate where pNDM-40-1 had integrated, whether all of the sequence was intact and to try to establish the mechanism of integration. Furthermore, seeing if transfer to other Enterobacteriaceae species occurred as readily, and whether maintenance also required integration into other replicons in these strain backgrounds, would have helped to better establish whether these plasmids cannot successfully replicate independently in Enterobacteriaceae. As the replicase and origin of replication of pNDM-BJ01-like plasmids are not known (Hu et al. 2012), defining these would be an important next step. This could be technically challenging if they were not functional in an *E. coli* background. However, demonstrating that the plasmid replicase could be functional cloned into a plasmid in a naturally transformable *Acinetobacter* spp. strain like *A. baylyi* ADP1 (Young et al. 2005) but not in *E. coli* would provide evidence suggesting that these plasmids could not be autonomously maintained in Enterobacteriaceae. If this were shown to be the case then this would provide an explanation as to why complete pNDM-BJ01-like plasmid sequences have not been identified in Enterobacteriaceae.

Thus, in summary, the early success (allowing for the uncertainties outlined above) of the spread of *bla*$_{NDM-1-like}$ genes could be attributed to a sequence of chance evolutionary events: a) the gene fusion resulting in the efficient promotion of the gene; b) the association with Tn*125* allowing efficient movement to new contexts in *Acinetobacter* spp.; c) the association with pNDM-BJ01-like plasmids allowing efficient dissemination to new species of bacteria including Enterobacteriaceae; d) the apparent tendency of the *bla*$_{NDM-1}$ context, once present in non-*Acinetobacter* GNB, to act as a hotspot for the insertion of new ISs/ Tns/ IS*CR*s, capable of moving

the immediate *bla*$_{NDM-1}$ context, to new genetic locations. What these key events are missing is any clear idea of how the dissemination amongst *Acinetobacter* spp. occurred. On the basis of the available evidence this seems to have been predominantly through pNDM-BJ01-like plasmids in China (Hu et al. 2012; Yang et al. 2012; Zhang et al. 2013b; Jones et al. 2015). However, “European” and Middle Eastern or North African isolates have predominantly been reported to have *bla*$_{NDM-1-like}$ on the chromosome, and no evidence answers the question as to how these strains originally may have acquired these genes (Kaase et al. 2011; Decousser et al. 2013; Espinal et al. 2013).

As far as the Indian subcontinent is concerned, despite being proposed to be the epicentre for the spread of NPGNB (Kumarasamy et al. 2010; Nordmann et al. 2011b; Dortet et al. 2014), very little detailed characterisation of *Acinetobacter* spp. has been carried out for isolates from this part of the world (Karthikeyan et al. 2010; Bharadwaj et al. 2012; Jones et al. 2014a). Although pNDM-32 and related plasmids were not found to be conjugative or mobilizeable, the hypothesis that natural transformation may be an important means of dissemination of foreign DNA to *A. baumannii* has recently been boosted by several strands of evidence, so these plasmids may still have been vectors which contributed to the spread *bla*$_{NDM-1}$ (Rumbo et al. 2011; Harding *et al.* 2013). Whether pNDM-BJ01-like plasmids are important to the spread of *bla*$_{NDM-1}$ between *Acinetobacter* spp. in other parts of South Asia, as well as China, clearly requires further data. The fact that two out of the five strains of *Acinetobacter* spp. studied here harboured pNDM-BJ01-like plasmids, in addition to an isolate described elsewhere, from a soldier returning from Afghanistan, at least suggests that the hypotheses that they are is worthy of further study.

6.5 What role have Enterobacteriaceae and "environmental" GNB including *V. cholerae* played in the subsequent spread of *bla*$_{NDM}$?

The subsequent dissemination between species of GNB is more complicated and thus harder to track and explain. *bla*$_{NDM\text{-}1\text{-}like}$ contexts in Enterobacteriaceae and other GNB, are very diverse, although they are more frequently associated with certain species, like *E. coli* and *K. pneumoniae* (Kumarasamy et al. 2010; Nordmann et al. 2011b; Dortet et al. 2014), and types of plasmid, for example IncA/C, IncHI1 and IncL/M plasmids (Carattoli 2013; Johnson and Woodford 2013). It may simply be that the combination of an integration hotspot upstream of *bla*$_{NDM\text{-}1\text{-}like}$ genes, encouraging multiple associations with new ISs, the selective advantage of a highly expressed carbapenemase and subsequent chance associations with broad host range MGEs, explains the wide dispersal and varied contexts of *bla*$_{NDM\text{-}1\text{-}like}$ genes.

It is already a well-established hypothesis that environmental bacteria and HGT in the environment may be an important source of resistance determinants in pathogenic bacteria (Stokes and Gillings 2011; Lupo *et al.* 2012; Woodford *et al.* 2014). It has been proposed here, and elsewhere, that the original spread of the progenitor of *bla*$_{NDM\text{-}1\text{-}like}$ genes into an *Acinetobacter* spp. background probably occurred from a high GC organism like a *Xanthomonas* spp. in the environment (Sekizuka et al. 2011). Similarly many other β-lactamases of clinical importance are thought to have been acquired from environmental species, with the proposed source of CTX-M type ESBLs from *Kluyvera* spp. being a prime example (D'Andrea et al. 2013). The potential for spread of β-lactamases and other genes of clinical importance within the bacterial gene pool, between environmental, human commensals and potentially pathogenic bacteria, has also been a growing area of

scientific interest (Stokes and Gillings 2011; Lupo et al. 2012; Djordjevic *et al.* 2013; Nesme and Simonet 2014). Ongoing dissemination could have important implications for genetic plasticity, and thus potential adaptation of phenotypes of GNB (Stokes and Gillings 2011).

Several studies and case reports have identified NPGNB isolates from the environment, wild and livestock animals, as well as in faecal screening and clinical samples (Perry et al. 2011; Walsh et al. 2011; Wang et al. 2012; Johnson and Woodford 2013; Wang et al. 2013; Ahammad et al. 2014). A recent review showed that colonising and environmental isolates represented a significant proportion of NPGNB reported globally (Berrazeg et al. 2014). Furthermore, some of the bacterial isolates from clinical and screening samples are organisms which are considered to have an environmental source, such as *P. aeruginosa*, *V. cholerae* and many of the *Acinetobacter* spp. (Darley et al. 2012; Hu et al. 2012; Carattoli *et al.* 2013; McGann et al. 2013). As alluded to at the beginning of the general discussion, however, it is an impractical undertaking to attempt to define genetic contexts in all of the "environmental" organisms that *bla*$_{\text{NDM-1-like}}$ genes have been described in. Of the many "environmental" organisms that our laboratory has identified, from a range of studies from the environment, colonisation of humans and clinical isolates, *V. cholerae* was selected as a potentially interesting model organism for reasons that are expanded on in Chapter 5.

The data presented in Chapter 5 demonstrated once again the great variety of genetic contexts associated with *bla*$_{\text{NDM-1}}$. On the one hand, there are clear evolutionary relationships between many of the local contexts *bla*$_{\text{NDM-1-like}}$ genes are found in from GNB but there are significant genetic re-assortments observed. The local contexts in the *V. cholerae* strains could well have evolved from a single

common source. The wider genetic contexts, however, demonstrated significant diversity, being associated with three distinct MGEs, in three strain backgrounds. Whether this is a chance observation, given the small number of isolates analysed, or reflects real evidence of significant spread between different strains of *V. cholerae*, would require further study. To our knowledge, there are no systematic studies designed to detect the presence of NPVC in environmental and clinical isolates, and there are limited data for other resistance determinants (Kitaoka et al. 2011). Setting up a collaboration to analyse this question, in a country where resistance rates in other pathogens are high and cholera is a significant clinical problem would, therefore, be of future interest.

Within the limitations acknowledged, the range of MGE *bla*$_{\text{NDM-1}}$ was found associated with in these strains was intriguing. The IncA/C plasmids have been established as important vectors of resistance genes, including *bla*$_{\text{NDM-1}}$ in Enterobacteriaceae, but it seemed highly likely that the plasmid demonstrated in *V. cholerae* 116-17a had acquired the *bla*$_{\text{NDM-1}}$ context in an independent event to that associated with any of the IncA/C plasmids containing the gene which have been sequenced from Enterobacteriaceae (Carattoli et al. 2012; Johnson and Lang 2012; Carattoli 2013). Furthermore these plasmids have been described in *V. cholerae* previously and been hypothesised to originate from aquatic species such as *V. cholerae* (Johnson and Lang 2012).

The other plasmid identified seemed to have a full complement of genes required for conjugative transfer, previous data suggested it could be transferred by conjugation *in vitro* (Walsh et al. 2011) and it was very closely related to a sequence identified from a different environmental *Vibrio* spp. The SGI-NDM-1 element is one of only two descriptions at present of *bla*$_{\text{NDM-1-like}}$ genes being in GEIs. Like IncA/C

plasmids, these elements have previously been shown to have mobile potential *in vitro*, although they require a conjugative element to mobilise them. SGIs have been found in *P. mirabilis*, including the other example of one of these elements harbouring *bla*$_{NDM-1}$, and in *Salmonella* spp (Siebor and Neuwirth 2013; Girlich et al. 2014). Thus the dynamics of sharing of IncA/C plasmids and SGIs between Enterobacteriaceae and *Vibrio* spp. could have significant evolutionary implications. Experiments to look at how widespread these elements are in *Vibrio* species, the efficiency of transfer between the different host backgrounds and the stability of IncA/C plasmids would be interesting in this regard.

From the data available currently it is not possible to conclude whether genetic contexts observed in *V. cholerae* were acquired from Enterobacteriaceae, whether Enterobacteriaceae have acquired these contexts from *Vibrio* spp. and other environmental species, or whether in fact there is a multi-directional sharing of these genetic contexts between different bacterial genera, in different ecological niches. As such it is not possible to draw firm conclusions about the importance of *Vibrio* spp. in the dynamics of environmental sharing of AMR genes. However, the ecological environments *V. cholerae* is associated with, it's apparent potential for genetic plasticity, acquisition and sharing of foreign DNA, combined with the data presented here, suggest that this species has significant potential in this regard and is worthy of further study.

A broader question stemming from the apparent importance of environmental bacteria in the dissemination of *bla*$_{NDM-1-like}$ genes, and the diversity of species involved, is to what extent this could be the case for other AMR genes. Studies have shown that many AMR genes identified in bacterial pathogens, and a diverse array of genes which are closely related to known AMR genes, can be identified in aquatic

and telluric environments, as well as in wild, livestock and companion animals (Lupo et al. 2012; Djordjevic et al. 2013; Nesme and Simonet 2014). ESBL producing Enterobacteriaceae have been frequently identified in animals, raw meat and the environment (Ewers *et al.* 2012; Liebana *et al.* 2013). There are fewer reported isolates of other carbapenemase genes from environmental and animal sources than there are for *bla*$_{\text{NDM-1-like}}$ genes, although this may simply reflect *bla*$_{\text{NDM-1-like}}$ genes being sought in the environment more frequently following the well-publicised reporting of contamination of the New Delhi environment with NPGNB (Walsh et al. 2011). However, a number of reports have identified environmental and animal associated organisms harbouring of *bla*$_{\text{KPC}}$, *bla*$_{\text{VIM}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{OXA-23}}$ and other carbapenemase genes (Guerra *et al.* 2014; Woodford et al. 2014).

6.6 What role has South Asia played in the spread resistance genes including *bla*$_{\text{NDM-1-like}}$ genes amongst GNB?

There are substantial geographic differences in the rates of resistance of bacterial pathogens globally. Despite limitations in global monitoring of AMR there is evidence of significantly higher rates of AMR in pathogenic GNB in parts of Southern Europe, South America and South Asia, when compared to North America and the rest of Europe (Hawkey and Jones 2009; Gales *et al.* 2012; Mendes *et al.* 2013; Jones *et al.* 2014b). Notwithstanding the initial negative response to the concerns raised about the spread of NPGNB in South Asia from India (Kumarasamy et al. 2010; Walsh and Toleman 2011b), it has since been acknowledged by clinicians and policy makers that AMR is a significant problem in India, culminating in the Chennai Declaration in 2012 (Ghafur *et al.* 2013). Praiseworthy for its intent, its honesty and its pragmatism, the Chennai Declaration is none-the-less a stark acknowledgement

of the extent of the challenge facing Indian health services with regards to AMR pathogens, which is conservative in its recommendations and unlikely to result in any substantial improvement in the situation for the foreseeable future. The road-map agreed suggests that, in the opinion of the experts involved in the meeting, control of over-the-counter antimicrobials was virtually non-existent and strict controls would not be implementable, most Indian hospitals had weak or absent infection control and antimicrobial stewardship programs, microbiology laboratories were not providing good quality standardised diagnostic services and there was no meaningful systems in place to monitor either antimicrobial usage or AMR bacterial pathogens.

Several studies have previously suggested that travel to geographic regions with a higher prevalence of ESBL producing Enterobacteriaceae, especially India, is a risk factor for colonisation or infection with these organisms (Freeman *et al.* 2008; Laupland *et al.* 2008; Tängdén *et al.* 2010; Peirano *et al.* 2011b). NPE have been isolated fairly frequently in most studies reporting data from India, Pakistan and Bangladesh (Kumarasamy et al. 2010; Perry et al. 2011; Islam et al. 2012; Johnson and Woodford 2013). The global dissemination of NPE is proposed to stem from travel to and from South Asia (Kumarasamy et al. 2010; Johnson and Woodford 2013; Jain et al. 2014; Peirano et al. 2014). However, some authors have disputed this point (Kant and Haldar 2010) and there is increasing evidence of spread occurring in some parts of the world which cannot be directly attributed to importation from South Asia (Johnson and Woodford 2013; Berrazeg et al. 2014). NPGNB have been identified from environmental sources in China (Zhang et al. 2013a) as well as in India (Walsh et al. 2011) and Bangladesh (M. Toleman, unpublished data), but few studies have reported on attempts to isolate organisms from the environment from

other parts of the world (Isozumi *et al.* 2012; Berrazeg et al. 2014; Woodford et al. 2014).

Earlier in the discussion it was proposed that the variability of genetic contexts, in terms of species, strains, MGEs and immediate contexts associated with *bla*$_{\text{NDM-1-like}}$ genes, seems to be rather greater than has been observed for some other important resistance genes. Potential reasons for the variability of *bla*$_{\text{NDM-1-like}}$ contexts have been discussed, based on the features of these genetic contexts. A further possibility is that the extent of spread, and thus the diversity of genetic contexts observed, has to do with environmental and healthcare conditions where NPGNB appears to be most prevalent, in South Asia.

Most South Asian countries are relatively poor. India may be one of the largest economies globally but this is partly a reflection of its large population (~1.2 billion people). In fact, about 32.7% of the population were estimated to live on less than $1 a day in 2012 (World Health Organization 2014). Within the region, health care systems are poorly resourced and infant mortality rates are high. For example WHO health statistics data for 2006-2013 suggest that India has only 7 physicians and 17.1 nursing and midwifery personnel per 10,000 population, compared to 27.9 physicians and 88.3 nursing and midwifery personnel per 10,000 in the UK (World Health Organization 2014). The infant mortality rates in 2013 for India and the UK were 41.4 and 3.9 per 1000 live births respectively. South Asian countries also have relatively poor standards of waste management and provision of safe, uncontaminated water (Cairncross 2003; Ensink *et al.* 2008; Ensink and van der Hoek 2009; Akter *et al.* 2014; Cowling *et al.* 2014). There is very little data on standards of infection control, cleaning and rates of healthcare associated infection from this part of the world (Mehta *et al.* 2007; Chakravarthy *et al.* 2014), although

anecdotally standards of infection control are poor in many hospitals, as would be expected in resource poor settings. Antimicrobial stewardship is also poor, with many drugs being widely available over the counter without a prescription (Kotwani and Holloway 2011; Banerjee *et al.* 2013), and sometimes being of questionable quality (Hadi *et al.* 2010). India is one of the major producers of generic pharmaceuticals for the global market, and several studies have highlighted high levels of contamination with drugs, including antibiotics in wastewater as a result of these industries (Fick *et al.* 2009; Singh *et al.* 2014).

It is hypothesised that all of these factors could contribute to high rates of resistance, the broad dissemination of *bla*$_{\text{NDM-1-like}}$ genes and other AMR determinants, and the variability of the associated genetic contexts. Poor regulation of antimicrobial usage in terms of appropriateness, dosage, duration of treatment and quality of medication could all encourage sub-lethal exposure in patients, encouraging colonisation with resistant bacterial flora in both community and hospital settings. Poor standards of infection control and hygiene could then lead to spread of resistant organisms between individuals in hospital and community settings. Human waste would as a result be more likely to contain both resistant flora and residual pharmaceutical products and their metabolites, contaminating the environment. In contaminated environments it is highly likely that some human commensals and pathogens are able to survive, and engage in exchange of genetic information by HGT, with organisms which are naturally found in the environment (Stokes and Gillings 2011).

Further contamination of the environment with by-products of industry could encourage AMR in bacteria and spread of MGEs, either by offering a selective advantage, from elements that harbour AMR and heavy metal resistance

determinants, or from other effects of these compounds. A striking result of one study was the very high rates concentrations in wastewater near factories in India producing pharmaceuticals of fluoroquinolones, especially ciprofloxacin (Larsson *et al.* 2007). It has been mentioned previously in the General Introduction and Chapter 5 that ciprofloxacin can induce the SOS response in some GNB and is associated with increased conjugation rates of SXT/ R391-like ICEs (Beaber et al. 2004). SOS responses are further associated with plasticity associated with integrons and mutation rates, so could increase genetic plasticity in other ways. It is not known what effect many pharmaceuticals, industrial by-products and drug metabolites might have, but it is reasonable to hypothesise that some are likely to have ecological effects relevant to AMR and genetic plasticity of bacteria.

Use of contaminated water supplies for bathing, washing and agricultural purposes (Ensink et al. 2008; Ensink and van der Hoek 2009), as occurs not infrequently in much of the developing world, including in South Asia, would then bring humans, their livestock and companion animals, and their crops back into contact with bacteria which have had the opportunity to engage in significant HGT under significant selection pressure. It is not argued that the conditions described are exclusive to South Asia, but simply that there are reasons to suppose that the situation may be worse in some parts of South Asia than in many other geographic locations.

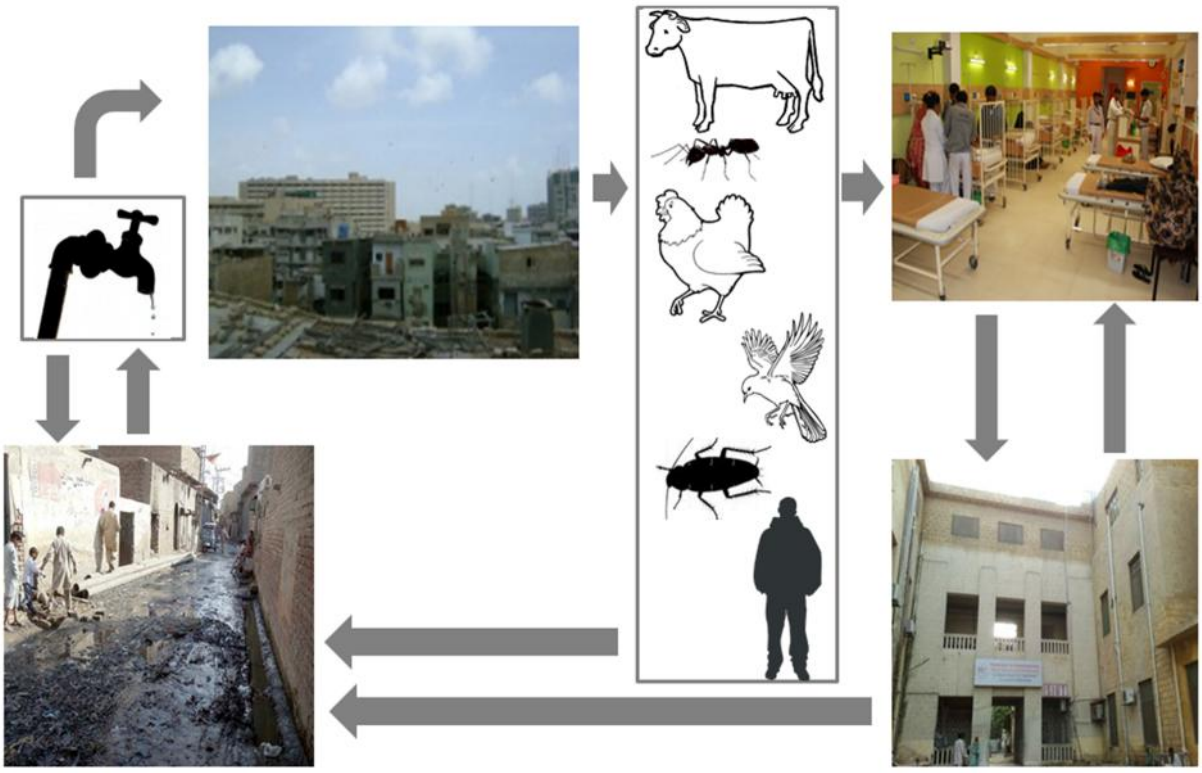


Figure 6.2 – Flow chart showing the potential transfer of bacteria, and the AMR genes and the MGEs they harbour between different hosts and environmental niches. Especially in resource poor settings where standards of hygiene and infection control are not high, and people may live in close proximity to animals, there is the potential spread of bacteria between different host species and environmental niches, including the hospital environment. Figure courtesy of T.R. Walsh.

A further factor that has been highlighted is the higher ambient temperatures in South Asia and other parts of the world with significant problems with resistance in GNB (Nordmann et al. 2011b). It may be that the ambient temperature encourages environmental growth of some important groups of bacteria, and even has effects on HGT. Walsh et al. reported higher transfer rates by conjugation when experiments were conducted at 30°C, than at 25 °C or at the standard 37°C used to culture many

bacterial pathogens and to conduct most conjugation experiments (Walsh et al. 2011). This paper further observed that 30°C fell within the range of mean high and low ambient temperatures in New Delhi, where environmental NPGNB had been isolated, for much of the year.

6.7 What are the implications of environmental spread of *bla*$_{\text{NDM-1-like}}$ genes and other AMR genes?

It is possible that the widespread dissemination of *bla*$_{\text{NDM-1-like}}$ genes, which encompasses GNB which are human pathogens, commensals and bacteria normally found in several environmental niches, is a fairly unique evolutionary event. It is also possible that this enzyme will not ever acquire the degree of clinical importance that has been established for other β-lactamases like CTX-M (D'Andrea et al. 2013), KPC and VIM (Tzouvelekis et al. 2012). However, it is feared that *bla*$_{\text{NDM-1-like}}$ genes are likely to become more prevalent in successful pathogenic strains. There are already sporadic examples where this has occurred, as with the dissemination of ST11 *K. pneumoniae* in Greece (Voulgari et al. 2014), ST15 *K. pneumoniae* in Nepal (Stoesser et al. 2014) and ST1 *A. baumannii* in Tamil Nadu reported here (Jones et al. 2014a).

If it is also the case that many of the resistance genes which have contributed to our problems with AMR in pathogens, also initially disseminated widely amongst environmental species prior to emergence as clinical challenges, then this has important implications. In an inter-linked, globalised world, preventing the spread of AMR is unlikely to be as simple as adopting good antimicrobial stewardship and infection control procedures within isolated healthcare settings. Instead far more wide reaching solutions may be necessary including careful control of antimicrobial

usage globally in human medicine, agriculture and aquaculture, as well as global resistance monitoring in key pathogens. Furthermore, attempts to better control environmental contamination with antibiotics and other chemicals, with provision in all parts of the world of clean and safe water supplies and proper sewage treatment would, amongst their many benefits, mitigate against the spread of AMR in the environment.

This presents a massive political, scientific and economic challenge. Currently many countries have limited or non-existent monitoring systems of clinical infections and AMR. No global monitoring system currently exists, and despite the efforts of various groups, the data on the presence of AMR and bacterial pathogens in the environment, livestock and companion animals is even more limited (World Health Organization 2001; Walsh and Toleman 2011b). These deficiencies are vital, since as human movement becomes ever less constrained by geography, practices in one geographical area which facilitate the spread of AMR pathogens within any ecological setting, could lead to spread into human flora, and then dissemination globally.

Our current position of ignorance, in the absence of any meaningful international monitoring, prevents us from assessing either the extent of the problem or planning a proportionate response. Is the experience with NPGNB the tip of an iceberg, which warns of impending doom, or actually not representative of the development of AMR in bacteria generally? Clarifying this will require considerable work and investment. Control of antimicrobial use in any of the industries which utilise them would in turn have major economic ramifications, and could impact on our ability to continue to feed the global population according to current patterns of consumption, let alone meet the increasing demand for meat which is likely to be a

consequence of the burgeoning middle class in countries like India and China. However, the alternative may be sleep walking into a post-antibiotic world, with profound implications for the delivery of healthcare in the future.

Appendices

Appendix 2.1 – Recipes for reagent stock solutions made up locally.

Reagents purchased from one of the following companies: Thermo Fisher Scientific, Waltham, USA; Sigma Aldrich, St. Louis, USA.

TBE Buffer (10×)

Tris (Fisher)	108 g
Boric Acid (Sigma)	54 g
Na2EDTA (Fisher)	7.44 g

Made up to 1 L with sterile distilled water. Autoclaved before use.

TE Buffer (10×)

Tris (Fisher)	12.1 g
Na2EDTA (Fisher)	3.72 g
HCl (Fisher)	2 M 30 ml

Made up to 1 L with sterile distilled water. Autoclaved before use.

ES Buffer (Deproteination Buffer)

Na lauroyl sarcosine (Sigma)	10 g
Na2EDTA (Fisher) pH 8	18.61g

Made up to 100 ml with sterile distilled water. Filter sterilised.

CS Buffer

Tris (Fisher) pH 8	12.1 g in 10 ml of sterile distilled water
Na2EDTA (Fisher) pH 8	3.72 g in 20 ml of sterile distilled water

Made up to 100 ml with sterile distilled water. Filter sterilised.

S1 Buffer

Na Acetate (Fisher)	2 g
Zn Acetate (Fisher)	0.1 g
HCl (Fisher)	to pH 7.5

25 ml sterile distilled water and 25 ml glycerol (Fisher)

Denaturing solution

NaCl (Fisher)	87g
NaOH (Fisher)	0.5M

Made up to 1L in sterile distilled water.

Neutralising solution

Tris (Fisher)	78.5g
NaCL (Fisher)	87g
Conc HCl (Fisher)	to pH 7.5

Tris and NaCl dissolved in 800mls of sterile distilled water, adjusted to pH 7.5 and made up to 1L.

20× Saline-Sodium Citrate (SSC) solution

NaCl (Fisher)	17.5g
Na_3citrate.H_2O (Fisher)	8.8g

Adjusted to pH 7.0 with 1M HCl and made up to 100mls in sterile distilled water.

Pre-hybridisation solution

20× SSC	6mls
5% Ficoll (Sigma)	400µL
5% Polyvinylpyrrolidone (Sigma)	400µL
10mg/ml Salmon testes DNA (Sigma)	300µL
UHT milk	400µL
10% SDS	1ml
Sterile distilled water	11.5mls

0.1M Tris HCl Buffer, pH 7.5

Tris (Fisher)	1.21g
HCl (Fisher)	to pH 7.5

Made up to 100mls in sterile distilled water.

CTAB/ NaCl solution

NaCl (Fisher)	4.1g
CTAB (Sigma Aldrich)	10g

NaCl dissolved 80ml water, CTAB slowly added while stirring and heating up to 65°C to dissolve. Made up to 100mls with water.

Appendix 2.2 – List of Culture Media

Columbia Blood Agar (CBA, E&O Laboratories Ltd, Bonnybridge, Scotland)

Used for primary culture and subculture of all isolates, and for plate mating assays.

Thiosulphate Citrate Bile Salts Sucrose (TCBS) Agar (E&O Laboratories Ltd)

Used for primary culture of *V. cholerae* isolates.

Luria Bertani (LB) Agar, Miller (Thermo Fisher Scientific, Waltham, USA)

Supplemented with antimicrobials for subculture and passage experiment.

LB Broth, Miller (Thermo Fisher Scientific)

Used for subculture, mating supplemented with antimicrobials for passage experiment.

Mueller Hinton (MH) Agar (E&O Laboratories Ltd)

Used for gradient strip susceptibility testing.

Brilliance™ UTI Clarity Agar (Oxoid Ltd, Basingstoke, UK)

Supplemented with antimicrobials for selective isolation in mating experiments.

Appendix 3.1 – Copy of Jones *et al.* AAC 2014;58 (7):4211-4213. (Jones et al. 2014a)

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Plasmid Carriage of bla_{NDM-1} in Clinical *Acinetobacter baumannii* Isolates from India

Lim S. Jones,[a,b] Mark A. Toleman,[a] Janis L. Weeks,[a] Robin A. Howe,[a,b] Timothy R. Walsh,[a] Karthikeyan K. Kumarasamy[c]

Cardiff University, Cardiff School of Medicine, University Hospital of Wales, Heath Park, Cardiff, United Kingdom[a]; Public Health Wales Microbiology Cardiff, University Hospital of Wales, Heath Park, Cardiff, United Kingdom[b]; Department of Microbiology, Dr ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Chennai, India[c]

NDM-1 probably emerged in *Acinetobacter* species prior to its dissemination among *Enterobacteriaceae*, and NDM-1-like enzymes are increasingly reported in *Acinetobacter* species. Here, we report on the genetic context of bla_{NDM-1} in the earliest known NDM-1-producing organisms, clinical isolates of *Acinetobacter* from India in 2005. These strains harbor bla_{NDM-1} plasmids of different sizes. The gene is associated with the remnants of the Tn*125* transposon normally associated with bla_{NDM-1} in *Acinetobacter* spp. The transposon has been disrupted by the IS*26* insertion and subsequent movement events.

Multidrug-resistant *Enterobacteriaceae* producing the New Delhi metallo-β-lactamase-1 (NDM-1) enzyme have been isolated around the world, with many cases linked to travel, especially to South Asia (1–3). Carbapenem resistance in the opportunistic pathogen, *Acinetobacter baumannii*, is mostly associated with OXA-type β-lactamases (4), but NDM-1-like enzymes have been increasingly reported (5–10). In all bacterial species, the bla_{NDM-1} gene is associated with at least one copy of a complete or partial IS*Aba125* gene, which provides bla_{NDM-1} with a strong promoter (11–13). IS*Aba125* almost certainly originates from *Acinetobacter* spp., and there is evidence that bla_{NDM-1} was probably formed by a fusion event between the aminoglycoside resistance gene, *aphA6*, and an ancestral carbapenemase in an *Acinetobacter* background (13), thus implying that bla_{NDM-1} spread to *Enterobacteriaceae* from *Acinetobacter* spp.

A small number of studies have reported a relatively high prevalence of NDM-1-producing *A. baumannii*, causing infections in intensive care patients in Indian hospitals (10, 14). There are also several case reports of colonization and infection with these organisms from European countries, many with epidemiological links with travel to North Africa or the Balkans (5, 6, 11), and from the Middle East (9). In China, bla_{NDM-1} has been frequently identified in *Acinetobacter* spp., including *A. baumannii*, both in clinical cases and from environmental sources (7, 15–17). The genetic context of bla_{NDM-1}-like genes in *Acinetobacter* spp. shows less variation than that observed in other genera. In most strains, bla_{NDM-1} is bracketed by two copies of IS*Aba125* to form a Tn*125* transposon. The content of Tn*125* is usually conserved, with the occasional exceptions resulting from truncation by insertion of other IS elements. Tn*125* is found inserted in several different gene locations, with direct repeats at either end indicating movement by transposition. All isolates from Europe and North Africa are chromosomally located, but in China, bla_{NDM-1} is reportedly found mostly on plasmids in *Acinetobacter* spp. other than *A. baumannii* (15–18).

We received 9 *Acinetobacter* isolates that were collected in 2005 from a hospital in Tamil Nadu, India. Most isolates were from patients receiving intensive care and were isolated from blood, pus, and respiratory secretions. The initial identification and sensitivity testing were performed using a BD Phoenix system (Becton, Dickinson, Franklin Lakes, NJ, USA), with supplementary

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Address correspondence to Lim S. Jones, jonesls5@cardiff.ac.uk.

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TABLE 1 Study isolates, specimen types, and antimicrobial MICs for *Acinetobacter* isolates

Isolate	Specimen type	MIC (μg/ml) for[a]:											
		Azt	Caz	Taz	Imp	Mem	Ami	Gent	Tob	Cip	Col	Rif	Tig
A. baumannii CHI-16	Blood	>16	≥256	>16/4	≥32	≥32	≤4	>4	2	≥32	≤1	6	2
A. baumannii CHI-18	Blood	>16	≥256	>16/4	≥32	≥32	>16	>4	>4	≥32	≤1	4	3
A. baumannii CHI-32	Blood	>16	≥256	>16/4	≥32	≥32	≤4	>4	2	≥32	≤1	6	2
A. baumannii CHI-34	Sputum	>16	≥256	>16/4	≥32	≥32	≤4	>4	>4	≥32	≤1	4	2
Acinetobacter sp CHI-40-1	Pus	>16	≥256	>16/4	≥32	≥32	>16	>4	4	≥32	≤1	≥256	0.75
A. baumannii CHI-40-2	Pus	>16	≥256	>16/4	≥32	≥32	>16	>4	>4	≥32	≤1	6	2
A. baumannii CHI-41	Sputum	>16	≥256	>16/4	≥32	≥32	>16	>4	>4	≥32	≤1	6	1.5
A. baumannii CHI-44	Endotracheal aspirate	>16	≥256	>16/4	≥32	≥32	>16	>4	>4	≥32	≤1	6	2
A. baumannii CHI-45-1	Endotracheal aspirate	>16	≥256	>16/4	≥32	≥32	>16	>4	>4	≥32	≤1	3	2

[a] MICs were determined by the BD Phoenix system except for ceftazidime, imipenem, meropenem, ciprofloxacin, rifampin, and tigecycline, which were determined by an Etest or a MIC test strip. Ami, amikacin; Azt, aztreonam; Caz, ceftazidime; Cip, ciprofloxacin; Col, colistin; Gent, gentamicin; Imp, imipenem; Mem, meropenem; Rif, rifampin; Taz, piperacillin-tazobactam; Tig, tigecycline; Tob, tobramycin.

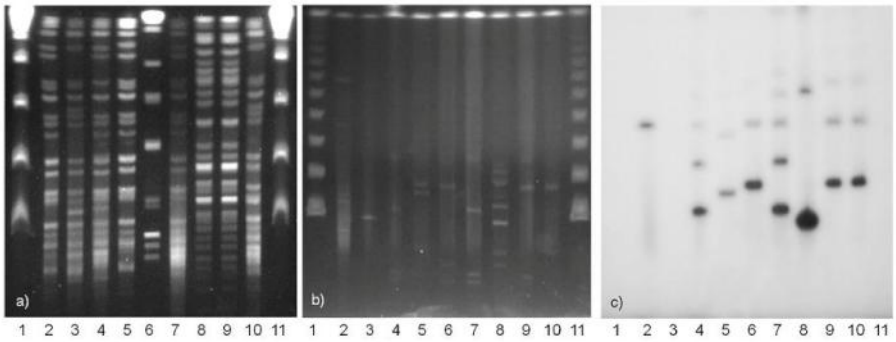

FIG 1 (a) PFGE of ApaI-digested genomic DNA. Lane 1, λ ladder (48.5-kb concatemers); lane 2, CHI-16; lane 3, CHI-18; lane 4, CHI-32; lane 5, CHI-34; lane 6, CHI-40-1; lane 7, CHI-40-2; lane 8, CHI-41; lane 9, CHI-44; lane 10, CHI-45-1; lane 11, λ ladder. (b) PFGE of S1 endonuclease-digested genomic DNA; (c) in-gel hybridization with ^{32}P-labeled bla_{NDM-1} gene probe. Lanes 1, λ ladder; lanes 2, *Klebsiella pneumoniae* KP506 (NDM positive control); lanes 3, *Escherichia coli* UAB190 (NDM negative control); lanes 4, CHI-16; lanes 5, CHI-18; lanes 6, CHI-32; lanes 7, CHI-34; lanes 8, CHI-40-1; lanes 9, CHI-40-2; lanes 10, CHI-45-1; lanes 11, λ.

sensitivity testing performed using the Etest (bioMérieux, LaPlane, France) or MIC test strip (Liofilchem, Roseto degli Abruzzi, Italy) method (Table 1). The confirmation of *A. baumannii* identification was performed by PCRs for bla_{OXA-51}-like genes. All isolates were bla_{OXA-51}-like positive by PCRs except CHI-40-1, and all were extensively drug resistant (19), with the *A. baumannii* isolates being resistant to all drugs tested except colistin and in some cases amikacin and tobramycin. Seven isolates were positive for bla_{NDM-1} by PCRs, with CHI-41 and CHI-44 being the only bla_{NDM-1}-negative isolates. All of the *A. baumannii* isolates carried bla_{OXA-23}-like genes, confirmed to be associated with IS*Aba1*, and thus having a strong upstream promoter, by PCR. All PCR amplicons were confirmed by sequencing.

A. baumannii isolates were then typed by the multilocus sequence typing (MLST) method described by Turton et al. (20) and by pulsed-field gel electrophoresis (PFGE) of ApaI-digested genomic DNA. All *A. baumannii* isolates producing NDM-1 by MLST were within group II, which corresponds with worldwide clone 1. The 2 bla_{NDM-1}-negative isolates were within group I. ApaI profiles were similar but not identical for all group II *A. baumannii* isolates and differed substantially from those for group I isolates (Fig. 1a) and CHI-40-1.

To investigate the genetic context of bla_{NDM-1}, we performed sequencing by primer walking in CHI-32, CHI-34, and CHI-45-1 using primers designed against the context in *A. baumannii* 161/07 (21). Gene probing was performed by in-gel hybridization of S1 nuclease and NotI PFGE gels. The gene probes were made using a random priming method with bla_{NDM-1}, IS*Aba125*, IS*26*, and IS*CR27* PCR products labeled with [^{32}P]CTP. As the primer walking PCR results were consistent for all three isolates studied, products were only fully sequenced for CHI-45-1. The full Tn*125* structure was present; however, IS*CR27* contains an IS*26* insertion (Fig. 2). The sequence analysis of PCR amplicons revealed that the fragment of IS*CR27* downstream of the IS*26* insertion is present both in its normal position and upstream of bla_{NDM-1}. The results of probing NotI gels with bla_{NDM-1}, IS*26*, and IS*CR27* suggest that it is the latter context which is more common. S1 gels demonstrated multiple plasmids in all *A. baumannii* isolates. The probing showed that bla_{NDM-1} was on multiple bands, ranging in size from ~45 kb to ~300 kb. We believe most of the larger bands represent

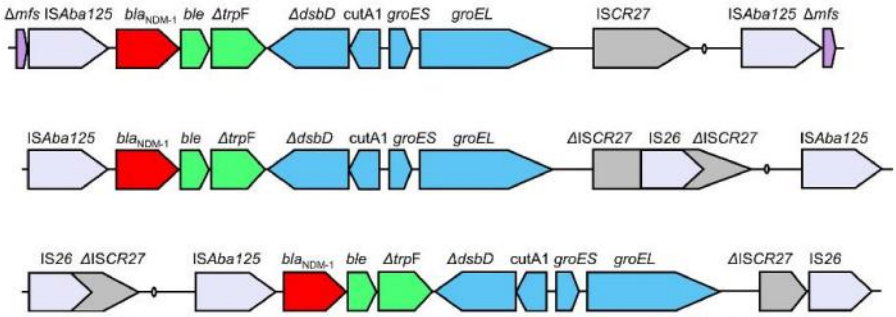

FIG 2 Gene maps of the genetic context of bla_{NDM-1} in *Acinetobacter* species. *aphA6* codes for 3′ phosphotransferase VI aminoglycoside-modifying enzyme, *ble* for bleomycin resistance protein, *trpF* for phosphoribosylanthranilate isomerase, *tat* for twin-arginine translocation pathway signal sequence domain protein, *cutA1* for periplasmic divalent cation tolerance protein, *groES* for cochaperonin, *groEL* for chaperonin, IS*CR27* for insertion sequence common repeat 27 transposase, *oriIS* for origin of insertion of IS*CR27*, and Δ*mfs* for major facilitator superfamily (MFS) metabolite/H^+ symporter. Arrows indicate the direction of the transcription of genes. The genes *tat*, *cutA1*, *groES*, and *groEL* are shaded in the same color because they are believed to be from a common source, with similar genes found in synteny in both *Xanthomonas* and *Pseudoxanthomonas* spp.

cointegrate formation rather than the presence of multiple plasmids carrying bla_{NDM-1}, since the bands increase in size by intervals of approximately the size of the smallest band for each isolate (Fig. 1b). Mating experiments were performed on plates and in broth. *A. baumannii* CHI-32, CHI-34, and CHI-45-1 were used as donors, and *Escherichia coli* UAB190 and *Acinetobacter pittii* AG3528 were used as recipients (both rifampin resistant). No transconjugants were obtained after multiple mating experiments.

The *A. baumannii* isolates analyzed in this study are among the earliest found to produce NDM-1, having been initially identified in 2005. This is the first time that the genetic context of *A. baumannii* isolates from the Indian subcontinent has been analyzed. The findings are compatible with the hypothesis that bla_{NDM-1} might have been disseminated from *Acinetobacter* to *Enterobacteriaceae* in South Asia. In these isolates, Tn*125* has been disrupted by IS*26*, and subsequent rearrangement has resulted in bla_{NDM-1} being within an IS*26* composite transposon, which might potentially mobilize bla_{NDM-1}. Otherwise, the genes usually found on Tn*125* are conserved, and so the genetic context is compatible with being the progenitor of the bla_{NDM-1} in many of the *Enterobacteriaceae* for which sequences are available. bla_{NDM-1} is located on plasmids in these isolates, which could facilitate mobilization of the gene to other bacterial species. The mating experiments suggest that the plasmids are nonconjugative, but they may be mobilizable with a helper plasmid. That these clinical isolates of *A. baumannii* producing NDM-1 were clonally related demonstrates the potential for bla_{NDM-1} establishing itself in successful strain backgrounds capable of being disseminated in the hospital environment and further compromising therapeutic options in the treatment of significant bacterial pathogens.

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We declare no conflict of interest.

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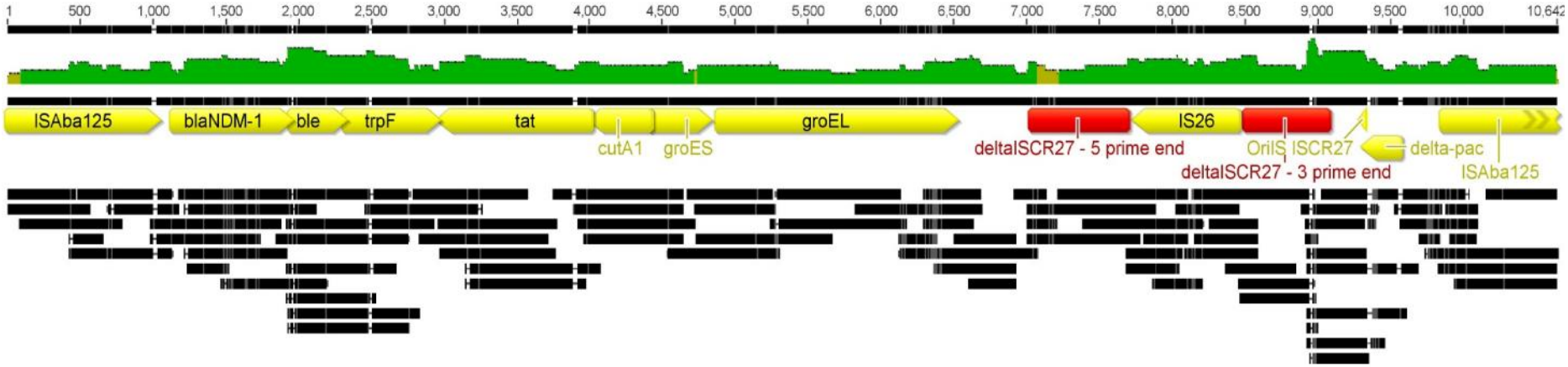
Appendix 3.2 – List of primers used for PCR and sequencing of PCR amplicons for *A. baumannii* isolates. All PCRs run using 0.2 µM of each primer at a Mg2+ concentration of 2.25 mM.

Primer	Sequence
32 c102F	TGCTCTGGCTGAAACGGTGA
32 c15F	CGCGCTTGACAGACCCTAGA
32 c162F	TGCACGCTGTACTGTTGCTC
32 c162R	TCACCAAGTCAGGTGCTCGT
32 c184R	ACTTCACCTTGAGGCTGACCA
32 c184R2	CGTAGTGCACGCATGACTTCAA
32 c248F	TCATTCCGACAGAGGTGTGC
32 c248R	TGCACACCTCTGTCGGAATG
32 c31F	GGATGGAGCTGTTTCAGGCG
32 c31R	GCAAGGTGCTGTGCACGGAT
32 c34 F	AGCGAGAAGGAAAATGTCCAGT
32 c39F	TTGTTGTTCGCAACGAGGGC
32 c39R	CCAGAATTAGCCCTCGTTGCG
32 c44F	TGCCAACGTTCTTCGCCATC
32 c44R	GTCACCAGACATGGCGCAAAGAA
32 c51 F	CCGGGTTTGTCGGAGAGTCA
32 c51F2	CCAAGGACAAACTTTGGAAGGC
32 c57 R2	ATGATGCAGTGGTGGGGAGA
32 c57F	TGCTTCCCACTCTGTGAGCC
32 c57R	TGCTAAACCACGCCAATCTGC
32 c68F	AAGGCACCATTTGGAAATTTACGGCA
32 c68R	TGCGCCACAGACACTGCAAA
32 c68R2	TTCGGTTTCTGTAAGCACAGC
32 c7R	CGGGGATATTTTTGTTGATTGCTGG
32 c7R2	ACCGGGTTTGTCGGAGACTT
32 c86F	CTCTCCGTGCCCAATCCTGT
32 c86R	TACACATCTGCGGCCAGCGT
32 c90F	CAGATCAGCCCGACCACCTT
32 c90R	TCCTACAAATCGCATCAACACCA
32c133gapF	TCAAACAACTCGAACGCGAA
32c164gapF	AGCGTGCCAATGAAATTCTACG
32c53gapR	GCGGTATAGACCCAGCCTGA
32c89gapR	CCGTGTCGATACTTTCCATCCA
34c28F	CATCCCTGTCGGTGTTGCTT
45c143F	GTGGGGTAAAGGCAGGGTTC
45c143F2	CCTCGATTGACCCGGAGTTG

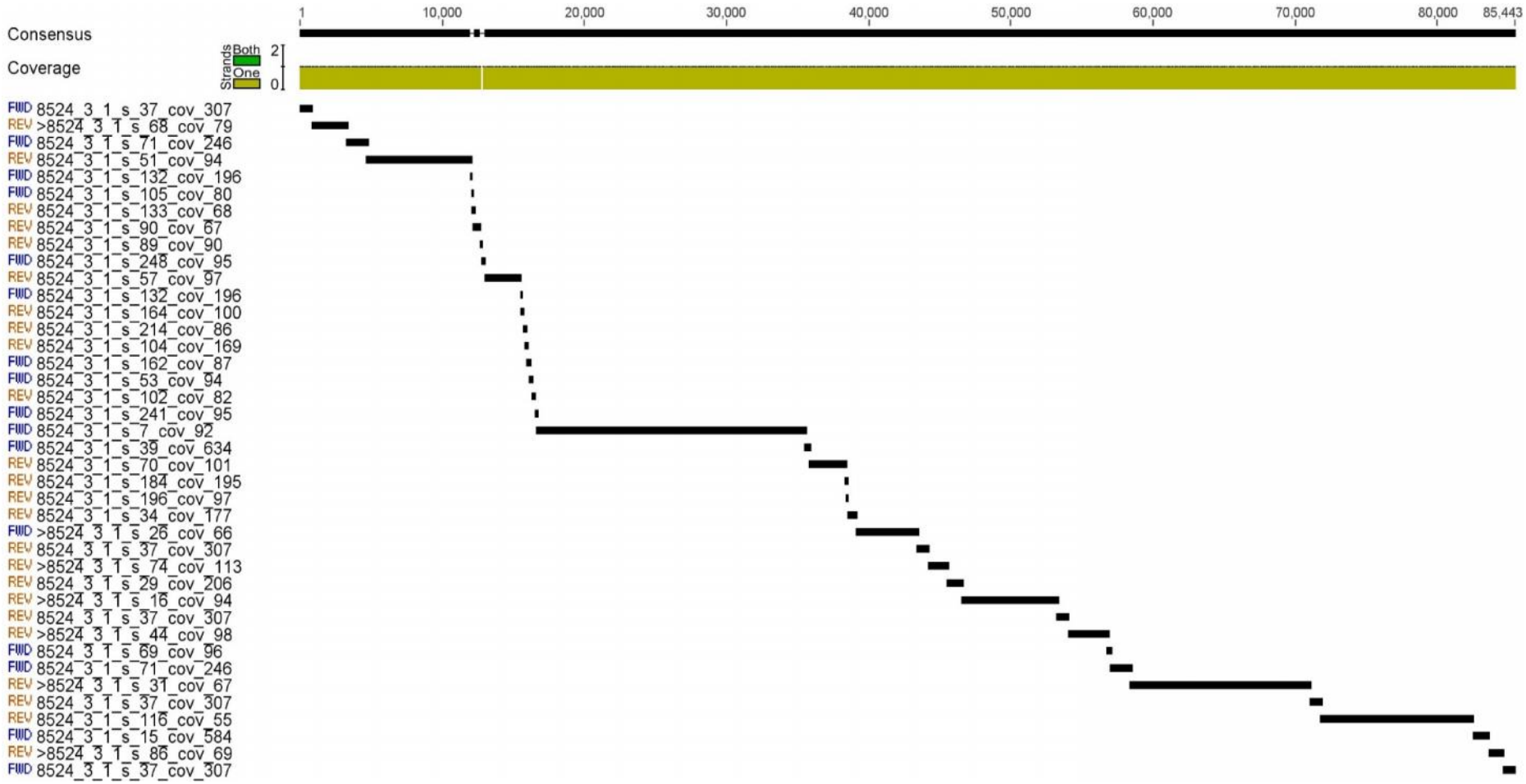
Primer	Sequence
45c143R	ACGCTCCGCCATAATCGTTC
45c202F	ACAGTGCCACTTTTATCGCT
45c202R	TCCACGCTTCACAATAACTGGA
45c27R	TTGAAGGACGCGCTGCTAAA
45c35F	TGTTCGAGCAGCTATAGAAA
45c35F2	TGGCCCTGATGATCCGCTTT
45c35R	CAAAAGACCACGCCCTCTCA
45c41F	GTCGTCCGCCGCATCTATTT
45c41R	CAGCGATAGGCCAGGTTCAG
45c9F	GCTTTTTGGGTTTGCGTCCC
45con27F	TGAAGGGTTCTGTTGGCACG
45intF	GTTAGCAGCACTGTGGCGTA
45intR	AGATGCACCAAAGCGCAGAA
45repAF	GTCTCTCCACGATGTGCGAA
45repAR	AATTGCGATCATTGCTGCTC
47c33 R	ACGCTCCGCCATAATCGTTC
aadA1 3F	CGGCAAAATCGCGCCGAAGG
armA FR	TCTGGAAAGGAGAAGGGAATGGAAGAG
armAFF	AGGATAGGCAGAATAGTAAGACCCCCA
BioNDM-MR	CCGTTGGAAGCGACTGCCCC
ble3F	CATGGTGGCATTGGTGAACGC
crff-r	CGCTTGAGCCGTTGCGCCC
CRIS F	GAGTGCCAAAACAGGGGGACCA
dctF	CAGGGCGCTCACCGGCATAG
groeES-3F	GGTGCTGGACAACGGCCAGG
groeES-F	TCGGCCACCGAGAAGCCGAT
groeES-R2	CCTGGCCGTTGTCCAGCACC
groEL1FR	GCTTCGGTGGTGATCATCAG
groel2ff	CGCGAGATCGTCACCAATGC
groEL-5F2	GCGCAGGCGATGGACAAGGT
groEL-gapF	TGAAGCGCGGCATCGACCAG
groEL-gapR	CGCGCAGGGCGATCTGGATG
groEL-MF	GGCGGCGTGGTGATTTCCGA
groEL-MR	GCCTTCACCGCGCAGACCTT
groES-5R1	ATGACCACGCGGTCGTGCAG
groESgapR	GCCGTCCAGCTTCACTTCGG
groSgapF	CGCCGGGTTTTTCCACGTCA
insE-3F	GCAACGGCTCAAGCGGGTCT
insE-3R	CGAAGTGGGCGAGGATGGCG
insE-5R	ACAGTGCTCGCAGACCACGC
IS125 5F3	CATAAGTGAAGCATTGGTATTTGGTGT
IS125 5R	TGTGACCACGTCTACGTCTAGC
is125-DOWN	GATCTTCAAAGTTAAGATGATGG

Primer	Sequence
IS125gapF	GCAAAGGCAGAATCAGTGCG
IS125-NDMF	TCGCATTTGCGGGGTTTTTAATGCT
IS125prom1	TGTCGCACCTCATGTTTGA
IS125prom2	TGTCGCACCTCATGTTTG
IS26gapF	GCACCGGCCTTCGCGTTTGA
IS26-gapFF	CACTCCGCGTTCAGCCAGCA
IS26-gapR	CGGGGCCGCACTGTCGATTT
IS26gapR2	AACGGCTGCGCTGGTACTGG
ISAb125 TRANSIS_Fb	CGAGCATTACCCAAGGGTGA
ISAb125 TRANSIS_R	AAACAACGGATCGCTTCAAC
ISAb125_5F	AACTTGAAGTGCGACATAAACCACCT
ISAb125_5F2	TCAAGAAAAGAAGGCTTTTCAGCCAG
ISAb125-3R	CGCATGTGCCTTTTTGCCAGGG
ISAba1 5R	ACAAGCATGATGAGCGCAAAGCAC
ISAba1 5R	ACAAGCATGATGAGCGCAAAGCAC
ISAba125-3R(2)	GCCAATCTAACGCCTTTCTAGGACGA
ISCR1 FR	TTTAAACGGGGCGGTGGGGC
ISCR1FF	GTCTGGTCGGGTTGGTGGCG
ISCR27-3R	CCGGTAGTGCGCTTGCTCCA
ISCR27-gap1F	GCCCGCTGCCCGAGTATGTG
iscr27-gap1R	GAAGCGCAACGGGTACGGGA
ISCR27-gap2R	ATTGCGCCACGGCGTCTTGA
Mph2FR	GAGCAGCGGCCTCTCCTTTG
ndm 3f2	ATCGACGGCACCGACATCGC
NDM_5R	CTCAGCTTCGCGACCGGGTG
ndm-1F	GAAGCTGAGCACCGCATTAG
ndm-1R	TGCGGGCCGTATGAGTGATT
ndm-3f	GCCATTCCGCCCCCGATAGC
NDM-5R2	GGGCGGATTTCACCGGGCAT
NDM-APHF	AGGAAAACTTGATGGAATTGCCCA
ndm-p1	CAGTTGCGGAGCTTTGAAGC
ndm-p2	CGCGTTAGATTGGCTTACAC
QACR	CGGATGTTGCGATTACTTCG
qacR ff	GACAACGGCGGAAGGGGCAA
tat-3R	GGCACCGCACCTCGGTCAAG
tat-gapF	CCAGGCCAGCGCGTCGTAAA
tat-gapR1	GTACCAGGGCTGCGCCGATG
tat-gapR2	GAGGGCAAGAGCCCACAGCC
trpF-gapF	TATCGCGGTGCCTTGCCGTG
trpF-R	CACGGCAAGGCACCGCGATA
VibIS26F	GCGATGAGGCAGCCTTTTGTCT

Appendix 3.3 – Geneious assembly of sequenced PCR amplicons used to initially define the context of *bla*$_{NDM-1}$ in *A. baumannii* CHI-45-1 by primer walking. Note that the presence of the full context below being present was also provided by a long range PCR using pimers NDM-3F and ISCRgap2R, linking the 3' end of *bla*$_{NDM-1}$ to the 3' fragment of IS*CR27*. Evidence for context 2 as shown in Figure 3.6 was provided by PCR with the primers NDM_5R and insE-3F, linking the 5' end of *bla*$_{NDM-1}$ to the 3' fragment of IS*CR27*.



Appendix 3.4 – Geneious assembly of contigs making up pNDM-32 from the WGS of *A. baumannii* CHI-32. Note that several contigs appear in the assembly more than once. Links between all contigs were confirmed by PCR and sequencing of amplicons, this procedure completely resolved the two assembly gaps shown in the figure.



Appendix 4.1 – Copy of Jones *et al.* AAC 2015;59 (2):923-929. (Jones et al. 2015)

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Characterization of Plasmids in Extensively Drug-Resistant *Acinetobacter* Strains Isolated in India and Pakistan

Lim S. Jones,[a,b] Maria J. Carvalho,[a] Mark A. Toleman,[a] P. Lewis White,[b] Thomas R. Connor,[c] Ammara Mushtaq,[d] Janis L. Weeks,[a] Karthikeyan K. Kumarasamy,[e] Katherine E. Raven,[f] M. Estée Török,[f] Sharon J. Peacock,[f,g] Robin A. Howe,[a,b] Timothy R. Walsh[a]

Cardiff University, Cardiff School of Medicine, University Hospital of Wales, Cardiff, United Kingdom[a]; Public Health Wales Microbiology Cardiff, University Hospital of Wales, Cardiff, United Kingdom[b]; Cardiff University, Cardiff School of Biosciences, Cardiff, United Kingdom[c]; Dow University of Health Sciences, Dow Medical College, Karachi, Pakistan[d]; Department of Microbiology, Dr ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Chennai, India[e]; University of Cambridge, Department of Medicine, Addenbrooke's Hospital, Cambridge, United Kingdom[f]; Wellcome Trust Sanger Institute, Hinxton, Cambridgeshire, United Kingdom[g]

The bla_{NDM-1} gene is associated with extensive drug resistance in Gram-negative bacteria. This probably spread to *Enterobacteriaceae* from *Acinetobacter* spp., and we characterized plasmids associated with bla_{NDM-1} in *Acinetobacter* spp. to gain insight into their role in this dissemination. Four clinical NDM-1-producing *Acinetobacter* species strains from India and Pakistan were investigated. A plasmid harboring bla_{NDM-1}, pNDM-40-1, was characterized by whole-genome sequencing of *Acinetobacter bereziniae* CHI-40-1 and comparison with related plasmids. The presence of similar plasmids in strains from Pakistan was sought by PCR and sequencing of amplicons. Conjugation frequency was tested and stability of pNDM-40-1 investigated by real-time PCR of isolates passaged with and without antimicrobial selection pressure. *A. bereziniae* and *Acinetobacter haemolyticus* strains contained plasmids similar to the pNDM-BJ01-like plasmids identified in *Acinetobacter* spp. in China. The backbone of pNDM-40-1 was almost identical to that of pNDM-BJ01-like plasmids, but the transposon harboring bla_{NDM-1}, Tn*125*, contained two short deletions. *Escherichia coli* and *Acinetobacter pittii* transconjugants were readily obtained. Transconjugants retained pNDM-40-1 after a 14-day passage experiment, although stability was greater with meropenem selection. Fragments of pNDM-BJ01-like plasmid backbones are found near bla_{NDM-1} in some genetic contexts from *Enterobacteriaceae*, suggesting that cross-genus transfer has occurred. pNDM-BJ01-like plasmids have been described in isolates originating from a wide geographical region in southern Asia. *In vitro* data on plasmid transfer and stability suggest that these plasmids could have contributed to the spread of bla_{NDM-1} into *Enterobacteriaceae*.

Acinetobacter baumannii is a successful nosocomial pathogen, and extensively drug-resistant strains are increasingly prevalent (1, 2). Other *Acinetobacter* spp. are found in the environment and can cause opportunistic infections (2). There is evidence that the gene encoding New Delhi metallo-β-lactamase-1 (NDM-1) evolved in an *Acinetobacter* background through fusion between the aminoglycoside resistance gene *aphA6* and a β-lactamase-encoding progenitor of bla_{NDM-1} (3). Subsequently, bla_{NDM-1} and its closely related variants have spread rapidly among many genera of Gram-negative bacteria (4, 5), and they are found on plasmids of several different incompatibility types and chromosomally (4–6). NDM enzymes hydrolyze all β-lactams except aztreonam and are commonly found with other resistance mechanisms, mediating resistance to almost all clinically available antimicrobials (5, 7).

bla_{NDM} genes are prevalent in clinical *Enterobacteriaceae* isolates in South Asia. Many cases of infection or colonization with NDM-producing *Enterobacteriaceae* around the world have been linked to travel to the Indian subcontinent (5, 8). Studies within Indian hospitals have identified NDM-1-producing *Acinetobacter* spp. causing infections in intensive care units (9–11). Similar cases have been reported in Europe, but most patients probably became colonized during travel to the Balkans or North Africa (12–14). In China, bla_{NDM-1} has been observed many times in several *Acinetobacter* spp., including *A. baumannii*, from clinical, environmental, and farm animal samples but is less commonly reported in *Enterobacteriaceae* (15–22).

The immediate genetic contexts of bla_{NDM} (the genes flanking bla_{NDM}) in *Acinetobacter* spp. are well conserved. An intact IS*Aba125* is normally present upstream (3, 14, 16). Downstream there are usually a conserved set of genes from the bleomycin resistance gene, *ble*, to IS*CR27*. Most examples have an intact IS*Aba125* further downstream from IS*CR27*, thus capturing the entire context in a Tn*125* transposon (13, 14, 16). In Chinese isolates, bla_{NDM-1} is usually found on plasmids, with all sequenced examples being closely related to pNDM-BJ01 from *A. lwoffii* WJ10621, despite being reported in many different *Acinetobacter* spp (15, 16, 19, 23). In most isolates identified outside Asia, bla_{NDM-1} is found on the chromosome (12–14).

The *Acinetobacter* plasmids from China share features which suggest that they could have contributed to the acquisition of bla_{NDM} by *Enterobacteriaceae*. We set out to see if similar plasmids were present in *Acinetobacter* spp. from India and Pakistan, where some studies have shown a high prevalence of *Enterobacteriaceae*

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Address correspondence to Lim S. Jones, JonesLS5@cardiff.ac.uk.
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TABLE 1 Bacterial isolates used in this study

Isolate	Species	Source	Location where isolated	Yr isolated
CHI-40-1	*Acinetobacter berezeniae*	Clinical isolate (pus)	Tamil Nadu, India	2005
73261-EC	*Acinetobacter haemolyticus*	Fecal screening	Karachi, Pakistan	2012
70114-EC	*Acinetobacter haemolyticus*	Fecal screening	Karachi, Pakistan	2012
69122-EW	*Acinetobacter haemolyticus*	Fecal screening	Karachi, Pakistan	2012
74312-EC	*Acinetobacter schindleri*	Fecal screening	Karachi, Pakistan	2012
73668-ECT	*Acinetobacter towneri*	Fecal screening	Karachi, Pakistan	2012

producing bla_{NDM}. We describe the whole-genome sequence (WGS) of an *A. bereziniae* isolate from Chennai, India, with such a plasmid. We further show that closely related plasmids carrying bla_{NDM-1} are present in *Acinetobacter* spp. isolated in Karachi, Pakistan. We also investigated the conjugation efficiencies and stabilities of these plasmids in different recipients to further explore their potential as vectors in the dissemination of bla_{NDM-1}.

MATERIALS AND METHODS

Bacterial strains studied. A full list of isolates used in this study is given in Table 1. *A. bereziniae* CHI-40-1 (11) was investigated in detail in the current study. Other *Acinetobacter* species isolates were from fecal screening samples collected at the Civil Hospital Karachi, Pakistan, in 2012. Samples from consecutive patients admitted to the hospital were collected at admission and discharge, and isolates growing on selective plates containing ertapenem were further analyzed. The isolates studied here represent all of the bla_{NDM-1}-positive *Acinetobacter* spp. isolated from 717 fecal swabs processed as of January 2013. *Escherichia coli* UAB190 (24) and *A. pittii* AG3528 were used as recipients in mating experiments.

Identification and antimicrobial susceptibility testing. Initial bacterial identification was performed by matrix-assisted laser desorption ionization–time of flight mass spectroscopy (MALDI-TOF) (Bruker, Billerica, MA, USA). Confirmation of the identification was by phylogenetic analysis of 16S rRNA gene sequences for Pakistan isolates, together with ribosomal multilocus sequence typing (rMLST) for CHI-40-1 (see Fig. S1 in the supplemental material). Antimicrobial susceptibility testing was performed by Etest (bioMérieux, LaPlane, France) and MIC test strip (Liofilchem, Roseto degli Abruzzi, Italy). Interpretation was according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (version 3.1).

WGS and analysis. *A. bereziniae* CHI-40-1 was sequenced using an Illumina HiSeq platform at the Wellcome Trust Sanger Institute, Cambridge. A unique index-tagged insert library was prepared to allow processing of the sample data following multiplex sequencing with other libraries on eight channels of an Illumina Genome Analyzer GAII cell to give 100-bp paired-end reads, as previously described (25). Reads were assembled *de novo* using the Velvet Assembly Tool (version 1.2.10) (26). Plasmid contigs were identified using Blast searches against the sequence of plasmid pNDM-BJ01 from *Acinetobacter lwoffii* strain WJ10621 (accession number JQ001791) (16). Links between contigs were confirmed by PCR and sequencing of amplicons. Detailed comparison between pNDM-40-1 and closely related plasmids was performed using nucleotide alignments created in Geneious (27). Antibiotic resistance genes were identified using RESFinder. Annotation was by transfer of annotations for genes with close nucleotide identity from reference sequences in Geneious.

PFGE and in-gel hybridization. Genomic DNA was prepared in 1% low-melting-point agarose plugs as described previously (28). Briefly, plugs were made using a bacterial cell suspension in Tris-EDTA (TE) buffer at a standard optical density of 1.8 to 2.0 at a wavelength of 600 nm (28). Plugs were treated with ApaI (Thermo Scientific, Waltham, MA, USA) or S1 nuclease (Thermo Scientific) and pulsed-field gel electrophoresis (PFGE) performed as described previously (28, 29). Autoradiographs were prepared by in-gel hybridization of pulsed-field gels with gene probes, made using a random primer method to label bla_{NDM-1} or *traA* PCR products with [^{32}P]CTP, as previously described (28).

Conjugation and passage experiments. Conjugation experiments were performed as described previously using a plate mating assay at 30°C (4), with *A. bereziniae* CHI-40-1 and *A. haemolyticus* 69122-EW as donors and *E. coli* UAB190 and *A. pittii* AG3528 (both rifampin resistant) as recipients. Selection was performed on Brilliance UTI Clarity agar (Oxoid Ltd., Basingstoke, United Kingdom) supplemented with rifampin (Sigma-Aldrich, St. Louis, MO, USA) for recipient selection, ampicillin (Sigma-Aldrich) and rifampin for UAB190 background transconjugants, or meropenem (AstraZeneca, London, United Kingdom) and rifampin for AG3528 background transconjugants. For each experiment, 5 isolated colonies were subcultured to selective media. Pure growths on subculture were tested by MALDI-TOF to confirm the species background, and the presence of bla_{NDM-1} was confirmed by PCR. Mating efficiency was calculated as the number of transconjugants per recipient cell. Transconjugants obtained from mating between CHI-40-1 and each recipient background were subjected to S1 PFGE and in-gel hybridization with bla_{NDM-1} and *traA*.

A passage experiment was performed on *A. bereziniae* CHI-40-1 and its transconjugants, *E. coli* UAB190$_{NDMP2}$ and *A. pittii* AG3528$_{NDMP1}$. Cultures from selective plates were inoculated into Luria-Bertani (LB) broth (Thermo Scientific) with and without antibiotic selection and incubated overnight at 37°C. The following day, cultures were reinoculated into a fresh broth with the same selection as the starting culture. Columbia blood agar plates (E&O Laboratories, Bonnybridge, Scotland) were inoculated daily to check purity, and cultures were stored each day in LB broth with 10% glycerol at −80°C. This procedure was repeated for 14 consecutive days. Antibiotic selection was with meropenem at 10 μg/ml for CHI-40-1 and AG3528$_{NDMP1}$ and at 1 μg/ml for UAB190$_{NDMP2}$. Stored cultures were investigated by S1 PFGE and probing for bla_{NDM-1} and *traA* as well as real-time quantitative PCR (qPCR) (see below).

PCR. A full list of PCR and sequencing primers used together with full PCR conditions is given in Table S2 in the supplemental material. The presence of plasmids similar to pNDM-BJ01 was confirmed by PCR with primers described by Hu et al. (16). Sequencing of PCR amplicons was used to resolve gaps in the pNDM-40-1 sequence and to primer walk the bla_{NDM-1} context in 69122-EW. PCR amplicons for sequencing were purified using the QIAquick gel extraction kit (Qiagen, Limburg, Netherlands) as per the manufacturer's instruction, and products were submitted to Eurofins MWG Operon (Ebersberg, Germany) for sequencing.

Real time-quantitative PCR (qPCR) was performed to quantify changes in bla_{NDM-1} and *traA* copy number present in bacterial cells over the course of the passage experiment. The single-copy chromosomal gene *rpoB* was used as the reference gene. Dual-labeled probes with fluorescent dye and quenchers were synthesized by Eurofins MWG Operon. bla_{NDM-1} and *traA* fluorescence cycle threshold (C_T) values were compared to *rpoB* C_T values, and quantification was performed by the $\Delta\Delta C_T$ method (30). Regression analysis was performed using Excel 2007. A validation experiment showed that ΔC_T values were linear over the range of values detected in the passage experiment. All experiments were performed in triplicate.

Nucleotide sequence accession numbers. Accession numbers for pNDM-40-1 from *A. bereziniae* CHI-40-1 and the partial sequence for pNDM-69122 from *A. haemolyticus* 69122-EW are KF702385 and LN611576, respectively. *A. bereziniae* CHI-40-1 assembly contigs are deposited under study accession number PRJEB7120, contig accession numbers CDEL01000001 to CDEL01000324.

TABLE 2 Antimicrobial MICs for *Acinetobacter* species recipients and transconjugants

Strain	MIC (mg/liter)[a]												
	ATM[b]	CAZ[b]	IPM	MEM	TZP	AMK	GEN	TOB	CIP	CST	FOF	SXT	TGC[b]
CHI-40-1	48 (R)	≥256 (R)	≥32 (R)	≥32 (R)	96 (R)	48 (R)	≥256 (R)	12 (R)	≥32 (R)	0.75 (S)	8	≥32 (R)	0.75 (R)
69122-EW	3 (S)	≥256 (R)	≥32 (R)	≥32 (R)	24 (R)	32 (R)	2 (S)	8 (R)	≥32 (R)	0.75 (S)	32	0.25 (S)	1 (R)
73668-ECT	16 (R)	≥256 (R)	≥32 (R)	≥32 (R)	48	6 (S)	192 (R)	ND	≥32 (R)	0.5 (S)	4	0.25 (S)	0.5 (I)
74312-EC	48 (R)	≥256 (R)	≥32 (R)	≥32 (R)	≥256 (R)	48 (R)	96 (R)	12 (R)	≥32 (R)	0.5 (S)	16	≥32 (R)	0.75 (R)
AG3528	16 (R)	3 (S)	0.75 (S)	0.75 (S)	2	2 (S)	0.75 (S)	0.75 (S)	4 (R)	1 (S)	16	0.125 (S)	ND
UAB190	0.125 (S)	0.25 (S)	0.38 (S)	0.047 (S)	2	2 (S)	8 (R)	1.5 (S)	0.006 (S)	0.5 (S)	3	0.094 (S)	ND
$AG3528_{NDMP1}$	16 (R)	≥256 (R)	≥32 (R)	≥32 (R)	96	3 (S)	0.75 (S)	0.75 (S)	3 (R)	1 (S)	16	0.125 (S)	ND
$UAB190_{NDMP2}$	0.064 (S)	≥256 (R)	24 (R)	4 (I)	256	2 (S)	8 (R)	1.5 (S)	0.006 (S)	0.5 (S)	3	0.094 (S)	ND

[a] Antimicrobial susceptibility results based on EUCAST pharmacokinetic/pharmacodynamic (PK/PD) non-species-specific breakpoints. R, resistant; I, intermediate resistance profile; S, sensitive; ATM, aztreonam; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; TZP, piperacillin-tazobactam; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; CST, colistin sulfate; FOF, fosfomycin; RIF, rifampin; SXT, co-trimoxazole; TGC, tigecycline; ND, not determined.

[b] Species-specific *Acinetobacter* species breakpoints not available.

RESULTS

Species identification, antimicrobial susceptibility, and resistance genes present in *A. bereziniae* CHI-40-1. The clinical *Acinetobacter* isolate from Chennai was found to be an *A. bereziniae* strain by rMLST. Five NDM-1-producing *Acinetobacter* isolates from the fecal screening study were obtained from five different patients and included three species (Table 1; see Fig. S1 in the supplemental material). Three *Acinetobacter haemolyticus* isolates were found to be representatives of a single strain by ApaI restriction digestion and PFGE. All strains were extensively drug resistant (Table 2). In keeping with this, genes associated with resistance to β-lactams (bla_{OXA-58}), aminoglycosides (*strA*, *strB*, and *aacC2*), macrolides [*msr*(E) and *mph*(E)], trimethoprim (*dfrA1*), and sulfonamides (*sul1* and *sul2*) were identified in *A. bereziniae* CHI-40-1.

Characterization of plasmid pNDM-40-1. S1 PFGE and bla_{NDM-1} probing showed that *A. bereziniae* CHI-40-1 harbored multiple plasmids, with bla_{NDM-1} present on plasmids corresponding to bands of ~45 kb and ~250 kb (Fig. 1a and b). However, bla_{NDM-1} was present on a single ApaI restriction fragment of ~45 kb (Fig. 1c and d).

The *de novo* assembly of the *A. bereziniae* CHI-40-1 WGS produced 324 contigs, with a mean GC content of 38% and a combined size of 4.78 Mb. A 45,827-bp plasmid harboring bla_{NDM-1}, pNDM-40-1, was closed by PCR and sequencing of amplicons. The GC content of the plasmid backbone is 36.2%, and that of the variable region (from IS*Aba14* to the end of Tn*125*, nucleotides [nt] 5427 to 16280) is 52.5%.

At the time of writing, complete sequences of nine pNDM-BJ01-like plasmids were available in GenBank. The backbone of pNDM-40-1 is 100% identical at the nucleotide level to those of pNDM-BJ01 (bases 1 to 5684 and 17987 to 47274, accession number JQ001791), pNDM-BJ02 (JQ060896), pAbNDM-1 (JN377410), and pXM1 (AMXH01000087). pNDM-AB (KC503911), pM131_NDM-1 (JX072963), and pNDM-Iz4b (KJ547696) exhibit minor differences from the backbone of pNDM-BJ01 and from one another. pNDM-AB differs the most, because of a 3.5-kb insertion containing the genes *traD* and *insB* and a putative methyltransferase gene (Fig. 2;

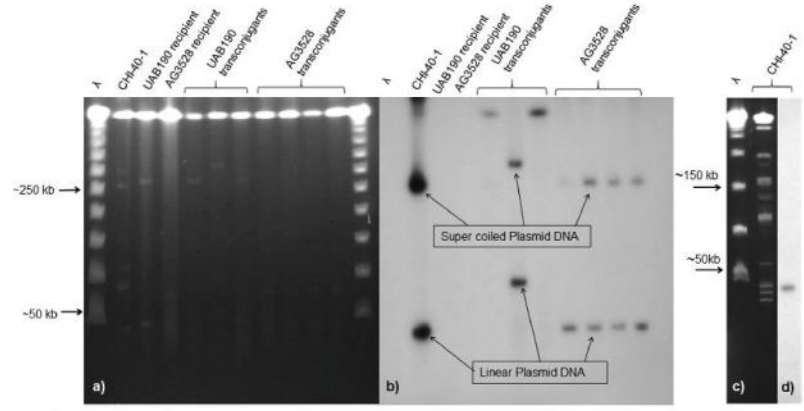

FIG 1 (a) Pulsed-field gel of S1 nuclease-digested genomic DNA from *A. bereziniae* CHI-40-1, recipients, and transconjugants; (b) in-gel hybridization with a bla_{NDM-1} gene probe; (c) pulsed-field gel of ApaI-digested genomic DNA from CHI-40-1; (d) in-gel hybridization with a bla_{NDM-1} gene probe. The molecular size marker is concatemers of λ of ~50 to 1,000 kb.

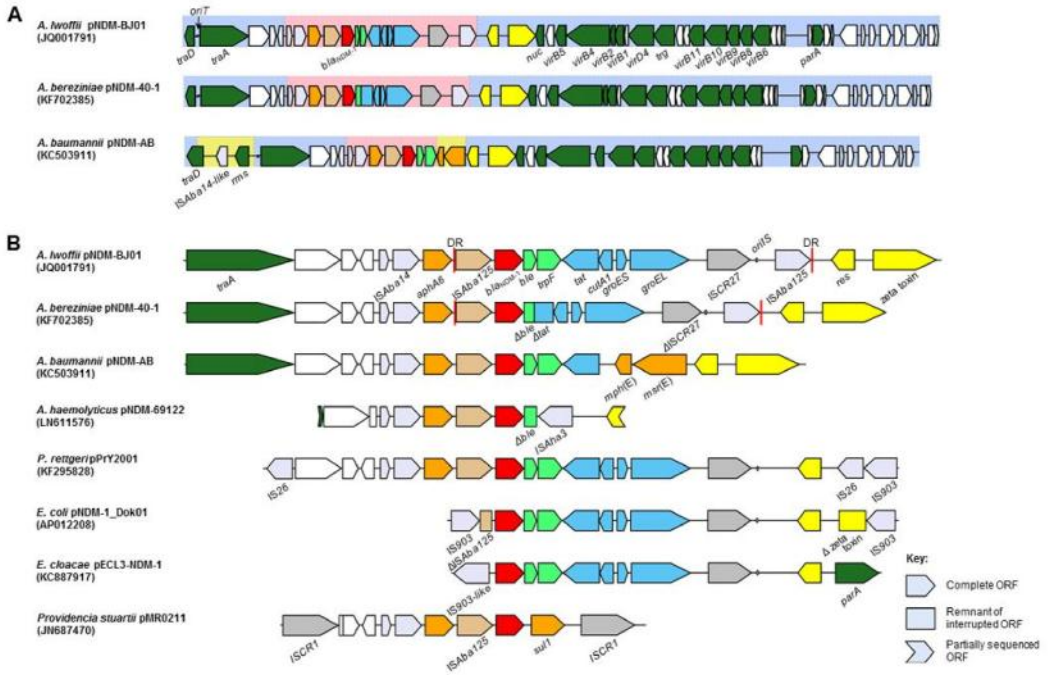

FIG 2 (A) Gene maps of plasmids pNDM-BJ01, pNDM-40-1, and pNDM-AB; (B) immediate bla_{NDM-1} context from pNDM-40-1, *A. haemolyticus* 69122-EW, and related sequences in *Acinetobacter* and *Enterobacteriaceae*. Open reading frames (ORFs) are color coded with the direction of transcription indicated by arrowheads, and truncated remnants of ORFs are shown as rectangles. Red, bla_{NDM-1}; orange, other antibiotic resistance; lime green, usually immediately downstream of bla_{NDM-1}; blue, from a common context in *Xanthomonas* and *Pseudoxanthomonas*; brown, IS*Aba125*; dark gray, IS*CR* transposases; light gray, other insertion sequence transposases; yellow, resolvase and zeta-toxin from pNDM-BJ01-like plasmids; dark green, named plasmid backbone genes; white, genes for hypothetical proteins. Regions with a light blue shaded background contain plasmid backbone with close identity among pNDM-BJ01-like plasmids. The pink shaded regions represent genes normally found in the bla_{NDM-1} context in *Acinetobacter* spp. Regions from pNDM-AB with a yellow background represent genes with no significant identity to those in pNDM-40-1. *oriT*, origin of transfer; *traD*, conjugal transfer gene; *traA*, MobA/L-type relaxase gene; *res*, resolvase gene; *nuc*, nuclease homologue; *virB1* to *-B11* and *virD4*, putative T4SS genes; *trg*, putative lytic transglycosylase gene; *parA*, putative plasmid partition gene; *rms*, putative type I restriction-modification system methyltransferase subunit gene; *aphA6*, aminoglycoside resistance gene; *ble*, bleomycin resistance gene; *trpF*, phosphoribosylanthranilate isomerase gene; *tat*, twin-arginine translocation pathway signal sequence domain gene; *cutA1*, periplasmic divalent cation tolerance gene; *groES*, cochaperonin gene; *groEL*, chaperonin gene; *oriIS*, origin of insertion of IS*CR27*; *res*, putative resolvase gene; *msr*(E) and *mph*(E), macrolide resistance genes.

see Table S3 in the supplemental material). These plasmids were all identified in isolates from China and were found in five different species, including *A. baumannii*. Additionally, several bla_{NDM-1}-negative *Acinetobacter* species sequences contain regions with significant identity to the backbones of pNDM-BJ01-like plasmids (see Fig. S3 in the supplemental material).

The bla_{NDM-1} gene in pNDM-40-1 is found within a Tn*125* transposon (Fig. 2), as with other pNDM-BJ01-like plasmids (12, 13, 16). Tn*125* in pNDM-40-1 has two deletions relative to Tn*125* in pNDM-BJ01: a 1,298-bp deletion from the 3′ end of *ble* to *tat* and a 150-bp deletion within IS*CR27* (see Fig. S3 in the supplemental material). pNDM-BJ02 and contig 5 from *Acinetobacter soli* TCM341 lack the 3′ IS*Aba125*, while in pAB-D499 and pM131_NDM-1, there is an IS*Aba11* inserted at the 3′ end of the element. In pNDM-AB, a large part of the context from *cutA1* to the 3′ IS*Aba125* is replaced by the macrolide resistance genes *msr*(E) and *mph*(E) (23). In all pNDM-BJ01-like plasmids, the aminoglycoside resistance gene *aphA6* and an IS*Aba14* element are found immediately upstream of Tn*125* (Fig. 2).

GenBank searches show that bla_{NDM-1} contexts in *Enterobacteriaceae* have high degrees of identity with the bla_{NDM-1} context from pNDM-BJ01-like plasmids. In most cases, this is restricted to genes that make up part of the full Tn*125* element harboring bla_{NDM-1}, with at least a fragment of the IS*Aba125* upstream of bla_{NDM-1} and the *ble* and *trpF* genes being present in almost all cases. Four sequences from *Enterobacteriaceae* with regions of close identity to the bla_{NDM-1} context in pNDM-BJ01, which included part of the plasmid backbone, were available at the time of writing (31) (Fig. 2). These were in the plasmids pPrY2001 from *Providencia rettgeri* (KF295828), pMR0211 from *Providencia stuartii* (JN687470), pNDM-1_Dok01 from *E. coli* (AP012208), and pECL3-NDM-1 from *Enterobacter cloacae* (KC887917). Plasmid pPrY2001 contains the most extensive region of identity. The sequence is nearly identical to that found in pNDM-BJ01 from the

far 3′ end of *traA* to the resolvase gene, with the main difference being the absence of the 3′ IS*Aba125*.

All pNDM-BJ01-like plasmids share a region with genes coding for a type IV secretion system (T4SS) involved in constructing the conjugation machinery and mediating conjugative transfer of plasmid DNA to recipient bacteria (16). In addition, all pNDM-BJ01-like plasmids contain genes proposed to code for a plasmid partition system (*parA*) and a putative zeta-toxin, which may contribute to plasmid stability through a toxin-antitoxin addiction system. It has not been possible to identify the replicase or the origin of replication of these plasmids. However, the wide range of replication strategies already described means that the lack of an identifiable replicase is not entirely surprising (6, 32).

The bla_{NDM-1} context in *A. haemolyticus*. PCR analysis revealed that the *A. haemolyticus* strain, but not the other two *Acinetobacter* species isolates from Karachi, contained several regions of a pNDM-BJ01-like plasmid backbone. The immediate bla_{NDM-1} context in *A. haemolyticus* 69122-EW was linked to pNDM-BJ01-like backbone genes *traA* upstream and the resolvase gene downstream (Fig. 2). The immediate context differed from that described in pNDM-BJ01 in that most of Tn*125* was missing.

A previously uncharacterized insertion sequence, IS*Aha3*, most similar to IS*Alw1* (95% amino acid [AA] identity between transposases), was inserted between *ble* and the putative resolvase gene. No direct repeats (DRs) were observed, but this was not uncommon for other closely related ISs deposited in ISFinder. It is possible that transposition of IS*Aha3* resulted in deletion of the sequence often found between *ble* and *res* and also resulted in the loss of one of the DRs (33). S1 PFGE and in-gel hybridization showed that bla_{NDM-1} was present on ~45-kb plasmids in *A. haemolyticus* 69122-EW, pNDM-69122.

Conjugative transfer and stability of plasmids harboring bla_{NDM-1}. Transconjugants were obtained from mating experiments with *A. bereziniae* CHI-40-1 and *A. haemolyticus* 69122-EW donors, in both *E. coli* UAB190 and *A. pittii* AG3528 recipients, at rates of 10^{-4} to 10^{-5} transconjugants per recipient cell. All putative transconjugants tested were found to be the recipient background species by MALDI-TOF and to be bla_{NDM-1} positive by PCR. MICs to all β-lactams except aztreonam were elevated in selected transconjugants (Table 2).

PCR analysis showed that the relaxase gene, *traA*, and other sections of the pNDM-BJ01-like backbone were present in all transconjugant colonies tested. In *A. pittii* AG3528 transconjugants, in-gel hybridization showed that both *traA* and bla_{NDM-1} were present on ~45-kb plasmids, as expected for pNDM-40-1. However, in *E. coli* UAB190 transconjugants, these genes were both present on either the chromosome or ~90-kb plasmids (Fig. 1b) (*traA* data not shown).

Probing of S1 PFGE gels of CHI-40-1, $UAB190_{NDMP2}$, and $AG3528_{NDMP1}$ over the course of a 14-day passage showed that bla_{NDM-1}-positive bands did not alter in size (see Fig. S4 in the supplemental material). The intensity of the bla_{NDM-1} bands in CHI-40-1 was similar over the course of the experiment with and without meropenem selection. The intensity of the bla_{NDM-1} bands for both transconjugant strains was stable with antibiotic selection but decreased significantly over the course of the passage without meropenem selection.

Regression analysis of $\Delta\Delta C_T$ values from qPCR experiments (see Fig. S5 in the supplemental material) showed statistically significant falls in the quantities of bla_{NDM-1} and *traA* template over the course of the passage experiment for transconjugant strains without meropenem selection. For the donor strain, CHI-40-1, there was little change. For all strains tested, bla_{NDM-1} and *traA* remained detectable throughout the 14-day passage experiment, even in the absence of antibiotic selection.

DISCUSSION

All the complete NDM-1 plasmid sequences from *Acinetobacter* spp. that we were able to identify in GenBank were from Chinese isolates and were similar to pNDM-BJ01. pNDM-BJ01 contains a single ApaI restriction site, in keeping with the ~45-kb bands present in both the S1 and ApaI gels. We conclude that CHI-40-1 harbors just one plasmid containing bla_{NDM-1} and that the 300-kb band represented residual supercoiled plasmid DNA.

The pNDM-BJ01-like plasmids have a GC content similar to that of most *Acinetobacter* species WGSs deposited in GenBank (~40%). All examples of these plasmids harboring bla_{NDM-1} have very high levels of identity with one another. The small number of related sequences not associated with bla_{NDM-1} are more distantly related to pNDM-BJ01-like plasmids but are also found exclusively in *Acinetobacter* spp. We propose that these findings are compatible with this plasmid lineage having evolved within the *Acinetobacter* genus and with the acquisition of bla_{NDM-1} being a relatively recent event.

Most descriptions of conjugative transfer of bla_{NDM-1} from *Acinetobacter* spp. *in vitro* are for isolates with pNDM-BJ01-like plasmids (15, 16, 19, 23). Conjugation rates were similar for the pNDM-BJ01-like plasmids studied here into *E. coli* and *A. pittii* recipients. The stability of both bla_{NDM-1} and *traA*, coding for the pNDM-BJ01-like relaxase, was similar in *E. coli* and *A. pittii* transconjugants. However, in the *E. coli* transconjugants studied in more detail, this apparently required recombination of pNDM-40-1, or part of it, with the chromosome or another plasmid. Although pNDM-40-1 was therefore no longer likely to function as an autonomously replicating plasmid, this still suggests a potential means by which bla_{NDM-1} contexts could have spread from *Acinetobacter* spp. to *Enterobacteriaceae*. This may explain why no complete pNDM-BJ01-like plasmid has yet been described in *Enterobacteriaceae*. However, sequence data now strongly suggest that at least some bla_{NDM-1} contexts in *Enterobacteriaceae* are derived from pNDM-BJ01-like plasmids, since several examples demonstrate high levels of identity with the plasmid backbone sequences which flank IS*Aba14*, *aphA6*, and Tn*125* containing bla_{NDM-1} (31).

Recent reports suggest that NDM-1-producing *Enterobacteriaceae* may be more common than originally demonstrated in China (21, 22), and it is possible that the spread of bla_{NDM-1} into *Enterobacteriaceae* could have initiated there. However, the high prevalence in some regions of the Indian subcontinent of NDM-producing *Enterobacteriaceae* has been well documented, and most reports of travel-associated colonization with NDM-producing bacteria involved the subcontinent (5, 8). It is therefore potentially significant that pNDM-BJ01-like plasmids have now been identified from India and Pakistan and from returning travelers to the region (34, 35). Insufficient data are available to establish whether these cases could be linked to recent spread from China or whether these plasmids are prevalent in a wider geographical region including the Indian subcontinent.

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We have no conflicts to declare.

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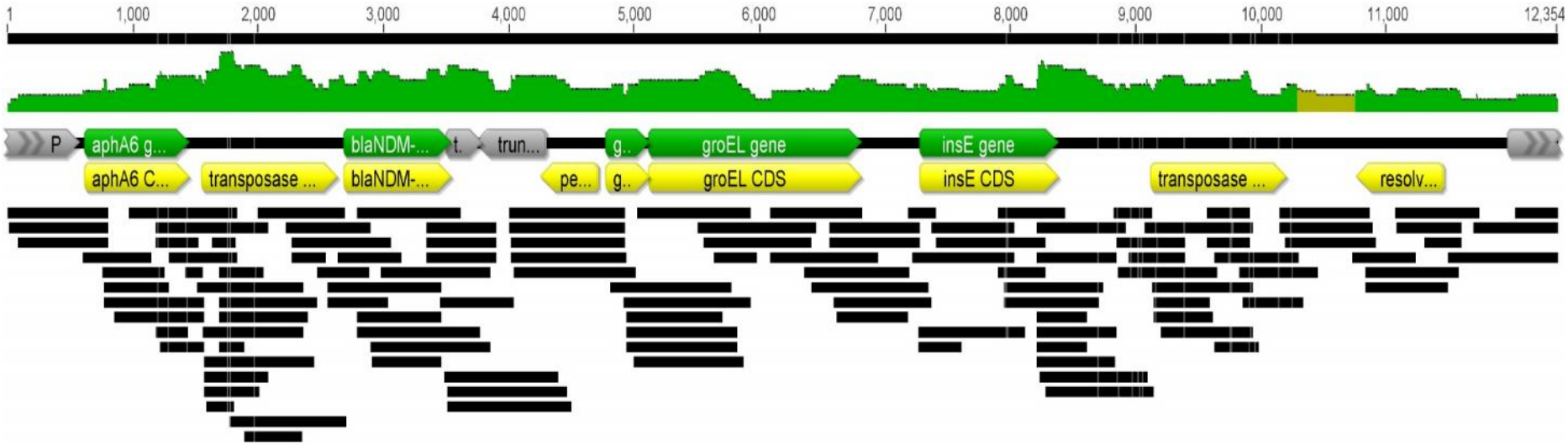
Appendix 4.2 – List of primers and probes used for PCR and sequencing of PCR products

Primer	Sequence	Use	Primer/ probe conc. (μM)	Mg^{2+} conc. (mM)
27F (Henriques *et al.* 2006)	AGAGTTTTGATCCTGGCTCAG	PCR and sequencing of 16sRNA locus	0.2	2.25
1492R (Henriques et al. 2006)	GGTTACCTTGTTACGACTT	PCR and sequencing of 16sRNA locus	0.2	2.25
800R (Karah *et al.* 2011)	CTACCAGGGTATCTAAT	Sequencing 16s rRNA locus	0.2	2.25
ndm-1F (Yong *et al.* 2009a)	GAAGCTGAGCACCGCATTAG	*bla*$_{NDM-1}$ detection and sequencing	0.2	2.25
ndm-1R (Yong et al. 2009a)	TGCGGGCCGTATGAGTGATT	*bla*$_{NDM-1}$ detection and sequencing	0.2	2.25
aphA6-5F	AATTGGTCAGTCGCCATCGG	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
IS125 5R	TGTGACCACGTCTACGTCTAGC	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
IS125gapF	GCAAAGGCAGAATCAGTGCG	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
NDM_5R	CTCAGCTTCGCGACCGGGTG	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
ndm-p1	CAGTTGCGGAGCTTTGAAGC	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
ndm-3f	GCCATTCCGCCCCCGATAGC	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
trpF-R	CACGGCAAGGCACCGCGATA	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
tat-3R	GGCACCGCACCTCGGTCAAG	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
tat-gapR1	GTACCAGGGCTGCGCCGATG	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
groEL-5F2	GCGCAGGCGATGGACAAGGT	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
groEL-MR	GCCTTCACCGCGCAGACCTT	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
ISCR27-gap2F	GGCAAGGTCGGCGGCTTCTC	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
ISCR27-gap2R	ATTGCGCCACGGCGTCTTGA	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
resF	AAAGACTGCCAAACGCCCTG	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
PN2F (Hu et al. 2012)	TAGATTCGATTCACGGCATA	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25
PN5R (Hu et al. 2012)	CGTCTTTGTAGCCTTTATCTC	PCR and sequencing *bla*$_{NDM-1}$ context	0.2	2.25

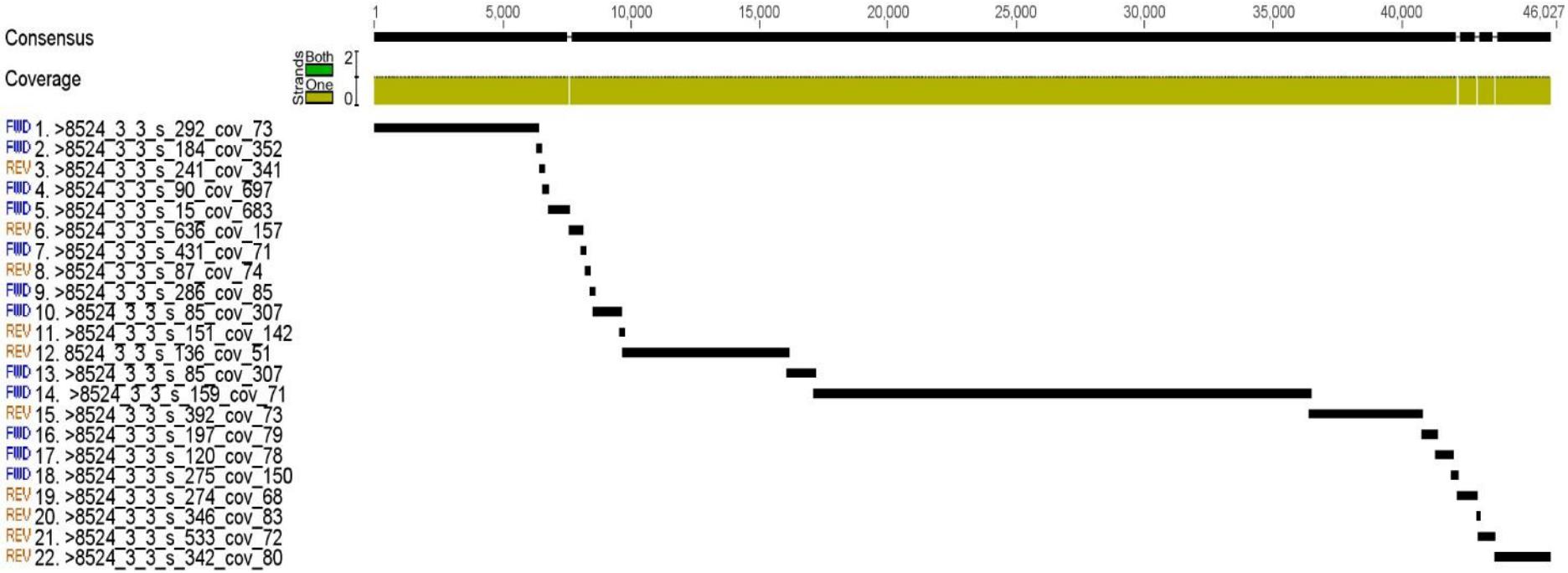
Primer	Sequence	Use	Primer/ probe conc. (μM)	Mg^{2+} conc. (mM)
ble3F	CATGGTGGCATTGGTGAACGC	PCR and sequencing bla_{NDM-1} context	0.2	2.25
res3F	TGCAAAACAAATTAACGCCCAGTCTGA	PCR and sequencing bla_{NDM-1} context	0.2	2.25
res-gapR	AGAAGGCGAGGATGAGGGACT	PCR and sequencing bla_{NDM-1} context	0.2	2.25
ISAba7like-FF	GCCAGTAACCATACGTAAAGAAAGACG	PCR and sequencing bla_{NDM-1} context	0.2	2.25
ISAba7like-RR	ATGCAACAAAGCCGTCGGGA	PCR and sequencing bla_{NDM-1} context	0.2	2.25
69122gapF	TGGTGATATAAAACGGCGAATTCAAACA	PCR and sequencing bla_{NDM-1} context	0.2	2.25
45c143R	ACGCTCCGCCATAATCGTTC	PCR and sequencing bla_{NDM-1} context	0.2	2.25
IS125 3R	CGCATGTGCCTTTTTGCCAGGG	PCR and sequencing bla_{NDM-1} context	0.2	2.25
aphA6-3R	TCAGCATTAAAAACCCCGCAAA	PCR and sequencing bla_{NDM-1} context	0.2	2.25
aphA6-5R	AGTCATGATGAGTTCAGGCACC	PCR and sequencing bla_{NDM-1} context	0.2	2.25
5PgapF1	TCAGCACTCAATTCAGCAAGTGT	PCR and sequencing bla_{NDM-1} context	0.2	2.25
5PgapF4	GTTGGTGGGTTGGTGTCTGT	PCR and sequencing bla_{NDM-1} context	0.2	2.25
5PgapF5	TCTGCCCCCATCAAAACGTG	PCR and sequencing bla_{NDM-1} context	0.2	2.25
5PgapR1	TAAACCGCCACCAACCGAAC	PCR and sequencing bla_{NDM-1} context	0.2	2.25
5PgapR3	TGGGACTTTTGGATTTGCGGA	PCR and sequencing bla_{NDM-1} context	0.2	2.25
orfA F	ACTGGGCCGCTTCAACCACA	Gap closure pNDM-40-1	0.2	2.25
p40-1gap1 F	ACGCTTTCCACGTTGCCCTGA	Gap closure pNDM-40-1	0.2	2.25
p40-1gap2F	TGCGGTTCTGCGGTCAGCTC	Gap closure pNDM-40-1	0.2	2.25
p40-1gap3F	TCAGAGCGACACCGCACGAA	Gap closure pNDM-40-1	0.2	2.25
p40-1gap4F	ACGGGGGAGTATGGGAAACT	Gap closure pNDM-40-1	0.2	2.25
p40-1gap5F	CTTGTAGGAATGTTGGCAGGGT	Gap closure pNDM-40-1	0.2	2.25
AphA6gap5R	AGTCATGATGAGTTCAGGCACC	Gap closure pNDM-40-1	0.2	2.25
p40-1gap2R	TTCGTGCGGTGTCGCTCTGA	Gap closure pNDM-40-1	0.2	2.25
p40-1gap3R	TCCCATACTCCCCCGTCATAGC	Gap closure pNDM-40-1	0.2	2.25
p40-1gap5R	AGGTACGCCAACGAAACAGC	Gap closure pNDM-40-1	0.2	2.25
PN11F(Hu et al. 2012)	AATGTGGTCTGCGGTGTA	Detection of pNDM-BJ01-like plasmids	0.2	2.25
PN11R(Hu et al. 2012)	GCCTGCTGTAACTTCTCAA	Detection of pNDM-BJ01-like plasmids	0.2	2.25

Primer	Sequence	Use	Primer/ probe conc. (μM)	Mg^{2+} conc. (mM)
PN6F (Hu et al. 2012)	TCAGGATTCACCCACCAT	Detection of pNDM-BJ01-like plasmids	0.2	2.25
PN6R (Hu et al. 2012)	GGCTCAAGACTACAACGATA	Detection of pNDM-BJ01-like plasmids	0.2	2.25
PN9F (Hu et al. 2012)	ATCTACGATCTTGCCTTGTT	Detection of pNDM-BJ01-like plasmids	0.2	2.25
PN9R (Hu et al. 2012)	CTTGTTCTGACGAGCCTAA	Detection of pNDM-BJ01-like plasmids	0.2	2.25
TraA F1	TGGTCAGCAAAACCCGCATGT	TraA quantification by qPCR	0.5	4
TraA R3	GGTTAGCCCATTCTAGGCGGGT	TraA quantification by qPCR	0.5	4
Tra Probe	TCCAGTAAACCCTGAAAAGGGCGGTGCGGGT	TraA quantification by qPCR	0.2	4
NDM RT F1	TGGGTCGAACCAGCAACCGC	NDM quantification by qPCR	0.25	4
ndm RT R1	TGCCGAGCGACTTGGCCTTG	NDM quantification by qPCR	0.25	4
NDM probe	ACCCCGGCCCCGGCCACACCAGT	NDM quantification by qPCR	0.2	4
rpoB Ac RT F1	ATGGCATACTCATATACCGA	*Acinetobacter* rpoB reference for qPCR	0.75 (40-1 probe) 0.5 (AG3 probe)	3 (40-1 probe) 4 (AG3 probe)
rpoB Ac RT R1	TGGAGACCGATATCTTCGCG	*Acinetobacter* rpoB reference for qPCR	0.75 (40-1 probe) 0.5(AG3 probe)	3 (40-1 probe) 4 (AG3 probe)
rpoB 40-1 probe	TGCCCCAAGTCATGCATGCTCCGTACTTGC	*A. bereziniae* rpoB reference for qPCR	0.2	3
RpoB AG3 probe	TGCCCCAAGTAATGGATGCACCGTACTTAC	*A. pittii* rpoB reference for qPCR	0.2	4
rpoB Ec F3	TCCTTTCTATCCAGCTTGACTCGT	*E. coli* rpoB reference for qPCR	0.25	4
rpoB Ec R3	CGCAGTTTAACGCGCAGCGG	*E. coli* rpoB reference for qPCR	0.25	4
RpoB Ec Probe	ACGTCAGCTACCGCCTTGGCGAACCGGTGT	*E. coli* rpoB reference for qPCR	0.2	4

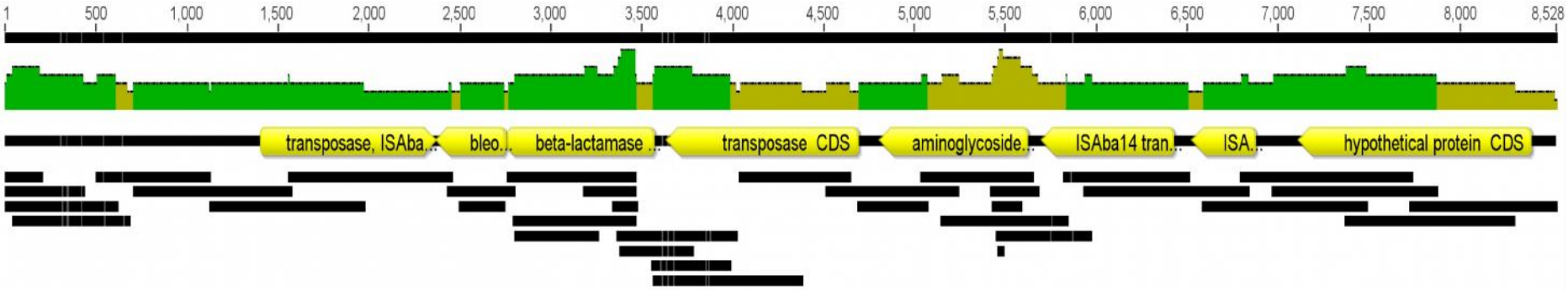
Appendix 4.3 – Geneious assembly of sequenced PCR amplicons obtained by primer walking of the immediate *bla*$_{NDM-1}$ context in *A. bereziniae* CHI-40-1. As the image is zoomed out bases with 100% identity to the consensus appear as black lines. Grey areas in the black lines indicate bases for which the assembly is not identical. However, there were no base pair ambiguities in the final assembly. The assembly used for the image file also contains an annotated version of the final assembly.



Appendix 4.4 – Geneious assembly of contigs making up pNDM-40-1 from *A. bereziniae* CHI-40-1. Note repetition of contig 85 cov 307. Contigs assembled sequentially and originally with PCR amplicons closing short gaps in sequence. PCR amplicons have been removed from the assembly in the image, leaving four short sequence gaps. Contig links and sequence gaps were closed by PCR and sequencing of amplicons.



Appendix 4.5 – Geneious assembly of sequenced PCR amplicons obtained by primer walking of the immediate *bla*$_{NDM-1}$ context in *A. haemolyticus* 69122-EW. As the image is zoomed out bases with 100% identity to the consensus appear as black lines. Grey areas in the black lines indicate bases for which the assembly is not identical. However, there were no base pair ambiguities in the final assembly. The assembly used for the image file also contains an annotated version of the final assembly.



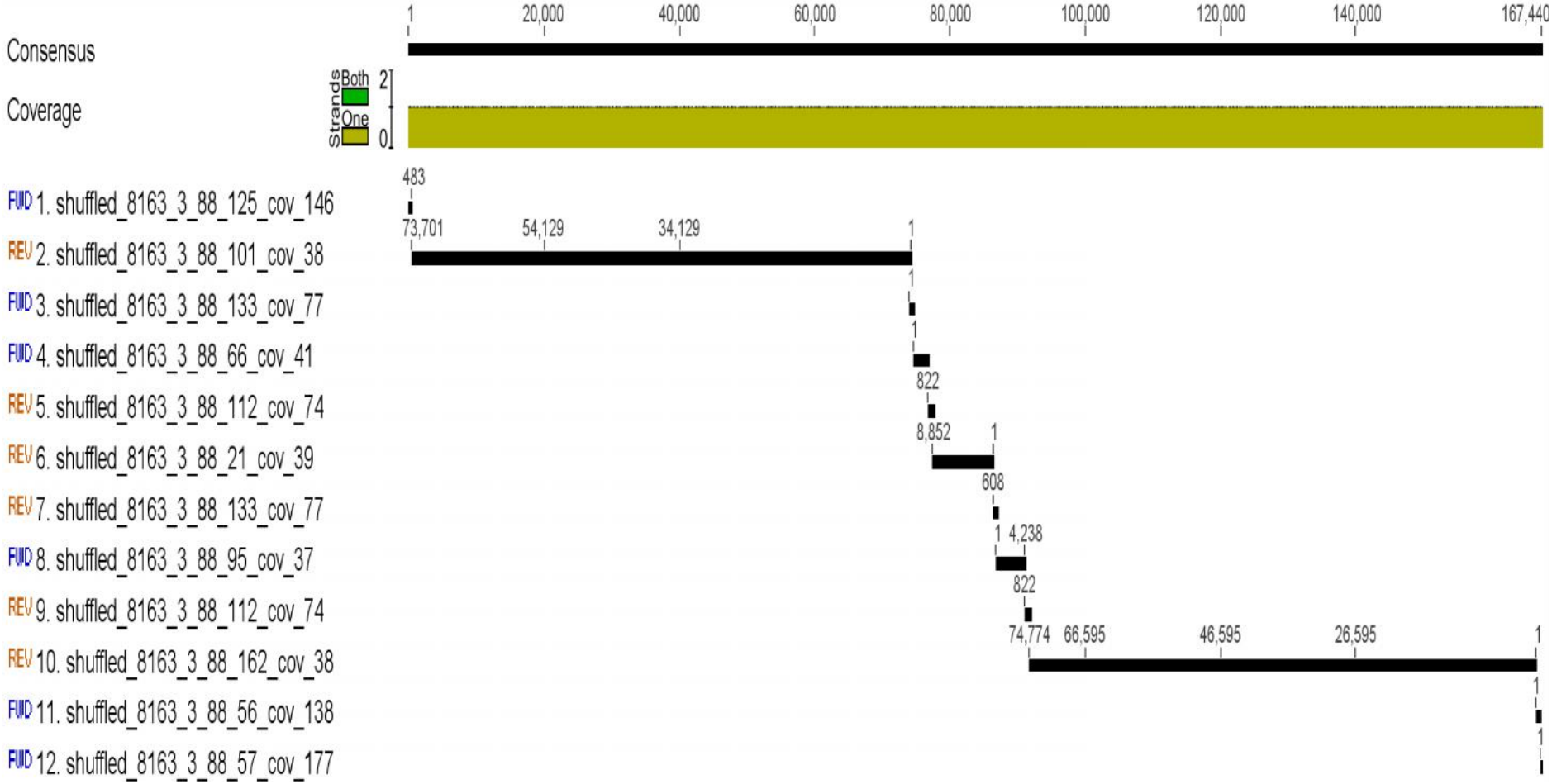
Appendix 5.1 – List of strains used to construct the whole genome phylogenetic tree of *Vibrio cholerae* isolates shown in Figure 5.4. Background colour of table cells matches the colour used to label each phylogenetic group in Figure 5.4.

Strain Name	Accession	Isolation place	Isolation Year	Group	Serotype
A46	ERS013160	N.I	1964	L1	Ogawa
A70	ERS013162	Bangladesh	1969	L1	Inaba
A49	ERS013161	N.I	1962	L1	Inaba
A68	ERS013171	Egypt	1949	L1	Inaba
A66	ERS013170	Bangladesh	1962	L1	Inaba
A61	ERS013169	India	1970	L1	Inaba
A60	ERS013168	Thailand	1958	L1	Inaba
A59	ERS013167	India	1970	L1	Inaba
A57	ERS013166	India	1980	L1	Ogawa
A51	ERS013165	Egypt	1949	L1	Ogawa
A50	ERS013164	Bangladesh	1963	L1	Ogawa
A76	ERS013163	Bangladesh	1982	L1	Inaba
A103	ERS013172	N.I	1990	L1	Inaba
A111	ERS013176	N.I	1990	L1	Inaba
A279	ERS013197	Sweden	1990	L1	Inaba
A389	ERS013203	Bangladesh(M)	1987	L1	Inaba
GP16	ERS013136	India	1971	L1	Inaba
O395_Combined	CP000626 / CP000627	India	1965	L1	Ogawa
A215	ERS013277	California	1985	L2	Inaba
MJ1236	CP001485/ CP001486	Bangladesh(M)	1994	L2	Inaba
MO10	AAKF03000000	India	1992	L2	O139
B33	ACHZ00000000	Mozambique	2004	L2	Ogawa
A219	ERS013194	Georgia	1986	L3	Inaba
A217	ERS013193	Louisiana	1986	L3	Inaba
A213	ERS013191	Georgia	1984	L3	Inaba
A209	ERS013190	Florida	1980	L3	Inaba
A325	ERS013280	Argentina	1993	L4	Inaba
TM11079-80	ACHW00000000	Brazil	1980	L4	Ogawa
M66	CP001233 / CP001234	Indonesia	1937	L5	No information
MAK757	AAUS00000000	Celebes_Islands	1937	L5	Ogawa
NCTC_8457	AAWD01000000	Saudi_Arabia	1910	L6	Inaba
V52(O37)	AAKJ02000000	Sudan	1968	L7	O37
BX330286	ACIA00000000	Australia	1986	L8	Inaba
116-17a	ERR180907	New Delhi, India	2011	-	non-01/ 0139
116-17b	ERR180911	New Delhi, India	2011	-	non-01/ 0139
116-14	ERR180910	New Delhi, India	2011	-	non-01/ 0139
BRV8	ERR180912	Bristol, UK	2011	-	non-01/ 0139

Appendix 5.2 – List of primers used to confirm final assembly of pNDM-116-17.

Note all primers used for PCR and sequencing. For PCR reactions all primers used at a final concentration of 0.2µM.

Primer name	Primer sequence
NDM 5R	CTCAGCTTCGCGACCGGGTG
V3 hypA R	GTGCCGGGATAACATCAATA
V3 c101 R	CAAAGTGCGGGTGCGTTATG
V3 c101F	CGAAGTAATCGCAACATCCGC
V3 c162 F	AACCAGATGAATATCGCCTC
V3 c162 R	CTACGATTCCGAAAACAAGG
V3 c21 F	CTTTCTTTTTCACGCACAGT
V3 c21 R	ATCGCAAGCATCTTTTAACG
V3 c66 F	GCGGATGAAAATTTAACGGT
V3 c66 R	CCAGTCAGAACAATCAATGC

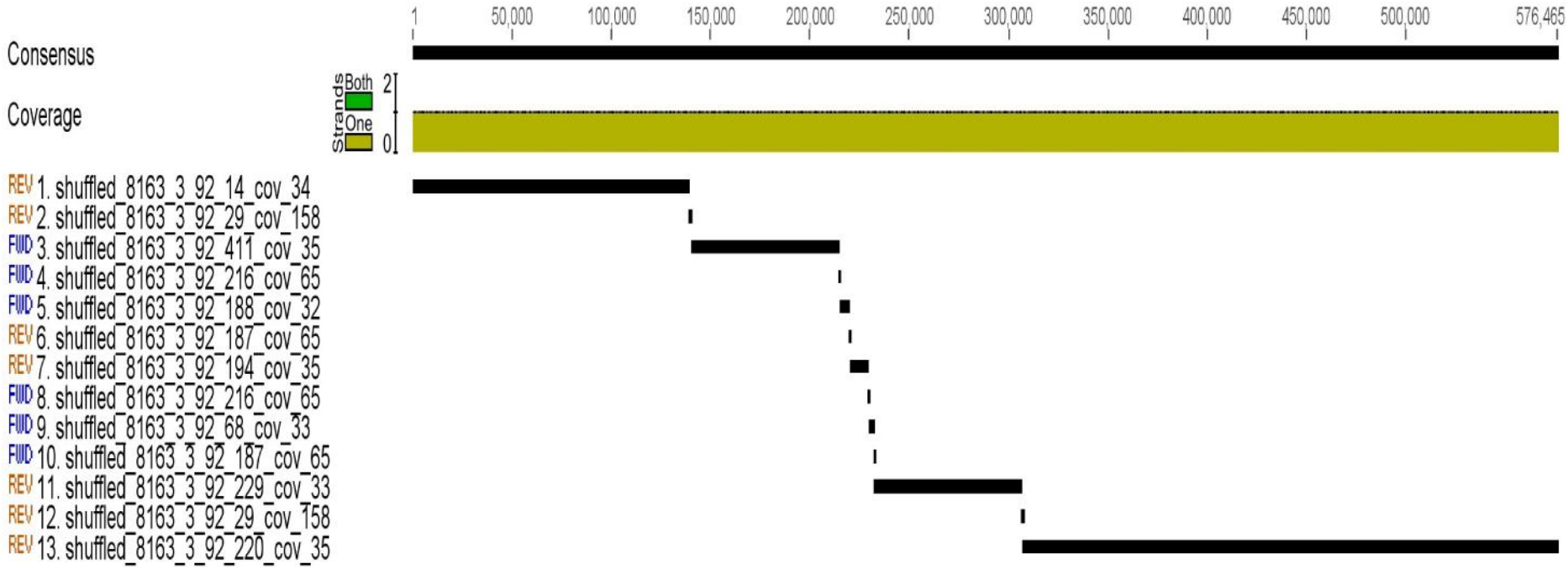


Appendix 5.3 – Geneious assembly of contigs making up pNDM-116-17 from *V. cholerae* 116-17. Note repetition of contigs 133 cov 77 and 112 cov 74. Contigs assembled sequentially and all links were later confirmed by PCR and sequencing of amplicons.

Appendix 5.4 – List of primers used to confirm location of chromosomal integration of pNDM-116-17 in *V. cholerae* 116-17b.

Other primers used to link contigs as shown in 5.2.

Primer name	Primer sequence
V4 c220F	AGTAAAGAGCTGCTTGGCGG
V4 c14R	ACCATAGATGCTGCTGTGCG
V3 c101R	CAAAGTGCGGGTGCGTTATG
V3 c162F	AACCAGATGAATATCGCCTC



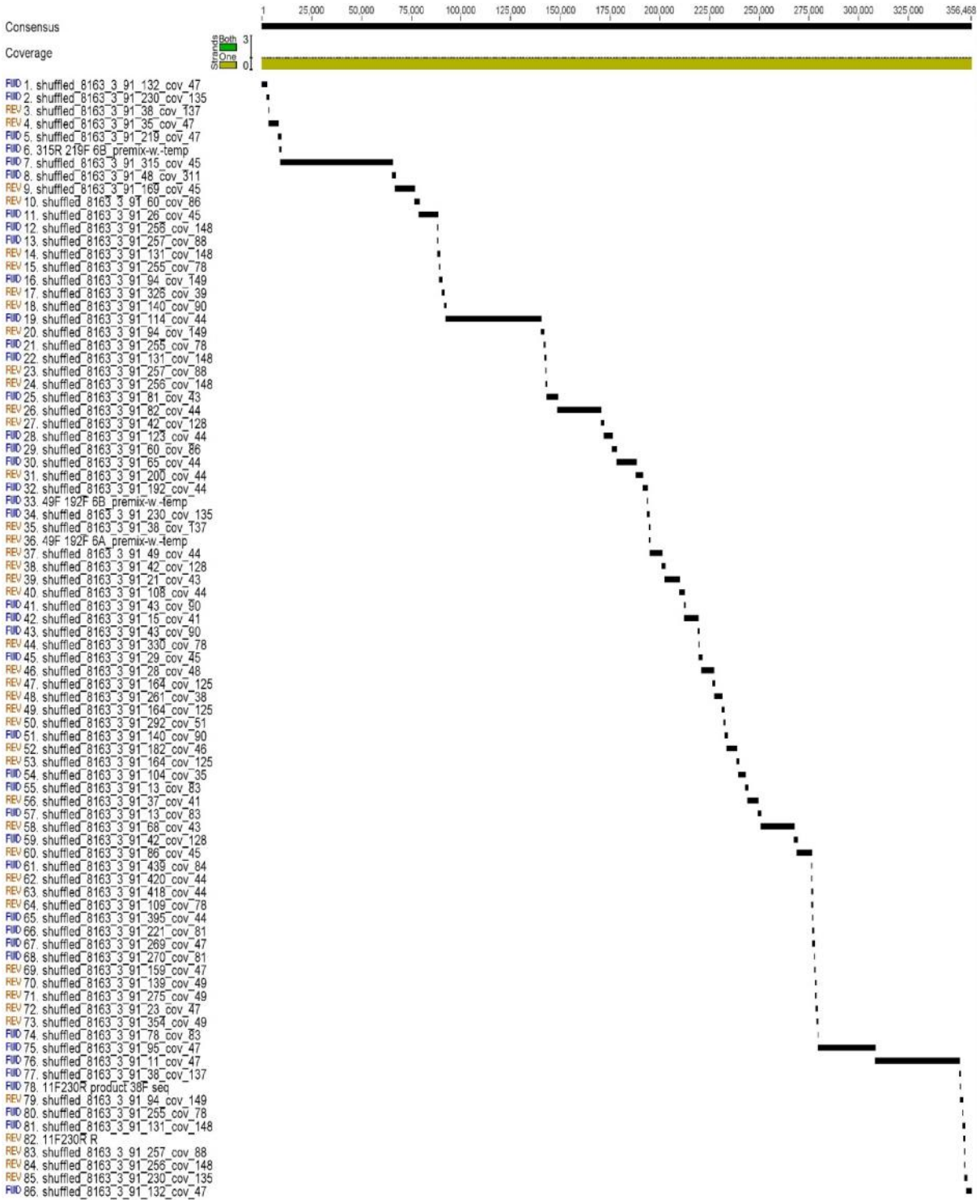
Appendix 5.5 – Geneious assembly of contigs from *V. cholerae* 116-17b, including contigs with identity to pNDM-116-17 flanked by contigs with *V. cholerae* chromosomal genes and SXT/R391-like ICE. Note repetition of contigs 216 cov 65 and 187 cov 65. Contigs assembled sequentially. Links between contigs with chromosomal/ ICE genes to pNDM-116-17 contigs confirmed by PCR and sequencing of amplicons.

Appendix 5.6 – List of primers used to confirm final assembly of pNDM-116-14.

For PCR reactions all primers used at a final concentration of 0.2µM.

Primer name	Primer sequence
aadA1 3F	CGGCAAAATCGCGCCGAAGG
aadA1FF	GAGATCACCAAGGTAGTCGG
IS26-gapFF	CACTCCGCGTTCAGCCAGCA
ISCR1FF	GTCTGGTCGGGTTGGTGGCG
QACR	CGGATGTTGCGATTACTTCG
V6 c104 R	ATTGGGCTGTCAGGAGCCGT
V6 c108 R	AGGTTTCGGAGGCCGGTGGT
V6 c108F	GCGGTCGGCACTGTAGCCAT
V6 c11 F	AACACGCCCAACACCCGCAG
V6 c11 R	CAACCGATCGACGCGGCCTT
V6 c114 R	ACGGGGTTGGGCTGGAGAGA
V6 c114 F	CGCAGTCGGCAGCACCTCAA
V6 c123 F	TGAATCCCGAATCGCCTTGCG
V6 c123 R	CACCAGGCACGACGGCGATT
V6 c131F	CTGTGAACAAGGTTCGGCGT
V6 c132 5R	GCCTGAGTTCGCTACTCTTCG
V6 c132 F	ATATCCGCGCAGCCCAGTGC
V6 c15 R	GTCGGCGGAGGTGAAAGCGG
V6 c15F	GCGCCCGACGCCCTCTCTAT
V6 c169 F	AGGCCCGAGTTGTCACCCCA
V6 c169 R	ACGGTAACGCCTTTCGGCGG
V6 c182F	ACGTTATCGCTGACCGGGGC
V6 c182R	GCTTGTTGGCGAATAGATGGCGT
V6 c192 R	CGCCGCAATACAGCACAGGGT
V6 c192F	GCAGGCAGTAGGTAATGATGCTGTG
V6 c200 F	CCCCGTGGGTCGACTCTGGT
V6 c200R	TGGGGCTGCGTAAACCACACG
V6 c21 F	ATAGTGGCCGGCGCGGTTTC
V6 c21 R	CGCGCAAACTCACTGACACTGG
V6 c219 F	ACGGGGGCTAGAGGGCGTTT
V6 c219 R	TGCTGCTACCCAAACTCCTTCGG
V6 c230R	GACGGGGCTTCCCAGAACAA
V6 c256F	TGCGTATTTCGGTGATTGCG
V6 c26 F	GCAGTGGTCAGGGCGTCGAG
V6 c26 R	CGCCGTTGTGTGCGATGCAG
V6 c261 F	GCCATCACCATCACCGGCGT
V6 c261 R	GCGCGTGCCGGATAAGCAGA
V6 c28F	CAGAGTTTGCGGCAGGGGCA
V6 c28R	GCCATCTCAGGGGTGGTGCC

Primer name	Primer sequence
V6 c292 F	TTGCCCCTGCTATTGTGCCTTT
V6 c29F	GGGCGACGACGGCTTCTGAG
V6 c315 F	GGCGGTGGGTCATCTCTTGCT
V6 c315 R	GACTGCGATCTACGCGGGGC
V6 c326 F	TGCGCATCAGAACGCCGAGA
V6 c326 R	CGGCCAAAGTCAATCCTGACGC
V6 c330R	CAACGTGCCCTGAACCGGGG
V6 c35 F	GCGCAGGAGCTGAGCCTTCTT
V6 c35 R	GGGGGCTTGGAGCGGTATGA
V6 c38F	TGACGCATCTCGCTTCTTGT
V6 c38R	AGAAGCGAGATGCGTCAAACGA
V6 C49 F	CGGCAACCCCGCGCTTTTAC
V6 c49 R	ACACCAACCTGCCCTAATTGTGGT
V6 c65 F	ACTCAACTTCATGCCGGTCAGC
V6 c65 R	TGTGCCCTTGGCCACCGTTC
V6 c68 F	TCTGGCACCCCTTCACTCTGGA
V6 c68 R	GTGTCGCCCAATGGCGCTCT
V6 c81 F	CGGTGTCGCTCGATACGCCC
V6 c81 R	ATACTGGCCGCAGCGTTGGT
V6 c82 F	CCCCAAAGAGTGCCAGCGCA
V6 c82 R	AAAGCGCCAGCTTCGGCACA
V6 c86 F	CGCCAGCCAGCTCAATGCCT
V6 c86 R	TGAACCAGGAGCCAGCACGA
V6 c94R	TCAAAAGTGGTCGCCTGGAC
V6 c95F	ACTCCAACAGAGGGTAGGAATCGGT
V6 c95R	CCGTTTGCTTCTGGGTTCGGT
V8 c214R	TAGTGCAGTTTGATCCTGAC

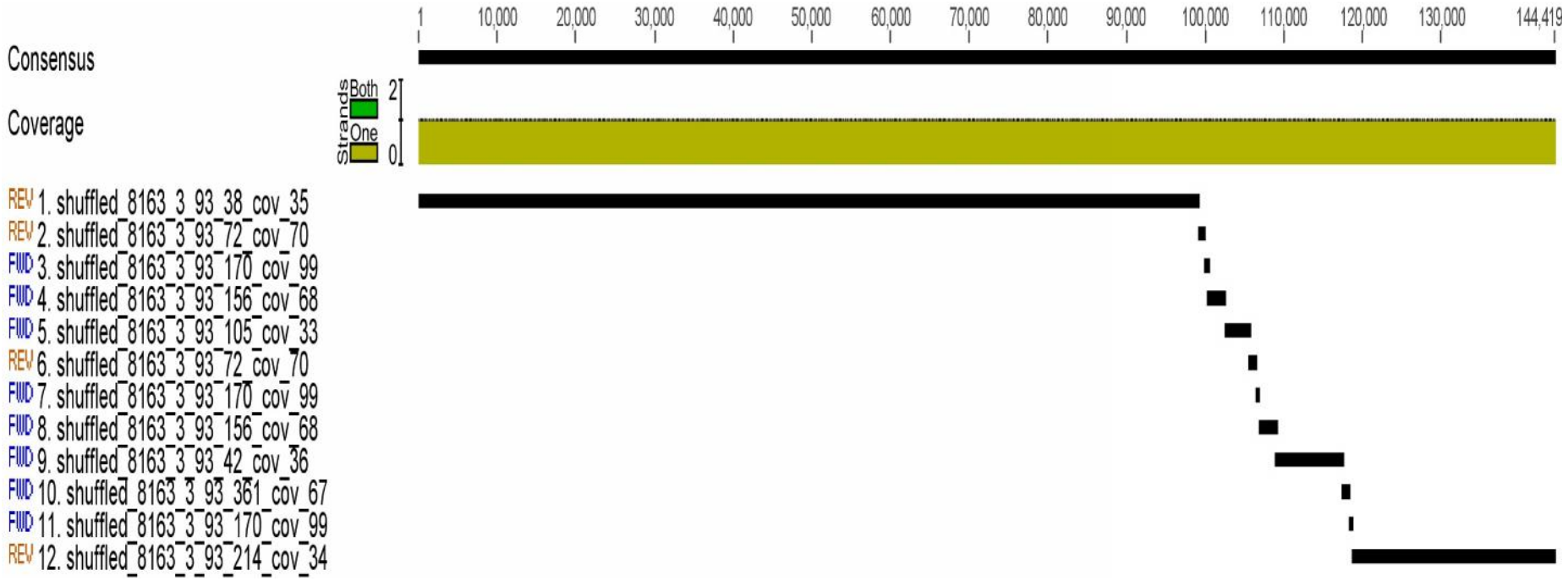


Appendix 5.7 – Geneious assembly of contigs making pNDM-116-14 from *V. cholerae* 116-14. Note that several contigs appear in the assembly more than once and a few PCR amplicons required to achieve assembly in Geneious are included in the figure. Links between all contigs were confirmed by PCR and sequencing of amplicons.

Appendix 5.8 – List of primers used to confirm assembly of chromosomal context of *bla*$_{NDM-1}$ in *V. cholerae* BRV8 including SGI-NDM-1.

Note all primers used for PCR and sequencing. For PCR reactions all primers used at a final concentration of 0.2µM.

Primer name	Primer sequence
aadA1 3F	CGGCAAAATCGCGCCGAAGG
IS26-gapR	CGGGGCCGCACTGTCGATTT
trpF-gapF	TATCGCGGTGCCTTGCCGTG
V8 c214R	TAGTGCAGTTTGATCCTGAC
V8 c38F	GTTTGATGTTATGGAGCAGC
V8 c42F	ATCGCAAGCATCTTTTAACG
V8 c42F	ACAACGAGTTAATGAAGCCT
y-sul1R	TCAAGAAAAATCCCATCCCC



Appendix 5.9 – Geneious assembly of contigs from *V. cholerae* BRV8 which include SGI-NDM-1. Note repetition of contigs 72 cov 70, 170 cov 99 and 156 cov 68. Contigs assembled sequentially and all links were later confirmed by PCR and sequencing of amplicons.

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