# The Role of International 'High-Risk Clones' in the Emergence of Metallo-β-Lactamase-Producing *Pseudomonas aeruginosa* in the UK

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# Abstract

*Pseudomonas aeruginosa* is a Gram-negative bacterium responsible for a wide range of opportunistic hospital-acquired infections. However metallo-β-lactamases (MBLs) encoding resistance to carbapenems and other β-lactam antibiotics – which are important agents for the treatment of *P. aeruginosa* infections – are increasingly reported in the species worldwide. This study aimed to characterise a comprehensive collection of MBL-producing *P. aeruginosa* referred to the UK national reference laboratory from 267 patients between 2003 and 2012. VIM-type MBLs predominated (91%) but a few IMP- and NDM-type enzymes were also identified. Diverse types were identified amongst these isolates using nine-locus variable number tandem-repeat (VNTR) analysis, but the majority (85%) belonged to six major complexes, corresponding to sequence types (STs) 111, 233, 235, 357, 654/964 and 773, respectively, by multi-locus sequence typing (MLST). These are all 'high-risk clones' frequently reported internationally as hosts of MBLs. $bla_{VIM}$- and $bla_{IMP}$-MBL genes were carried in diverse class 1 integron structures; the six most common integron structures (I-VI) carried $bla_{VIM-2}$ (n=5) or $bla_{VIM-6}$ (n=1) genes; four were associated with single STs (I, ST111; III, ST773; IV, ST654/964; V,ST357) while two integrons types (II,VI) were seen in multiple STs. Five of these six integrons were located on the *P. aeruginosa* chromosome, while one (VI) was located on a plasmid. VIM-encoding genomic islands were characterised in representatives of STs 111, 233 and 235 using whole genome sequencing (WGS) methods. SNP analysis achieved greater discrimination among MBL-producing representatives of STs 111 and 235. CRISPR-Cas bacterial defence systems were absent from all sequenced representatives of STs 111 and 235 but were present in single representatives of STs 233, 357, 654 and 773. Overall, 'high-risk clones' are important in the spread of MBL-producing *P. aeruginosa* in the UK. Future work should focus on further elucidating the reasons for the success of these clones, to better understand how to limit the spread of these multi-drug resistant organisms.

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# Publications relating to this work

## Journal articles

**Wright LL**, Turton JF, Livermore DM, Hopkins KL, Woodford N. Dominance of international "high-risk clones" among metallo-β-lactamase-producing *Pseudomonas aeruginosa* in the UK. *J Antimicrob Chemother* 2015; **70**: 103–10.

Turton JF, **Wright L**, Underwood A, Witney AA, Chan Y, Al-Shahib A, Arnold C, Doumith M, Patel B, Planche TD, Green J, Holliman R, Woodford N. High-resolution analysis by whole genome sequencing of an international lineage (ST-111) of *Pseudomonas aeruginosa* associated with metallo-carbapenemases in the United Kingdom. *J Clin Microbiol* 2015; **53**: 2622–31.

**Wright LL**, Turton JF, Hopkins KL, Livermore DM, Woodford N. Genetic environment of metallo-β-lactamase genes in *Pseudomonas aeruginosa* isolates from the UK. *J Antimicrob Chemother* 2015; **70**:3250-58.

## Conference papers

**Wright LL**, Turton JF, Livermore DM, Hopkins KL, Woodford N. Phylogenetic analysis of metallo-β-lactamase-producing *Pseudomonas aeruginosa* in the UK**.** Abstract: C2-1597, *53rd ICAAC,* Denver, 2013. Abstract: C2-1597 (Poster)

**Wright LL**, Turton JF, Livermore DM, Hopkins KL, Woodford N. Analysis of metallo-β-lactamase-encoding class 1 integrons in *Pseudomonas aeruginosa* isolates from the UK. *24th ECCMID*, Barcelona, 2014. Abstract: P0988 (Poster)

**Wright LL**, Doumith M, Turton JF, Hopkins KL, Livermore DM, Woodford N. Whole genome sequencing of metallo-β-lactamase-producing *Pseudomonas aeruginosa* belonging to the ST235 high-risk clone. *55th ICAAC*, San Diego, 2015. Abstract: C2-1670 (Talk)

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# 1 Introduction

## 1.1 *Pseudomonas* genus

The genus *Pseudomonas* was first proposed by Migula in 1894 as a bacterial group of "cells with polar organs of motility".[1] Over the next 70 years a large number of diverse strictly aerobic Gram-negative rods that were motile by one or several polar flagella, were placed in the genus.[2] DNA and RNA hybridisation studies in the 1960s and 70s, identified at least five main sub-divisions sharing little sequence homology.[1,2] This diversity was confirmed by sequencing of 16S rRNA, and housekeeping genes such as *rpoB* and, consequently, since the 1980s many former *Pseudomonas* species were moved into other new or existing genera, including *Burkholderia*, *Comamonas*, *Pandoraea*, *Ralstonia*, and *Stenotrophomonas.*[2]

Despite these reclassifications, there are still some 225 species in the genus *Pseudomonas* according to the List of Prokaryotic Names with Standing in Nomenclature[3] (http://www.bacterio.net/pseudomonas.html; last accessed 20th November 2015). They predominantly occupy soil- and water-associated environments and are noted for their metabolic and physiological flexibility.[4]

Phenotypic features of these *Pseudomonas* spp. are described in the current edition of the Bergey's Manual of Bacteriology, and include cell morphology, cell wall composition, pigment types, nutritional and metabolic characteristics, and susceptibility to different compounds.[2,5] However, these phenotypic features do not discriminate absolutely between *Pseudomonas* spp. and those of other genera, and so genotypic methods including 16S and *rpoB* gene sequencing are increasingly necessary to identify new species.[2,5]

Seven main subgroups were identified in the genus, namely the *Pseudomonas aeruginosa*, *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*, *Pseudomonas pertucinogena*, *Pseudomonas putida*, *Pseudomonas stutzeri* and *Pseudomonas syringae* groups.[2] *P. aeruginosa*, the type species, and common opportunistic human pathogen, is in an important member of the genus and is discussed in more detail later in this chapter. Other well-studied species are the plant pathogen *P. syringae*, of which there are many different pathovars each infecting different plant species, the saprotrophic organism *P. putida*, and the insect pathogen *Pseudomonas entomophilia*, which is lethal to *Drosophila melanogaster.*[4]

Members of the genus have diverse applications in industry. Some are used as biocontrol agents in agriculture (e.g. *P. fluorescens* and *P. putida*),[4,6] or as plant growth promoters (e.g. *P. fluorescens and P. stutzeri*).[4] Several are being investigated for their ability to produce insecticides, for example, the Fit toxin identified in strains of *P. fluorescens*, and *P. chlororaphis* is active against insect pests including the tobacco budworm *Heliothis virescens* and the greater wax moth *Galleria mellonela*.[7] Members of the genus are also useful bioremediation agents due to their ability to degrade aromatic hydrocarbons; for example, *P. putida* can degrade benzene, toluene and ethylbenzene[8] and *P. aeruginosa* strains have been isolated from soil and groundwater contaminated with petroleum by-products.[4]

## 1.2 *Pseudomonas aeruginosa* general biology

*P. aeruginosa* infection (though not the organism) was recognised in the clinical setting as early as 1850, by Sedillot, as a result of the blue-green discolouration of wound dressings that it causes.[9] Pyocyanin, the pigment responsible for this colour

was first extracted by Fordos in 1860,[9] while the earliest description of the culture of the organism was by Gessard in 1882.[10] The species was referred to by several different names, including *Bacillus pyocyaneus* and *Bacterium aeruginosa*, before finally being classified as *P. aeruginosa*.[1,11]

*P. aeruginosa* grows well in a variety of conditions including at temperatures up to 42$^{o}$C, and is noted for its sweet grape-like odour, as well as the pyocyanin (blue-green) and pyoverdin (yellow-green) pigments, produced by most, but not all strains.[12] Other distinguishing features include the species inability to ferment lactose and positive oxidase and catalase test results.[12] Colonies are often large and round, although their morphology can vary; some representatives, particularly from cystic fibrosis patients with chronic lung infections, display a mucoid colony phenotype.[12] The species has minimal nutritional requirements and utilises a wide range of organic compounds as energy and carbon sources. As with other *Pseudomonas* spp., *P. aeruginosa* is tolerant of many environmental conditions, grows particularly well in moist environments, and interacts with a wide range of hosts, including plants, nematodes, fruit flies and mammals.[4,13] It is well known as an opportunistic human pathogen, as discussed below.

The *P. aeruginosa* genome is large in size, compared to other Gram-negative bacteria, varying between 6-7 Mb and having a high GC content (~67%).[14] Like other members of its genus, the species is noted for its high level of recombination, and the acquisition of genes is thought to have played an important part in its evolution, conferring the ability to thrive in diverse environments.[4] *P. aeruginosa* genomes have a mosaic structure including a conserved core interspersed with blocks of

accessory genes. A recent estimate, based on available genome sequences, suggest there are around 5233 core genes, accounting for around 88% of the average genome.[14]

## 1.3 *P. aeruginosa* as an opportunistic pathogen

*P. aeruginosa* infections are rare in healthy individuals, but the species is an important human opportunistic pathogen, and infections are particularly prevalent in hospital settings. *P. aeruginosa* was the second most-frequently-isolated Gram-negative species, after *Escherichia coli*, in a recent point prevalence survey of health-care-associated infections in Europe, by the European Centre for Disease Prevention and Control (ECDC), where it accounted for 8.3% of infections.[15] *P. aeruginosa* infections are less common in community settings but can include keratitis, dermatitis, folliculitis, otitis externa, osteomyelitis and septic arthritis.[16]

### 1.3.1 Common *P. aeruginosa* infections in the hospital setting

Hospital-acquired *P. aeruginosa* infections are largely seen in the most vulnerable patients such as the immunocompromised, critically ill, or injured. While infections can affect almost any part of the body, the respiratory tract is the most common site, while urinary tract, blood, and wound infections are often seen. Frequent examples include ventilator-associated pneumonia in intensive care patients, wound infections in burns patients, urinary tract infections associated with indwelling devices such as catheters, and bacteraemias.[13,16] The species is also one of the principal causes of chronic lung infection in cystic fibrosis patients and of those with bronchiectasis and chronic obstructive pulmonary disease.

### 1.3.2 *P. aeruginosa* hospital outbreaks

*P. aeruginosa* can colonise many surfaces in hospitals such as taps, showers, ventilators and catheters. This colonisation can be an important source of infection in a clinical setting.[16] There are many reports of *P. aeruginosa* hospital outbreaks, often associated with contaminated water outlets; other environmental sources (e.g. ventilators, bronchoscopes) and, occasionally, colonised health-care workers are implicated.[16,17] The exact routes of transmission between these sources and patients are not always apparent.[17] A variety of infection prevention and control measures are usually implemented to bring outbreaks under control including improved hand hygiene, screening and isolation of affected patients, and decontamination of potential environmental sources.[16]

Recent examples of UK outbreaks include those that occurred in several neo-natal critical care units in Northern Ireland in late 2011 and early 2012, resulting in four deaths.[18] Investigations found that tap components from the affected hospitals were contaminated with the same *P. aeruginosa* strain seen in patients at each site suggesting that these water outlets could have acted as an environmental sources of the infections, although the presence of other sources could not be ruled out.[18]

### 1.3.3 Virulence factors

*P. aeruginosa* produces a wide variety of virulence factors that help it to evade human host defences.[13,19] These include flagella and pili, which are involved in motility and adhesion and can also elicit an inflammatory response; a type 3 secretion system, which is involved with the injection of toxic proteins into host cells; secreted proteases, which can contribute to tissue damage; and

lipopolysaccharide (LPS), which is a major component of the outer membrane (as in other Gram-negative bacteria) and is inherently toxic.[19] Like many other bacteria, *P. aeruginosa* has a quorum sensing mechanism which co-ordinates adaptation to its environment;[13] this mechanism can regulate many virulence factors and also contributes to the formation of biofilms. These biofilms are complex communities of bacteria that enable attachment and persistence in the environment. They are also important for the establishment of chronic infections in the lungs in cystic fibrosis patients.[20] Biofilms are hard to eradicate due to a variety of factors including (i) the poor penetration of disinfectants, antibiotics and effectors of the host's immune system, and (ii) the presence of slow-growing bacteria that are not killed by antibiotics.[19,21]

## 1.4 General resistance mechanisms of *P. aeruginosa*

### 1.4.1 Commonly used antibiotics

*P. aeruginosa* is intrinsically resistant to many antimicrobial agents, due to interactions between several underlying mechanisms. One of the principal mechanisms is the organisms low outer membrane permeability compared with other Gram-negative species, thus reducing transport of many antibiotics across the membrane.[22] *P. aeruginosa* also expresses several efflux pumps including the MexAB-OprM system,[23] which can rapidly exclude many diverse molecules, including some antibiotics, from the bacterial cell. In addition, *P. aeruginosa* has a chromosomally-encoded AmpC β-lactamase.[24] As a result of these mechanisms, the species is naturally resistant to many antibiotics and therapeutic options are generally limited to certain aminoglycosides, fluoroquinolones and β-lactams (table

1.1, figure 1.1). A combination of mutational and acquired resistance mechanisms can lead to resistance to almost all antimicrobial agents[22] and as a last resort, against multi-resistant strains, polymyxin antibiotics are sometimes used. This section will cover the major agents used to treat *P. aeruginosa* infections and the prevalent resistance mechanisms of the species to these antibiotics; these are summarised in table 1.1.

**Table 1.1:** Resistance mechanisms of *P. aeruginosa* against major antibiotic classes used to treat infections with the species

| Class | Examples | Resistance mechanisms | |
|---|---|---|---|
| | | Chromosomal | Acquired enzymes |
| **Aminoglycosides** | amikacin, gentamicin, tobramycin | • Increased efflux (particularly MexXY-OprM pump)<br>• Reduced cytoplasmic membrane transport | • Aminoglycoside-modifying enzymes (AMEs)<br>• 16S rRNA methylases (very rare) |
| **Fluoroquinolones** | ciprofloxacin, levofloxacin | • Mutations in quinolone resistance determining region (QRDR) of *gyrA*, *gyrB*, *parC*, *parE* genes<br>• Increased efflux (MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM pumps) | • Aminoglycoside acetyltransferase variant *aac(6')-Ib-cr* (reduces susceptibility to ciprofloxacin) |
| **β-Lactams** | | • Increased efflux; particularly MexAB-OprM pump (resistance to all β-lactams except imipenem)<br>• Overexpression of AmpC β-lactamase (resistance to all β-lactams except the carbapenems and carbenicillin)<br>• Lower outer membrane permeability due to OprD porin loss (resistance only to imipenem and reduced susceptibility to meropenem) | • Acquired penicillinases e.g. TEM-1/2, PSE-1/4 (resistance only to penicillins)<br>• Extended spectrum β-lactamases e.g. PER-1, VEB-1 (resistance to all β-lactams except carbapenems)<br>• Carbapenemases (resistance to most β-lactams including carbapenems; see also Section 1.5.3):<br>– Class A (e.g. KPC, GES-5)<br>– Class B /MBLs (e.g. VIM, IMP, NDM; these enzymes do not confer resistance to aztreonam)<br>– Class D (e.g. OXA-48-like; these enzymes do not confer resistance to the cephalosporins) |
| Carboxypenicillins | carbenicillin, ticarcillin | | |
| Ureidopenicillins | piperacillin | | |
| 3rd generation Cephalosporins | ceftazidime | | |
| 4th generation Cephalosporins | cefepime, cefpirome | | |
| Monobactams | aztreonam | | |
| Carbapenems | imipenem, meropenem, doripenem | | |
| **Polymyxins** | colistin, polymyxin B | • Mutations affecting LPS biosynthesis | |

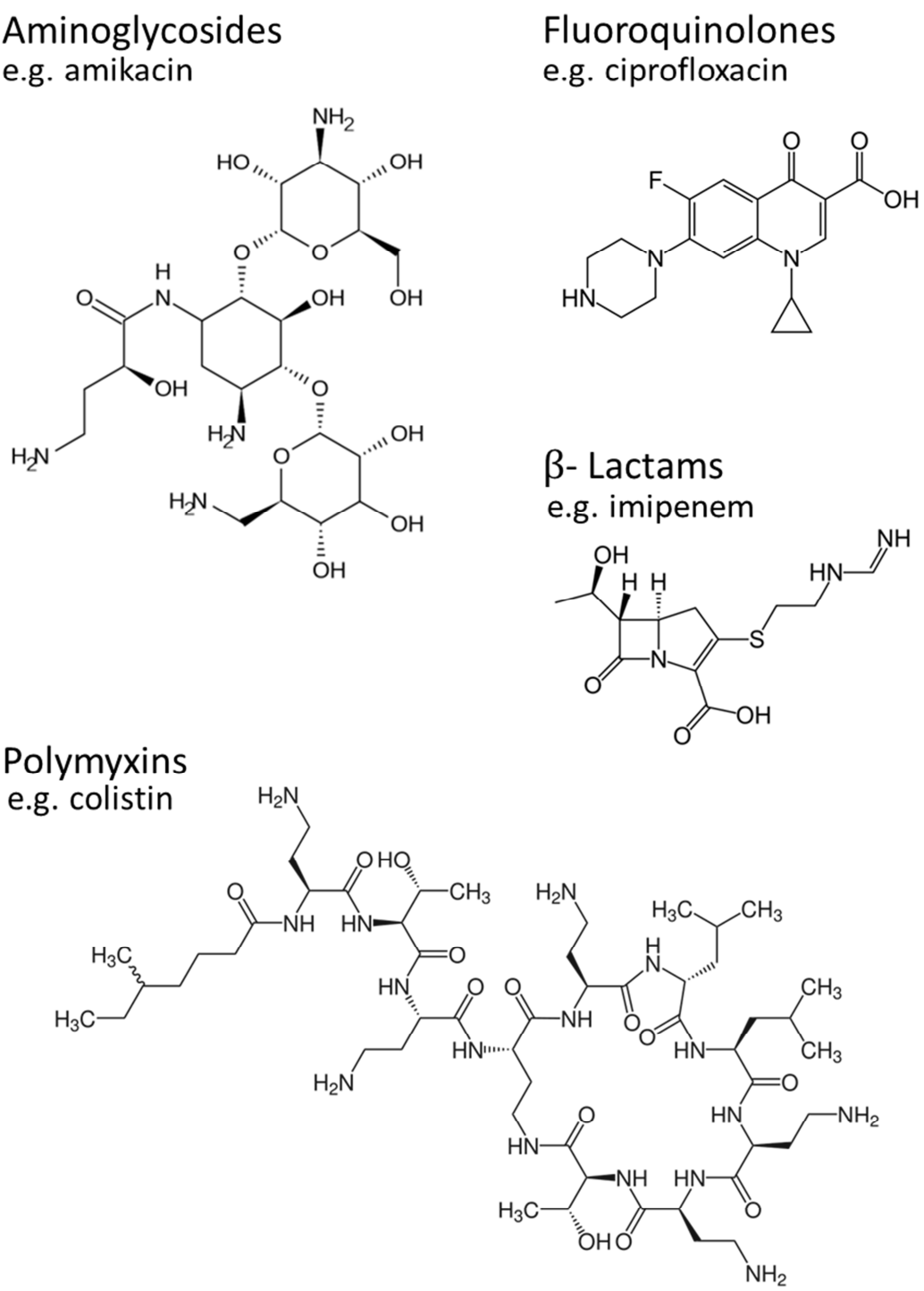



**Figure 1.1:** Chemical structures of main antibiotic classes used to treat *P. aeruginosa* infections

### 1.4.2 **Aminoglycosides**

Aminoglycosides are bactericidal antibiotics that disrupt protein synthesis by binding to the 30S subunit of the bacterial ribosome, causing misreading.[25] Members of this class that are most commonly used – often in combination with β-lactams – to treat *P. aeruginosa* infections include amikacin (figure 1.1), gentamicin and tobramycin. Increased efflux, particularly associated with the MexXY-OprM efflux pump, is a major mechanism of resistance to aminoglycosides in *P. aeruginosa*.[26] Reduced cytoplasmic membrane transport, thought to be related to PhoP and PhoQ activity also contributes to resistance to aminoglycosides, possibly as a result of modifications of the LPS component of the outer membrane. [27]

Acquired mechanisms are less commonly seen, but include aminoglycoside-modifying enzymes (AMEs) belonging to three main classes; acetyltransferases (AACs), nucleotidyltransferases (ANTs) and phosphotransferases (APHs)[25] with members of all three AME classes described in *P. aeruginosa.*[27] Although none of the AMEs confers resistance to all antibiotics in the class, multiple enzymes can be present in the same strain and, in combination, can do so. Acquired genes encoding 16S rRNA methylases such as RmtA and RmtD, are reported, albeit rarely, in the species, in Japan and Brazil respectively, and confer pan-aminoglycoside resistance.[28,29]

### 1.4.3 **Fluoroquinolones**

Fluoroquinolones are inhibitors of DNA synthesis by their interactions with the bacterial DNA gyrase and topoisomerase IV enzymes. Members of this class used to treat *P. aeruginosa* infections include ciprofloxacin (figure 1.1) and levofloxacin.

Major mechanisms of resistance are mutations in the quinolone resistance determining region (QRDR) of genes encoding the DNA gyrase (*gyrA* or *gyrB*) or topoisomerase IV (*parC* or *parE*) enzymes.[26] Mutations causing overexpression of efflux pumps, including the MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM pumps can also reduce susceptibility.[26] The only acquired fluoroquinolone resistance determinant seen in *P. aeruginosa* is *aac(6')-Ib-cr*[30] encoding the AAC(6')-Ib-cr enzyme; this has two amino acid substitutions compared with AAC(6')-Ib, which allow it to acetylate some fluoroquinolones (including ciprofloxacin but not levofloxacin), in addition to aminoglycosides.[25,31] Other plasmid-mediated quinolone resistance genes, such as *qnr*-type, *oqxAB* and *qepA* that are seen in Enterobacteriaceae are not reported in *P. aeruginosa.*

### 1.4.4 β-Lactams

β-Lactams are a large class of antibiotics that target the bacterial D-alanyl-D-alanine transpeptidase enzymes (also known as penicillin-binding proteins), thus inhibiting cross-linking of the peptidoglycan bacterial cell wall. *P. aeruginosa* is intrinsically resistant to many β-lactams due to its low membrane permeability relative to other bacterial species, constitutive expression of efflux pumps, and a chromosomally-encoded AmpC-type β-lactamase.

Members of this antibiotic class that do usually remain active against *P. aeruginosa* are limited to carboxypenicillins (e.g. carbenicillin), ureidopenicillins (e.g. piperacillin), some third- (e.g. ceftazidime) and all fourth-generation (e.g. cefepime) cephalosporins, monobactams (e.g. aztreonam) and carbapenems (e.g. imipenem, meropenem and doripenem but not ertapenem).[26] Structures of these antibiotics

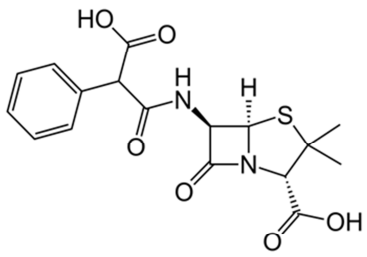
are shown in figure 1.2. Carbapenems are particularly important as they have the broadest spectrum of activity against Gram-negative bacteria; they are discussed further in Section 1.5.

Mutations affecting the species' intrinsic resistance mechanisms are a major cause of resistance to 'antipseudomonal' β-lactams. Mutants that overexpress the chromosomal AmpC-type β-lactamase are generally resistant to all β-lactams, except for the carbapenems and, to a large extent, carbenicillin.[9] Mutations leading to increased expression of efflux pumps, particularly the MexAB-OprM system, can also cause resistance to most β-lactams including the carbapenems meropenem and doripenem, but not imipenem. Reduced membrane permeability via loss of the OprD porin, confers resistance to imipenem and reduces meropenem susceptibility.[9]

Acquired β-lactamases can also confer resistance although they are uncommon in the species compared with Enterobacteriaceae. They include (i) acquired penicillinases (e.g. TEM-1/2 PSE-1/4) conferring resistance to the penicillin antibiotics, (ii) extended-spectrum β-lactamases (ESBLs) (e.g. PER-1, VEB-1, LCR-1), which compromise all β-lactam antibiotics except carbapenems, and (iii) carbapenemases (e.g. VIM, IMP, NDM, KPC, OXA-48-like), which hydrolyse most or all β-lactams including carbapenems (although OXA-48-like enzymes do not confer resistance to cephalosporins, and aztreonam is stable against VIM, IMP and NDM types). Carbapenemases, though still rare, are increasingly being seen in *P. aeruginosa*[32] and will be covered in more detail in the following sections.

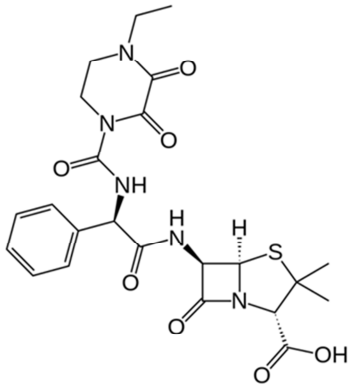
Carboxypenicillins

e.g. carbenicillin



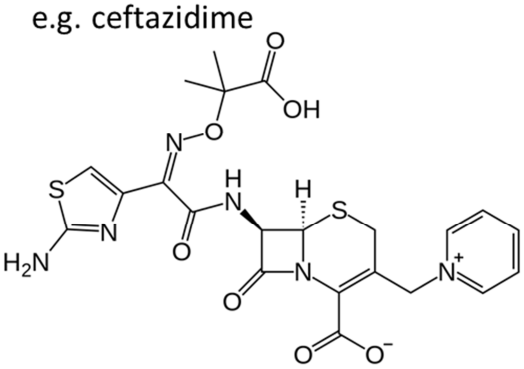
Ureidopenicillins

e.g. piperacillin



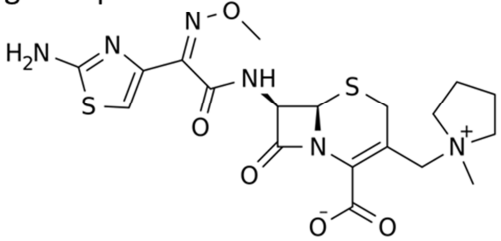
3rd generation cephalosporins

e.g. ceftazidime



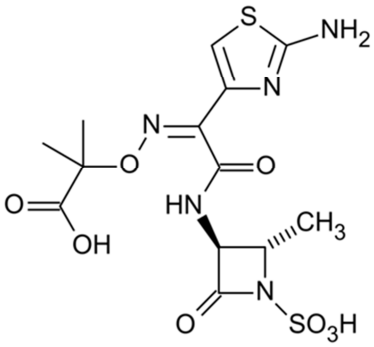
4th generation cephalosporins

e.g. cefepime



Monobactams

e.g. aztreonam



Carbapenems

e.g. imipenem

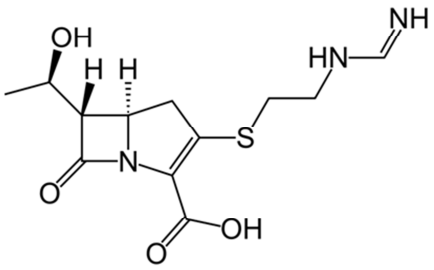


**Figure 1.2:** Structures of β-lactam antibiotics used to treat *P. aeruginosa* infections

### 1.4.5 **Polymyxins**

Colistin (polymyxin E) and polymyxin B (figure 1.1) disrupt the bacterial outer membrane by binding to LPS. They were first used in the 1940s but, due to their neurotoxic and nephrotoxic side-effects, their intravenous use for treatment of *P. aeruginosa* infections was largely abandoned in the 1960s, once aminoglycosides and carbenicillin became available. Colistin is used as a nebulised drug for CF patients and is then considered relatively safe as there is little absorption across the lungs to other tissues. The intravenous formulations have also been readopted in recent years due to the increase of multi-drug resistant infections.[33] Although susceptibility rates to these agents in *P. aeruginosa* remain high, resistance has been reported, generally due to mutations affecting LPS integrity and modification of its lipid A moiety.[34,35]

## 1.5 Resistance mechanisms to carbapenems in *P. aeruginosa*

### 1.5.1 Carbapenem antibiotics

The general structure of the carbapenem antibiotics is shown in figure 1.3. They differ from other β-lactams by the lack of a sulphur atom in the thiazolidinic ring, and in having a 6' hydroxyethyl side chain rather than an amino-acyl substituent. These modifications increase stability to most β-lactamases.[36]

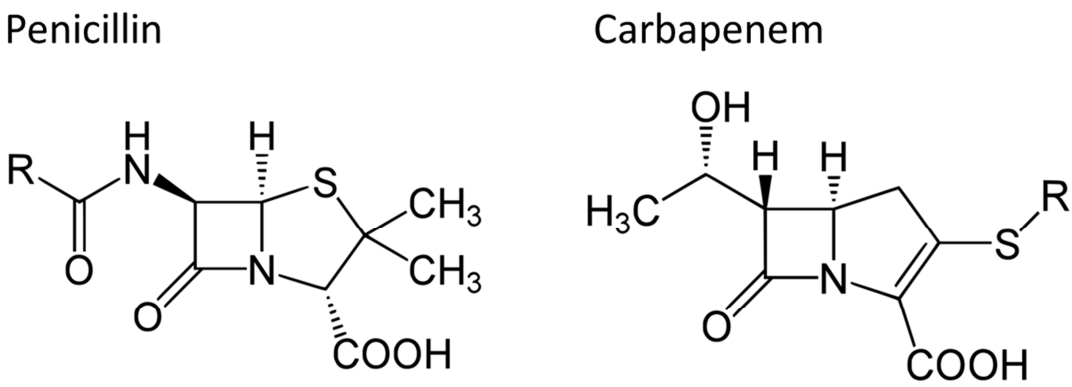



**Figure 1.3:** General structure of the penicillin and carbapenem antibiotics

Carbapenems are broad-spectrum agents, often considered as 'antibiotics of last resort' and used to treat serious infections. The first drug to be approved for use was imipenem, in the 1980s, followed by meropenem, ertapenem and more recently doripenem. Ertapenem is not active against *P. aeruginosa*, possibly owing to increased efflux and/or decreased uptake, due to its large side chain.[37] However, imipenem, meropenem and doripenem are important agents against this species.[36]

Carbapenem resistance in *P. aeruginosa* is increasingly seen worldwide. Data from the European Antimicrobial Resistance Surveillance Network (EARS-Net) for bloodstream isolates from 2013 (figure 1.4) show a clear resistance gradient across

Europe, with resistance rates ranging from 2.9% in Denmark, to 60.5% in Romania; the rate in the UK was 5.2%.

Carbapenem resistance and *Pseudomonas* spp. is one of the key drug-bug combinations recommended for monitoring of resistance in the UK Five Year Antimicrobial Resistance Strategy 2013 to 2018.[38] Resistance rates are higher in chronic *P. aeruginosa* infections, such as in the lungs of cystic fibrosis patients, which progressively become resistant to multiple antibiotics including carbapenems.[39,40]

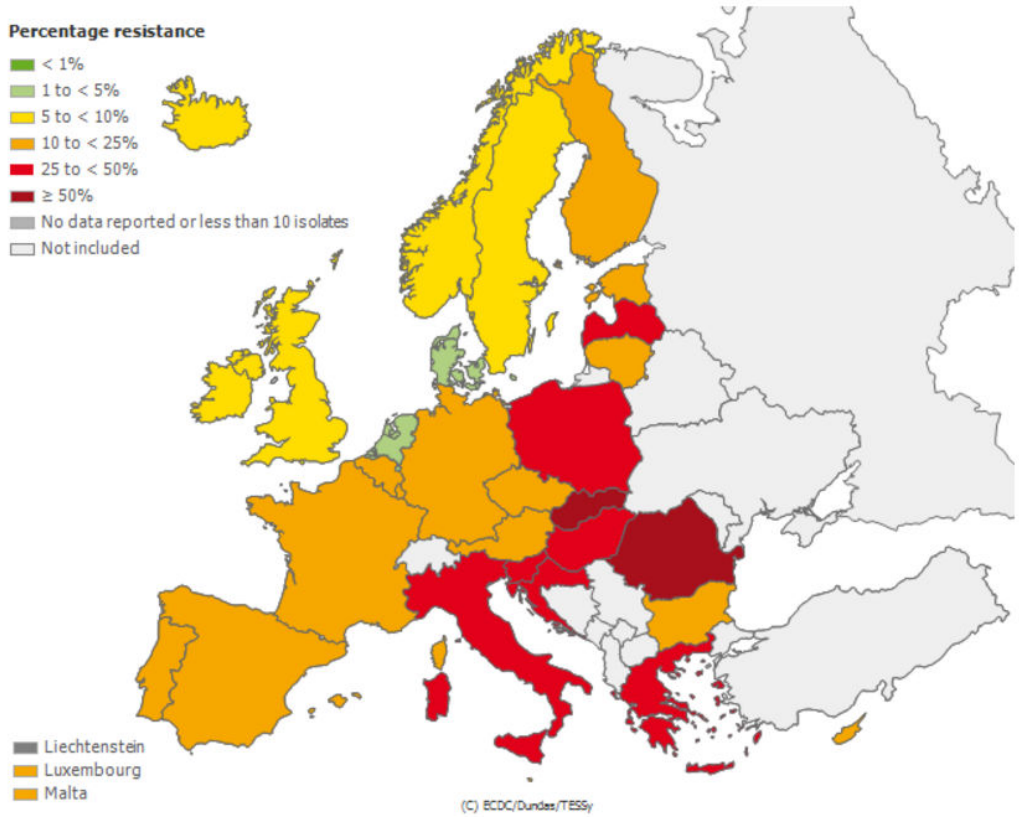



**Figure 1.4:** Proportion of carbapenem resistant *P. aeruginosa* isolates in participating countries of the European Antimicrobial Resistance Surveillance Network (EARS-Net) in 2013. Map obtained from the EARS-Net Antimicrobial Resistance Interactive Database (ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/database.aspx; last accessed on 3rd November 2015).

### 1.5.2 Resistance mechanisms to carbapenems

Imipenem resistance in *P. aeruginosa* is most commonly due to reduced permeability, as a result of mutations causing loss of the OprD porin, while meropenem- and doripenem-resistant strains generally have a combination of porin loss and upregulated efflux, possibly coupled with constitutive AmpC expression.[26] Acquired carbapenemases are, however, increasingly being seen in the species worldwide.[22] These enzymes, which also confer resistance to most other β-lactam antibiotics, pose a growing and major problem in *P. aeruginosa* as they frequently occur in isolates already resistant to multiple other drug classes, leaving few treatment options.

### 1.5.3 Carbapenemases

Acquired carbapenemases are seen in three of the four Ambler β-lactamase classes (A, B and D) and are discussed below. Carbapenemases of all these three classes have been found in *P. aeruginosa* isolates with those belonging to class B being the most common.

#### 1.5.3.1 Class A carbapenemases

Class A enzymes are serine carbapenemases; they include KPC enzymes and those GES variants that have carbapenemase activity. Genes encoding KPC enzymes are found on transposable elements and transferable plasmids and are mostly seen in Enterobacteriaceae, particularly *Klebsiella pneumoniae* where they were discovered.[41] However *P. aeruginosa* producing KPC-2 carbapenemases are reported to be widespread in Latin America,[42–44] the Caribbean[45,46] and China[47] although they have not been reported in this species in the UK or the rest of Europe

nor in mainland North America. GES variants with carbapenemase activity include GES-5, which has been recently detected in *P. aeruginosa* from an outbreak in Spain[48], and in sporadic isolates from India,[49] Turkey[50], South Africa[51], Brazil [52] and China.[53] A few *P. aeruginosa* producing GES-5 carbapenemases have also been submitted to Public Health England's (PHE) Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, from UK labs, over recent years (unpublished data). Other GES variants with carbapenemase activity are seen in *P. aeruginosa* and include GES-18 in Belgium[54], GES-6 in Portugal,[55] and GES-20, which was found to be widespread in the species in a recent multi-centre study in Mexico[56].

#### 1.5.3.2 Class D carbapenemases

Some class D (OXA) enzymes, also serine carbapenemases, are active against carbapenems. Several, including OXA-23, -40 and -58 are commonly seen in *Acinetobacter baumannii*, with OXA-40 and OXA-198 occasionally seen in *P. aeruginosa*.[57,58] The OXA-48 carbapenemase, which is increasingly seen in Enterobacteriaceae has not been reported in *P. aeruginosa*, but the closely related enzyme, OXA-181, was recently seen in *P. aeruginosa* isolates submitted to AMRHAI, from a patient who was hospitalised in India (unpublished data).

#### 1.5.3.3 Class B carbapenemases

Class B carbapenemases, also known as metallo-β-lactamases (MBLs), are widespread worldwide in *P. aeruginosa*, and are the most commonly reported carbapenemases in the species. These enzymes have a $Zn^{2+}$ ion at their active site and can be distinguished from serine carbapenemases due to strong inhibition of

their activity by metal chelators, such as EDTA. MBLs are widely scattered in Gram-negative species worldwide and can hydrolyse almost all β-lactam antibiotics including carbapenems, the exception being aztreonam to which *P. aeruginosa* is generally non-susceptible (according to EUCAST guidelines) for other reasons. A wide variety of MBLs are reported in *P. aeruginosa* as outlined in the following section.

## 1.6 Metallo- β-lactamases

Comprehensive epidemiological data for the worldwide occurrence and spread of MBLs in *P. aeruginosa* are not available and knowledge instead relies upon reports and surveys from particular countries or hospitals. The enzymes most widely seen globally in the species belong to the VIM and IMP families[32], while other enzymes including the NDM, SPM, AIM, GIM and FIM MBLs are generally restricted to smaller geographical areas. MBL families and their epidemiology are outlined below.

### 1.6.1 IMP-type metallo-β-lactamases

IMP-1 was the first MBL to be reported in *P. aeruginosa* from Japan in 1991[59] Since then, a total of 53 different IMP alleles have been officially described in different species (www.lahey.org/Studies/other.asp; last accessed 19th November 2015). A phylogenetic tree showing the relationship between these enzymes is presented in figure 1.5. These enzymes vary in sequence, but with little effect on their spectrum of resistance. Most variants are reported only in small geographical areas,[32] but a few were seen across wider geographical ranges in *P. aeruginosa,* as well as other Gram-negative species. These include IMP-1 which, in addition to Japan, has been described in *P. aeruginosa* isolates from Singapore, Thailand, Brazil and Iran,[52,60–62]

and IMP-7, which has been reported in the species in Canada, Singapore, and central Europe.[63–66] The enzymes are predominantly reported in Asian countries, and although several IMP variants were seen amongst *P. aeruginosa* in Europe, they are largely infrequent and geographically scattered and may represent imports from areas where the enzymes are more common.[67] There are several reports of IMP-7-producing *P. aeruginosa* in Poland, the Czech Republic and Slovakia[65,68,69] suggesting that organisms encoding these enzymes may be circulating in central Europe.

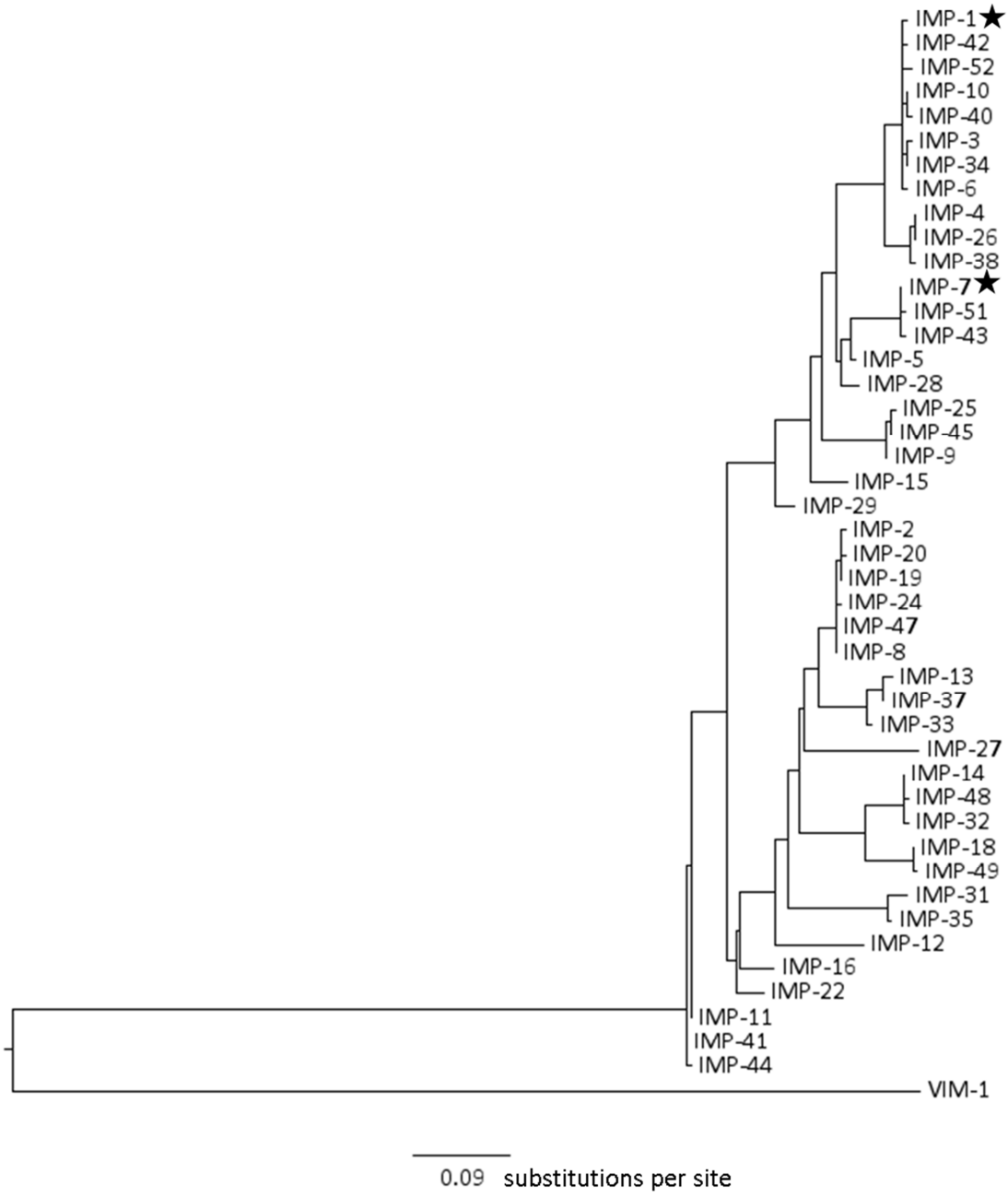



**Figure 1.5:** Maximum-likelihood tree showing members of the IMP family of MBLs for which sequence data are available. Amino acid sequences were taken from GenBank using accession numbers detailed on the Lahey website (www.lahey.org/Studies/other.asp; last accessed 19th November 2015). The VIM-1 sequence is included in the tree as an outgroup. The IMP alleles most commonly seen in *P. aeruginosa* are indicated by a star. Sequences were not available for IMP-17, IMP-23, IMP-36, IMP-39, IMP-46, IMP-50 or IMP-53.

### 1.6.2 **VIM-type metallo-β-lactamases**

The VIM-1 enzyme was first reported in 1999 in a *P. aeruginosa* isolate from Italy[70] and since then a total of 46 VIM-MBL variants have been assigned (www.lahey.org/Studies/other.asp; last accessed 19th November 2015) from *P. aeruginosa* and other Gram-negative species. The relationship between these enzymes is shown in figure 1.6. As with IMP-type MBLs, some variants, notably VIM-2, are more widely reported than others. Three major subgroups are described, as shown in figure 1.6.[71] Subgroup 1 includes the VIM-1 and VIM-4 variants that are commonly seen among *P. aeruginosa* in Europe, with VIM-1 having been reported in Italy, France and Greece[72,73] while VIM-4 has been described in five European countries (Belgium, Greece, Hungary, Poland and Sweden)[74–79] and also in Iran.[62] The most commonly seen enzyme in subgroup 2 is VIM-2, which is widespread worldwide, particularly in *P. aeruginosa*, and was the most commonly detected enzyme in a recent study of carbapenem-non-susceptible *P. aeruginosa* isolates from 14 European countries.[80] VIM-2 enzymes have also been reported in *P. aeruginosa* from Canada,[81] Colombia,[82] Japan,[83] Korea,[84] Thailand,[61] Saudi Arabia,[85] Singapore,[64] South Africa[86] and the USA.[87] VIM-7 is the sole member of subgroup 3 and has currently only been described in *P. aeruginosa* isolates in the USA.[88,89]

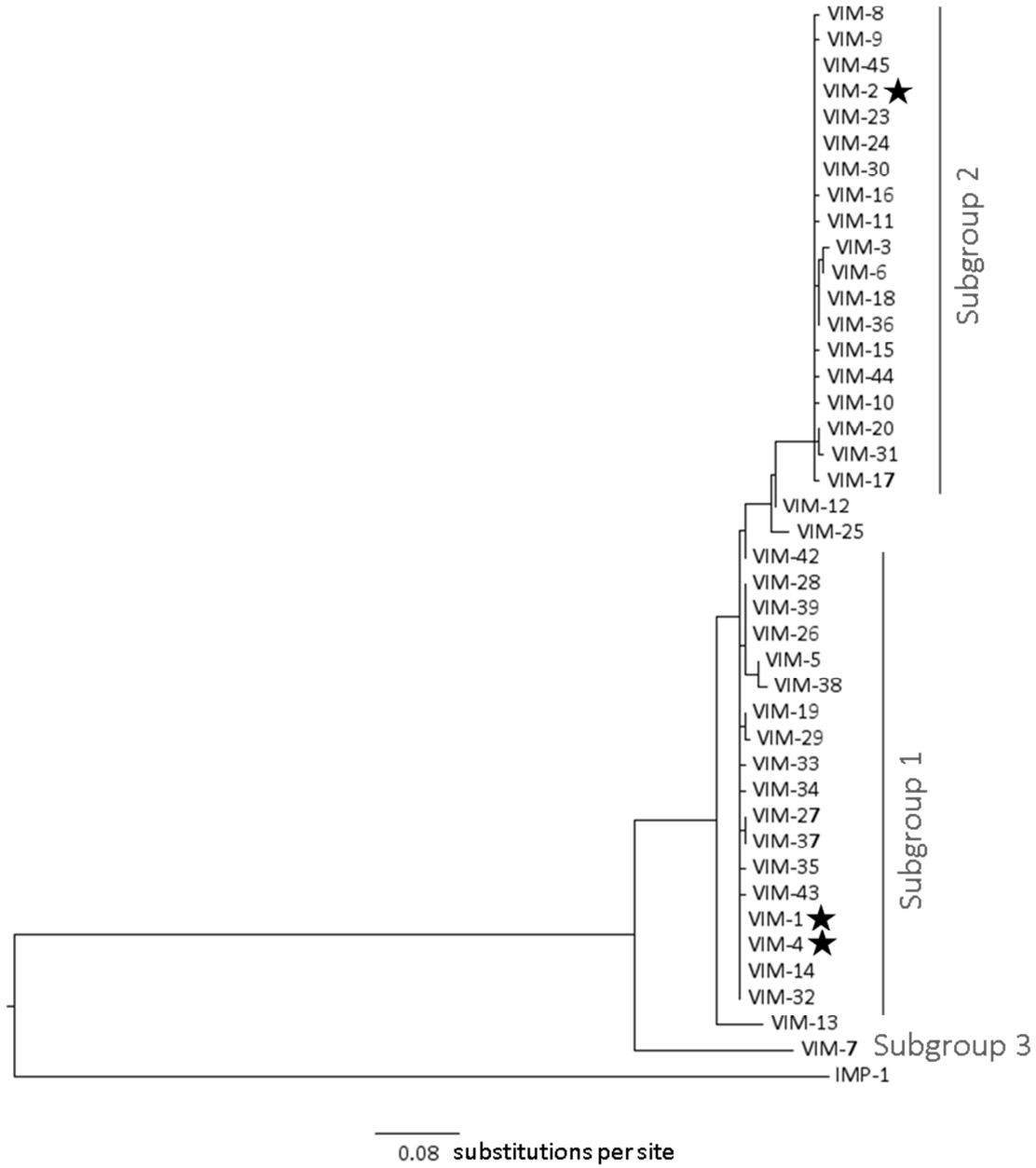



**Figure 1.6:** Maximum-likelihood tree showing members of the VIM family of MBLs for which sequence data are available. Amino acid sequences were taken from GenBank using accession numbers detailed on the Lahey website (www.lahey.org/Studies/other.asp; last accessed 19th November 2015). The IMP-1 sequence is included as an outgroup. VIM alleles commonly seen in *P. aeruginosa* are indicated by a star. Sequences were not available for VIM-20, VIM-21, VIM-22, VIM-40, VIM-41 or VIM-46.

### 1.6.3 Other metallo-β-lactamases

The NDM-1 MBL is believed to have emerged in India or Pakistan, and has become widely scattered in Enterobacteriaceae elsewhere.[90] There are currently 16 different alleles described (www.lahey.org/Studies/other.asp; last accessed 19th November 2015) of which NDM-1 remains the most widely reported. There are several reports of *P. aeruginosa* isolates producing NDM MBLs in the Indian subcontinent,[91,92] and it is suspected to be endemic in the Balkans region,[93] with reports of the enzyme in other countries being associated with travel to these regions.[94,95] Other MBLs reported in *P. aeruginosa* isolates remain largely restricted to the countries where they were originally found. They include SPM-1, which has become widespread in Brazil[96,97] with only isolated cases seen elsewhere, from patients previously hospitalised in Brazil; these include an isolate from a Swiss patient[98] and an isolate received by AMRHAI in 2013 (unpublished data). Reports of *P. aeruginosa* with the AIM, GIM and FIM MBLs are restricted to Australia, Germany and Italy respectively.[99–101]

## 1.7 Typing of *P. aeruginosa*

As discussed above, *P. aeruginosa* infections are common in hospital settings, with outbreaks often seen. Discrimination between *P. aeruginosa* strains is therefore necessary to inform epidemiological studies. Typing can help to establish whether a temporal increase in *P. aeruginosa* cases is due to the spread of a specific strain or the accumulation of sporadic cases, informing appropriate infection control interventions. Typing is also used for longer term surveillance and can help to identify types that may cause difficult-to-treat infections. Finally, typing can be used

to describe the population genetics of bacteria when applied to large numbers of isolates from diverse sources.

A variety of techniques are utilised for the typing of *P. aeruginosa* isolates with the most widely used including serotyping, pulsed-field gel electrophoresis (PFGE), variable number tandem-repeat (VNTR) analysis and multi-locus sequence typing (MLST). More recently, whole-genome sequencing (WGS) has been adopted for the molecular typing of several organisms including *P. aeruginosa* and offers a high level of discrimination. These typing methods, and their respective advantages and disadvantages will be discussed below.

### 1.7.1 Serotyping

Serotyping involves the detection of diverse O-antigens of the LPS, based on their reaction with particular anti-sera, and was previously the main reference method used for typing of many bacteria including *P. aeruginosa*.[102] However, the method only offers crude discrimination compared with molecular methods, and some strains are not typable due to nonagglutination or polyagglutination.[103] For these reasons its use has fallen into decline in recent years.

### 1.7.2 Pulsed-field gel electrophoresis (PFGE)

This method has been widely used and involves the digestion of DNA using specific rare-cutting restriction enzymes, most commonly *Spe*I for *P. aeruginosa*,[104–106] and separation of the resulting fragments by PFGE. The DNA fingerprints thereby obtained can be compared for different isolates to determine their similarity. It is highly discriminatory, and useful for the investigation of single-hospital outbreaks.

However, it is labour intensive, has a long turnaround time and results are difficult to compare between laboratories due to differences in methodologies.

### 1.7.3 Variable number tandem-repeat analysis (VNTR)

VNTR analysis involves PCR amplification of multiple repetitive regions within the genome, known as tandem repeats. Due to a high mutation rate in these regions, resulting from strand slippage, deletion or insertion of repeat units often occurs. The variation in the length of these repeat regions can be detected by PCR using primers spanning the repeats and thereby sizing the resulting amplicons. Several VNTR schemes are described for *P. aeruginosa,* each using a different selection of tandem-repeat loci. They include a 15-locus scheme,[107] two nine-locus schemes,[104,108] and a recently described 16-locus scheme, which incorporates loci from the two nine-locus schemes.[109] VNTR offers shorter turnaround times than PFGE. It has a similarly high level of discrimination, and is useful for outbreak investigation.[104] Due to the numerical nature of the resulting profiles it is easier to compare results between laboratories than for PFGE, although there is still the potential for interpretative differences, and as different laboratories may use different VNTR schemes, published data are not always readily comparable.

### 1.7.4 Multi-locus sequence typing (MLST)

MLST is increasingly used, with schemes being developed for many bacterial species in recent years. An MLST scheme for *P. aeruginosa* was proposed in 2004 by Curran et al.[110] This technique involves sequencing seven housekeeping genes, with alleles of each gene being assigned a unique allele number, and each unique combination of alleles being used to define a sequence type (ST). STs sharing five or more of the

seven alleles are categorised as clonal complexes. The *P. aeruginosa* MLST scheme has a relatively low level of discrimination, with several VNTR or PFGE types often represented within a single ST.[106,111] The method is more expensive than VNTR but it is readily transferable between laboratories and allows easy comparison of isolates in multiple hospitals, nationally or internationally.

### 1.7.5 Whole-genome sequencing (WGS)

The most definitive method for comparing isolates to each other is WGS. Unlike VNTR and MLST, which focus on specific areas of the genome, this method offers an overview of the whole genome, by the use of analyses such as single nucleotide polymorphism (SNP) analysis or whole genome MLST.[112] A major advantage is that other aspects of the organism's biology can be investigated in parallel, for example by seeking the presence of resistance or virulence determinants. The main barriers to the use of WGS methods in a clinical setting are high cost and the specialist knowledge needed for bioinformatic analyses of the resulting data. However, with improved technology and automated analysis pipelines, the method is fast becoming cheaper and accessible for a wide range of applications including for typing and outbreak investigation.

## 1.8 *P. aeruginosa* 'high-risk clones'

### 1.8.1 Population structure of *P. aeruginosa*

In 1993, Smith et al. described bacterial population structures ranging from panmictic (i.e. with a random association between loci, due to a high level of recombination) to clonal, with an intermediate epidemic structure.[113] MLST analysis of *P. aeruginosa* isolates has indicated that the species has a non-clonal epidemic

population structure i.e. it is predominantly panmictic but with occasional successful epidemic clones.[110,114] This is consistent with several reports that have shown that, in general, clinical *P. aeruginosa* isolates are indistinguishable from environmental isolates in terms of typing data. For example, a recent study of isolates submitted to AMRHAI from UK hospitals showed considerable overlap between representatives from patient and environmental sources in the UK with the prevalent types found in diverse sources and locations.[115] Pirnay and colleagues also investigated the population structure of *P. aeruginosa* using representatives collected from diverse environments in 30 countries over a 125 year time period. They confirmed the non-clonal epidemic structure, finding there was no specific disease habitat or geographical selection and that most isolates were diverse, but with occasional successful clones.[103]

This population structure is illustrated in figure 1.7, showing an eBURST[116] population snapshot based on all types seen in the *P. aeruginosa* MLST database (http://pubmlst.org/paeruginosa/; last accessed 20th November 2015), where most STs identified are singletons but with some clonal complexes consisting of closely related strains. Frequently-seen clones include clone C (ST17) and PA14 (ST253) as well as the Liverpool epidemic strain (ST146) commonly isolated from the lungs of cystic fibrosis patients.[117] Additionally some successful lineages are termed 'high-risk clones' and are thought to play a major role in the spread of resistance in *P. aeruginosa*, as described in the following section.[118]

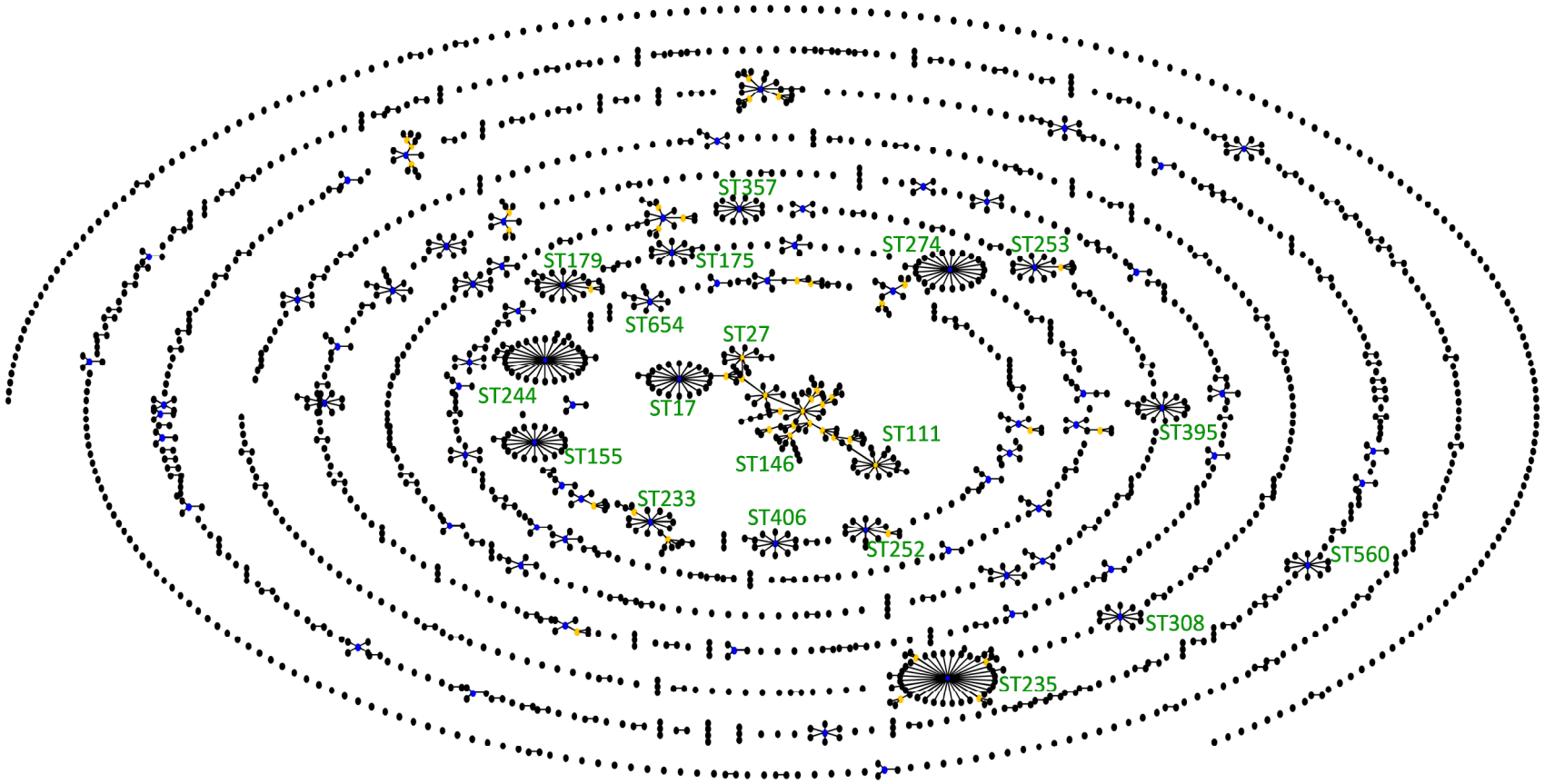



**Figure 1.7:** Population snapshot of the 2226 sequence types (ST) on the *P. aeruginosa* PubMLST database (http://pubmlst.org/paeruginosa/; last accessed 20th November 2015). The eBURST diagram was generated using the eBURST software v3 (available at http://eburst.mlst.net/default.asp).[116] The algorithm identifies groups (termed clonal complexes) of related STs in a population and predicts the founding ST of each group. Each dot represents a different ST while lines connect single locus variants; blue dots show predicted primary founders, yellow dots show subgroup founders and black dots indicate all other STs. Founders of prevalent clonal complexes are labelled.

### 1.8.2 Metallo-β-lactamases and 'high-risk clones'

'High-risk clones' are frequently associated with the carriage of diverse resistance genes including MBLs. A good early example is a multi-drug resistant clone of serotype O12 that was first reported in the 1980s, and is represented exclusively in clinical isolates. It has been associated with the expression of a variety of different resistance genes in European countries including the penicillinase PSE-1.[119,120] The introduction of MLST has allowed the comparison of STs of multi-drug-resistant *P. aeruginosa* across the world and, although acquired MBLs are recorded in diverse STs, a large proportion of MBL-producing isolates belong to a few 'high-risk clones'.[118] The most common host STs worldwide include ST111, ST235 and ST175 and isolates belonging to these clones often, but not exclusively, carry diverse resistance genes including MBLs. The worldwide distribution of these major lineages will be discussed in this section and is illustrated in figure 1.8.

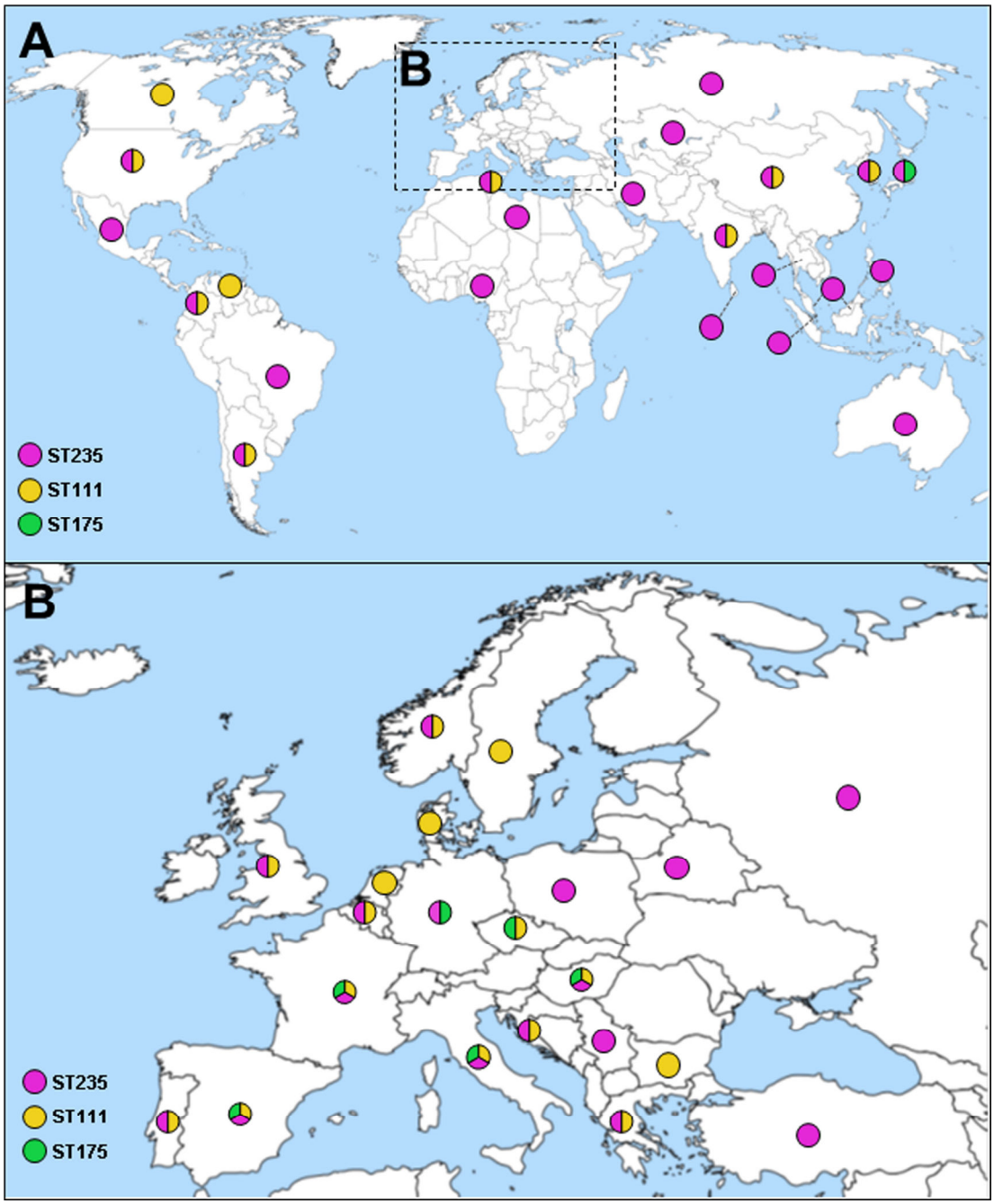



**Figure 1.8:** Worldwide reports of *P. aeruginosa* 'high-risk clones' of sequence types (STs) 111, 235 and 175 carrying varied acquired resistance genes including $bla_{MBL}$. Figure taken from Oliver *et al*.[117]

### 1.8.3 **ST111 'high-risk clone'**

ST111 is frequently described amongst MBL-producing *P. aeruginosa*, with isolates of this ST commonly reported to be of serotype O12, which has been recognised as a common host of multi-resistance in Europe since the 1980s (see above).[120,121] ST111 has been particularly associated with $bla_{VIM-2}$ genes in Europe, and was the most common type in a nationwide study of MBL-producing *P. aeruginosa* in The Netherlands in 2010-11,[121] as well as being responsible for outbreaks affecting hospitals in central Greece[122] and Italy.[123] ST111 isolates carrying $bla_{VIM-2}$ genes have also been seen in smaller numbers in other European countries including Sweden,[124] Croatia,[125] Spain,[126] Germany,[127] Belgium[80] and Portugal.[80] Outside of Europe, ST111 was one of the most common types identified in a study of MBL-producing *P. aeruginosa* in Colombia, again associated with $bla_{VIM-2}$.[128] Isolates of ST111 have also been reported to carry a variety of other MBL genes including $bla_{VIM-4}$ in Hungary,[74] $bla_{VIM-1}$ in Greece[129] and $bla_{IMP-13}$ in France,[130] while an outbreak of ST111 isolates carrying $bla_{GIM-1}$ was recently reported in Germany.[131] An ST111 isolate from Colombia reportedly harboured a KPC-2 carbapenemase along with $bla_{VIM-2}$.[132] Aside from carbapenemases, other resistance determinants reported among ST111 isolates include $bla_{VEB-1}$ ESBL and $bla_{PSE-1}$ penicillinase genes.[30,133]

### 1.8.4 **ST235 'high-risk clone'**

ST235 has been widely reported around the world associated with a variety of MBLs. *P. aeruginosa* of ST235 are reported to belong to serotype O11, which was previously associated, though not exclusively, with multi-drug resistant

isolates.[103,124,134,135] This ST was the most commonly identified among MBL-producing *P. aeruginosa* in a study of five Mediterranean countries[106] and an ST235 clone with $bla_{VIM-2}$ was found to be widespread across Russia, Belarus and Kazakhstan in a study conducted between 2002-2010.[136] The ST was also associated with $bla_{VIM-2}$ genes in Spain,[126] Croatia,[125] Germany,[127] Greece,[129] Malaysia and Sri Lanka.[137] Other $bla_{VIM}$ alleles associated with ST235 isolates including $bla_{VIM-4}$ in Hungary,[74] Norway,[124] and Belgium,[75] and $bla_{VIM-13}$ in Spain.[138] $bla_{IMP}$ alleles are more commonly seen in the ST in Asia with $bla_{IMP-6}$-carrying ST235 organisms causing outbreaks in Japan[139] and South Korea.[140] There are single reports of ST235 isolates carrying $bla_{NDM-1}$ and $bla_{SPM-1}$ MBL genes in France,[94] and Brazil,[97] respectively. ST235 *P. aeruginosa* are also reported amongst KPC-2 and GES-5 carbapenemase producers in Colombia[128] and Spain, respectively, as well those carrying $bla_{PSE-1}$ ESBL and $bla_{PER-1}$ penicillinase genes.[48,75]

### 1.8.5 **ST175 'high-risk clone'**

ST175 has been described as a 'high-risk clone'[117] and is particularly widespread in Spain where it is often multi-drug resistant, with one Spanish hospital reporting a large outbreak of a VIM-2-MBL-producing ST175 clone.[141,142] An outbreak involving a VIM-2-positive ST175 clone was also described in Germany.[143] In addition to MBL-producing strains, members of the ST also include strains not producing MBLs, including those isolated from France, [144] the Czech Republic[145] and Spain,[146] which are frequently multi-drug resistant due to mutations causing OprD inactivation, AmpC overproduction or increased efflux.[117]

### 1.8.6 Other prevalent clones among MBL producers

While ST111, ST235 and to a lesser extent ST175 are the most widely reported clones among MBL-producers, others have been repeatedly associated with these acquired enzymes and are also considered as 'high-risk clones'.[117] Notable lineages include ST277 carrying *bla*$_{SPM-1}$, widespread in Brazil,[97] although not often detected elsewhere and ST233, associated with *bla*$_{VIM-2}$-positive isolates in several European and African countries and the USA[30,124,147–150] as well as a *bla*$_{IMP-1}$ variant in a Singaporean isolate.[64] Other examples include ST654 reported in a *bla*$_{VIM-2}$-carrying Swedish isolate (thought to be imported from Tunisia)[124] and with *bla*$_{IMP-1}$ and *bla*$_{IMP-26}$ in Singapore;[64] ST357 associated with *bla*$_{VIM-2}$ or *bla*$_{IMP-7}$ genes in a hospital in the Czech Republic,[68] and *bla*$_{IMP-7}$ in Poland;[65] and ST773 reported among *bla*$_{VIM-2}$-carrying Indian isolates.[137]

## 1.9 Genetic environment of MBL genes

In order to devise effective control measures it is important to understand how MBL genes are mobilised and spread through bacterial populations. *bla*$_{VIM}$ and *bla*$_{IMP}$ genes are commonly located in class 1 integrons that often also contain genes conferring resistance to other antibiotic classes, such as aminoglycosides.[151] This section will discuss the structure and origins of integrons along with mechanisms by which they may be mobilised.

### 1.9.1 Integron structure and function

Integrons are gene-capture systems that were first described in the late 1980s, and have since been observed in many Gram-negative bacterial species.[152] They share three main features: (1) an integrase gene, *intI*, (2) an adjacent recombination site

(*attI)* recognised by the integrase and (3) a promoter upstream of this integration site (figure 1.9). Together, these features are able to capture and express gene cassettes, which are small circular elements that consist of a recombination site (*attC*) along with an open reading frame. Insertion of new gene cassettes is catalysed by the integrase, which recognises the *attI* and *attC* sites, in the integron and gene cassettes, respectively, and promotes recombination between them. Metagenomics studies show that these gene cassettes are abundant in a variety of environments and, based on the sequence of their encoding proteins, they are thought to have originated from diverse bacterial species.[153] Hundreds of integron families have been identified, based on phylogeny of their *intI* genes, and they are estimated to be present in more than 15% of sequenced bacterial genomes where they are thought to have played a role in bacterial adaptation and evolution.[153] Only integron classes 1-5 are associated with the carriage of resistance genes in clinical isolates, with class 1 integrons being the most commonly seen in Gram-negative pathogens and opportunists including *P. aeruginosa.*[153]

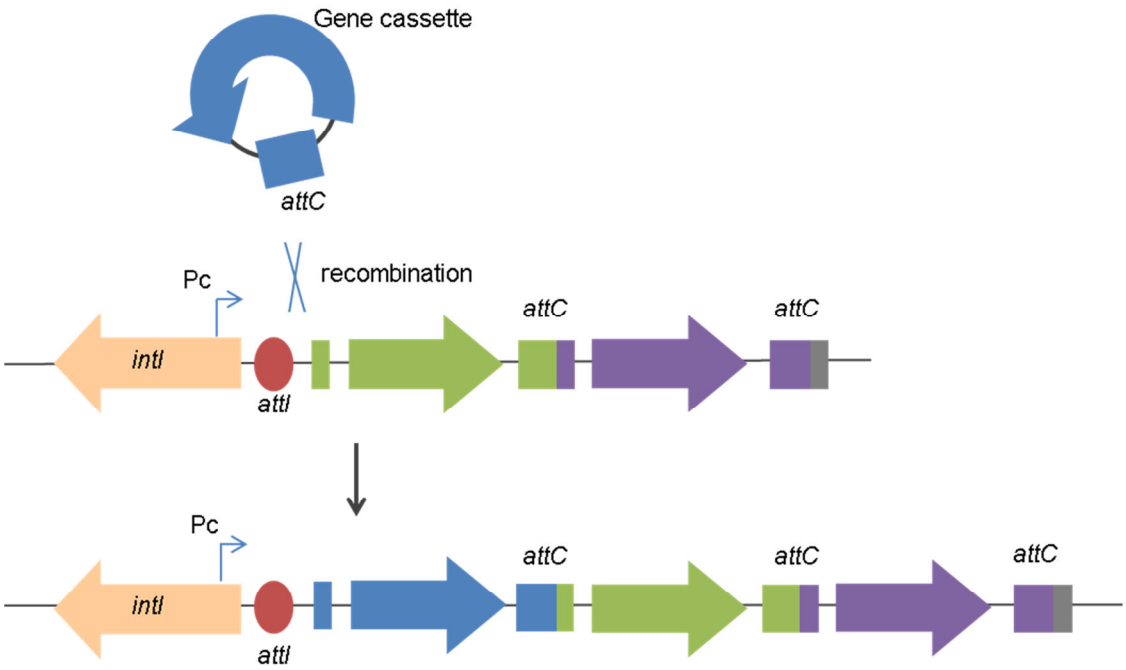



**Figure 1.9:** Integron structure. Integrons consist of an integrase gene (*intI*), an adjacent recombination site (*attI*) and a promoter, which can drive expression of integrated genes (Pc). Gene cassettes, consisting of a promoterless gene and a recombination site (*attC*), are 'captured' and inserted into the integron array by the integrase between the existing *attI* and *attC* sites.

### 1.9.2 Class 1 integrons

Class 1 integrons often carry one or more antibiotic resistance genes, and diverse gene cassette arrays have been characterised, often with genes encoding resistance to multiple antibiotic classes, such as β-lactams and aminoglycosides, and including the MBL genes $bla_{VIM}$ and $bla_{IMP}$. Clinically-relevant class 1 integrons are thought to have emerged in recent years, driven by the selective pressure of antibiotics, as discussed further in Section 4.1.

### 1.9.3 Mobilisation of class 1 integrons

Integrons cannot mobilise themselves, but are often located within transposon structures which, in turn, may be located on plasmids, or on mobilisable genomic islands within the chromosome. In the Enterobacteriaceae, class 1 integrons are often found on plasmids that can be easily transferred between strains by conjugation, but few studies have determined the genomic location of MBL-encoding integrons in *P. aeruginosa.*[151] Recent studies indicate that, although they can be present on plasmids in the species,[154] they are more often located in the chromosome, often carried in large genomic islands that may also contain other resistance or virulence associated genes.[30,147] More work is needed to better understand the structures that contain these integrons, their genomic location and whether they are mobilised between strains and this will be discussed further in Section 4.1.

## 1.10 Reports of MBL-producing *P. aeruginosa* in the UK

MBL-producers account for a small proportion of carbapenem resistant *P. aeruginosa* in the UK, with mutational mechanisms being more common. In a nationwide survey of bacteraemia isolates in the UK in 2012 by the British Society for Antimicrobial Chemotherapy (BSAC) 23/218 (11%) *P. aeruginosa* isolates were non-susceptible to imipenem but only one carried an MBL.[155] Nevertheless, both the number of MBL-producing *P. aeruginosa* isolates submitted to AMRHAI (two in 2003 *vs.* 52 in 2012) as well as the number of hospital laboratories referring MBL-producing isolates (two in 2003 *vs.* 35 in 2012) have steadily increased over recent years. In addition, there are a few reports of MBL-producing *P. aeruginosa* causing

outbreaks in the UK. These include an outbreak of a VIM-producing strain belonging to serotype O15, also carrying a VEB-1a ESBL, which was reported at a hospital in the North-West of England with fifteen isolates collected between 2003 and 2007.[156] VIM-2-producing strains were also reported in a prolonged hospital-wide outbreak at a London site in 2005 to 2011 affecting 85 patients, with a smaller outbreak affecting four patients at the haematology unit at a hospital in the south of England between 2009 and 2010.[157]

In contrast to data available for other countries, at the start of this PhD project there was little information on the molecular epidemiology of MBL-producing *P. aeruginosa* in the UK. In particular, there were no MLST data to allow producer strains to be compared with those seen internationally. Additionally, although the MBLs $bla_{VIM}$ and $bla_{IMP}$ were known to exist as part of class 1 integrons in the species, little was known about their wider genetic context and the extent to which they might move between strains.

## 1.11 Aims and Objectives

The main aims of this thesis were therefore to investigate the molecular epidemiology of MBL-producing *P. aeruginosa* in UK hospitals and to determine the genetic context of the MBL genes they encode, to better understand how they might be horizontally transferred between strains. This was achieved by characterising a collection of MBL-producing *P. aeruginosa* isolates submitted to the UK national reference laboratory (AMRHAI) between 2003 and 2012.

The specific objectives were:

- To investigate the importance of international 'high-risk clones', sporadic isolates and local outbreak strains in the dissemination of MBL-producing *P. aeruginosa* in the UK by typing members of our collection (Chapter 3).
- To determine the genetic context of the MBL genes, by studying the integron structures encoding them and their plasmid and/or chromosomal location (Chapter 4).
- To explore the value of whole-genome sequencing methods in providing further discrimination between closely related isolates, including investigation of the acquired resistance genes that they carry (Chapter 5).

# 2 General materials and methods

This chapter will outline the bacterial isolates used in this study, along with the general methods used to study them. Methods relating to specific chapters are discussed therein.

## 2.1 Bacterial isolates

### 2.1.1 MBL-producing *P. aeruginosa* study population

Three-hundred and thirty-four MBL-positive isolates were identified, as below, amongst *P. aeruginosa* isolates referred from UK hospital laboratories to AMRHAI between 2003 and 2012 for susceptibility testing, investigation of resistance mechanisms, and/or strain typing.

The process used to select isolates is shown in figure 2.1. Isolates were identified as MBL-producers by PCR, as described in Section 2.2.4; this PCR was undertaken by AMRHAI staff at the time of referral for 307 isolates whilst the remaining 27 MBL-producers were identified by retrospective screening (this study) of 121 isolates originally submitted only for typing between 2009 and 2012 that had similar VNTR types to those previously found to carry MBLs (accepting variants at up to three VNTR loci).

Of these 334 MBL-positive isolates, 130 were previously typed by nine-locus VNTR as part of the reference service, as described in Section 3.2.1. In this study an additional 161 MBL-producing *P. aeruginosa* isolates were subjected to nine-locus VNTR analysis, whilst the remaining 43 isolates were no longer viable in AMRHAI's archives.

The total of 334 MBL-positive isolates were from 267 patients, and VNTR profiles remained consistent when multiple isolates were received from the same patient. Accordingly, to reduce bias, one isolate per patient was retained for further study, leaving 267 non-duplicate-patient isolates; these were from 89 UK laboratories.

The 89 referring laboratories mapped into 11 main regions, which were further sub-divided into 18 sub-regions in the UK (figure 2.2). In this thesis, individual referring laboratories were assigned codes consisting of their respective region and a unique number within this region, in the format "region_number" (e.g. North West_1).

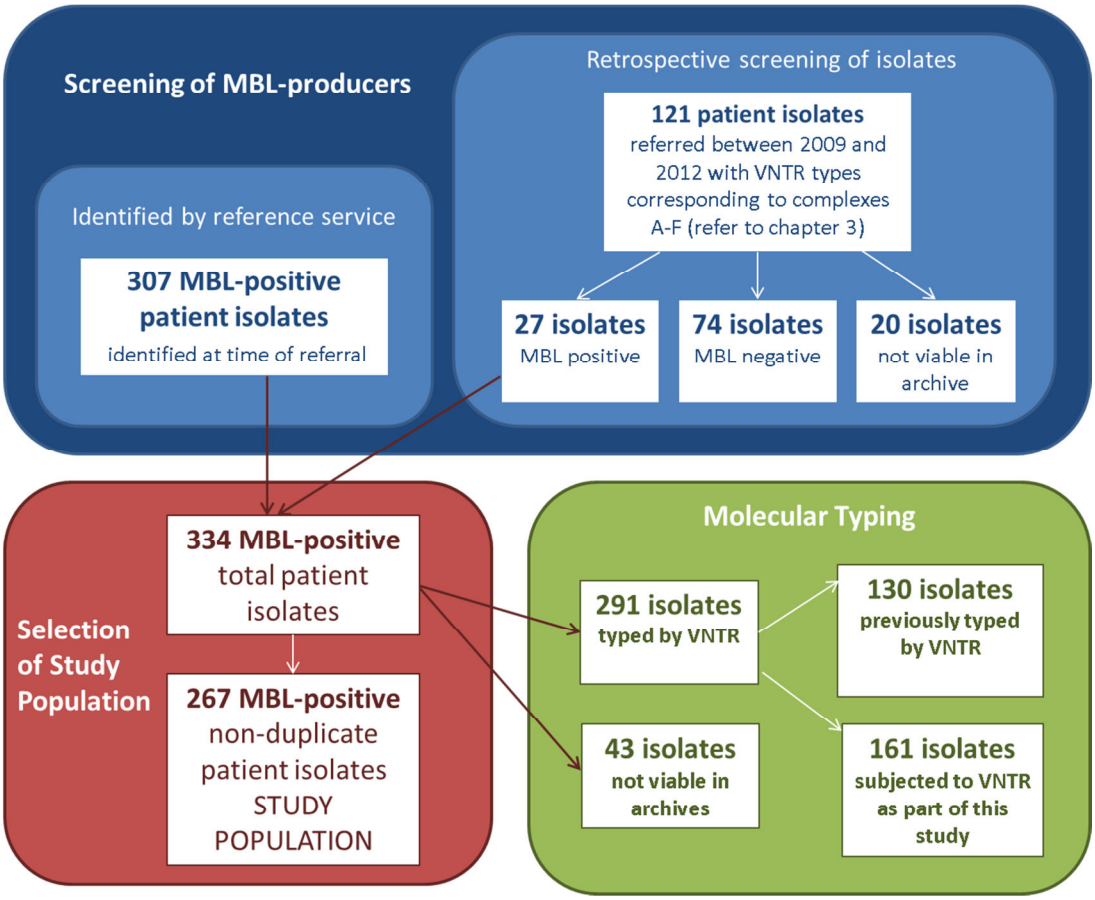



**Figure 2.1:** Selection of MBL-producing *P. aeruginosa* isolates for further study


| | Sub-region | Region |
|---|---|---|
| 1 | Scotland | Scotland |
| 2 | North East | Northern and Yorkshire |
| 3 | Yorkshire and Humber | |
| 4 | Cumbria and Lancashire | North West |
| 5 | Greater Manchester | |
| 6 | Cheshire and Merseyside | |
| 7 | Lincolnshire, Leicestershire, Nottinghamshire and Derbyshire | East Midlands |
| 8 | West Midlands | West Midlands |
| 9 | Wales | Wales |
| 10 | Avon, Gloucestershire and Wiltshire | South West |
| 11 | Devon, Cornwall and Somerset | |
| 12 | Thames Valley | South East |
| 13 | Hampshire, Isle of Wight and Dorset | |
| 14 | Sussex, Surrey and Kent | |
| 15 | London | London |
| 16 | Bedfordshire, Hertfordshire and Northamptonshire | East of England |
| 17 | Norfolk, Suffolk, Cambridgeshire and Essex | |
| 18 | Northern Ireland | Northern Ireland |

**Figure 2.2:** Regions and sub-regions of the UK referred to in this study

### 2.1.2 BSAC Bacteraemia Surveillance Programme *P. aeruginosa* isolates

As a comparator group, all 209 *P. aeruginosa* isolates collected in 2011 as part of the BSAC Bacteraemia Surveillance Programme (http://www.bsacsurv.org/) were studied. Thirty-nine hospitals in the UK or Irish Republic submitted up to seven non-duplicate *P. aeruginosa* isolates from blood samples of bacteraemia patients between the 1st January and 31st December 2011. These isolates, along with patient demographic and minimum inhibitory concentration (MIC) data were provided by the BSAC.

### 2.1.3 MBL-producing *Pseudomonas* spp. other than *P. aeruginosa*

Twenty-three isolates of MBL-producing *Pseudomonas* spp. other than *P. aeruginosa* were submitted to AMRHAI between 2003 and 2012, the same time period as the MBL-producing *P. aeruginosa*. The 23 isolates were referred from 12 different UK hospital laboratories; 21 were isolated from patient specimens, while the remaining three isolates were from the hospital environment.

## 2.2 General methods

### 2.2.1 Culture

Isolates were cultured on nutrient agar and incubated at 37°C overnight (16-20 hours). Where required, liquid cultures were prepared in nutrient broth and incubated at 37°C overnight (16-20 hours), with shaking.

### 2.2.2 Minimum inhibitory concentration (MIC) determination

MICs were previously determined by the BSAC agar dilution method[158] and are interpreted using EUCAST breakpoints (www.eucast.org/clinical_breakpoints/). MICs were determined by AMRHAI staff, as part of the reference service or the BSAC Bacteraemia Surveillance Programme.

### 2.2.3 PCR and Sanger Sequencing

#### 2.2.3.1 Preparation of DNA templates for PCR

Crude DNA extracts were prepared by suspending 3-5 colonies from overnight culture plates in 100 µl PCR-grade water and vortexing for 10 seconds. The suspension was heated at 95 $^{o}$C for 5 minutes and centrifuged for 2 minutes at 15500 rcf (g).

#### 2.2.3.2 PCR setup

Primers were obtained from Sigma-Aldrich (Poole, UK). Primer stocks were made up at 100µM and stored at -20$^{o}$C and 10 µM working stocks were made up as necessary. In general, PCR was set up using the *Taq* DNA Polymerase Kit (Qiagen, Sussex, UK). In some cases, as indicated, PCR reactions were set up using the QIAgility robot (Qiagen), in which case the *Taq* DNA Polymerase Kit (Qiagen) was used as above except that Qiagen *Taq* Polymerase was replaced with the Platinum *Taq* DNA Polymerase (Invitrogen, Paisley, UK). Final concentrations of reagents used were 1 x PCR Buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each primer and 1 U *Taq* Polymerase per 25 µl reaction. Q solution (which helps in the amplification of GC rich DNA templates and those with high levels of secondary structure) from the *Taq* DNA Polymerase Kit (Qiagen) was used at 1 x concentration

for some PCRs, as indicated in the relevant methods sections. Long-range PCRs were carried out with the LongRange PCR Kit (Qiagen). Final concentrations of reagents were as above, except that 2.5 mM $MgCl_2$ and 500 µM of each dNTP were used. Primers used are shown in table 2.1 and cycling conditions, which varied for each PCR, are described in the respective methods sections.

**Table 2.1:** Primers used in this study

| Primer | Primer sequences (5'-3') | Target | Reference |
|---|---|---|---|
| IMP mp fwd | GGAATAGAGTGGCTTAATTCTC | *bla*$_{IMP}$ | [159] |
| IMP mp rev II | CCAAACCACTACGTTATCT | *bla*$_{IMP}$ | [159] |
| VIM mp fwd | GATGGTGTTTGGTCGCATA | *bla*$_{VIM}$ | [159] |
| VIM mp rev II | CGAATGCGCAGCACCAG | *bla*$_{VIM}$ | [159] |
| SPM mp fwd | AAAATCTGGGTACGCAAACG | *bla*$_{SPM}$ | [159] |
| SPM mp rev II | ACATTATCCGCTGGAACAGG | *bla*$_{SPM}$ | [159] |
| GIM mp fwd | TCGACACACCTTGGTCTGAA | *bla*$_{GIM}$ | [159] |
| GIM mp rev II | AACTTCCAACTTTGCCATGC | *bla*$_{GIM}$ | [159] |
| SIM-1-F | TACAAGGGATTCGGCATCG | *bla*$_{SIM}$ | [159] |
| SIM-1-R | TAATGGCCTGTTCCCATGTG | *bla*$_{SIM}$ | [159] |
| NDM-F | GGGCAGTCGCTTCCAACGGT | *bla*$_{NDM}$ | [160] |
| NDM-R | GTAGTGCTCAGTGTCGGCAT | *bla*$_{NDM}$ | [160] |
| ms172L | GGATTCTCTCGCACGAGGT | ms172 (VNTR) | [104] |
| ms172R | TACGTGACCTGACGTTGGTG | ms172 (VNTR) | [104] |
| ms211L | ACAAGCGCCAGCCGAACCTGT | ms211 (VNTR) | [104] |
| ms211R | CTTCGAACAGGTGCTGACCGC | ms211 (VNTR) | [104] |
| ms213L | CTGGGCAAGTGTTGGTGGATC | ms213 (VNTR) | [104] |
| ms213R | TGGCGTACTCCGAGCTGATG | ms213 (VNTR) | [104] |
| ms214L | AAACGCTGTTCGCCAACCTCTA | ms214 (VNTR) | [104] |
| ms214R | CCATCATCCTCCTACTGGGTT | ms214 (VNTR) | [104] |
| ms217L | TTCTGGCTGTCGCGACTGAT | ms217 (VNTR) | [104] |
| ms217R | GAACAGCGTCTTTTCCTCGC | ms217 (VNTR) | [104] |

**Table 2.1 continued**

| Primer | Primer sequences (5'-3') | Target | Reference |
|---|---|---|---|
| ms222L | AGAGGTGCTTAACGACGGAT | ms222 (VNTR) | [104] |
| ms222R | TGCAGTTCTGCGAGGAAGGCG | ms222 (VNTR) | [104] |
| ms207L * | ACGGCGAACAGCACCAGCA | ms207 (VNTR) | [104] |
| ms207R | CTCTTGAGCCTCGGTCACT | ms207 (VNTR) | [104] |
| ms209L* | CAGCCAGGAACTGCGGAGT | ms209 (VNTR) | [104] |
| ms209R | CTTCTCGCAACTGAGCTGGT | ms209 (VNTR) | [104] |
| ms61L* | CTTGCCGTGCTACCGATCC | ms61 (VNTR) | [104] |
| ms61R | CCCCCATGCCAGTTGC | ms61 (VNTR) | [104] |
| acsA-F | ACCTGGTGTACGCCTCGCTGAC | *acsA* (MLST amplification) | [110] |
| acsA-R | GACATAGATGCCCTGCCCCTTGAT | *acsA* (MLST amplification) | [110] |
| aroE-F | TGGGGCTATGACTGGAAACC | *aroE* (MLST amplification) | [110] |
| aroE-R | TAACCCGGTTTTGTGATTCCTACA | *aroE* (MLST amplification) | [110] |
| guaA-F | CGGCCTCGACGTGTGGATGA | *guaA* (MLST amplification) | [110] |
| guaA-R | AACGCCTGGCTGGTCTTGTGGTA | *guaA* (MLST amplification) | [110] |
| mutL-F | CCAGATCGCCGCCGGTGAGGTG | *mutL* (MLST amplification) | [110] |
| mutL-R | CAGGGTGCCATAGAGGAAGTC | *mutL* (MLST amplification) | [110] |
| nuoD-F | ACCGCCACCCGTACTG | *nuoD* (MLST amplification) | [110] |
| nuoD-R | TCTCGCCCATCTTGACCA | *nuoD* (MLST amplification) | [110] |
| ppsA-F | GGTCGCTCGGTCAAGGTAGTGG | *ppsA* (MLST amplification) | [110] |
| ppsA-R | GGGTTCTCTTCTTCCGGCTCGTAG | *ppsA* (MLST amplification) | [110] |
| trpE-F | GCGGCCCAGGGTCGTGAG | *trpE* (MLST amplification) | [110] |
| trpE-R | CCCGGCGCTTGTTGATGGTT | *trpE* (MLST amplification) | [110] |
| acsA-SF | GCCACACCTACATCGTCTAT | *acsA* (MLST sequencing) | [110] |
| acsA-SR | AGGTTGCCGAGGTTGTCCAC | *acsA* (MLST sequencing) | [110] |

**Table 2.1 continued**

| Primer | Primer sequences (5'-3') | Target | Reference |
|---|---|---|---|
| aroE-SF | ATGTCACCGTGCCGTTCAAG | *aroE* (MLST sequencing) | [110] |
| aroE-SR | TGAAGGCAGTCGGTTCCTTG | *aroE* (MLST sequencing) | [110] |
| guaA-SF | AGGTCGGTTCCTCCAAGGTC | *guaA* (MLST sequencing) | [110] |
| guaA-SR | GACGTTGTGGTGCGACTTGA | *guaA* (MLST sequencing) | [110] |
| mutL-SF | AGAAGACCGAGTTCGACCAT | *mutL* (MLST sequencing) | [110] |
| mutL-SR | GGTGCCATAGAGGAAGTCAT | *mutL* (MLST sequencing) | [110] |
| nuoD-SF | ACGGCGAGAACGAGGACTAC | *nuoD* (MLST sequencing) | [110] |
| nuoD-SR | TGGCGGTCGGTGAAGGTGAA | *nuoD* (MLST sequencing) | [110] |
| ppsA-SF | GGTGACGACGGCAAGCTGTA | *ppsA* (MLST sequencing) | [110] |
| ppsA-SR | GTATCGCCTTCGGCACAGGA | *ppsA* (MLST sequencing) | [110] |
| trpE-SF | TTCAACTTCGGCGACTTCCA | *trpE* (MLST sequencing) | [110] |
| trpE-SR | GGTGTCCATGTTGCCGTTCC | *trpE* (MLST sequencing) | [110] |
| 5'CS | GGCATCCAAGCAGCAAG | 5'cs of class 1 integrons (i*ntI1* gene) | [161] |
| 3'CS_1 | AAGCAGACTTGACCTGA | 3'cs of class 1 integrons | [161] |
| 3'CS_2 | CGGATGTTGCGATTACTTCG | 3'cs of class 1 integrons | [162] |
| Tni-CF | CGATCTCTGCGAAGAACTCG | *tniC* gene of Tn*402*-like 3' region | [162] |
| VIM2004A | GTTTGGTCGCATATCGCAAC | $bla_{VIM}$ gene | [163] |
| VIM2004B | AATGCGCAGCACCAGGATAG | $bla_{VIM}$ gene | [163] |
| VIM-2A | ATGTTCAAACTTTTGAGTAA | $bla_{VIM}$ gene (sequencing) | [164] |
| VIM-2B | CTACTCAACGACTGAGCG | $bla_{VIM}$ gene (sequencing) | [164] |
| IMP Fwd | GAAGGYGTTTATGTTCATAC | $bla_{IMP}$ gene | [163] |
| IMP Rev | GTAMGTTTCAAGAGTGATGC | $bla_{IMP}$ gene | [163] |
| dhfr2_F | CCACCTTTGGCTTCGGAGAT | *dhfr2* gene | This study |
| dhfr2_R | TGTGCAATACCAACCGACGA | *dhfr2* gene | This study |

**Table 2.1 continued**

| Primer | Primer sequences (5'-3') | Target | Reference |
|---|---|---|---|
| Sul-R | CCGACTTCAGCTTTTGAAGG | *sul1* gene of class 1 integron | [165] |
| aacA29AB1 | GAAAGAACAAGACGCTGCCG | *aacA29a* or *b* genes | [166] |
| aacA29AB2 | ACTTGCGGTGCGTGATGACC | *aacA29a* or *b* genes | [166] |
| aacA4-F | GACCTTGCGATGCTCTATG | *aacA4* gene | [167] |
| aacA4-R | CAGCAACTCAACCAGAGC | *aacA4* gene | [167] |
| OXA10like-F | GGTGTCATAAAGAATGAGCAT | *oxa-10* like gene | [167] |
| OXA10like-R | TCCATGTTAAAGGCGAAAAAGT | *oxa-10* like gene | [167] |
| AACA7-F | AGCATTGGGCTCGTGGTCG | *aacA7* gene | [84] |
| AACA7-R | GACACCTCCGTGAATCCAG | *aacA7* gene | [84] |
| aacA7-F2 | GCTTCCGGAAACCGTTGTGA | *aacA7* gene | This study |
| strAstrB-F | TATCTGCGATTGGACCCTCTG | *strAstrB* genes | [124] |
| strAstrB-R | CATTGCTCATCATTTGATCGGCT | *strAstrB* genes | [124] |
| NDM-orfF | ATGGAATTGCCCAATATTATG | $bla_{NDM}$ | This study |
| NDM-orfR | TCAGCGCAGCTTGTCGGCCA | $bla_{NDM}$ | This study |
| CM7 | AACCAGTTCCGCGTTGGCCTGG | *rpoB* | [168] |
| CM31B | CCTGAACAACACGCTCCGGA | *rpoB* | [168] |
| PA5101_F | AATCCGGTAATGCTCCTCGC | *PA5101* gene | This study |
| PA5101_R | ATGCAGTGACCTTCGGCTAC | *PA5101* gene | This study |
| VIMGI-LH_R | GGGAAATCAGGGATCGGAGC | Left hand portion of genomic island A | This study |
| VIMGI-RH_F | TGCCACGATCAAGGGATTCG | Right hand portion of genomic island A | This study |
| PA2229_F | CCTGGATGCCCTTCTCACC | *PA2229* gene | This study |
| PA2229_R | CTGGTGCTGAAGTTCTCGCC | *PA2229* gene | This study |
| ST111-GI-J1_R | GATCAGTACCTCCTGCGACG | Left hand junction of genomic island B | This study |
| ST111-G1-J2_F | TTCTGAGACGACCCCAAGTT | Right hand junction of genomic island B | This study |

**Table 2.1 continued**

| Primer | Primer sequences (5'-3') | Target | Reference |
|---|---|---|---|
| endA_F | GCTACTCGGGAAATCGTGTCG | *endA* | This study |
| endA_R | TATTGCTGGGGTTTCTGCGG | *endA* | This study |
| PACS171b-J1_R | TTTCGCTAAGCCCGCAACTA | phage integrase gene of PACS171b-like genomic island | This study |
| PACS171b-J2_F | TGAGATGGGCCTGGTGGTAT | transposase gene of PACS171b-like genomic island | This study |
| glykin_F | GAACCCCCTACTCTGGCAAC | glycerate kinase gene | This study |
| glykin_R | GCGAAGGGAGGTTGGATGAA | glycerate kinase gene | This study |
| urf2_R | CGGAATACGTCGAGCACTTCT | *urf*2Δ of Tn*6249* | This study |
| TnpATn6249_F | TCGATTGGTTGCAAAGCGTC | *TnpA* of Tn*6249* | This study |
| cse3_For | ATGTACCTGACCAGACTGACCCTTGATCCTCGCAGCG | *cse3* gene (Type I-E CRISPR-Cas) | [169] |
| cse3_Rev | GGCTCAGCAGGCCACAGCCGAAAGCCTTG | *cse3* gene (Type I-E CRISPR-Cas) | [169] |
| csy1_FOR | ATGACCTCTCCCCTCCCAACGCCTACG | *csy1* gene (Type I-F CRISPR-Cas) | [169] |
| csy1_REV | TCAGTCACGCTCATCTTCGAGTATCTCC | *csy1* gene (Type I-F CRISPR-Cas) | [169] |

* indicates primers that were fluorescently labelled at their 5'end with either 6-FAM (ms207L), NED (ms209L) or VIC (ms61L)

#### 2.2.3.3 Analysis of PCR amplicons

Amplicons were analysed on 1.5% agarose gels, except for PCRs targeting integrons, which were sized on 1% agarose gels. DNA ladders Hyperladder 50 bp or Hyperladder 1 kb (both Bioline, London, UK) were used depending on the expected product size.

#### 2.2.3.4 Sanger Sequencing

Amplicons were treated with ExoSAP-IT (Affymetrix, Santa Clara, USA). Two µl of ExoSAP-IT was added per 5 µl of amplicon and the mixture was incubated at 37$^{o}$C for 15 minutes followed by inactivation at 80$^{o}$C for 15 minutes. Following Exo-SAP-IT treatment, 1.5 µl amounts of the PCR products were combined with 0.5 µl of the relevant primer (10µM) and 4 µl PCR-grade water, and submitted to the Genomic Services and Development Unit at PHE Colindale for Sanger sequencing. Resulting sequences were analysed using Bionumerics software v6.1 (Applied Maths, Sint-Martens-Latem, Belgium). Primers used for sequencing are shown in table 2.1 and referred to in the relevant methods sections.

### 2.2.4 Detection of MBL genes

Genes encoding VIM-, IMP-, SPM-, GIM-, and SIM-type MBLs were sought by multiplex PCR, as detailed by Ellington et al.[159] using primer pairs IMP mp Fwd with IMP mp rev II, VIM mp fwd with VIM mp rev II, SPM mp fwd with SPM mp rev II, GIM mp fwd with GIM mp rev II, and SIM-1-F with SIM-1-R, respectively (see table 2.1). Genes for NDM-type MBLs were sought using a single PCR using primers NDM-F and NDM-R, as previously described.[160] For both PCRs cycling conditions were

95°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 56°C for 40 seconds and 72°C for 40 seconds and a final extension step of 72°C for 5 minutes.

## 2.2.5 Whole genome sequencing

### 2.2.5.1 DNA extraction

Genomic DNA was extracted using the GeneJet Genomic DNA Purification Kit (Thermo Scientific, Loughborough, UK) according to manufacturer instructions. DNA concentration was estimated using the Qubit dsDNA BR Assay Kit on the Qubit Fluorometer (Life Technologies, Paisley, UK). DNA quality was estimated using the Nanodrop ND-1000 Spectrophotometer (Thermo Scientific) with 260 nm/ 280 nm absorbance readings between 1.8 and 2.0 deemed acceptable.

### 2.2.5.2 Illumina Sequencing

DNA was diluted to between 10-30 ng/µl before submission to the Genomic Services and Development Unit, PHE Colindale for Illumina sequencing, where the following steps were carried out. Library preparation was performed using the Nextera XT DNA Sample Preparation Kit (Illumina, Cambridge, UK). Quality checks were performed on the libraries thereby generated using the LabChip GX instrument (Perkin Elmer, Beaconsfield, UK) to determine the average fragment size and the KAPA Universal Library Quantification Kit (KAPA Biosystems, London, UK) to determine the concentration of clusterable material for loading onto the Illumina HiSeq. The library was clustered onto a HiSeq Flow Cell using The TruSeq Rapid Duo Sample Loading Kit on a cBot instrument before analysis was carried out on a HiSeq instrument, on 'Rapid Run' mode using TruSeq Rapid PE Cluster v2 Kit and TruSeq Rapid 144 SBS v2 Kit (200 cycles) (all Illumina). The resulting fastq reads

were trimmed using Trimmomatic 0.32,[170] and the trimmed fastq files were used in the analyses described in the following chapters.

#### 2.2.5.3 MinION sequencing

MinION sequencing was carried out as part of the MinION access programme (Oxford Nanopore Technologies, Oxford, UK) at the University of East Anglia (UEA), Norwich (run 1) or PHE, Colindale (run 2), using the latest protocols and reagents available at the time the work was undertaken. The reagents used were from the Nanopore Sequencing Kit SQK-MAP004 (run 1) or SQK-MAP005 (run 2), both Oxford Nanopore Technologies, except where otherwise specified. Run 1 was carried out by Justin O'Grady, Solomon Mwaigwisya and Katarzyna Schmidt at UEA, Norwich while run 2 was carried out by the author, with the help of Jane Turton, at PHE, Colindale.

Shearing was performed on extracted DNA in a g-TUBE (Covaris, Massachusetts, USA) using 1.5 µg DNA in a total volume of 81 µl at 6000 rpm (run 1) or 4200 rpm (run 2), to yield fragments of around 8 kb or 20 kb respectively, in an Eppendorf 5424 centrifuge (this centrifuge was recommended by Oxford Nanopore Technologies, as different centrifuges have varying ramp speeds, which can affect shearing). For run 1 PreCR treatment was carried out using the PreCR Repair Treatment Kit (New England BioLabs, Hertfordshire, UK). Eighty µl of fragmented DNA, 6 µl nuclease-free water, 10 µl 10 x Thermopol Buffer, 1 µl 10x $NAD^+$, 1 µl 10 mM dNTPs and 2 µl PreCR Repair Mix were combined, mixed gently and incubated at 37$^{o}$C for 30 minutes. The DNA was cleaned up using Agencourt AMPure XP Beads (Beckmann-Coulter, High Wycombe, UK): these were first resuspended by

vortexing, then 100 µl were added to the reaction, and incubated at room temperature for 5 minutes to bind to DNA. The suspension was spun down briefly at 13000 rcf and pelleted on a magnetic rack, with the supernatant aspirated once it became clear and colourless. The beads were then washed twice with 200 µl of freshly prepared 70% ethanol. The tube was briefly spun down as before and replaced on the magnet before residual ethanol was aspirated. The pellet was resuspended in 81 µl 10 mM Tris-HCl pH 8.5 and incubated for 2 minutes at room temperature. The beads were then re-pelleted on the magnet and the eluate removed once clear and colourless. This eluate (run 1), or the fragmented DNA without PreCR treatment (run 2) were quantified using the Qubit Fluorometer as before and 1 µg DNA in a total volume of 80 µl was taken forward for the end-repair step. Here, the 80 µl of DNA solution was combined with 10 µl End-Repair Buffer, 5 µl End-Repair Enzyme Mix, both from the NEBNext End Repair Module Kit (New England BioLabs), and 5 µl DNA CS (ligation control DNA) provided in the Nanopore Sequencing Kits. The reagents were mixed and incubated at room temperature for 20 minutes. The reaction was cleaned up with the Agencourt AMPure XP Beads as before except that the DNA was eluted into a final volume of 26 µl of 10 mM Tris-HCl pH 8.5 (run 1) or nuclease-free water (run 2). The dA-tailing reaction was set up using the NEBNext dA Tailing Module (New England BioLabs). Twenty-five µl of the end-repaired DNA was combined with 3 µl 10x dA-Tailing Buffer and 2 µl dA-Tailing Enzyme (Klenow Fragment). The reaction was cleaned up with the Agencourt AMPure XP Beads as before except that the DNA was eluted into a final volume of 31 µl of 10 mM Tris-HCl pH 8.5 (run 1) or nuclease-free water (run 2).

Thirty µl of the dA-tailed DNA were mixed with 10 µl Adapter Mix, 10 µl HP Adapter and 50 µl Blunt/TA Ligase Mastermix, in a 1.5 ml Protein LoBind Tube (Eppendorf, Hamburg, Germany). The mixture was briefly spun down in a centrifuge at 13000 rcf and incubated for 10 minutes at room temperature. His-Tag Dynabeads (Life Technologies) were prepared by pelleting 10 µl on the magnet, aspirating the supernatant and washing twice with 200 µl 1 x Bead Binding Buffer, before resuspending the washed and pelleted beads in 2x Bead Binding Buffer. These washed beads were added to the 100 µl adapter ligated DNA in a Protein LoBind Tube, mixed carefully by pipetting and incubated at room temperature for five minutes. The beads were pelleted on the magnetic rack and the supernatant aspirated; subsequently the pelleted beads were washed twice with 200 µl of 1 x Bead Binding Buffer. The tube was spun down briefly and placed back on the magnetic rack for 1-2 minutes before the remaining Bead Binding Buffer was aspirated. The library was eluted from the beads by resuspending in 25 µl Elution Buffer, incubating at room temperature for 10 minutes, pelleting the beads on the magnet and transferring the eluate to a new tube. One µl of this pre-sequencing mix was quantified on the Qubit fluorimeter to ensure that at least 100 ng DNA was still present.

A new MinION Flow Cell was inserted into the MinION (both Oxford Nanopore Technologies), which was connected to a laptop with the MinKNOW software (Oxford Nanopore Technologies) then started. Before loading the library onto the MinION Flow Cell 150 µl 1x Running Buffer was prepared (75 µl 2 x Running Buffer, 3 µl Fuel Mix, both from the Nanopore sequencing kit, and 72 µl nuclease-free

water) and loaded onto the MinION Flow Cell, this was repeated after 10 minutes. The library was prepared for loading by combining 75 µl 2 x Running Buffer, 60 µl nuclease-free water, 3 µl Fuel Mix, and 12 µl of the library to a final volume of 150 µl. This prepared library was loaded onto the MinION Flow Cell. Additional library was loaded onto the MinION Flow Cell, as before, after approximately 16 and 24 hours (run 2 only).

Reads were analysed using the Metrichor program using Workflow 2D Basecalling v 1.14 (Oxford Nanopore Technologies). This software generates fast5 files, which include both the raw data and the resulting base-calls in fastq format. Sequence data from these fast5 files of multiple reads were extracted to a multi-fasta file using a simple script (carried out by Justin O'Grady or Jack Turton). The resulting fasta file was used for the analyses described in later chapters.

# 3 Molecular epidemiology

## 3.1 Introduction

As described in Chapter 1, *P. aeruginosa* 'high-risk clones', including STs 235, 111 and 175 have been identified worldwide and are frequently associated with the carriage of diverse resistance genes, including MBL genes. However, before this study began there were few data available on the molecular epidemiology of MBL-producing *P. aeruginosa* in the UK despite increasing numbers being referred to AMRHAI over recent years.

### 3.1.1 Selection of typing methods

When this study commenced VNTR analysis was the main method used for the typing of *P. aeruginosa* at AMRHAI, having replaced PFGE in 2009. As discussed in Section 1.7, VNTR is a highly discriminatory typing method well suited to outbreak investigation and so was chosen as the primary typing method here. MLST was chosen as a secondary typing method since although it is less discriminatory than VNTR, it is more helpful for comparison of isolates from diverse locations including lineages reported internationally.

### 3.1.2 Aims

The overall aim of this part of the study was to investigate the molecular epidemiology of MBL-producing *P. aeruginosa* in the UK. Specific aims were: (1) to investigate the population structure of MBL-producing *P. aeruginosa* in the UK, using VNTR and MLST, including assessing the prevalence of internationally reported 'high-risk clones' in the UK and (2) to investigate whether any prevalent MBL-producing types represent clonal expansion of strains that had acquired MBLs, or the repeated acquisition of MBL genes by *P. aeruginosa* lineages that are

common anyway in serious infections; this was achieved by VNTR typing of a comparator population of isolates from the BSAC Bacteraemia Surveillance Programme.

This chapter first describes the study population in terms of the patient characteristics and submitting hospitals. It then describes the typing results by VNTR and MLST, and compares these data to typing data for the comparator population of isolates from the BSAC Bacteraemia Surveillance Programme.

## 3.2 Methods

### 3.2.1 Variable number tandem repeat (VNTR) analysis

Nine-locus VNTR analysis was performed as previously described by Turton et al.[104] Primer pairs (table 3.1, table 2.1), targeted nine VNTR loci (ms172, ms211, ms213, ms214, ms217, ms222 ms207, ms209 and ms61). For loci ms207, ms209 and ms61, fluorescently-labelled forward primers were used. PCR was set up as described in Section 2.2.3 on the QIAgility robot (Qiagen). Cycling conditions were 94$^{o}$C for 3 minutes, followed by 35 cycles of 94$^{o}$C for 30 seconds, 60$^{o}$C for 30 seconds and 72$^{o}$C for 45 seconds and a final extension step of 72$^{o}$C for 10 minutes. PCR products for six loci with 54- to 115-bp repeats (ms172, ms211, ms213, ms214, ms217 and ms222) were sized on 1.5% agarose gels run at 150 V for 2 hours. Fluorescently-labelled PCR products corresponding to the remaining three loci (ms207, ms209 and ms61) with 6-bp repeats, were combined with molecular grade water at a dilution of 1:40 in a total volume of 200 µl and 1 µl of each combined dilution was added to 10 µl Hi-Di formamide, and 0.5 µl Genescan LIZ-600 Size Standard (both Applied Biosystems, Foster City, USA). The amplicons were sized by fragment analysis on an ABI sequencing instrument by the GSDU, PHE Colindale and traces analysed using Peak Scanner v2 (Applied Biosystems). Repeat numbers for each locus were determined by comparing amplicon sizes with a table of expected sizes (PHE data). Data were analysed and minimum spanning trees produced using Bionumerics Software v6.1 (Applied Maths).

**Table 3.1:** Primers and repeat lengths for the nine VNTR loci

| VNTR loci | Primer 1[a] | Primer 2[a] | VNTR repeat length (bp) |
|---|---|---|---|
| ms172 | ms172L | ms172R | 54 |
| ms211 | ms211L | ms211R | 101 |
| ms213 | ms213L | ms213R | 103 |
| ms214 | ms214L | ms214R | 115 |
| ms217 | ms217L | ms217R | 109 |
| ms222 | ms222L | ms222R | 101 |
| ms207 | ms207L | ms207R | 6 |
| ms209 | ms209L | ms209R | 6 |
| ms61 | ms61L | ms61R | 6 |

[a]refer to table 2.1 for primer sequences

### 3.2.2 Multi-Locus Sequence Typing (MLST)

MLST was undertaken as described by Curran et al.[110] PCR and sequencing of the seven housekeeping genes *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE* was carried out using the primers described in table 3.2; sequences of the primers are shown in table 2.1. Cycling conditions were 95$^{o}$C for 5 minutes, followed by 35 cycles of 95$^{o}$C for 1 minute, 55$^{o}$C for 1 minute and 72$^{o}$C for 1 minute, and a final extension step of 72$^{o}$C for 10 minutes. Sequencing of the PCR products was carried out as described in Section 2.2.3.4. Sequences were analysed using Bionumerics Software v6.1 (Applied Maths) and sequence types (STs) assigned using the *P. aeruginosa* MLST database (http://pubmlst.org/paeruginosa/).

**Table 3.2:** Primers used for MLST

| MLST loci | Amplification | | Sequencing | |
|---|---|---|---|---|
| | **Primer 1**[a] | **Primer 2**[a] | **Primer 1**[a] | **Primer 2**[a] |
| *acsA* | acsA-F | acsA-R | acsA-SF | acsA-SR |
| *aroE* | aroE-F | aroE-R | aroE-SF | aroE-SR |
| *guaA* | guaA-F | guaA-R | guaA-SF | guaA-SR |
| *mutL* | mutL-F | mutL-R | mutL-SF | mutL-SF |
| *nuoD* | nuoD-F | nuoD-R | nuoD-SF | nuoD-SF |
| *ppsA* | ppsA-F | ppsA-R | ppsA-SF | ppsA-SF |
| *trpE* | trpE-F | trpE-R | trpE-SF | trpE-SF |

[a]refer to table 2.1 for primer sequences

### 3.2.3 **Sequencing of MBL genes**

Sequencing was carried out as described in Section 2.2.3.4. For sequencing of $bla_{VIM}$ and $bla_{IMP}$ MBL genes, primers specific to $bla_{VIM}$ (VIM2004A, VIM2004B) or $bla_{IMP}$ (IMP_Fwd, IMP_Rev) were used together with those specific to class 1 integrons (5'CS, 3'CS_1, TniCF). Sequencing of $bla_{NDM}$ genes was performed with primers NDM-orfF and NDM-orfR. Primer sequences are shown in table 2.1.

## 3.3 Results

### 3.3.1 Patient and isolate characteristics

As described in Chapter 2, 267 *P. aeruginosa* non-duplicate-patient isolates were selected for study. The number of MBL-producing *P. aeruginosa* isolates submitted to AMRHAI increased steadily over the ten-year period from two in 2003 to 52 in 2012, as did the number of hospital laboratories referring isolates to us, with two in 2003 and 35 in 2012 (figure 3.1). Of the total 89 referring laboratories, 83 submitted isolates from fewer than ten patients, with 71 submitting only one to two isolates each. The remaining six laboratories submitted between 13 and 31 isolates each, collectively accounting for 43% (116/267) of all source patients, with isolates from each hospital being submitted over long periods of six to nine years.

Patient demographic and isolation site data were extracted from the PHE laboratory information management system, and were based on information provided by referring laboratories. Data for the 267 non-duplicate-patient isolates are presented in table 3.3. Ages of the patients ranged from 0-94, mean 54 yrs. Sixty percent were male, 35% female; for 5% gender was not stated. Predominant isolation sites were urine (24%), respiratory tract (18%), wounds (17%), blood (13%), and indwelling devices (7%), whereas few were from skin (3%) and faecal (3%) specimens.

*bla*$_{VIM}$ genes alone were detected in 243 isolates (91%), *bla*$_{IMP}$ in 22 (8.2%), and *bla*$_{NDM}$ in one (0.4%); one further isolate had both *bla*$_{VIM}$ and *bla*$_{NDM}$. The specific *bla*$_{MBL}$ alleles seen are described in Section 3.3.2.5.

MIC data were available for 224/267 isolates tested as part of the AMRHAI's reference service; the distributions of MICs among the isolates are shown in table 3.4. The majority of MBL-producing *P. aeruginosa* isolates were resistant to almost all tested antibiotics. The main exceptions were aztreonam to which 69% of tested isolates remained intermediate, or for one isolate susceptible, and colistin, to which all isolates remained susceptible.

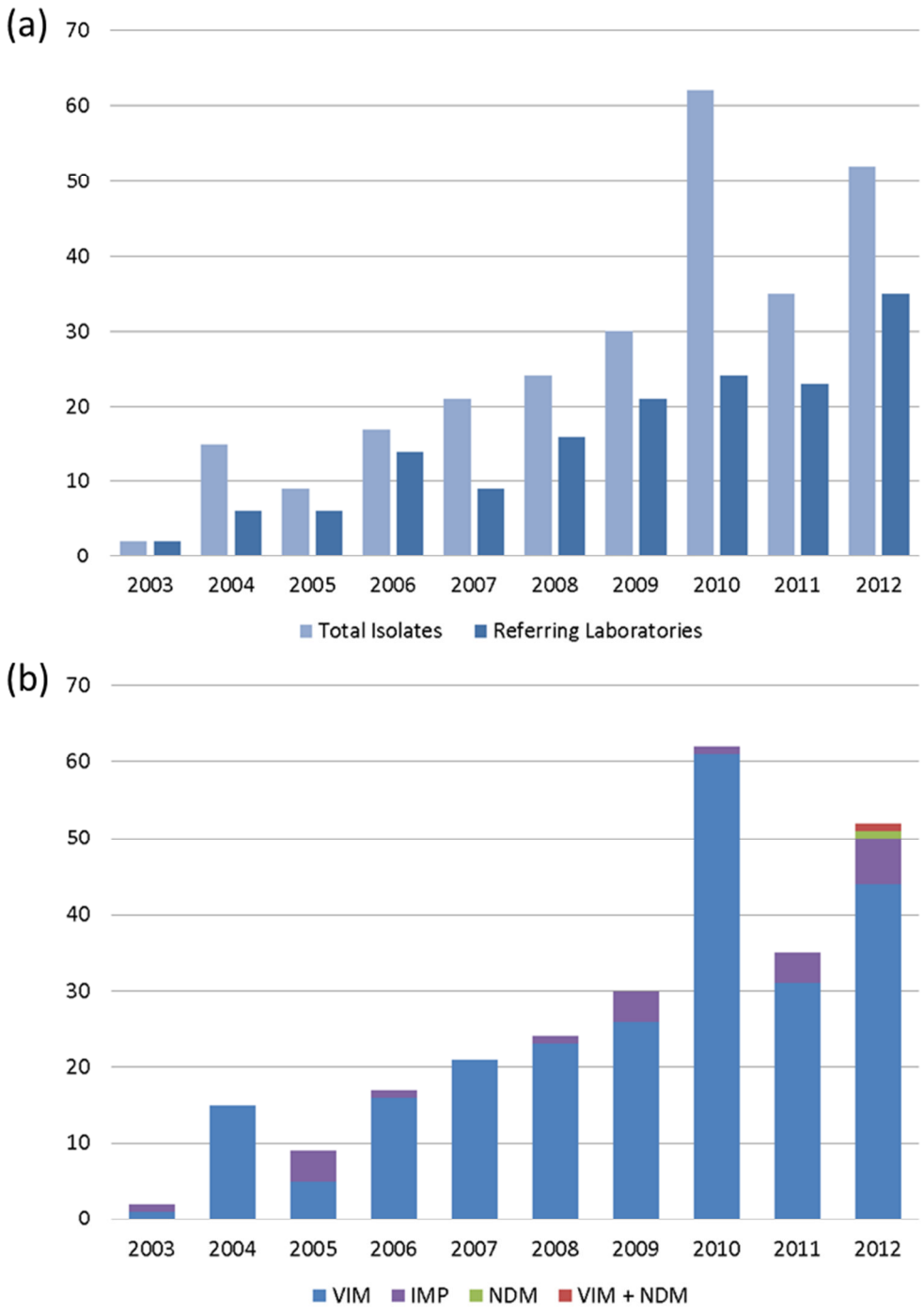



**Figure 3.1:** (a) Numbers of isolates and referring laboratories and (b) proportions of different MBL classes among the 267 non-duplicate-patient MBL-producing *P. aeruginosa* referred to the UK reference laboratory for each year from 2003 to 2012.

**Table 3.3:** Demographic and isolation site data for MBL-producing *P. aeruginosa* isolates (n=267)

| | **Number of patients (%)** |
|---|---|
| **Age (years)** | |
| 0-4 | 12 (4) |
| 5-19 | 6 (2) |
| 20-39 | 36 (13) |
| 40-49 | 29 (11) |
| 50-59 | 39 (15) |
| 60-69 | 58 (22) |
| 70-79 | 48 (18) |
| 80+ | 11 (4) |
| Not available | 28 (10) |
| | |
| **Gender** | |
| Female | 94 (35) |
| Male | 160 (60) |
| Not available | 13 (5) |
| | |
| **Isolation site** | |
| Urine | 65 (24) |
| Respiratory | 47 (18) |
| Wound | 45 (17) |
| Blood | 35 (13) |
| Indwelling device | 19 (7) |
| Skin | 9 (3) |
| Faeces | 8 (3) |
| Others | 20 (8) |
| Not available | 19 (7) |

**Table 3.4:** MIC distributions for MBL-producing *P. aeruginosa* isolates (n=267)

| Antibiotic | Number of isolates with MIC (mg/L) | | | | | | | | | | | ND | %S | %I | %R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ≤0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | ≥128 | | | | |
| Amikacin | | | | 1 | 3 | 7 | 10 | 25 | 28 | 61 | 86 | 46 | 9.5 | 11.3 | 79.1 |
| Gentamicin | | | 1 | 1 | 7 | 9 | 7 | 11 | 32 | 153* | | 46 | 8.2 | - | 91.8 |
| Tobramycin | | 1 | 1 | 1 | 1 | 1 | 1 | 11 | 26 | 177* | | 47 | 2.3 | - | 97.7 |
| Piperacillin-tazobactam | | | | | | | 1 | 18 | 44 | 60 | 96 | 48 | 8.7 | - | 91.3 |
| Ceftazidime | | | | | | 2 | 3 | 44 | 49 | 37 | 86 | 46 | 2.3 | - | 97.7 |
| Imipenem | | | | | | 1 | 4 | 7 | 18 | 68[a] | 126 | 43 | 0.4 | 1.8 | 97.7 |
| Meropenem | | | | 1 | | 6 | 12 | 13 | 24 | 168* | | 43 | 0.4 | 2.7 | 96.9 |
| Aztreonam | | | 1 | | 1 | 14 | 54 | 80 | 20 | 9 | 38 | 50 | 0.4 | 68.7 | 30.9 |
| Ciprofloxacin | 2 | 5 | 6 | | 1 | 5 | 3 | 199* | | | | 46 | 5.9 | 0 | 94.1 |
| Colistin | | | 7 | 181 | 32 | | | | | | | 47 | 100 | - | 0 |

S, susceptible; I, intermediate; R, resistant; ND: not determined. Cells shaded dark grey represent resistant isolates, light grey represents intermediate, and white susceptible

*Isolates with MIC ≥ indicated value

[a]includes 22 isolates for which the MICs were reported as >32 mg/L

### 3.3.2 Typing of MBL-producing isolates

#### 3.3.2.1 VNTR typing

Among the 267 non-duplicate-patient isolates, 232 had VNTR data already available or generated in this study (figure 3.2). A minimum spanning tree for these 232 isolates is shown in figure 3.3. The remaining 35 isolates were not available in the archive for retrospective VNTR typing, but 19 had previously-determined PFGE profiles (not shown) identical to those of isolates with known VNTR types and collected over the same time period at the same hospital. These isolates were therefore assumed to belong to the corresponding VNTR complex and are included, along with the 232 VNTR-typed isolates in table 3.6, as shown in figure 3.2. The remaining 16/35 unavailable isolates had no typing data, or belonged to PFGE types unique to their respective hospital; these latter isolates, from 12 laboratories, are excluded from table 3.6; all carried $bla_{VIM}$.

Six VNTR complexes (designated A-F) accounted for 86% of the 251 isolates with VNTR data available or inferred from PFGE data (table 3.5, figure 3.3). Owing to their predominance, isolates belonging to these complexes became the focus of further study. Isolates belonging to each of the six VNTR complexes were referred from nine to 25 laboratories each. Single VNTR types persisted at a few sites for prolonged periods ranging from seven months to nine years (table 3.6), as detailed in the following sections.

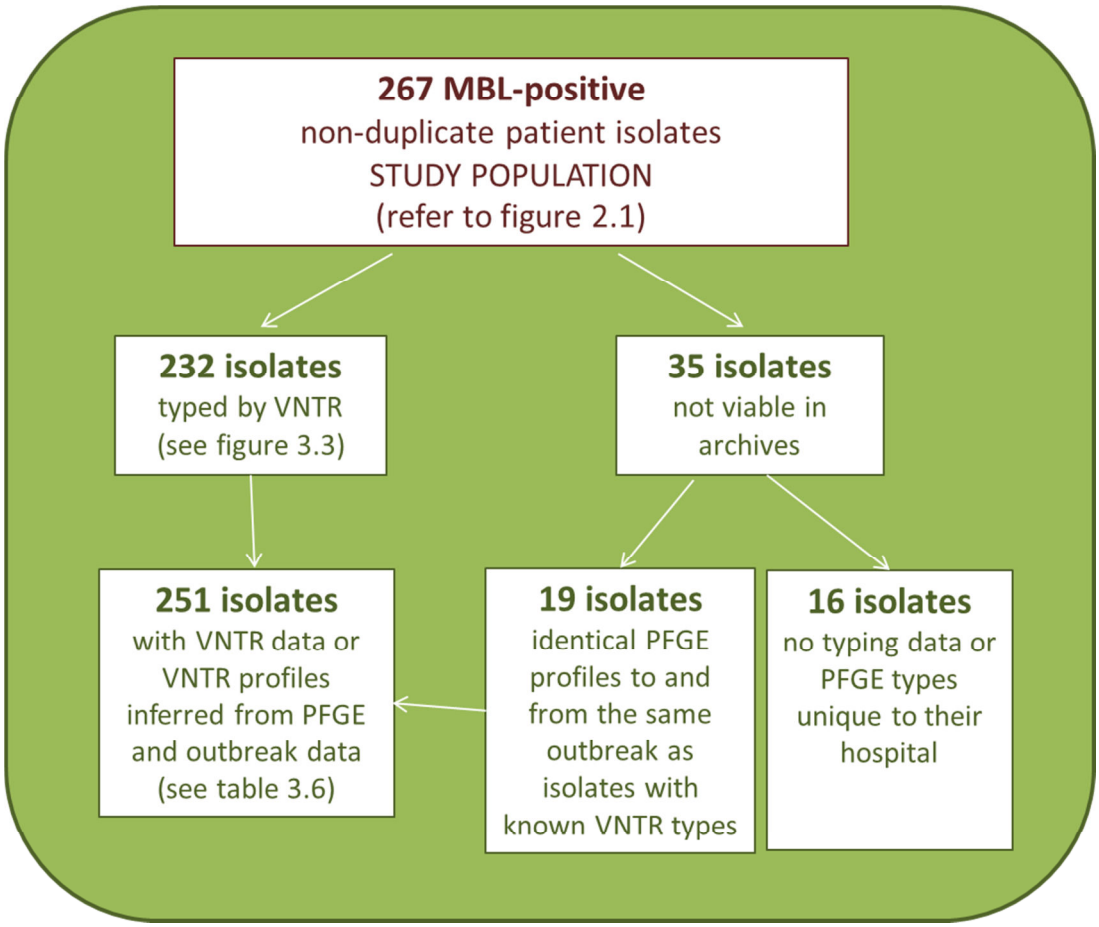



**Figure 3.2:** Isolates typed by VNTR from the study population of 267 non-duplicate-patient MBL-producing *P. aeruginosa* isolates.

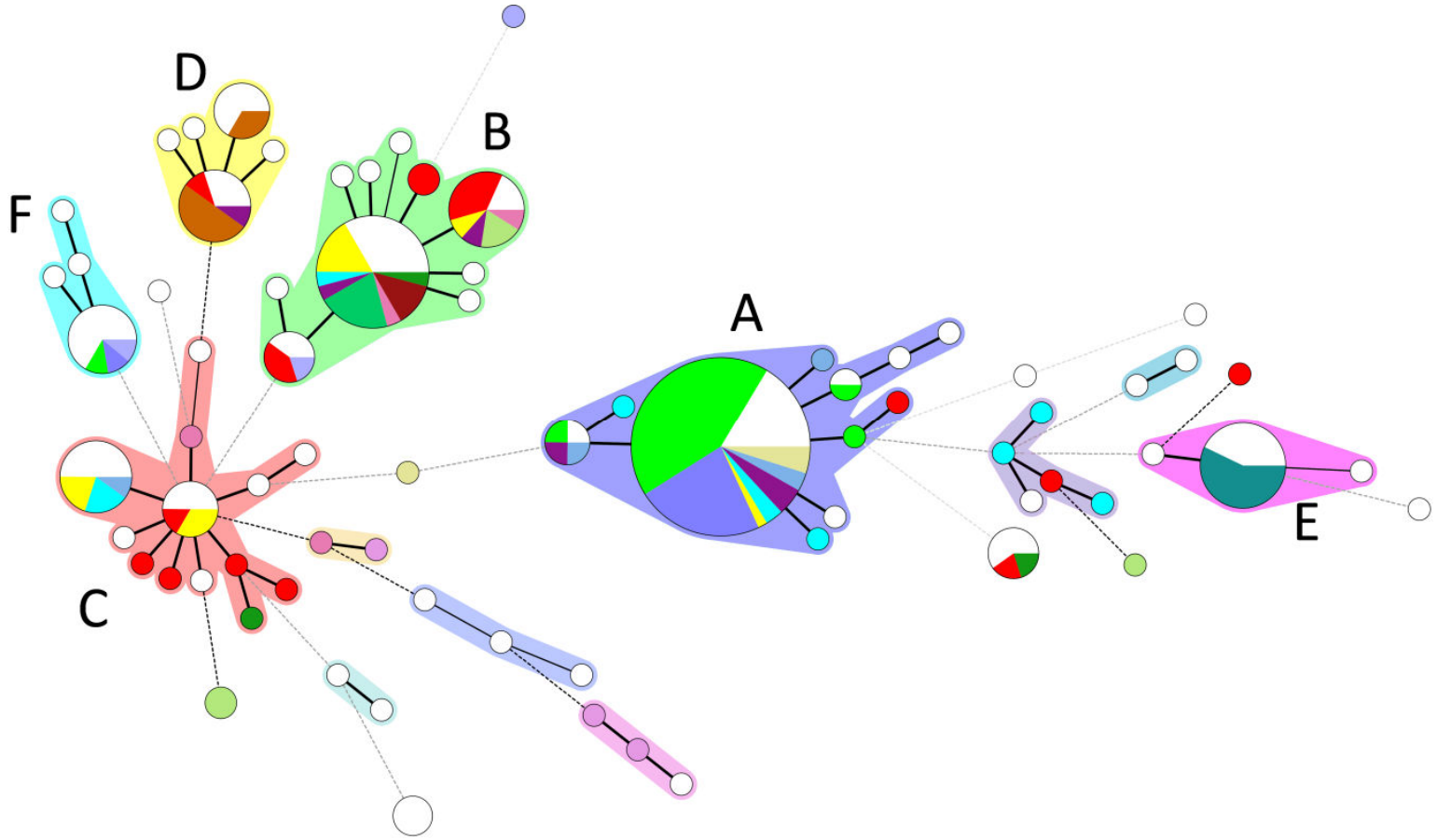



**Figure 3.3:** Minimum spanning tree, based on clustering at the first eight VNTR loci for MBL-positive *P. aeruginosa*, with one isolate included per patient (n=232). The six main complexes A-F are labelled. Coloured segments of the circles indicate laboratories that submitted three or more isolates, white segments represent laboratories submitting one or two isolates. The diameters of the circle are relative to the number of isolates with the VNTR profile. Shading indicates complexes. Thick solid lines represent single-locus variants; thin solid lines and dotted lines represent multi-locus variants**.**

**Table 3.5:** VNTR complexes identified among the MBL-producing *P. aeruginosa* isolates (n=251)

| VNTR Complex | VNTR type [a] | No. of different VNTR profiles | MLST type(s) (no. of isolates tested) | No. of isolates[b] | No. of submitting laboratories | MBLs detected (no. of isolates) |
|---|---|---|---|---|---|---|
| **A** | 11,3,4,3,2,2,x,4,x | 6 | ST111 (11) | 75 | 25 | VIM (70)<br>IMP (5) |
| **B** | 13,3,6,4,5,1,x,2,x | 16 | ST235 (18) | 52 | 25 | VIM (46)<br>IMP (6) |
| **C** | 12,3,4,5,3,1,x,2,x | 11 | ST233 (10) | 26 | 16 | VIM (26) |
| **D** | 11,3,2,15,3,1,x,3,x | 6 | ST654 (10), ST964 (1) | 19 | 11 | VIM (17)<br>IMP (1)<br>NDM (1) |
| **E** | 13,2,1,5,2,3,6,x,x | 7 | ST357 (9) | 30 | 9 | VIM (30) |
| **F** | 12,4,6,5,3,1,10,x,x | 3 | ST773 (5) | 13 | 11 | VIM (13) |
| **Others** | Diverse | 26 | Not done | 36 | 25 | VIM (25)<br>IMP (10)<br>VIM and NDM (1) |

[a] x represents loci where the repeat number varies among isolates within a complex

[b] one isolate per patient was included; these numbers include four isolates (complex B), 14 isolates (complex E) and one isolate (complex F) where the MBL-positive organisms were no longer available in the archive for VNTR analysis, but which were previously found to share a PFGE profile, and are from the same hospital outbreak as other isolates in the respective complex. Isolates with a PFGE profile corresponding to complex A were also received from an additional 39 patients at London_17; these are not included here as they had not been screened for MBL genes and were no longer available in AMRHAI's archives.

**Table 3.6:** Geographical and temporal distribution of MBL-producing *P. aeruginosa* belonging to the six major complexes (215 isolates) among referring laboratories

| **VNTR Complex** | **Major Contributors (≥ 5 referred isolates)** | | | | **Minor Contributors** |
|---|---|---|---|---|---|
| | Referring laboratory[a] | Number of isolates[b] | Time period over which isolates referred | MBL genes detected | |
| A (n=75) | London_17 [c] | 29 (39%) | 80 months | all with *bla*$_{VIM}$ | 2 laboratories with 4 representatives each |
| | Wales_1 | 13 (17%) | 18 months | all with *bla*$_{VIM}$ | 2 laboratories with 3 representatives each |
| | | | | | 19 laboratories with 1 representative each |
| B (n=52) | London_7 | 8 (15%) | 87 months | all with *bla*$_{VIM}$ | 1 laboratory with 3 representatives |
| | London_13 | 8 (15%) | 7 months | all with *bla*$_{VIM}$ | 6 laboratories with 2 representatives each |
| | Scotland_2 | 6 (12%) | 27 months | all with *bla*$_{VIM}$ | 15 laboratories with 1 representative each |
| C (n=26) | London_7 | 5 (19%) | 27 months | all with *bla*$_{VIM}$ | 3 laboratories with 2 representatives each |
| | London_13 | 4 (15%) | 18 months | all with *bla*$_{VIM}$ | 11 laboratories with 1 representative each |
| D (n=19) | South East_6 | 7 (37%) | 36 months | all with *bla*$_{VIM}$ | 2 laboratories with 2 representatives each |
| | | | | | 8 laboratories with 1 representative each |
| E (n=30) | North West_15 | 22 (73%) | 103 months | all with *bla*$_{VIM}$ | 8 laboratories with 1 representative each |
| F (n=13) | No major contributors | | | | 2 laboratories with 2 representatives each |
| | | | | | 9 laboratories with 1 representative each |

[a]referring laboratories are coded in the format 'UK region_number'.

[b]one isolate per patient is included; these numbers include four isolates (complex B), 14 isolates (complex E) and one isolate (complex F) that were not available in the archive for VNTR analysis, but which shared a PFGE profile and are from the same hospital outbreak as other isolates in the complex.

[c] Isolates were also received from an additional 39 patients at London_17 with a PFGE type corresponding to complex A. These are not included here as they had not been screened for MBL production and were no longer available in AMRHAI's archives.

#### 3.3.2.2 MLST analysis

Sixty-four organisms, representing the variation in VNTR profile within each of the six main complexes, were selected for MLST analysis. Isolates belonging to complexes A, B, C, D, E and F were found to belong to ST111, ST235, ST233, ST654/ST964, ST357 and ST773, respectively (table 3.5). Ten of 11 MLST-typed complex D isolates belonged to ST654, but one belonged to ST964; this is a single locus variant (SLV) differing only in the *acsA* allele, where ST964 has allele 145 with a single C to T substitution compared with allele 17 in ST654.

#### 3.3.2.3 Distribution of isolates from each complex among referring laboratories

Table 3.6 shows the distribution of each of the major complexes among referring laboratories. The largest group of isolates was complex A (corresponding to ST111; VNTR type 11,3,4,3,2,2,x,4,x, where x is variable), with 75 representatives. It included isolates from 25 laboratories spread across the UK, with few epidemiological links between them. More than half of the isolates came from long term 'outbreaks' at London_17 and Wales_1, referring 29 and 13 isolates scattered over seven-year (2006-2012) and 18-month (2010-2011) periods, respectively, all with $bla_{VIM}$. Most other complex A isolates (28/33) also had $bla_{VIM}$, but five, from two laboratories, had $bla_{IMP}$. VNTR profiles were highly conserved amongst all complex A isolates, with most only differing by repeat numbers between six and eight at locus 61. For nine isolates the referring laboratory had provided recent patient travel history; only two of these patients had recently travelled, both to Greece.

Complex B (ST235; 13,3,6,4,5,1,x,2,x) was the second largest group, comprising 52 isolates referred from 25 different laboratories. Major contributors included London_13, referring eight *bla*$_{VIM}$-positive isolates over a seven-month period in 2004. Scotland_2 referred six *bla*$_{VIM}$-carrying isolates; five of these six were received over a five month period in 2008-2009 while the remaining isolate was collected 27 months after the initial representative, in 2010. Another laboratory (London_7) also submitted complex B isolates carrying *bla*$_{VIM}$ from eight patients scattered over an eight-year period, albeit with three of these isolates received over a four-month period in 2010. The remaining 30 complex B isolates were from 22 laboratories, each submitting one to three representatives; 24 of these diverse-source representatives had *bla*$_{VIM}$, and six had *bla*$_{IMP}$. Six of 16 patients with corresponding travel data had travelled recently to Italy (three patients), the United Arab Emirates, Thailand or India.

Twenty-six isolates belonged to complex C (ST233; 12,3,4,5,3,1,x,x,x). These all carried *bla*$_{VIM}$ and were from 16 laboratories, which submitted one to five representatives each. They included persistent ST233 lineages at London_7 and London_13, referring five or four isolates scattered over 27- and 18-month periods, respectively. Of ten isolates with patient travel data, three patients had recently travelled, to South Africa, Saudi Arabia or Kuwait.

Nineteen isolates from 11 laboratories belonged to complex D (ST654/964; 11,3,2,15,3,1,x,3,x). Seven *bla*$_{VIM}$-positive representatives were submitted from South East_6 over a three year period (2009-2012), although four of the seven were submitted over a two-week period in 2010. One or two isolates were referred from

each of the remaining ten laboratories; ten isolates had *bla*$_{VIM}$, one had *bla*$_{IMP}$, and one *bla*$_{NDM}$. Corresponding patient travel data were available for five isolates; two VIM-producing isolates were associated with travel to Russia or the Indian subcontinent, while the isolate producing an NDM enzyme was associated with patient travel to India.

Most of the 30 complex E isolates (ST357; 13,2,1,5,2,3,6,5,x), all with *bla*$_{VIM}$, were from an outbreak at North West_15, with 22 isolates referred over a nine-year period, though 12/22 were submitted in 2007. Each of the remaining eight complex E isolates was referred from a different laboratory. Patient travel data were provided for seven of the isolates; three patients had recently travelled to Egypt, India, or both Dubai and India.

Finally, 13 isolates, all with *bla*$_{VIM}$, belonged to complex F (ST773; 12,4,6,5,3,1,10,x,x) and were received from 11 laboratories in diverse areas of the UK. Patient travel history was provided for five isolates, with patients having travelled to India (three patients), or an unknown location (one patient), while the one remaining patient had not recently travelled.

The 14% of isolates (n=36) that did not belong to any of these six major complexes were diverse, representing 26 different VNTR profiles. They were referred from 24 laboratories across the UK, each submitting one to three isolates each; 26 had *bla*$_{VIM}$, 10 had *bla*$_{IMP}$ and one had both *bla*$_{VIM}$ and *bla*$_{NDM}$ genes.

Among the six laboratories that submitted more than ten isolates, three were dominated by representatives of a single VNTR complex while the remaining three

submitted diverse types; timelines showing examples of each situation are shown in figure 3.4. The three laboratories submitting isolates belonging to predominantly one type included London_17 (figure 3.4) where 29/31 isolates referred over a seven-year period belonged to complex A (ST111). The other two such laboratories were North West_15, with all 22 isolates referred over nine years belonging to complex E (ST357) and Wales_1 where 14/15 isolates referred over six years belonged to complex A (ST111).

The three laboratories contributing more than ten isolates with diverse types represented included London_7 (figure 3.4) and London_12, referring 19 and 15 isolates, respectively, over seven- and eight-year periods. Isolates belonging to complexes A, B and C (STs 111, 235, 233) were seen at both laboratories and London_7 also referred an isolate belonging to ST654. The remaining laboratory, London_13, had temporally-separate outbreaks (lasting seven to 18 months) of MBL-positive isolates belonging to complexes B and C (STs 235 and 233), with eight and four representatives, respectively, separated by a six-year gap; an ST111 isolate was also received from the laboratory.

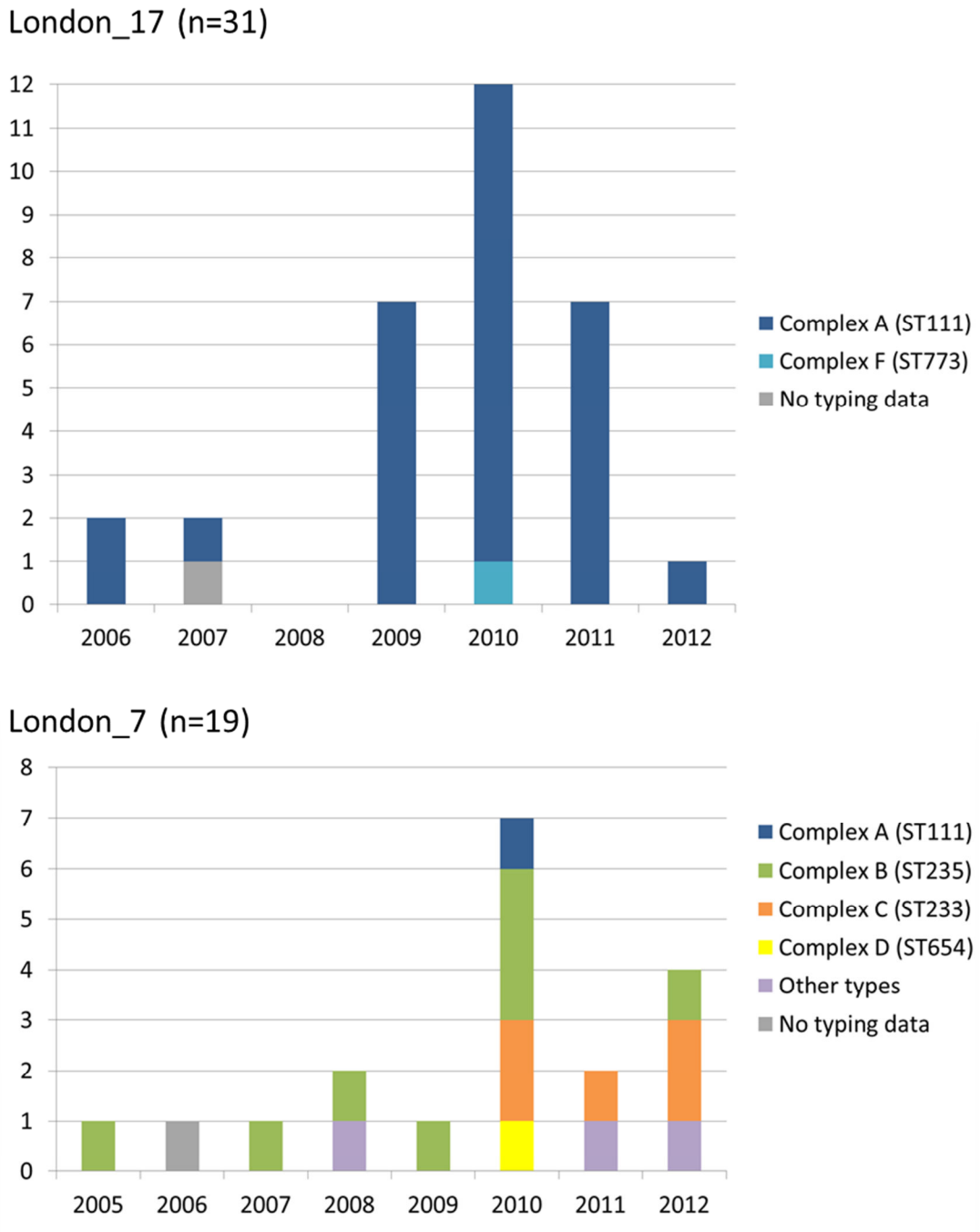



**Figure 3.4:** Time plots showing the prevalence of different VNTR complexes at London_17 and London_7. Laboratories referred isolates of a single predominant VNTR type (London_17) or of diverse types (London_7). Pre-2010 isolates were also received from an additional 39 patients at London_17 with a PFGE type, corresponding to complex A. These are not included here as they had not been screened for MBL production and were no longer available in AMRHAI's archives, but are likely to be representatives of the same strain.

#### 3.3.2.4 Regional distribution of MBL-positive isolates

The distribution of the 267 non-duplicate patient isolates among sub-regions of the UK is shown in figure 3.5. London accounted for 47% of these organisms, with MBL-positive isolates referred from 28 London-region laboratories; all six major complexes (A-F) were represented in the capital, and nine other VNTR types were seen. Four of these 28 London laboratories were among the six sites that submitted more than 10 isolates, as described above. Twelve per cent (31/267) of isolates were referred from six different laboratories in the Greater Manchester sub-region. Isolates belonging to complex E (ST357) predominated there, mostly from the nine-year persistence at North West_15, although isolates belonging to complexes A, B and D (STs 111, 235 and 654) were also seen at the five remaining laboratories. In the Sussex, Surrey and Kent sub-region half of the 18 representatives, received from five sites, belonged to complex D (ST654) with seven of these nine received from South_East_6; the remaining 9/18 isolates belonged to complexes B and C (STs 235 and 233) or had other VNTR profiles. Wales accounted for 6% (15/267) of isolates, mostly (13/15) from the outbreak of ST111 at Wales_1, although one ST111 isolate was from another laboratory and one ST773 isolate was referred from the region. Finally, the 12 (4%) isolates submitted from five laboratories in Scotland variously belonged to complexes A, B, C and F (STs 111, 235, 233 and 773). Fewer than ten isolates were referred from each of the remaining UK sub-regions.

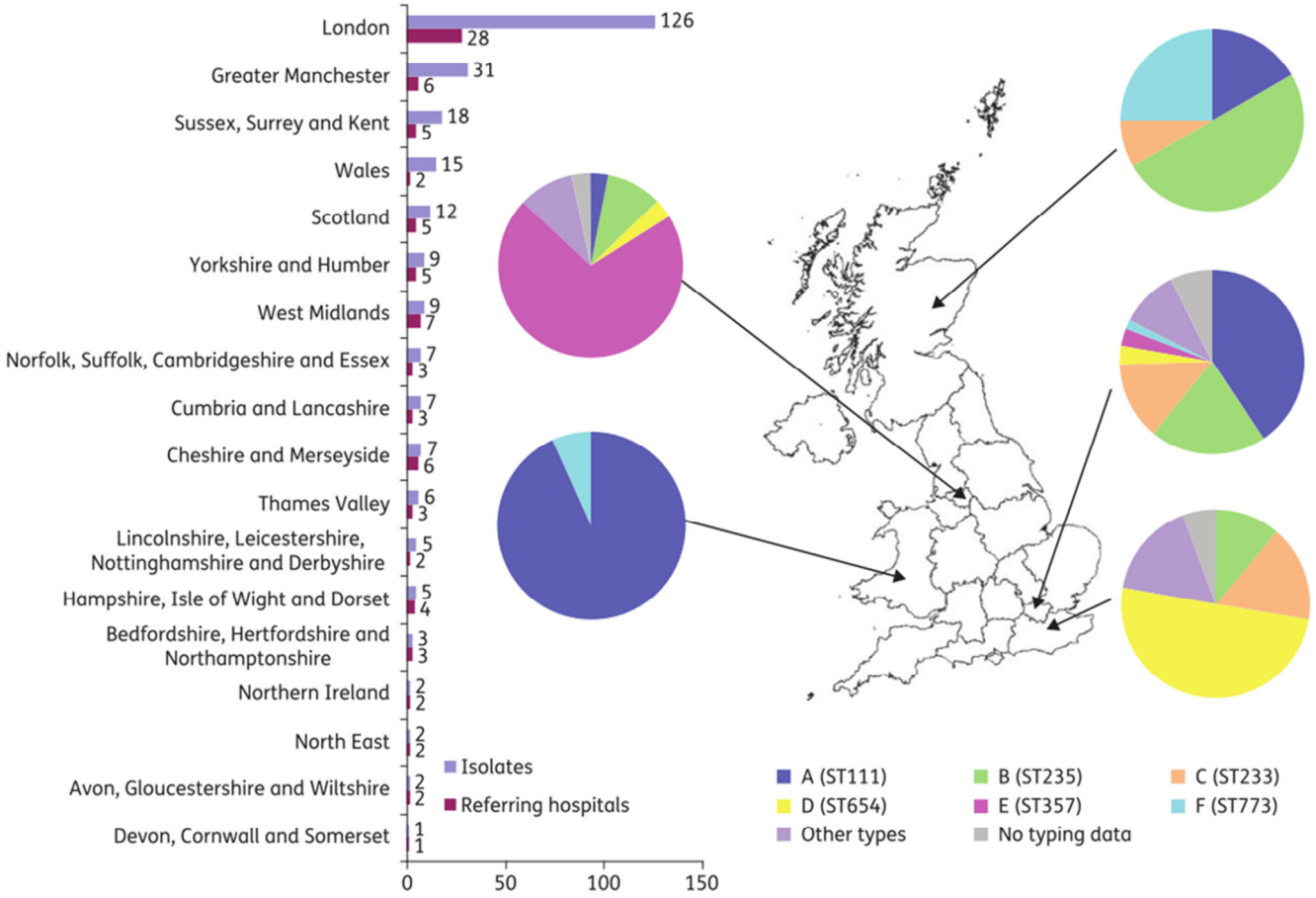



**Figure 3.5:** Geographical sources of isolates in the UK, and distribution of the six main complexes (A-F) in each of the five sub-regions referring more than ten isolates. The sub-regions represented in this map are described in figure 2.2.

#### 3.3.2.5 Sequencing of MBL genes

Isolates belonging to complexes C, E and F (STs 233, 357 and 773) consistently carried *bla*$_{VIM}$, while complex A, B and D isolates (STs 111, 235 and 654/964) variously carried *bla*$_{VIM}$, *bla*$_{IMP}$, or *bla*$_{NDM}$ genes.

In order to assess the diversity of MBL gene alleles within the six main complexes, representatives were selected to cover the VNTR variation. In complexes A, C, D, E and F (STs 111, 233, 654, 357 and 773) all *bla*$_{VIM}$-positive representatives had *bla*$_{VIM-2}$ alleles, whereas eight isolates selected from complex B (ST235) variously had *bla*$_{VIM-1}$ (two isolates), *bla*$_{VIM-2}$ (four isolates), *bla*$_{VIM-4}$ (one isolate) or *bla*$_{VIM-6}$ (one isolate). Amongst eight representatives of the 22 *bla*$_{IMP}$-positive isolates, *bla*$_{IMP-1}$ (three isolates), *bla*$_{IMP-7}$ (two isolates), *bla*$_{IMP-10}$ (one isolate) and *bla*$_{IMP-13}$ (two isolates) alleles were seen. Both *bla*$_{NDM}$-positive isolates had the *bla*$_{NDM-1}$ allele.

### 3.3.3 Typing of comparator *P. aeruginosa* bacteraemia isolates

As described in Section 2.1.2, the comparator set comprised 209 *P. aeruginosa* isolates collected in the UK and Ireland as part of the BSAC Bacteraemia Resistance Surveillance Programme in 2011. Patient demographic and MIC data, provided by the BSAC, are presented in tables 3.7 and 3.8. Sixty percent of patients were male and 40% female, while the most prevalent age groups were 60-69 (23%), 70-79 (27%) and 80+ (18%). Most isolates remained susceptible to all tested antibiotics. Only 4.8% were resistant to the carbapenems imipenem and meropenem, while 6.7% were resistant to doripenem. None of these isolates carried MBLs, indicating

that their carbapenem resistance was likely mediated by mutations leading to OprD inactivation, AmpC hyperproduction or increased efflux.

All 209 isolates were typed by VNTR and showed far greater diversity than the MBL producers, with 136 different VNTR profiles represented (figure 3.6). Two isolates had a VNTR profile consistent with complex B (ST235) and one had a profile indicative of complex C (ST233); the remaining 206 isolates did not share VNTR profiles with any of the major complexes identified amongst the MBL-producing isolates.

**Table 3.7:** Patient demographic data for BSAC bacteraemia isolates from 2011 (n=209)

| Demographic | Number of patients (%) |
|---|---|
| **Age (years)** | |
| 0-4 | 10 (5) |
| 5-19 | 5 (2) |
| 20-39 | 12 (6) |
| 40-49 | 13 (6) |
| 50-59 | 26 (12) |
| 60-69 | 48 (23) |
| 70-79 | 56 (27) |
| 80+ | 37 (18) |
| Not available | 2 (1) |
| | |
| **Gender** | |
| Female | 83 (40) |
| Male | 126 (60) |

**Table 3.8:** MIC distributions for 209 *P. aeruginosa* isolates from the BSAC Bacteraemia Resistance Surveillance Programme in 2011

| Antibiotic | Number of isolates with MIC (mg/L) | | | | | | | | | | | %S | %I | %R |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ≤0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | ≥128 | | | |
| Gentamicin | 3 | 2 | 35 | 122 | 36 | 5 | 2 | | 1 | | 3 | 97.1 | - | 2.9 |
| Ceftazidime | | | 6 | 71 | 98 | 20 | 10 | 1 | 3* | | | 98.1 | - | 1.9 |
| Imipenem | 3 | 13 | 26 | 110 | 35 | 4 | 8 | 10* | | | | 91.4 | 3.8 | 4.8 |
| Meropenem | 42 | 70 | 47 | 20 | 12 | 8 | 3 | 6 | | 1* | | 91.4 | 3.8 | 4.8 |
| Doripenem | 98 | 49 | 36 | 9 | 3 | 11 | 1 | 1 | 1* | | | 91.9 | 1.4 | 6.7 |
| Piperacillin-tazobactam | | | | 2 | 31 | 125 | 29 | 12 | 6 | 1 | 3 | 95.2 | - | 4.8 |
| Ciprofloxacin | 73 | 83 | 17 | 19 | 3 | 3 | 3 | 5 | 1 | 1 | 1 | 82.8 | 9.1 | 8.1 |
| Colistin | | 2 | 6 | 199 | 2 | | | | | | | 100 | - | 0 |

S, susceptible; I, intermediate; R, resistant. Cells shaded in dark grey represent resistant isolates, light grey represent intermediate, and white susceptible.

*Isolates have an MIC of ≥ the indicated value

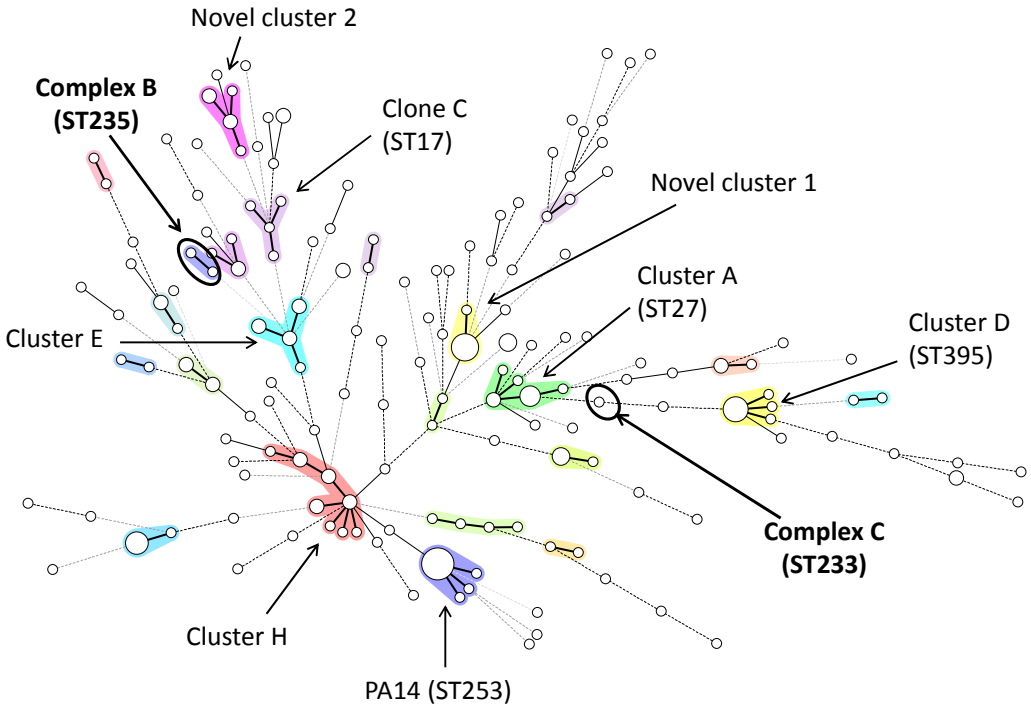



**Figure 3.6:** Minimum spanning tree based on clustering at the first eight VNTR loci for *P. aeruginosa* isolates from the BSAC Bacteraemia Resistance Surveillance Programme (n=209). The diameter of the circle is relative to the number of isolates with that VNTR profile. Shading indicates complexes. Thick solid lines represent single locus variants while thin solid lines and dotted lines represent multi-locus variants. Isolates corresponding to VNTR complexes B and C (STs 235 and 233, respectively) are shown in black circles. Isolates corresponding to previously reported clones as described by Martin et al.[115] are indicated and had the following VNTR profiles; Clusters A (8,3,4,5,2,3,5,2,x), D (10,3,5,5,4,1,3,x,x,), E (11,4,5,2,2,1,x,2,x) and H (12,5,1,5,2,2,x,x,x), Clone C (11,6,2,2,1,3,7/8,2/3,x), and PA14 (12,2,1,5,5,2,x,5,x). Two novel clusters are indicated with VNTR profiles 12,5,5,5,4,3,7,6,x and 12,8,2,2,4,3,5,1,x, respectively.

## 3.4 Discussion

Most (86%) of the MBL-producing *P. aeruginosa* isolates referred to the UK reference laboratory between 2003 and 2012 belonged to six VNTR complexes, corresponding to internationally recognised 'high-risk clones', STs 111, 235, 233, 357, 654 and 773, and the majority had VIM-type MBLs. These findings are in striking contrast to the general population structure of *P. aeruginosa*, as described in Chapter 1, where a recent UK study[115] shows considerable diversity, with overlap between environmental isolates and those causing clinical infection, although with a few prevalent clusters in diverse locations. This general diversity was confirmed by typing isolates from the BSAC Bacteraemia Surveillance Programme.

The six major complexes seen here among MBL-producers were not prevalent amongst MBL-negative isolates in this previous UK study, [115] nor were they prevalent in our comparator collection of (largely susceptible) *P. aeruginosa* isolates from the BSAC Bacteraemia Resistance Surveillance Programme, where they accounted for just 1.4% (3/209) of isolates. Rather, these 'high-risk clones' seem to represent a distinct subset of *P. aeruginosa* lineages, which may be successful precisely due to a particularly strong ability to acquire and/or maintain resistance genes compared with the general *P. aeruginosa* population. Notably, the reference laboratory has also received representatives corresponding to these six major complexes that carry non-metallo-carbapenemases, including a few ST235 isolates carrying $bla_{\text{GES-5}}$ and two isolates from a single patient with a VNTR profile corresponding to ST773 and both carrying $bla_{\text{OXA-181}}$ (unpublished AMRHAI data). Moreover, in three of these six complexes, we encountered isolates that variously

had either *bla*$_{VIM}$ or *bla*$_{IMP}$ genes, and in one complex (B; ST235), several different *bla*$_{VIM}$ alleles, indicating that organisms belonging to the same complex had independently acquired different genes.

As described in detail in Section 1.8, 'high-risk clones' with STs 111 and 235 are among the most commonly reported lineages amongst MBL-producing *P. aeruginosa* worldwide. These lineages, corresponding to complex A (ST111) and complex B (ST235), were also the most prevalent amongst our UK MBL-producing *P. aeruginosa*, accounting for 30% and 21% of isolates, respectively.

Complex A (ST111) was the largest group in the MBL-producing collection, and most isolates were *bla*$_{VIM}$-positive, with all sequenced representatives carrying *bla*$_{VIM-2,}$ as most commonly reported for ST111 in other European countries,[80,121–127] although we did also receive five representatives with *bla*$_{IMP-1}$ or *bla*$_{IMP-13}$. Although 25 laboratories referred isolates belonging to the complex, it was dominated by representatives from long-term 'outbreaks' at London_17 and Wales_1, lasting 7 years and 18 months, respectively. VNTR profiles within this complex were highly similar even when referred from different hospitals, with most differing only at locus 61, the most variable of the nine VNTR loci.[104]

Isolates belonging to complex B (ST235) had more diverse VNTR profiles than those in complex A. Representatives came from 25 referring laboratories, with no more than eight from any single site, and no known epidemiological links between most isolates. Although most carried *bla*$_{VIM}$, diverse alleles were identified (*bla*$_{VIM-1}$, $_{-2}$, $_{-4}$ and $_{-6}$); this variation is in contrast to the situation in Russia, Belarus and Kazakhstan, where an ST235 clone consistently carried *bla*$_{VIM-2}$ as part of the same

integron structure.[136] Together with the VNTR diversity, this carbapenemase diversity, including enzymes belonging to different VIM subgroups, suggests multiple imports and/or acquisitions of *bla*$_{VIM}$ and *bla*$_{IMP}$ alleles by ST235 *P. aeruginosa* have occurred in UK hospitals.

The other four major complexes (C-F) also correspond to 'high-risk clones,' previously reported as hosts for MBLs. We consistently saw *bla*$_{VIM-2}$ in complex C (ST233) isolates. ST233 representatives with *bla*$_{VIM-2}$ have likewise been found in Norway (imported from Ghana),[124] Japan,[171] South Africa,[86] the USA,[147] and Romania,[172] though a ST233 organism was reported to carry a *bla*$_{IMP-1}$ variant in Singapore.[64] ST654 (complex D) has been reported in Sweden (imported from Tunisia) carrying *bla*$_{VIM-2}$ genes,[124] in Singapore carrying *bla*$_{IMP-1}$ and *bla*$_{IMP-26}$,[64] and in Argentina carrying a KPC carbapenemase.[42] Here we predominantly saw *bla*$_{VIM-2}$, in ST654 but one isolate had *bla*$_{NDM-1;}$ a gene strongly associated with India, to which the patient had travelled. The sole ST964 isolate (also complex D/ST654-related) had *bla*$_{IMP-1}$, as also reported in this ST in Singapore.[64] We saw only *bla*$_{VIM-2}$ in representatives of complex E (ST357), referred from nine different laboratories over a nine-year period, but this ST has previously been reported with *bla*$_{VIM-2}$ or *bla*$_{IMP-7}$ genes in a hospital in the Czech Republic,[68] whilst a few ST357 isolates with *bla*$_{IMP-7}$ were reported in Poland.[65] Finally, ST773 (complex F) has been recently reported among Indian isolates carrying *bla*$_{VIM-2}$.[137] Interestingly, the geographically scattered complex F isolates tested here also all had *bla*$_{VIM-2}$ and four were from patients who had recently travelled to India; travel history for the other nine patients was not available.

The remaining VNTR profiles, accounting for 14% of MBL producers, were diverse, and probably represent multiple separate acquisitions of *bla*$_{VIM}$ and *bla*$_{IMP}$ genes either de novo from an undefined source species or as a result of horizontal exchange of genetic elements between *P. aeruginosa* strains.

Although comprehensive epidemiological data are lacking, VIM-type MBLs clearly are the predominant carbapenemases seen in *P. aeruginosa* in Europe, with only sporadic isolation, and/or local spread of strains producing IMP- or NDM- types (see Section 1.6), although some regional spread of the latter has been reported in the Balkan region.[93]

The 22 isolates harbouring *bla*$_{IMP}$ genes belonged to diverse VNTR types and had varied *bla*$_{IMP}$ alleles (*bla*$_{IMP-1}$, $_{-7}$, $_{-10}$ or $_{-13}$) with no identifiable epidemiological links among most isolates. Just two *bla*$_{NDM-1}$-positive isolates were found, both with different VNTR types. These IMP- and NDM-MBL-producing isolates may have been imported from outside the UK, or acquired locally, with limited spread of IMP-producers at a few sites. Unfortunately, data on patient travel were not available for most isolates, but single isolates carrying *bla*$_{NDM}$ and *bla*$_{IMP}$ were from patients who had travelled to India and Pakistan, respectively.

MBL-positive *P. aeruginosa* were referred from around half the hospital laboratories in the UK, with all six major complexes found in multiple UK regions. Referral of suspect isolates to the AMRHAI reference unit is not mandatory, and some likely MBL producers were no longer viable in AMRHAI's archives, so the numbers reported here under-estimate the true incidence of infection and colonisation by MBL-producing *P. aeruginosa* in the UK.

MBL-producers were rare at most referring sites, but a few sites did have persistent problems with single clones. These included the previously reported outbreak of ST357 *P. aeruginosa* at North West_15[156] where the strain, which also produced a VEB-1a extended-spectrum-β-lactamase, was thought to have been imported via a patient transferred from an Indian hospital, but to have acquired the VIM-MBL locally in the UK. In addition, the persistence of ST111 and ST654 at London_17 and South East_6, respectively, were previously reported[157]. The ST111 strain was seen in more than 89 patient isolates at London_17 between 2005 and 2012,[157] of which 29 were included here. The patients were dispersed across different hospital wards and scattered over the seven-year period, suggesting a widespread environmental source of the organism was more likely than patient-to-patient transfer. Possible environmental sources were investigated as part of this previous study[157] and while all samples of the water supply remained negative, some samples from waste outlets, as well as the hospital sewer, were positive. On this basis, the wastewater system was suggested as the source of these infections, with blockages and subsequent backflow of waste potentially contaminating clinical areas. Alternatively, the wastewater system could have simply been contaminated from infected patients, and it remains possible that there was another unidentified reservoir of the organism in the hospital that caused this long-term problem.

In contrast to this persistence of a single strain at a site over a long period, two laboratories (London_7 and London_12) referred MBL-positive isolates of diverse types over seven and eight year periods, respectively, implying that MBL-producing *P. aeruginosa* were introduced repeatedly. Since these 'high-risk clones' are

reported amongst MBL-producing *P. aeruginosa* worldwide, it is important not to assume that isolates with the same VNTR or ST profile are directly related unless this view is supported by epidemiological links between affected patients; it is just as likely that cases could represent repeated imports of the same clone from different sources.

In summary, internationally reported 'high-risk clones' with STs 111, 235, 233, 654, 773 and 357 were important in the increase in MBL-producing *P. aeruginosa* in the UK. These clones represent a distinct subset of isolates, as they were not prevalent in our comparator collection of mostly carbapenem susceptible isolates from the BSAC Bacteraemia Surveillance Programme, and appear to have a strong ability to acquire and/or maintain resistance genes or the mobile elements containing them. Possible reasons for this could include that these lineages have either stable genetic structures by which they can acquire new genes, or biological features that mean that they are more likely to acquire or maintain diverse horizontally-transferred genetic elements. To explore this more fully I went on to characterise the class 1 integron structures that usually contain these $bla_{\text{MBL}}$ genes and the wider genetic environment of these elements including their chromosomal or plasmid location. This work is described in the following chapter.

# 4 Genetic environment of MBL genes

## 4.1 Introduction

Chapter 3 explored the molecular epidemiology of MBL-producing *P. aeruginosa* isolates in the UK and showed that most of these organisms belonged to international 'high-risk clones'. As described in Section 1.9 the literature indicates that MBL genes $bla_{VIM}$ and $bla_{IMP}$ are usually located in class 1 integrons, which often also contain genes conferring resistance to other antibiotic classes, such as aminoglycosides. Class 1 integrons may in turn be located on mobilisable genetic elements, such as transposons, plasmids or genomic islands. It is important to determine the genetic environment of the MBL genes, including the integrons and mobile genetic elements containing them, to understand how they are able to spread among bacterial strains.

### 4.1.1 Origin of clinically relevant class 1 integrons

The class 1 integrons that are commonly seen among clinical isolates usually share two structures, the 5' and 3' conserved sequences (5'CS and 3'CS), often with diverse arrays of resistance gene 'cassettes' between them (figure 4.1). The 5'CS consists of the class 1 integrase gene (*intI1*), the *attI1* recombination site, and the promoter (Pc) while the 3'CS consists of fused *qacEΔ1* and *sul1* genes, conferring resistance to quaternary ammonium compounds and sulphonamides respectively, followed by an open reading frame, *orf5*, of unknown function. Class 1 integrons, containing the 5' and 3'CS, are commonly found as part of Tn*21*-like structures[173] (figure 4.1); they are thought to have emerged as a result of a series of random events, with selection pressures during the antibiotic era, favouring their proliferation.[174]

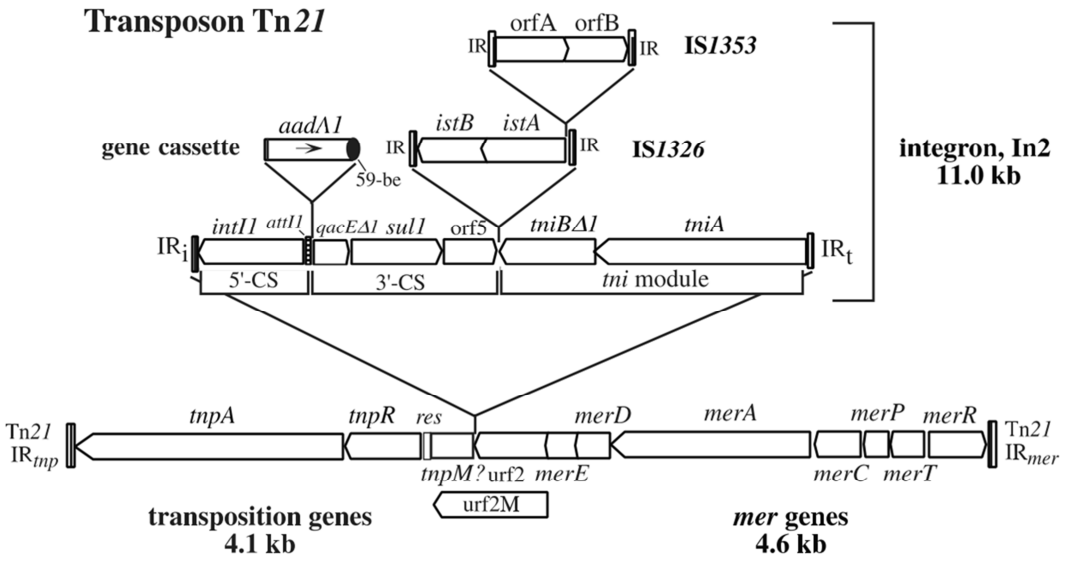



**Figure 4.1:** Tn*21* transposon structure. Figure taken from Liebert et al.[173]

Metagenomics studies of DNA from various environmental sources have discovered diverse class 1 integrase genes, some sharing as little as 92% nucleotide identity.[175] However the integrase genes of clinically-relevant class 1 integrons are almost always identical to one another, suggesting that they may be descended from a single ancestor.[153,175,176] Structures with similar *intI1* genes to clinical class 1 integrons are found on the chromosome of many members of the *Betaproteobacteria,* where they are generally species-specific.[153] On this basis, the ancestor of the clinically-relevant class 1 integrons is thought to have been captured from the chromosome of a member of the *Betaproteobacteria* by a Tn*5090*-like transposon (also known as a Tn*402*-like transposon).[153] Tn*5090* is a member of the Tn*5053* transposon family and readily inserts at the resolution (*res*) sites often found in plasmids.[177] So, once the Tn*5090*-like transposon had acquired the integron it was likely to be incorporated into a variety of plasmids, which then

allowed it to be mobilised between different bacteria. This Tn*5090*-like structure is thus thought to have been incorporated into a plasmid-encoded transposon, similar to Tn*21*. Such a structure, with the integron additionally having acquired a *qacE* gene, may have been selected by the use of quaternary ammonium compounds as hospital disinfectants in the early 1930s. Subsequently, insertion of a *sul1* gene, truncating *qacEΔ1*, could have been selected for by the introduction of the sulphonamides, an early synthetic antibacterial class, in the late 1930s. Around the same time a deletion event is thought to have resulted in the loss of the Tn*5090* transposition function (including loss of the *tniC* and part of the *tniB* genes), leading to the Tn*21*-like structures that are now commonly seen in association with diverse resistance gene cassettes.[173]

While this classical class 1 integron structure containing both the 5' and 3'CS, is the most common in Gram-negative bacterial species from clinical sources, including *P. aeruginosa,*[153] other variants lacking the 3'CS, are described in the literature, as shown in figure 4.2. Some have functional Tn*5090*-like transposon genes, including *tniC*, in their 3' region, as first reported amongst *P. aeruginosa* isolates in the USA[87] and subsequently in India,[162] Russia,[136] Norway,[178] and Tanzania.[179] These are thought to be descended from the same Tn*5090*-like ancestor, as the classical class 1 integron.[162] Another atypical class 1 integron structure contains a *sul1* gene and partial 5'CS in its 3' region, but lacks the *qacEΔ1* gene, and was associated with downstream Tn*5501* and Tn*5393* genes, as found in a *P. aeruginosa* isolate from Sweden.[124]

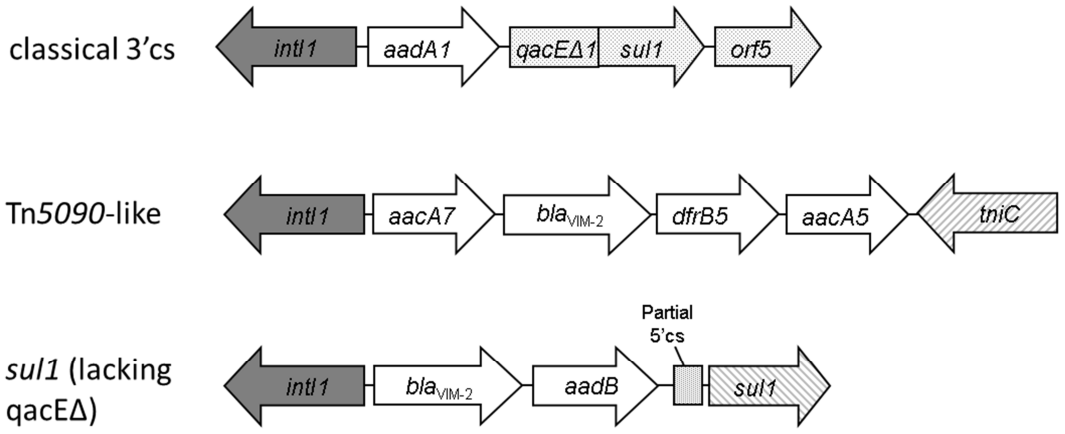



**Figure 4.2:** Examples of class 1 integrons with different 3' structures that have been seen in clinical isolates of *P. aeruginosa*. Not to scale.

### 4.1.2 Genetic context of class 1 integrons

Similar class 1 integron structures are found in different bacterial strains and species so mobilisation is thought to play an important part in the spread of the resistance genes they contain. However the mechanisms responsible for this lateral transfer are not well understood. As discussed in Section 1.9, the MBL-encoding class 1 integrons seen in *P. aeruginosa* have sometimes been localised on plasmids. For example, Li and colleagues described a 24-kb plasmid including a $bla_{VIM-7}$-containing class 1 integron from a *P. aeruginosa* isolate in the USA,[89] whilst a ~45-kb $bla_{VIM-2}$-containing plasmid was found in a clinical *P. aeruginosa* isolate from France,[164,180] and the $bla_{IMP-9}$ gene was located on a large ~450-kb plasmid in *P. aeruginosa* isolates from China.[181]

However, in many cases MBL-encoding plasmids cannot be found in MBL-producing *P. aeruginosa*, therefore it is thought that these and other acquired resistance genes may be more commonly located on the chromosome in this species.[151] With

the increased availability of whole-genome sequencing (WGS) technology, several recent studies have shown that these resistance genes can be carried within genomic islands - large structures present in chromosomal regions that show evidence of being acquired by horizontal gene transfer.

In *P. aeruginosa*, these structures include a $bla_{VIM-2}$-containing genomic island inserted in the chromosomal *PA5101* gene of an ST233 *P. aeruginosa* from an outbreak in the USA (figure 4.3).[147] This island contained two class 1 integrons carrying genes conferring resistance to multiple antibiotics along with a mercury resistance operon. Another example is a $bla_{VIM-1}$-containing genomic island found in an ST235 *P. aeruginosa* strain isolated in Italy (figure 4.4), in which the $bla_{VIM-1}$ gene was located within the transposon Tn*6249*, itself located within a disrupted glycerate kinase gene situated within a PA143/97 (PACS171b-like) genomic island; this in turn, was inserted within the chromosomal *endA* gene.[182] The Tn*6249* transposon contains two class 1 integrons which, in addition to the $bla_{VIM-1}$ gene, carried genes conferring resistance to aminoglycosides and chloramphenicol.

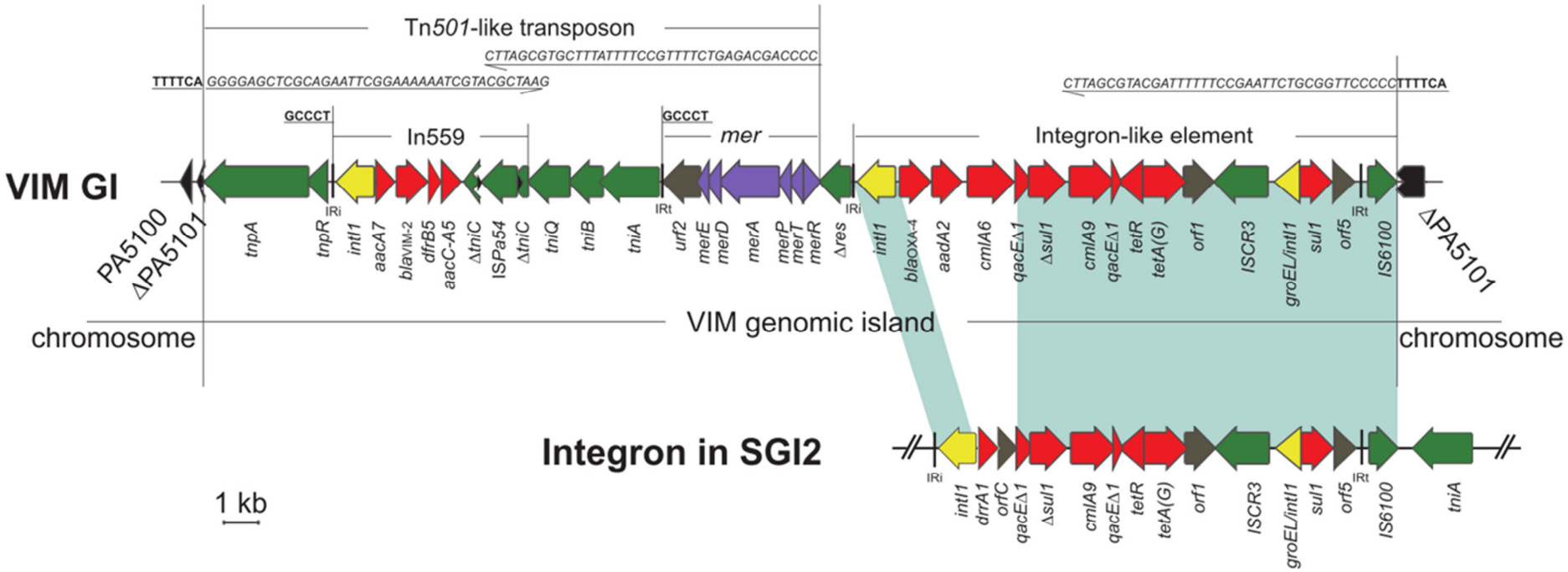



**Figure 4.3:** *bla*$_{VIM-2}$ containing genomic island (VIM GI) found in ST233 *P. aeruginosa* from the USA (GenBank accession number KJ463833), indicating the similarity of part the genomic island to an integron present in *Salmonella* genomic island 2 (SGI2). Figure taken from Perez et al. [147]

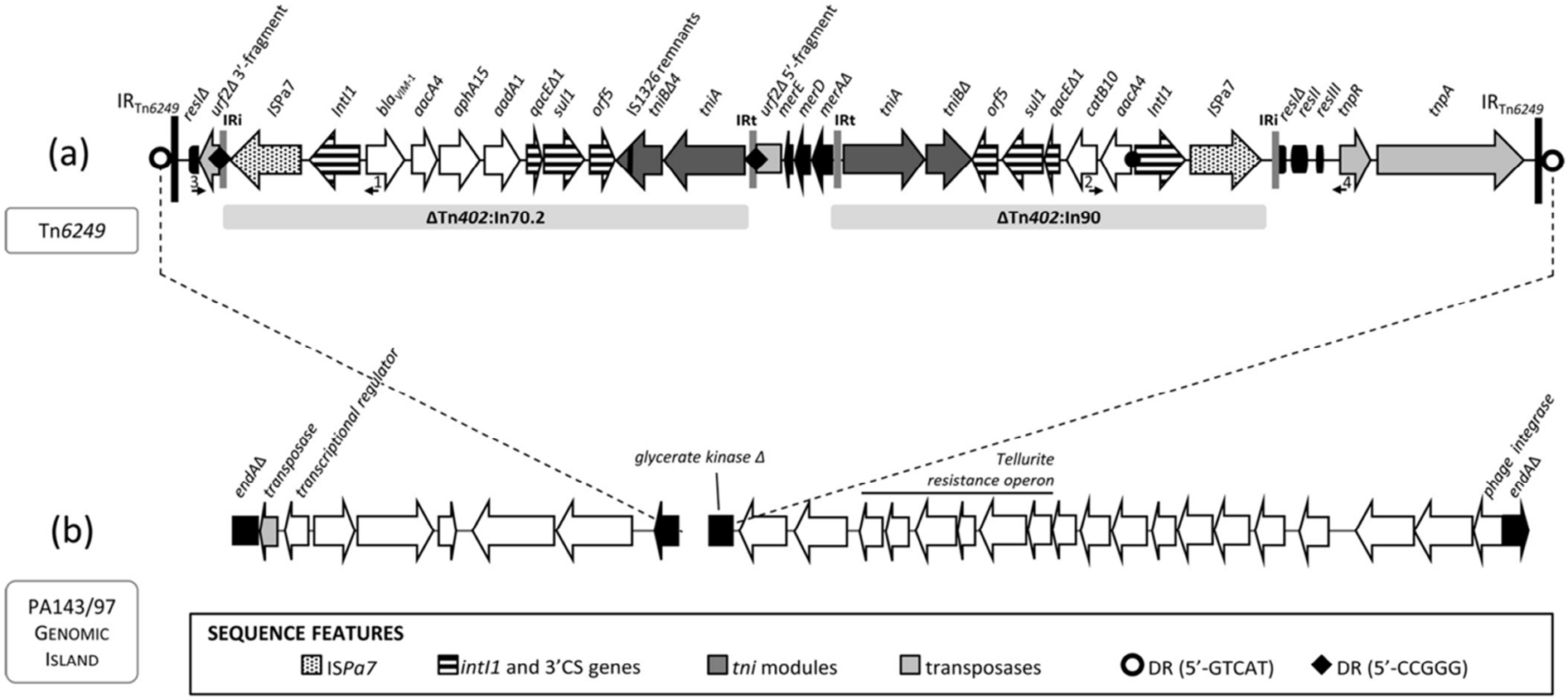



**Figure 4.4:** *bla*$_{VIM-1}$-containing genomic island found an ST235 *P. aeruginosa* strain isolated in Italy (GenBank accession number: LK054503). The *bla*$_{VIM-1}$ containing island is located within the disrupted glycerate kinase gene, which is in turn located within a PACS171b-like genomic island (PA143/97) inserted in the *endA* gene of the *P. aeruginosa* chromosome Figure taken from Di Pilato et al.[182]

Similar genomic islands, or segments of genomic islands, have been found in epidemiologically diverse bacteria, of the same or different species, suggesting that horizontal transfer of these structures can occur. For example, the *bla*$_{VIM-2}$-containing island shown in figure 4.3 contained an integron with similarity to part of *Salmonella* genomic island 2.[147] The mechanisms by which these genomic islands may be mobilised between different strains and species are only beginning to be understood. It is apparent that they often contain transposons or similar mobile genetic elements, such as insertion sequence common regions (ISCR), which could mobilise the DNA to a mobile genetic element capable of lateral transfer, such as a conjugative plasmid. However, some genomic island structures can mobilise themselves, either alone or with the help of other elements, by conjugation or transduction.[183] These include bacteriophage, integrative and conjugative elements (ICE), and integrative and mobilisable elements (IME), which share some similar features and are described below.

Bacteriophages are viruses that infect and replicate in bacterial cells. Bacteriophage genomes can exist either as an extrachromosomal form or in some cases integrated into the bacterial chromosome as latent 'prophage'. During transduction bacteriophages may transfer sections of the host genome to another bacterial cell, and were shown to mediate the transfer of antibiotic resistance genes in several bacteria. For example, P22 phages were reported to mobilise multiple resistance genes in *Salmonella*[184] and bacteriophage-mediated transfer of *tetM* and *ant(2")-I* resistance genes was demonstrated in enterococci.[185]

ICE are a diverse, and growing class of elements that are able to self-excise and integrate into the genome, like bacteriophage, but which are also capable of conjugation, similarly to conjugative plasmids.[186] In addition to genes necessary for these functions, ICE contain diverse accessory genes, including acquired antibiotic resistance determinants, and are found in the genomes of many bacteria. ICE that have been described in *Pseudomonas* spp. include the genomic island PAPI-1, which encodes genes involved in virulence and biofilm formation, and $ICE_{clc}$, which is involved in chlorcatechol degradation.[187] Several Tn*4371*-like ICEs have been found in *P. aeruginosa*, carrying a variety of accessory genes including those encoding RND efflux and arsenate resistance pumps;[188] they include $ICE_{Tn4371}$6061 which was found on the chromosome of a Brazilian *P. aeruginosa* isolate carrying $bla_{SPM}$, although this MBL gene was associated with an IS*CR4* element, not a class 1 integron.[189]

Further genomic island structures, called IME have also been described. These share some features with ICE, such as the ability to integrate and excise themselves, but require a helper element, such as a conjugative plasmid, for cell-to-cell transfer.[186] As with ICE, IMEs encode diverse accessory functions, with several having been associated with antibiotic resistance genes.[190] For example the *Salmonella* genomic island 1 includes a complex class 1 integron encoding multiple resistance genes and can excise itself from the genome into a circular form that can be mobilised *in trans* with the help of an IncC plasmid, after which the element can integrate into the genome.[191]

### 4.1.3 **Determining the genetic context of class 1 integrons**

Many studies have characterised class 1 integron gene cassette arrays by PCR targeting conserved 5' and 3' regions.[151] While this information can be useful to inform epidemiological studies, it does not give any information on the wider genetic environment of the elements.

A few studies have attempted to determine the wider genetic context of these elements. Their localisation within plasmids can be determined by attempting plasmid transfer by transformation or conjugation. However, not all plasmids transfer well using these methods, due to factors such as their large size, low replicon number, or because they do not encode conjugation functions. S1 nuclease digestion and hybridisation studies have also been used to detect plasmids, particularly large ones.[192] S1 nuclease cuts only single-stranded DNA, as is present transiently in supercoiled plasmids, due to torsional stress of the molecule, and thus converts plasmids to linear DNA. The enzyme does not so frequently cut chromosomal DNA. Consequently, after S1 nuclease treatment, linearised plasmid DNA can be separated from relatively intact chromosomal DNA by PFGE. The linearised plasmid can also be reliably sized with reference to a DNA ladder, in contrast to a supercoiled plasmid, where its secondary structure affects migration. Hybridisation of the separated products from the S1 nuclease digest with a $bla_{MBL}$-specific probe can then be used to determine whether the MBL gene is located on the plasmid.

A similar approach can be used to locate genes on the chromosome. The technique, first described by Liu et al.,[193] involves the use of the I-*ceu* I restriction enzyme,

which acts on ribosomal RNA genes and thus cuts bacterial chromosomal DNA at a few sites. For example I-*ceu* I digests *P. aeruginosa* DNA at four sites, resulting in four chromosomal fragments. As with S1 nuclease, separation of I-*ceu* I digestion products by PFGE, followed by hybridisation, can be used to localise an MBL gene to a chromosomal fragment.

Whole genome sequencing methods are increasingly available and have also been used to determine the structure of antibiotic-resistance-encoding genomic islands and plasmids, including those in *P. aeruginosa*.[147,182] Short-read technologies, such as Illumina sequencing are often used, but it can be problematic to assemble the repetitive regions often found in integron-containing structures, confounding attempts to determine their genetic context. Long-read technologies such as the MinION (Oxford Nanopore Technologies) or PacBio RS II (Pacific Biosciences, California, USA) are more expensive and, in the case of MinION, have lower accuracy than the short-read technologies, but can be used to scaffold the more accurate short reads. For example, Ashton and colleagues recently used a combination of Illumina and MinION reads to determine the structure and chromosomal location of a 24-kb antibiotic resistance island in *Salmonella*.[194]

### 4.1.4 **Aims**

The overall aim of the work described in this chapter was to investigate the genetic context of the MBL genes in our collection of MBL-producing *P. aeruginosa* isolates. Specific aims were (1) to characterise the $bla_{VIM}$- and $bla_{IMP}$-containing class 1 integron structures found in the MBL-producing *P. aeruginosa* isolates, including the sequencing of prevalent integrons, and (2) to investigate the wider genetic

environment of these integrons including their chromosomal and/or plasmid locations.

## 4.2 Methods

### 4.2.1 Detection of class 1 integrons

The strategy for the amplification of MBL-encoding class 1 integrons is illustrated in figure 4.5. Integrons were sought by two PCRs, initially using primers specific to the 5' or 3' CS (5'CS with 3'CS_1 3'CS_2), together with *bla*$_{VIM}$- and *bla*$_{IMP}$- specific primers (VIM-2004A, VIM2004B, IMP Fwd or IMP Rev). Where no amplicon was generated using reverse primers specific to the 3'CS together with an MBL-gene-specific forward primer, atypical class 1 integron structures were investigated. Tn*5090*-like class 1 integrons (which lack the 3'CS), and structures containing the *sul1* gene, but not *qacEΔ1*,[124] were sought using a reverse primer specific to *tniC* (Tni-CF) or *sul1* (Sul-R), respectively, together with an MBL-gene-specific forward primer (figure 4.5). Primer sequences are shown in table 2.1. Cycling conditions were 94$^{o}$C for 3 minutes, followed by 35 cycles of 94$^{o}$C for 1 minute, 55$^{o}$C for 1 minute and 72$^{o}$C for 5 minutes, and a final extension step of 72$^{o}$C for 15 minutes; the long elongation times were to allow for amplification of large integrons.

Resulting PCR products were sized on 1% agarose gels. For isolates where the amplicon sizes were similar for both the 5' and 3' regions of the integron, the amplicons were digested using AccI or SmlI restriction enzymes (New England BioLabs) at 37$^{o}$C or 55$^{o}$C, respectively, and electrophoresed on 1.5% agarose gels; resulting fingerprints were compared visually. Variable regions of frequently-detected integrons were sequenced using the primers described above for the amplification of class 1 integrons as well as additional primers targeting MBL or other genes contained within the integrons (VIM-2A, VIM-2B, dhfr2_F, dhfr2_R,

aacA29AB1, aacA29AB2, aacA4-F, aacA4-R, OXA10like-F, OXA10like-R, AACA7-F, AACA7-R, aacA7-F2, strAstrB-F and strAstrB-R). All primer sequences are shown in table 2.1.

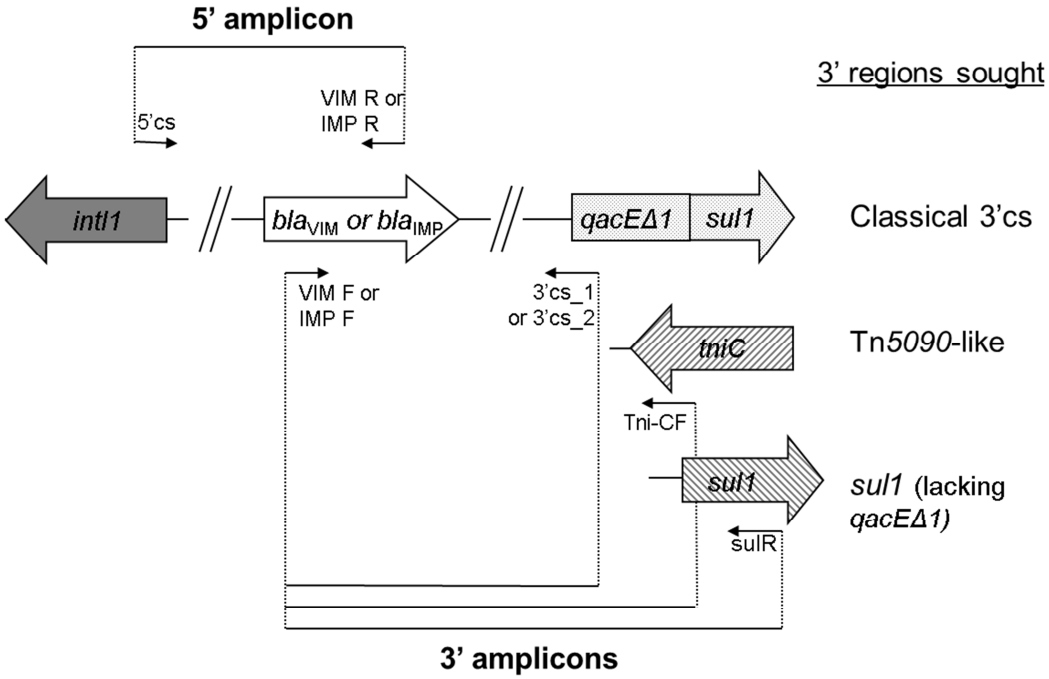



**Figure 4.5:** Strategy for the amplification of different class 1 integron 3' regions

### 4.2.2 *rpoB* gene sequencing

A region of the *rpoB* gene was amplified using primers CM7 and CM31B (see table 2.1), as described by Mollet and colleagues.[168] Cycling conditions were 94$^{o}$C for 3 minutes, followed by 35 cycles of 94$^{o}$C for 30 seconds, 60$^{o}$C for 30 seconds and 72$^{o}$C for 45 seconds, and a final extension step of 72$^{o}$C for 10 minutes. Amplicons were sequenced as described in Section 2.2.3.4, using the same primers. Resulting sequences were analysed in Bionumerics Software v6.1 (Applied Maths) and compared with sequences in our database, including those for type strains for most of the species found clinically, and strains detailed by Ait Tayeb and colleagues,[195] to identify them to species level.

### 4.2.3 Plasmid transformations

Overnight cultures of MBL-producing *P. aeruginosa* strains were prepared in nutrient broth. Plasmid extractions were carried out using the QIAprep Spin Miniprep Kit (Qiagen), according to the manufacturer's instructions.

Transformations were attempted by electroporation into the DH5α *E. coli* strain (Thermo Scientific). Briefly, DH5α cells were thawed on ice and 20 µl cells were mixed with 2 µl of the plasmid extract in a 0.5 ml tube and kept on ice. The mixture was added to a 0.1 cm Electroporation Cuvette (Bio-Rad, Hemel Hempsted, UK) and pulsed at 1.8 kV on the Gene Pulser Xcell Electroporation System (Bio-Rad). After pulsing, 0.5 ml of Super-Optimal Broth with Catabolite Repression (SOC broth; Bioline) was immediately added to the cuvette and the contents transferred to a 15 ml conical tube, which was incubated at 37˚C with shaking for approximately one hour. To select any transformants, 100 µl of the broth mixture was spread on to

Luria-Bertani (LB) agar plates containing 100 mg/L ampicillin and incubated at 37˚C overnight. Any resulting colonies were screened for the presence of the carbapenemase gene by PCR, as described in Section 2.2.4.

### 4.2.4 S1 nuclease and I-*ceu* I digests

S1 nuclease and I-*ceu* I digests and hybridisation were carried out as first described by Barton et al.[192] and Liu et al.,[193] respectively, as outlined below.

#### 4.2.4.1 Preparation of agarose plugs

Overnight cultures were grown on nutrient agar. Bacterial suspensions were made in 1 ml of SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.5) to a turbidity of 2.3-2.7 MacFarland units. An equal volume of bacterial suspension was mixed with 2% Macrosieve Low Melt Agarose (Flowgen, Nottingham, UK) in SE buffer at 56$^{o}$C and dispensed into the assembled mould, which was kept at 4$^{o}$C until plugs were set. The agarose plugs were then removed to bijou bottles and incubated overnight at 37$^{o}$C with shaking in 3 ml of first lysis buffer (6 mM Tris, 100 mM EDTA, 1M NaCl, 0.5% w/v Brij 58, 0.2% w/v sodium deoxycholate, 0.5% N-lauroyl sarcosine, 1 mM $MgCl_2$) containing 500 µg/ml lysozyme. The lysis buffer was then replaced with 3 ml alkaline lysis buffer (1% (w/v) N-lauroyl sarcosine, 0.5 M EDTA, pH 9.5) containing 60 µg/ml proteinase K and incubated overnight at 56$^{o}$C with shaking. The plugs were then washed three times at 4$^{o}$C using 3 ml TE buffer (10 mM Tris, 10 mM EDTA, pH 7.5), for a minimum of 30 minutes per wash. After the third wash the buffer was removed and replaced with a further 2 ml TE buffer. The plugs were stored at 4$^{o}$C until use.

#### 4.2.4.2 Digestion of plugs

Whole plugs were digested using S1 nuclease (Thermo Scientific) or I-*ceu* I (New England BioLabs). These plugs were first equilibrated in 1x S1 Buffer or 1x CutSmart Buffer for 20 minutes. The buffer was then removed and replaced with 1x S1 Buffer containing 0.08 U/µl S1 nuclease or 1x CutSmart Buffer containing 0.04 U/µl I-*ceu* I enzyme and incubated at 37$^{o}$C for 45 minutes or 3 hours, respectively. At the end of the incubation period 10 µl of 0.5 M EDTA was added to stop the reaction.

#### 4.2.4.3 PFGE running conditions

Digested DNA in the plugs was separated by PFGE on a 1.2% agarose gel on CHEF DRII apparatus (Bio-Rad). The Lambda Ladder PFG Marker (New England BioLabs) was used to size the fragments. Gels were run at 6 V for 24 hours at 12$^{o}$C with switch times of 20-120 seconds.

#### 4.2.4.4 In-gel hybridisation

In-gel hybridisation was carried out as described by Khan and Nawaz.[196] The gel was washed twice in distilled water for 10 minutes, to remove salts, and then dried between two sheets of blotting paper in a hybridisation oven at 55$^{o}$C overnight. The dried gel was then rehydrated in distilled water for 10 minutes resulting in a thinner, stronger gel able to withstand subsequent steps. The DNA was denatured by immersing the gel in 100 ml denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 30 minutes, washing it twice in distilled water for 5 minutes and neutralising in 100 ml neutralisation buffer (0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl). The DNA was cross-linked to the gel by placing on a UV transilluminator for 5 minutes. The gel was incubated at hybridisation temperature (42$^{o}$C) with gentle shaking for 2 hours in 15 ml per 100

$cm^2$ of pre-heated DIG EasyHyb (Roche, Sussex, UK), in a sealed plastic container in the water bath. An 801-bp DIG-labelled $bla_{VIM}$ probe was prepared using primers VIM-2A and VIM-2B (table 2.1) and the PCR DIG Probe Synthesis Kit (Roche), according to the manufacturer's instructions. Cycling conditions were 95°C for 2 minutes, followed by 30 cycles of 95°C for 10 seconds, 55°C for 30 seconds and 72°C for 2 minutes and a final extension step of 72°C for 7 minutes. The probe was denatured at 95°C for 5 minutes, followed by rapid cooling on ice immediately prior to use; then 2 µl of probe per ml was added to 50 ml pre-heated Hybridisation Solution (Roche). The gel was incubated with this mixture at 42°C overnight with gentle shaking. Stringency washes were carried out twice with ample 2x SSC, SDS 0.1% for 5 minutes at room temperature, followed by twice with 0.2x SSC, SDS 0.1% for 15 minutes at 68°C, both with gentle shaking.

The following steps were all carried out at room temperature with gentle shaking unless otherwise stated. First, the gel was washed with 100 ml of 1x Wash Buffer (Roche) for 5 minutes, followed by incubation in 100 ml of Blocking Solution (Roche) for 30 minutes. The Anti-DIG-AP Conjugate (Roche) was centrifuged at 15700 rcf for 5 minutes and diluted 1:5000 in blocking solution; the gel was incubated together with this antibody mixture for 30 minutes. Following this, the gel was washed twice, for 15 minutes each, in 100 ml Washing Solution (Roche) and equilibrated in 100 ml Detection Buffer (Roche) for 5-10 minutes. After transferring to a clean container approximately 40 ml of freshly prepared NBT/BCIP (Roche) was poured on to the gel, which was incubated in the dark without shaking until the colour had developed sufficiently (2 hours – overnight). This colour reaction was stopped by washing the gel in distilled water for 15 minutes.

### 4.2.5 Whole genome sequencing (WGS) data analysis for detection of genomic islands

Genomes were sequenced using Illumina and MinION technology, as described in Section 2.2.5. Genome assemblies were carried out using tools available on the PHE instance of the Galaxy server (https://galaxyproject.org/). Assemblies of Illumina fastq reads only were produced using SPAdes version 2.5.0 (http://bioinf.spbau.ru/spades) with the following settings:

Single cell: No,

Careful correction: Yes,

Use rectangle correction for repeat resolution: No,

Number of threads: 16,

Number of iterations for read error correction: 1,

K-mers to use; 21, 33, 55, 77

For hybrid assemblies, the multi-fasta files containing MinION reads were used in combination with fastq files produced by Illumina sequencing using SPAdes version 3.1.1 (http://bioinf.spbau.ru/spades); settings were as above, except that the 'supply long reads' option was enabled to allow the inclusion of the multi-fasta file containing the MinION reads in the analysis.

MinION reads were mapped to the Illumina-only or SPAdes assemblies using the Map with LAST tool version 1.0 (http://last.cbrc.jp/), also on the PHE galaxy server; default settings were used as follows:

Use custom match/mismatch score matrix: False,

Type of alignment: local,

Match score: 1,

Mismatch cost: 1,

Gap existence cost: 1,

Gap extension cost: 1,

Insertion existence cost: 1,

Insertion extension cost: 1,

Minimum score for gapped alignments: 40

The LAST output in the resulting text files was converted to BLAST format using the maf-convert.py script and parsed as previously described[194] (this was carried out by Philip Ashton, Gastrointestinal Bacteria Reference Unit, PHE). Each resulting .xml file detailed each of the MinION reads and corresponding contigs of the assembly to which the read mapped as well as the position of MinION read in relation to the contig. The contig containing the *bla*$_{VIM}$ gene for each assembly was located by a BLAST search. The parsed map with LAST data were used to manually scaffold other contigs shown to be linked to the *bla*$_{VIM}$-encoding contig by MinION reads, to obtain information on the genomic location of the *bla*$_{VIM}$ gene in the bacterial chromosome.

### 4.2.6 **PCR detection of genomic islands**

Four different multiplex PCRs were designed to amplify the left- and right-hand chromosomal junctions of three different *bla*$_{VIM}$-encoding genomic islands (referred to as genomic islands A, B and C; see below), which were previously described[147,182] (figures 4.3 and 4.4) or were identified as part of this work. Figure 4.6 shows the strategy for investigation of each of the three *bla*$_{VIM}$ containing genomic islands; primers used in the four PCRs are described below and their sequences are shown in table 2.1. For all PCRs, the cycling conditions were 94$^{o}$C for 3 minutes, followed by 30 cycles of 94$^{o}$C for 30 seconds, 60$^{o}$C for 30 seconds and 72$^{o}$C for 1 minute, with a final extension step of 72$^{o}$C for 5 minutes. Q solution was included in the reaction mix for these PCRs, as described in Section 2.2.3.

To detect genomic island A, isolates were screened for the junctions of a previously-described[147] *bla*$_{VIM-2}$ containing genomic island insertion within the chromosomal *PA5101* gene (figure 4.3; GenBank accession number KJ463833). The multiplex PCR, designated PCR 1, used primers targeting the *PA5101* gene (PA5101_F and PA5101_R) and the left-hand (VIMGI-LH_R) and right-hand (VIMGI-RH_F) regions of the genomic island.

Isolates were screened for a second *bla*$_{VIM-2}$ containing genomic island (genomic island B), identified during this study (as described in Section 4.3.5.2) using multiplex PCR 2. Primers targeted the *PA2229* chromosomal gene (PA2229_F and PA2229_R) and the left-hand (ST111-GI-J1_R) and right-hand (ST111-GI-J2_F) regions of the genomic island.

Selected isolates were screened for the insertion sites of a previously described[182] $bla_{VIM-1}$-containing structure described in a ST235 *P. aeruginosa* isolate (genomic island C; figure 4.4; GenBank accession number LK054503) using two different multiplex PCRs (PCRs 3 and 4) designed as part of this work. PCR 3 targeted the insertion of a PACS171b-like genomic island (primers PACS171b_J1_R and PACS171b_J2_F) in the chromosomal *endA* gene (primers endA_F and endA_R); PCR 4 targeted the junctions of the Tn*6249*-like transposon (primers urf2_R and TnpATn6249_F) within the glycerate kinase gene (primers glykin_F and glykin_R) of this PACS171b-like genomic island.

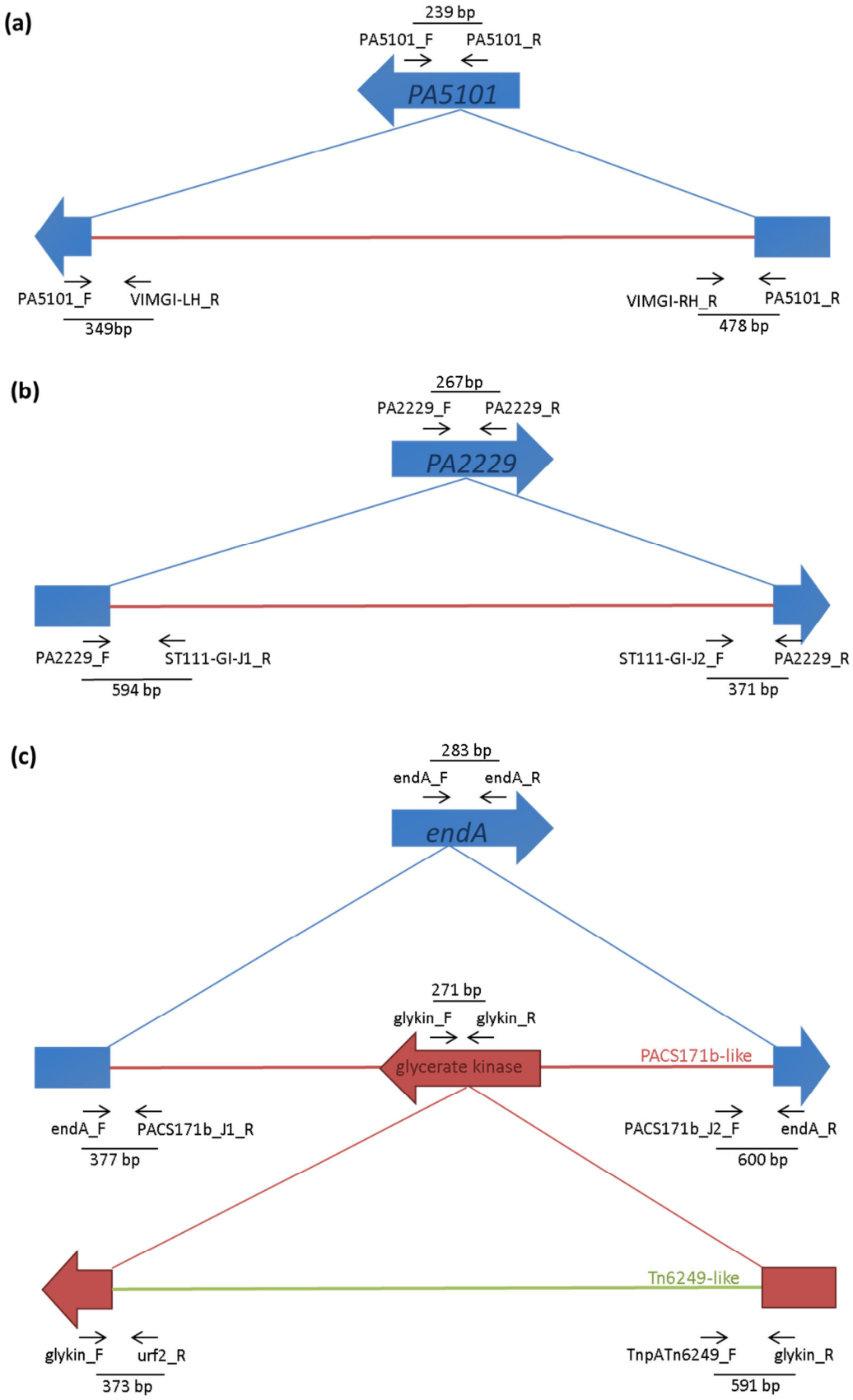



**Figure 4.6:** Strategy for PCR-based investigation of (a) genomic island A, (b) genomic island B and (c) genomic island C. Primer sequences are shown in table 2.1.

### 4.2.7 **Long-range PCR**

Long-range PCR was carried out as described in Section 2.2.3.2 to check the linkage of genomic-island-associated *bla*$_{VIM}$ genes identified in this study with chromosomal genes. Primers PA5101_F, PA2229_R or glykin_F, targeting the chromosomal genes in which the genomic islands were inserted were used together with VIM2004B, which was specific to the *bla*$_{VIM}$ gene (see table 2.1 for primer sequences). Cycling conditions were 93$^{o}$C for 3 minutes, followed by 30 cycles of 95$^{o}$C for 30 seconds, 60$^{o}$C for 30 seconds and 68$^{o}$C for 10 minutes, no final extension step was required. Q solution was included in the reaction mix for all long-range PCRs, as described in Section 2.2.3.2.

## 4.3 Results

### 4.3.1 Integrons found amongst *P. aeruginosa* isolates

Amplicons were obtained using a primer specific for the 5'CS together with another specific for the appropriate $bla_{MBL}$ gene from all of the 218/266 non-duplicate $bla_{VIM}$- or $bla_{IMP}$-carrying isolates of *P. aeruginosa* that could be recovered from AMRHAI's archives. One hundred and nineteen of these 218 isolates had an MBL-encoding integron with the 3'CS, 65 had Tn*5090*-like 3' regions and 17 had the *sul1* gene but lacked *qacEΔ1*; the 3' region could not be amplified using any primer combinations for the remaining 17 isolates.

A total of 42 different $bla_{VIM}$-containing integrons were seen amongst the 196 $bla_{VIM}$-positive isolates, detected in one to 58 isolates each; these isolates were referred from 74 hospital laboratories over the ten-year period, with individual sites referring one to 21 isolates each. Eight integron profiles were detected amongst the 22 IMP-producers, referred from 15 different laboratories over the ten-year period, each referring one to five isolates each. Five $bla_{IMP}$ integrons had the 3'CS, two had Tn*5090*-like 3' regions and for one the 3' region could not be amplified.

#### 4.3.1.1 Prevalent integron structures

Six integron profiles (designated I-VI), all containing $bla_{VIM}$, were each seen in more than five isolates. Each of these gave consistent restriction patterns on digestion with *AccI* or *SmlI* enzymes and these six integrons became the focus of further study. Representatives were selected for sequencing of the integron variable region: five of those studied contained $bla_{VIM-2}$ cassettes, whilst one contained a $bla_{VIM-6}$ cassette. Sequences of the variable regions of these integrons were

deposited in GenBank (accession numbers KR337988-KR337993), and their structures are shown in figure 4.7.

A minimum spanning tree and a table showing the distribution of these six major integron types among isolates belonging to the main VNTR complexes (A-F), described in Chapter 3, are presented in figure 4.8 and table 4.1, respectively. Table 4.2 shows the distribution of the integrons I-VI among different referring laboratories and maps showing this geographical distribution are presented in figure 4.9; these data are discussed further in the following sections. Most integrons were carried by representatives from multiple laboratories across the UK, the exception being integron VI, only seen at a single site, as detailed below.

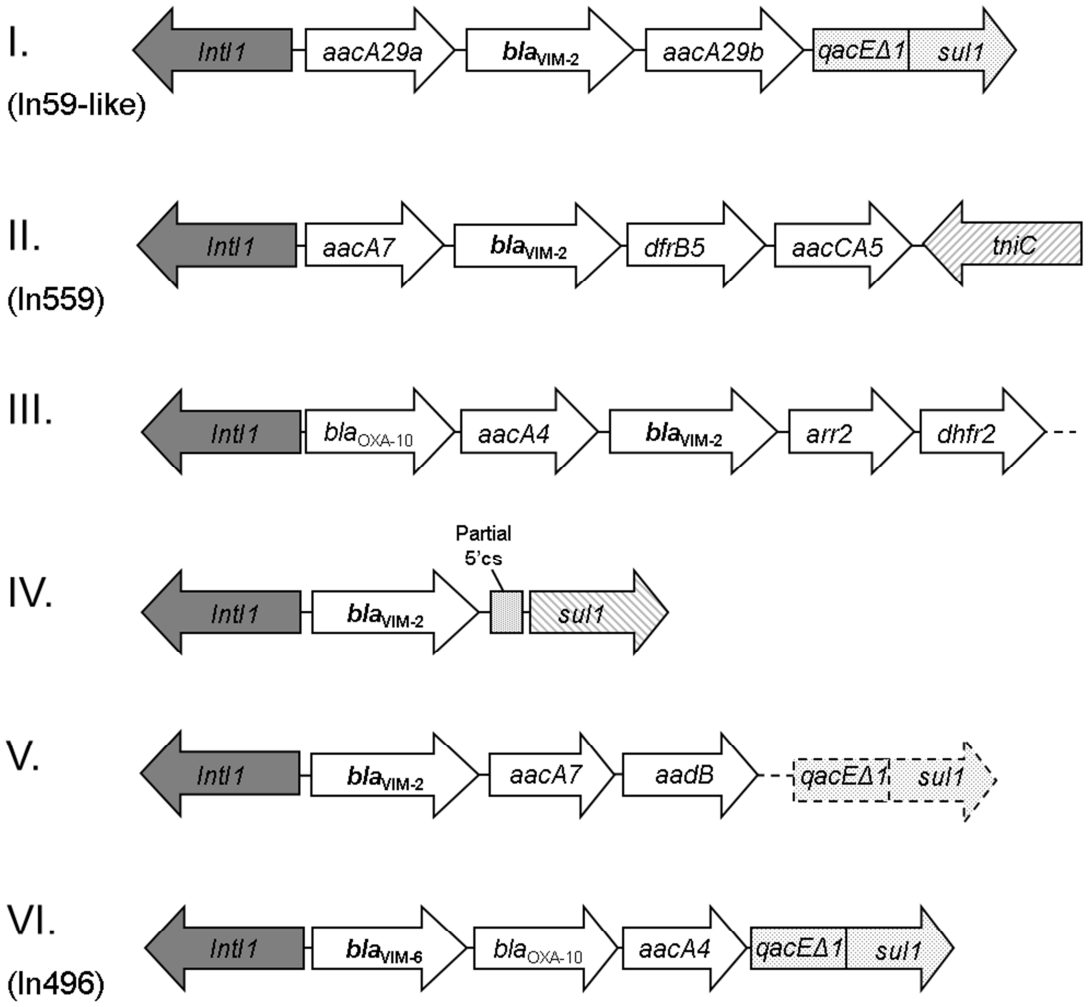



**Figure 4.7:** Schematic diagrams of MBL-encoding class 1 integron variable regions identified in more than five isolates each (not to scale). Only partial integron structures could be obtained for integrons III and V with regions not confirmed with sequence data indicated by dotted lines.

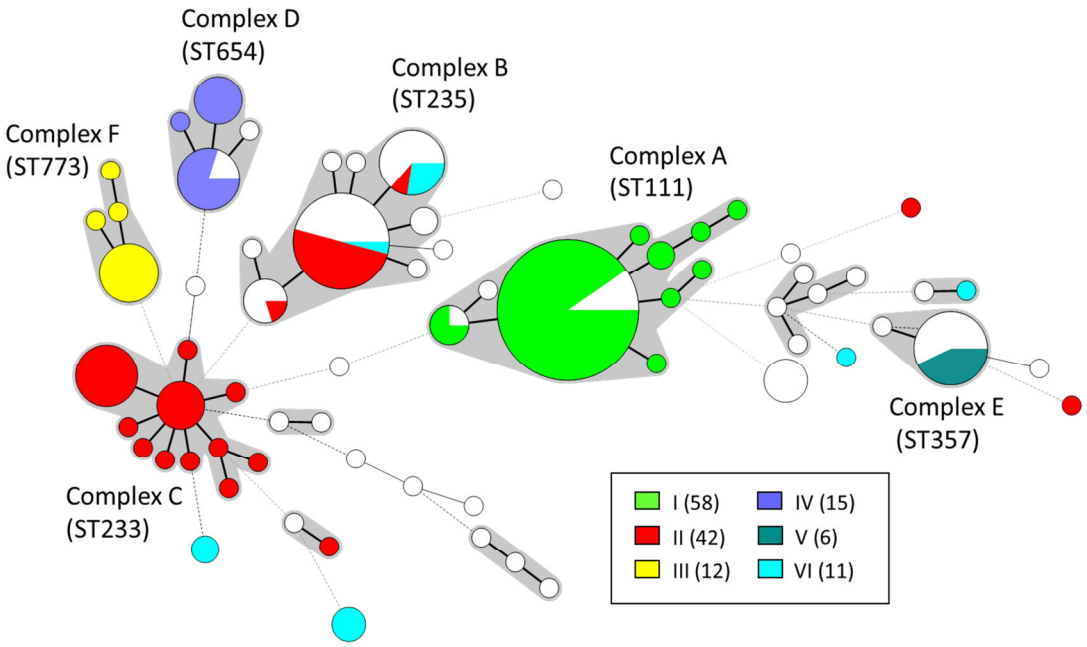



**Figure 4.8:** Minimum spanning tree, based on clustering at the first eight VNTR loci for the 218 MBL-producing isolates still viable in the archives for study of their integron content. The six main complexes A-F and their corresponding STs are labelled. The size of the circle is relative to the number of isolates with that VNTR profile. Coloured segments of the circles represent the six main *bla*$_{VIM}$-containing integron structures (I-VI), and white circles represent isolates that did not have one of the main integron types. Grey shading indicates complexes. Thick solid lines represent single locus variants; thin solid lines and dotted lines represent multi-locus variants.

**Table 4.1:** Integrons detected amongst isolates belonging to each of the VNTR complexes (n=218)

| VNTR complex (corresponding ST) | Total isolates | Number of submitting laboratories | MBLs detected | Major integron profiles seen[a] | Other integron profiles detected |
|---|---|---|---|---|---|
| **A (ST111)** | 65 | 25 | VIM (60)<br>IMP (5) | I (58; 89%) | Four integron profiles (two *bla*$_{VIM}$- and two bla$_{IMP}$-containing) amongst remaining seven isolates |
| **B (ST235)** | 47 | 25 | VIM (41)<br>IMP (6) | II (14; 30%)<br>VI (4; 9%) | 15 integron profiles (13 *bla*$_{VIM}$- and two bla$_{IMP}$-containing) amongst remaining 29 isolates |
| **C (ST233)** | 25 | 15 | VIM (25) | II (25; 100%) | None |
| **D (ST654/ ST964)** | 18 | 11 | VIM (17)<br>IMP (1) | IV (15; 83%) | Three integron profiles (two *bla*$_{VIM}$- and one bla$_{IMP}$-containing) amongst remaining three isolates |
| **E (ST357)** | 16 | 9 | VIM (16) | V (6; 38%) | Eight integron profiles (all *bla*$_{VIM}$-containing) amongst remaining 10 isolates |
| **F (ST773)** | 12 | 11 | VIM (12) | III (12, 100%) | none |
| **Other types** | 35 | 25 | VIM (25)<br>IMP (10) | II (3; 9%)<br>VI (7; 20%) | 19 integron profiles (15 *bla*$_{VIM}$-, four bla$_{IMP}$) amongst remaining 25 isolates |

[a]percentages in brackets represent the proportion of the total number of isolates in each complex that carried each of the main integrons.

**Table 4.2:** Geographical data for the major VNTR complex/ integron combinations

| Integron | Complex (ST) | No. of isolates | No. of laboratories | Overall time frame | Major contributors (≥3 isolates) | Minor contributors (1-2 isolates) |
|---|---|---|---|---|---|---|
| **I** | A (ST111) | 58 | 20 | 7 years (2006-2012) | London_17; 21 representatives over 80-month period<br>Wales_1; 13 representatives over 18-month period<br>London_26; 4 representatives over 45-month period<br>London_12; 3 representatives over 23-month period | One laboratory referring two isolates<br>Fifteen laboratories referring one isolate |
| **II** | B (ST235) | 14 | 6 | 9 years (2004 - 2012) | London_13; 5 representatives over 3-week period[a]<br>Scotland_4; 5 representatives over 114-month period | Four laboratories referring one isolate |
| | C (ST233) | 25 | 15 | 5 years (2008-2012) | London_7; 5 representatives over 27-month period<br>London_13; 4 representatives over 18-month period[a] | Three laboratories referring two isolates<br>Ten laboratories referring one isolate |
| | Others | 3 | 3 | 6 years (2006-2011) | None | Three laboratories referring one isolate |
| **III** | F (ST773) | 12 | 11 | 7 years (2006-2012) | No major contributors | One laboratory referring two isolates<br>Ten laboratories referring one isolate |
| **IV** | D (ST654/ ST964) | 15 | 7 | 9 years (2004 – 2012) | South East_6; 7 representatives over 36-month period | Two laboratories referring two isolates<br>Four laboratories referring one isolate |
| **V** | E (ST357) | 6 | 1 | 8 years (2003 – 2010) | North West_15; 6 representatives over 103-month period | none |
| **VI** | B (ST235) | 4 | 3 | 7 years (2006-2012) | None | One laboratory referring two isolates[b]<br>Two laboratories referring one isolate |
| | Others | 7 | 4 | 2 years (2010-2012) | South East_7; 3 representatives over 20-month period | One laboratory referring two isolates<br>Two laboratories referring one isolate |

[a] isolates of ST235 and ST233 carrying integron II were received from London_13 in 2004, and 2010-2012 respectively

[b] these two isolates also came from South East_7

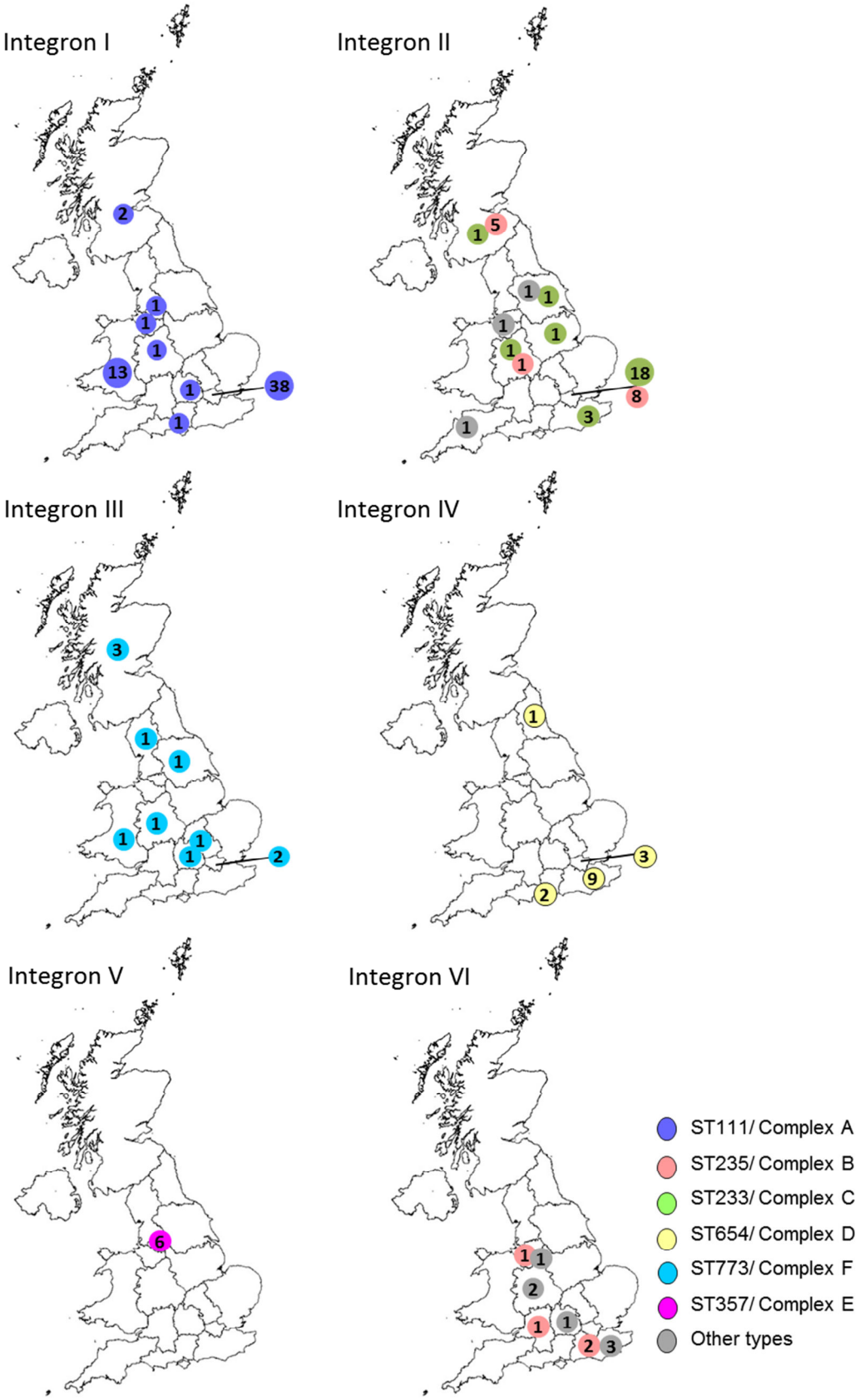



**Figure 4.9:** Distribution of isolates carrying each of the six main integrons across the main sub-regions of the UK. Coloured circles denote the ST/VNTR complexes (see Chapter 3) with numbers of isolates of the respective ST from each sub-region indicated. The sub-regions represented in these maps are explained in figure 2.2.

Integron I was an In59-like structure, differing from In59 (GenBank: AF263519)[197] by substitutions in non-coding regions, and contained a $bla_{VIM-2}$ gene flanked by two highly similar aminoglycoside resistance genes, *aacA29a* and *aacA29b*, followed by the 3'CS. This integron was only seen in ST111 (complex A) isolates where it was present in 58/65 (89%) representatives. The integron was sequenced for nine of these isolates and all had the same In59 variant. The 58 isolates carrying the In59-like integron were collected at 20 laboratories over seven years, and included all of the ST111 outbreak representatives at London_17 and Wales_1, referred over seven-year and 18-month periods.

Sequencing showed integron II to be identical to In559 (GenBank: DQ522233). Besides $bla_{VIM-2}$ this element also contained *aacA7*, *dfrB5* and *aacCA5* gene cassettes and had a Tn*5090*-like 3' region. All 25 isolates belonging to ST233 (complex C), referred from 15 laboratories, carried integron II as did 14/47 (28%) isolates belonging to ST235 (complex B) and three isolates that did not belong to any of the main VNTR complexes. Affected sites included London_7 with five ST233 representatives referred over a 27-month period and Scotland_4 submitting five ST235 isolates over a two-year period. London_13 referred four ST233 and five ST235 isolates over 18-month and three-week periods respectively, with these 'outbreaks' separated by six years.

'Integron' III was an integron-like element containing $bla_{OXA-10}$, *aacA4*, *arr2* and *dhfr2* gene cassettes along with $bla_{VIM-2}$, but with a 3' region that could not be amplified with any primer combination. This element was seen in all 12 isolates

belonging to ST773 (complex F), referred from 11 different laboratories over a seven-year period, each submitting only one or two isolates.

Integron IV had an unusual 3' region consisting of a partial 5'CS followed by a *sul1* gene. Fifteen of the 18 isolates belonging to complex D (ST654 and its close relative ST964) carried integron IV. These were referred from seven laboratories over a nine-year period. Referring sites included South East_6, from which seven representatives were received over a three-year period; the remaining six laboratories referred only one or two isolates.

Integron V, which contained $bla_{VIM-2}$, *aacA7* and *aadB* gene cassettes, yielded amplicons using primers targeting both the 5' and 3'CS together with those targeting the $bla_{VIM-2}$ gene, but the 3' region could not be confirmed due to mixed sequencing reads using primers targeting this region. All six isolates carrying integron V belonged to ST357 (complex E) and were referred from the same laboratory (North West_ 15) over a seven-year period, with three submitted in 2010.

Sequencing of integron VI showed that it was identical to the previously-reported In496 (GenBank FM994936).[198] This integron was seen in four ST235 (complex B) isolates from three laboratories and seven additional isolates of four different VNTR types, not belonging to any of the major complexes. These seven included four isolates referred from South East_7 belonging to ST235 (complex B) or two different VNTR types for which the ST was not determined. Two of the four isolates, each having a different VNTR type, were from patients on the same hospital ward in 2010 and separated by a 10-week period.

#### 4.3.1.2 Distribution of integrons amongst the major VNTR complexes

One to 17 different integron profiles were seen within each of the six major ST/VNTR complexes. Complexes C and F (corresponding to STs 233 and 773) had a single integron type present in all representatives. In ST111 (complex A, n=65), integron I was found in 89% of isolates, with two different *bla*$_{VIM}$- and two different *bla*$_{IMP}$-encoding integrons seen among the remaining 11% (n=7); all three of the identified 3' regions were represented among the integrons of these latter seven isolates. Among ST654/964 (complex D, n=18) representatives, 83% harboured integron IV; one further ST654 isolate carried an otherwise identical integron with an additional *aadB* cassette present following the *bla*$_{VIM-2}$ gene, while one carried a unique *bla*$_{VIM}$-containing integron with a 3'CS; the other, an ST964 organism had a *bla*$_{IMP}$-containing integron with the 3'CS.

Isolates belonging to the remaining two complexes, B and E (ST235, n=47 and ST357, n=16), harboured diverse integrons, with 17 and nine different types seen in these complexes, respectively. In ST235 (complex B), 13 different *bla*$_{VIM}$- and two different *bla*$_{IMP}$-encoding integrons were seen among the 29 representatives (62%) not carrying the main integron types (II and VI); none of these diverse integrons was seen in more than four isolates belonging to this complex. The majority of these diverse integrons had the 3'CS, but one *bla*$_{VIM}$-containing integron had a Tn*5090*-like region and none of the major 3' regions was detected for one *bla*$_{IMP}$-containing integron. Eight *bla*$_{VIM}$-containing integrons were seen among the 10 (62%) ST357 (complex E) isolates not harbouring integron V; these were received from nine different laboratories; four had the 3'CS and four had Tn*5090*-like 3' regions.

Integrons II and VI, respectively, also were seen in 9% and 21% of isolates not belonging to the main VNTR complexes but, otherwise, the integrons in these isolates were diverse. Among the remaining 70% (n=25) of these isolates, 15 from 12 laboratories carried different *bla*$_{VIM}$-containing integrons while four *bla*$_{IMP}$-containing integrons were seen in the remaining 10 isolates, each associated with a different VNTR type; eight integrons had the 3'CS, nine had Tn*5090*-like 3' regions and no 3' region could be amplified for integrons of the remaining two isolates.

### 4.3.2 Integrons seen in *Pseudomonas* spp. besides *P. aeruginosa.*

Twenty-three isolates belonging to *Pseudomonas* spp. other than *P. aeruginosa* were submitted to AMRHAI between 2003 and 2012 (as detailed in Section 2.1.3); 17 carried *bla*$_{VIM}$ genes while six carried *bla*$_{IMP}$. Sixteen of these 23 isolates were viable in the archives and were studied, as described in later chapters, to investigate their potential as an environmental reservoir for MBL-containing integrons. These 16 isolates were referred from ten different UK hospital laboratories; 15 were isolated from patient specimens, while the remaining isolate was from a hospital sink. Isolates were identified to species level by sequencing of their *rpoB* gene, as described in Section 4.2.2 (figure 4.10). Several species belonged to the *P. putida* group, i.e. *P. mosselii* (seven isolates), *P. monteilli* (one isolate), and other members of the *P. putida* group not closely related to any of the reference strains (six isolates). A further two isolates belonged to the *P. aeruginosa* group; one was *P. oleovorans*, while the other was *P. alcaligenes*.

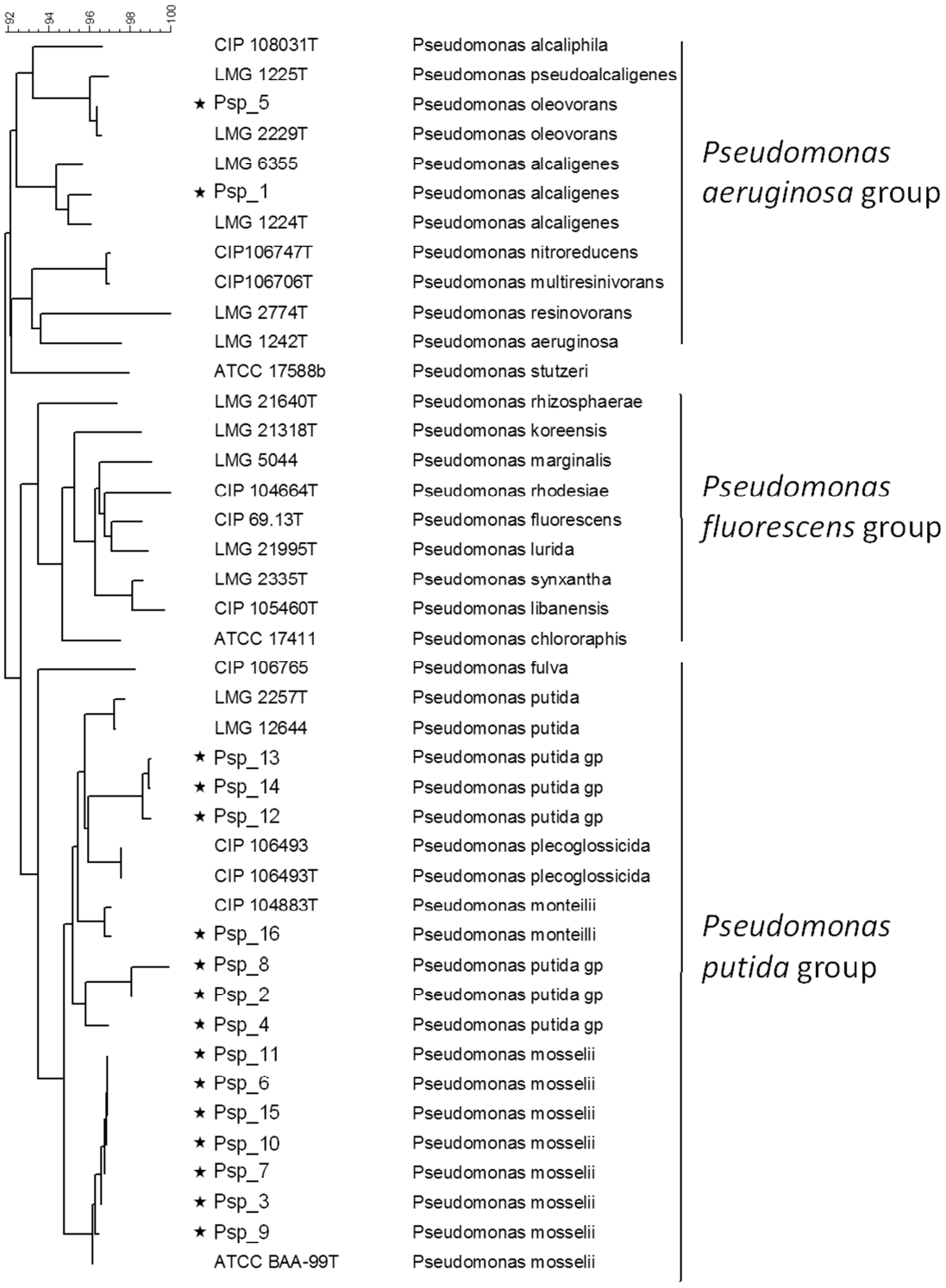



**Figure 4.10:** Neighbour-joining tree showing the relationship of *rpoB* gene sequences between MBL-producing isolates of non-*aeruginosa Pseudomonas* spp. described in this study (indicated by a star) and reference strains.

Nine different *bla*$_{VIM}$-containing and four different *bla*$_{IMP}$-containing integron structures were seen among these 16 non-*aeruginosa* isolates; none of these corresponded to the major integron structures (I-VI). Five isolates nevertheless had one of the less common integron profiles (identical in amplicon size and restriction pattern) seen among the MBL-producing *P. aeruginosa*.

Only one of these latter five *Pseudomonas* spp. isolates shared an integron profile with an MBL-producing *P. aeruginosa* isolate from the same hospital. This was a *P. mosselii* isolate cultured from a hospital sink at London_12 in 2004, which shared a *bla*$_{VIM}$-encoding integron profile with four MBL-producing *P. aeruginosa* isolates from different patients, two of them from London_12, also in 2004; one collected one week, and the other nine months before the *P. mosselii* isolate. The other two *P. aeruginosa* isolates with this integron profile were submitted from other London hospitals in 2009 and 2010.

The remaining four *Pseudomonas* spp. isolates that shared integron profiles with *P. aeruginosa* representatives were not linked in time and place to these *P. aeruginosa* isolates. A *bla*$_{VIM}$-positive *P. monteilli* isolate shared an integron profile with a *P. aeruginosa* strain isolated from a different hospital; both were isolated in 2012. A *P. putida* group isolate carrying *bla*$_{VIM}$ and submitted in 2010 shared the same integron profile as five *P. aeruginosa* isolates submitted between 2008 and 2012; none of these was from the same hospital as the *P. putida* group strain. A *bla*$_{VIM}$-positive *P. putida* group isolate submitted in 2011 shared an integron profile with a *P. aeruginosa* isolate from a neighbouring London hospital received one year previously. Lastly, a *P. putida* group isolate carrying *bla*$_{IMP}$ had a similar integron

profile to five *P. aeruginosa* isolates submitted from four different laboratories between 2005 and 2011.

### 4.3.3 Plasmid transformations

Attempts to transform plasmids from single representative isolates of each of the main integron/ST combinations (integron I/ST111, integron II/ST235, integron II/ST233, integron III/ST773, integron IV/ST654, integron V/ST357, and integron VI/ST235) into the *E. coli* DH5α strain were consistently and repeatedly unsuccessful. A plasmid carrying $bla_{VIM-2}$ from a *P. aeruginosa* strain previously isolated at AMRHAI (Paer_pl1), tested as a control, was successfully transferred.

### 4.3.4 S1 nuclease and I-*ceu* I hybridisation

Representatives of each of the main integron/ST combinations, as above, were subjected to S1 nuclease and I-*ceu* I digest followed by in-gel hybridisation with a VIM-specific probe, as described in Section 4.2.4. The control strain (Paer_pl1) was also included. The resulting gels are shown in figure 4.11.

For the control strain (Paer_pl1), as expected, the VIM probe hybridised to a plasmid band, of approximately 300 kb, in the S1 nuclease digest. Similarly, for a representative of ST235 with integron VI (Paer_LW_50), the VIM-probe hybridised with a c.450-kb plasmid band from the S1 nuclease digest. In contrast, for representatives of ST773 with integron III (Paer_LW_167), ST654 with integron IV (Paer_LW_121) and ST357 with integron V (Paer_LW_197), the VIM-probe hybridised with chromosomal bands of the I-*ceu* I digest, indicating that the $bla_{VIM}$ gene was located on the chromosome.

The VIM-probe did not hybridise to any plasmid or chromosomal bands for the representatives of ST111 with integron I (Paer_LW_238) or either ST235 (Paer_LW_13) or ST233 (Paer_LW_109) with integron II; these isolates were investigated further using other methods.

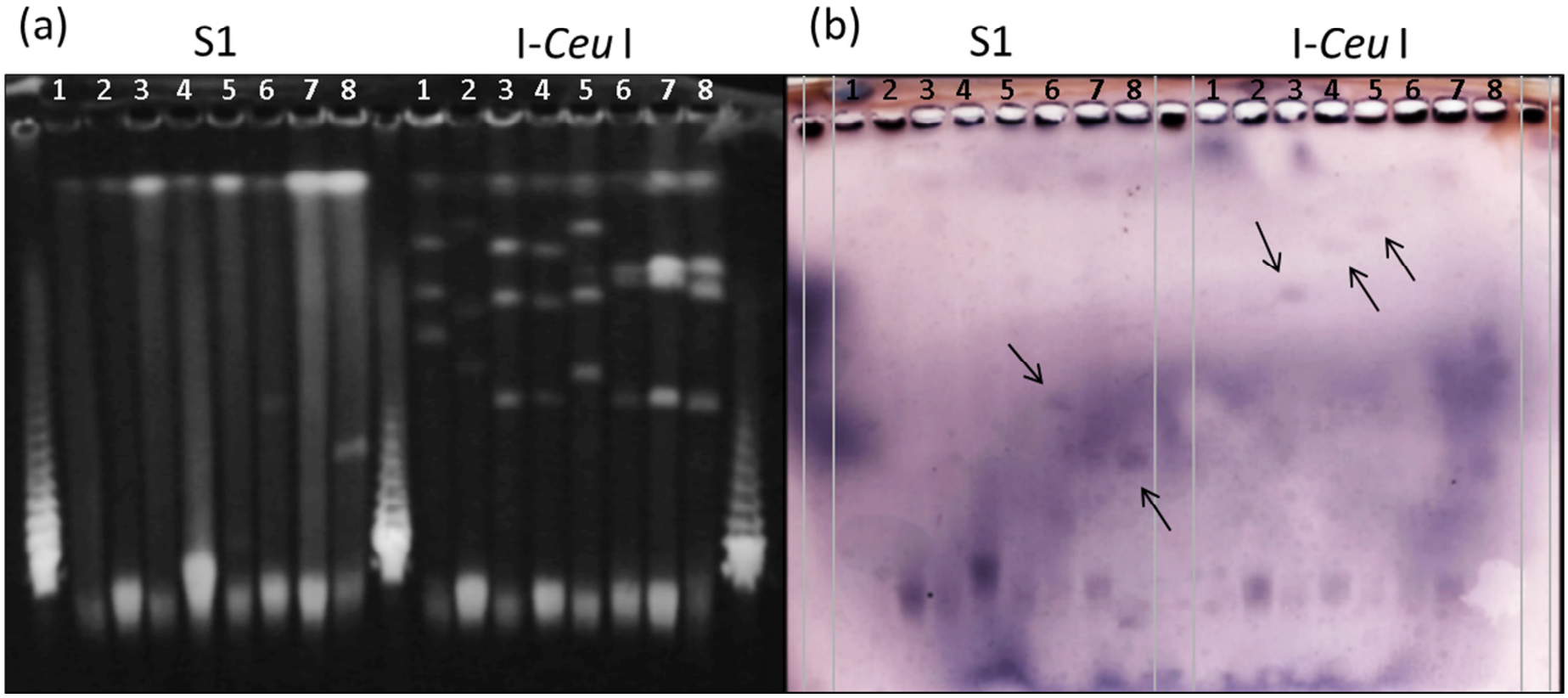



**Figure 4.11:** S1 and I-*Ceu* I digests; (a) digestion products separated by gel electrophoresis. (b) after in-gel hybridisation with a VIM-specific probe. Arrows indicate hybridisation. Representative isolates used are as follows; ST111/Integron I (lane 1), ST233/Integron II (lane 2), ST773/Integron III (lane 3), ST654/Integron IV (lane 4), ST357/Integron V (lane 5), ST235/Integron VI (lane 6), ST235/Integron II (lane 7), Control strain with VIM plasmid (lane 8). The Lambda Ladder PFG Marker was used to size the fragments.

### 4.3.5 Identification of genomic islands

#### 4.3.5.1 Genomic island structure carrying integron II in ST233 isolates

Integron II (In559) was previously identified in a genomic island (genomic island A) inserted in the *PA5101* gene on the chromosome of an ST233 *P. aeruginosa* isolate from the USA (figure 4.3).[147] Long-range PCR (as described in Section 4.2.7) using primers PA5101_F and VIM2004B on a representative of ST233 (Paer_LW_109) from our collection resulted in an amplicon of the expected size (6266 bp) based on the genomic island identified in the US isolate,[147] confirming that the $bla_{VIM-2}$ gene was linked to the chromosomal *PA5101* gene in this isolate (figure 4.12a).

A multiplex PCR (PCR 1) was carried out, as described in Section 4.2.6 and figure 4.6a, on all 42 isolates with integron II from this study to seek any evidence of a similar insertion of genomic island A into the *PA5101* gene (figure 4.12b). Amplicons were not obtained using primers targeting the intact chromosomal *PA5101* gene (PA5101_F and PA5101_R) for any of the 25 ST233 (complex C) isolates. However, amplicons were obtained for these 25 isolates using primers targeting *PA5101* together with the left-hand (VIMGI-LH_R) and right-hand (VIMGI-RH_F) junctions of the genomic island, indicating a similar insertion in the *PA5101* gene as for this previous US study;[147] 24/25 produced amplicons of the expected size (349 and 478 bp) while the remaining isolate gave a smaller product for the right-hand junction due to a 164-bp deletion in the *PA5101* gene. None of the remaining 17 isolates with integron II (belonging to ST235 or to other VNTR types) yielded products to indicate an insertion within *PA5101.* However, the 14 ST235 (complex B) isolates with integron II yielded a single 239-bp amplicon corresponding to an intact

*PA5101*, as did the two isolates not belonging to any of the main VNTR complexes (figure 4.12b). The final isolate, which also did not belong to any of the major VNTR complexes, yielded no amplicon with any primer combination.

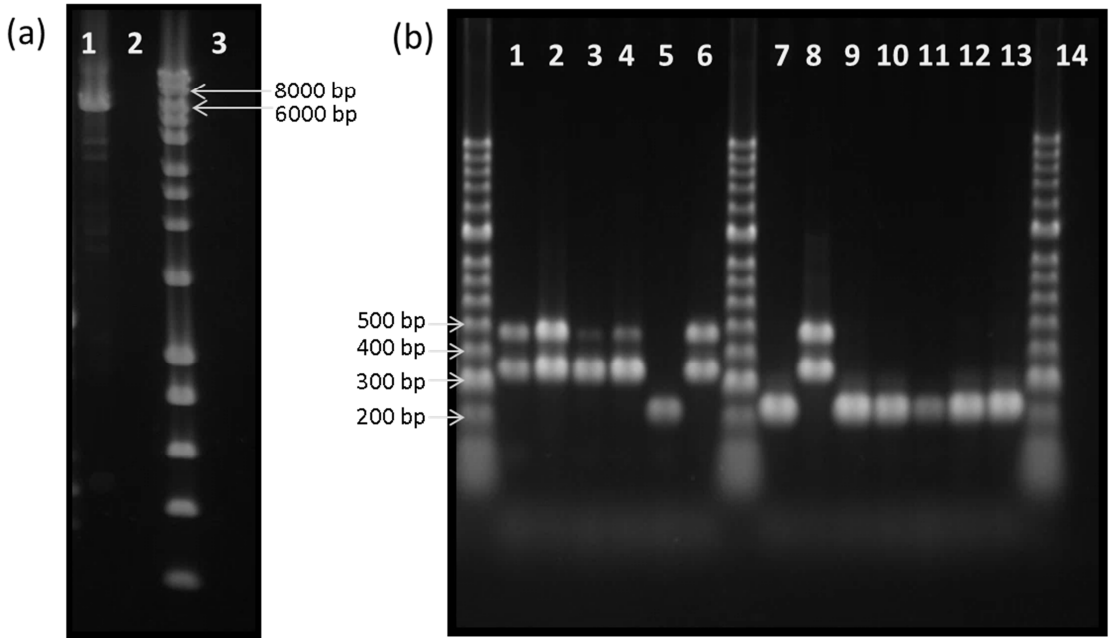



**Figure 4.12: (a)** Long PCR, confirming the linkage the *bla*$_{VIM}$ gene with the chromosomal *PA5101* gene using primers PA5101_F and VIM2004B; shown are integron II (In559) carrying ST233 (lane 1), and ST235 (lane 2) representatives and no template control (lane 3) **(b)** Example of amplicons obtained for PCR 1 (detection of genomic island A). Representatives carrying integron II (In559), belonging either to ST233 (lanes 1-4, 6 and 8) or ST235 (lanes 5 and 7), are shown. Amplicons for representatives carrying the other main integron structure (I or III-VI) are also indicated as follows: ST111/integron I, lane 9; ST773/integron III, lane 10; ST654/integron IV, lane 11; ST357/integron V, lane 12; ST235/integron VI, lane 13. The no template control is in lane 14.

#### 4.3.5.2 Genomic island structure carrying integron I in ST111 isolates

A representative of ST111 carrying integron I (Paer_LW_238) was selected for further investigation of the genetic location of the integron within the genome by WGS. Sequencing reads were obtained from two separate Illumina runs, and were combined, giving an average genome coverage of approximately 44x. *De novo* assembly of these Illumina reads, using SPAdes as described in Section 4.2.5, resulted in 166 contigs and an N50 (the contig length for which 50% of the entire assembly is contained within contigs of equal or greater value) of 100 kb, as shown in table 4.3. The $bla_{VIM-2}$ gene was found on a 1044-bp contig, but the analysis gave no information about its wider environment or its chromosomal or plasmid location.

**Table 4.3:** Assembly statistics for SPAdes assemblies of the ST111 representative

| Assembly | No. of contigs | Total length of contigs | N50 (bp) | Minimum contig size (bp) | Maximum contig size (bp) |
|---|---|---|---|---|---|
| **SPAdes (Illumina only)** | 166 | 7135311 | 100452 | 88 | 464511 |
| **SPAdes (hybrid)** | 92 | 7180261 | 162575 | 210 | 499444 |

To overcome this failure, long reads were obtained using MinION technology, as described in Section 2.2.5.3. These reads were used together with the short Illumina reads, to produce a hybrid assembly using SPAdes, as described in Section 4.2.5. This hybrid assembly had 92 contigs (table 4.3), of which two (called node 33; 71384 bp and node 66; 9204 bp) mapped to part of the integron I (In59-like) structure. The MinION reads were mapped to these contigs using Map with LAST,

and parsed as described in Section 4.2.5. Mapping results were checked manually and a total of 58 and 12 overlapping MinION reads were identified which, together, mapped to the full length of nodes 33 and 66, respectively, confirming that the contigs were correctly assembled. These included two MinION reads that each mapped to both the contigs (nodes 33 and 66), confirming that these were adjacent and approximately 460 bp apart. The MinION reads that spanned the two contigs also mapped to the full length of the integron I (In59) sequence obtained by PCR and Sanger sequencing of the same isolate; the 460-bp section of DNA corresponding to the *aacA29a* cassette. Thus these two contigs were assembled together with the integron I Sanger sequence, giving the c. 81-kb chromosomal fragment illustrated in figure 4.13.

Annotation of this 81-kb fragment revealed that integron I was part of a 28.5-kb genomic island inserted within a homologue of the *PA2229* gene of *P. aeruginosa* PA01. Integron I was contained within a truncated Tn*21*-like transposon including Tn*5090*-like *tniA* and *tniB*Δ transposon genes and a partial *mer* operon encoding mercury resistance. Downstream of this region the sequence had 99% nucleotide identity to Tn*4661* (NG_036554),[199] a Tn*4651*-like replicative transposon. Replicative transposons, such as this, can insert into their target site and replicate, forming an intermediate structure known as a cointegrate.[199] Homologous recombination at the resolution (*rst*) site separates the original and target sequences resulting in a single copy being present in both the original and target locations. The Tn*4661*-like structure seen here included the genes necessary for cointegrate formation (*tnpA* and *tnpC*) and resolution (*tnpS* and *tnpT*), as well as the

intact *rst* sequence (figure 4.13). Inverted repeats (iR) of Tn*4661* were present at either side of the Tn*4661*-like sequence, as reported previously,[199] with an identical sequence also at the junction of the chromosome with the Tn*21*-like portion of the genomic island. An 11-bp portion of the *PA2229* gene (CGGNGGTACCT) was also present on either side of the genomic island, indicative of the target-site duplication produced by a transposition event.

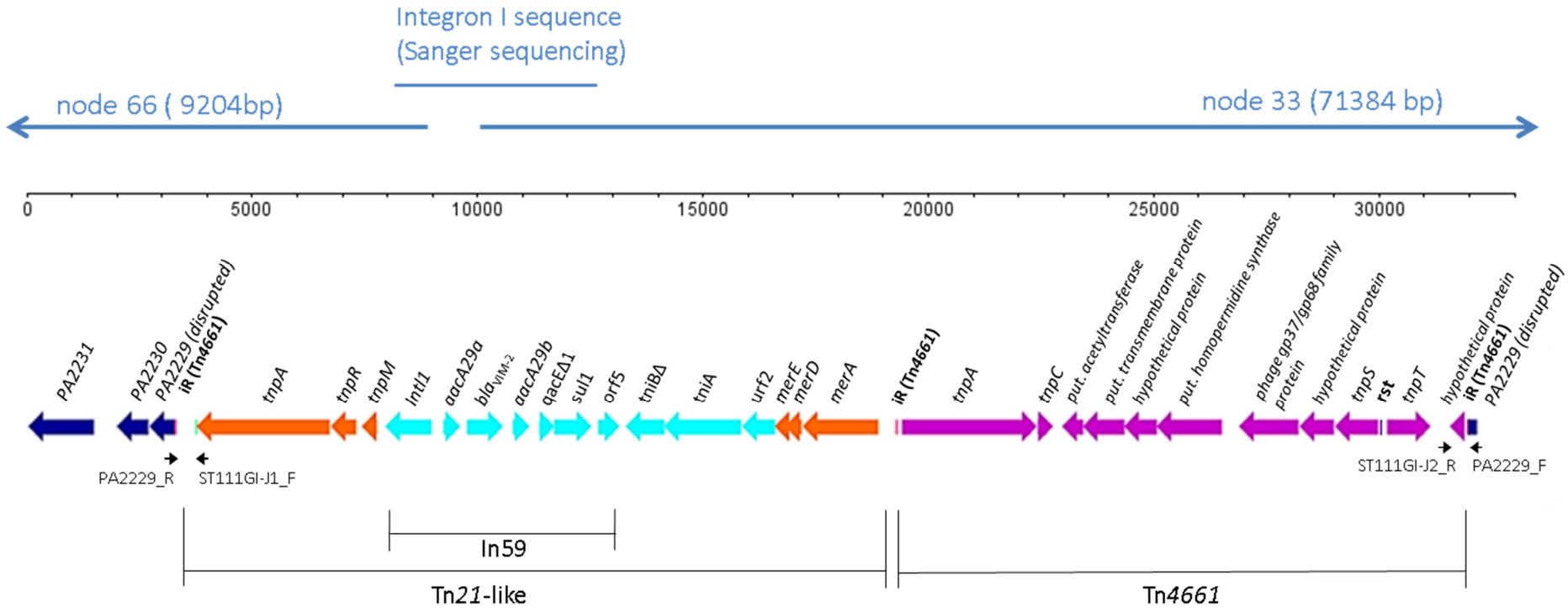



**Figure 4.13:** Structure of the integron I (In59)-containing genomic island (genomic island B) found in an ST111 isolate (Paer_LW_238). The relative positions of the Illumina hybrid contigs (designated nodes 33 and 66) and integron I sequence (obtained by PCR and Sanger sequencing) that were together used to assemble the sequence of the genomic island are indicated. The positions of the inverted repeat (iR) (GGGGTCATGCCGAGATAAGGGGAAAATTCATTCATTTGGAATGTAAG) and the resolution (*rst*; CAAAAACGGCTGCTTCGCGGTCGTTTCCCAGTACAC) sequences of Tn*4661* are also shown.

Long-range PCR on the same representative of ST111 using primers PA2229_R and VIM2004B, as described in Section 4.2.7, resulted in an amplicon of the expected size (7154 bp), confirming that the *bla*$_{VIM}$ gene was linked to the chromosomal *PA2229* gene (figure 4.14a).

A multiplex PCR (PCR 2) was designed, as described in Section 4.2.6 and figure 4.6b, to amplify the chromosomal junctions of genomic island B, using primers targeting the chromosomal *PA2229* gene (PA2229_F and PA2229_R) together with those targeting the island (ST111-GI-J1_R and ST111-GI-J2_F). PCR of 56/58 integron I (In59-like)-containing ST111 isolates yielded two amplicons of expected sizes (594 and 371 bp), indicating that a similar genomic island to that in the fully characterised isolate Paer_LW_238, was inserted in the same location in their genomes (figure 4.14b). The remaining 2/58 ST111 isolates carrying integron I, and 6/7 of the ST111 isolates carrying other *bla*$_{VIM}$- or *bla*$_{IMP}$-containing integrons yielded a single predicted 371-bp PCR product corresponding to the junction of the Tn*4661*-like region of the genomic island with the chromosomal *PA2229* gene (primers PA2229_F and ST111GI-J2_R), but no product corresponding to the junction of the *PA2229* gene with the Tn*21*-like part of the genomic island (primers PA2229_R and ST111GI-J1_F), indicating that Tn*4661* insertions were also present in these isolates. The final ST111 isolate, with a unique *bla*$_{VIM}$-containing integron, yielded a single predicted 267-bp amplicon corresponding to that expected for an intact *PA2229* gene (primers PA2229_F and PA2229_R) as did single representatives of other ST/ integron combinations (ST235/ integron II, ST233/integron II, ST654/integron IV, ST773/integron II, ST357/integron V and ST235/integron VI) indicating that they lacked insertions in the *PA2229* gene (figure 4.14b).

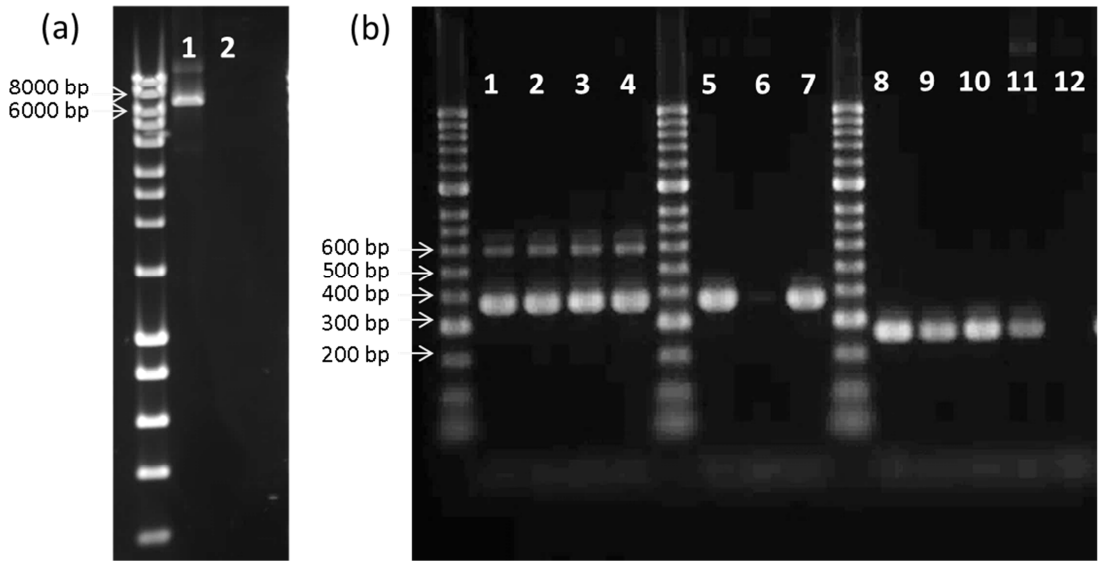



**Figure 4.14: (a)** Long-range PCR confirming the linkage of the *bla*$_{VIM}$ gene with the chromosomal *PA2229* gene, using primers PA2229_R and VIM2004B, for the integron I (In59)- carrying ST111 representative (lane 1), and the no-template control (lane 2) **(b)** Example of amplicons obtained for PCR 2 (detection of genomic island B) for ST111 isolates carrying integron I (lanes 1-4), ST111 isolates carrying other MBL encoding integrons (lanes 5-7); these had either one or two amplicons corresponding to the junctions of the chromosome with the genomic island and indicating an insertion is present in the chromosomal *PA2229* gene. Those obtained from isolates belonging to STs 235 (lane 8), 233 (lane 9), 654 (lane 10) and 773 (lane 11) are also shown and indicate that no insertion was present in the *PA2229* gene. The no template control is shown in lane 12.

#### 4.3.5.3 Genomic island structure carrying integron II in ST235 isolates

The genomic location of integron II (In559) in a representative of ST235 (Paer_LW_13) was similarly investigated by WGS. Illumina reads were obtained from two separate runs and the data combined, giving an average genome coverage of 36x. *De novo* assembly using SPAdes, as described in Section 4.2.5, resulted in an assembly of 129 contigs with an N50 of 127 kb (table 4.4). $bla_{VIM-2}$ was located on a 4661-bp contig (designated node 84), which also contained *dfrB5* and *aacC-A5* genes. This approach however gave no information about the wider environment of the MBL gene nor its chromosomal or plasmid location.

**Table 4.4:** Assembly statistics for SPAdes assemblies of the ST235 representative

| Assembly | No. of contigs | Total length of contigs | N50 (bp) | Minimum contig size (bp) | Maximum contig size (bp) |
|---|---|---|---|---|---|
| **SPAdes (Illumina only)** | 129 | 6661853 | 126926 | 81 | 316935 |
| **SPAdes (hybrid)** | 82 | 6703780 | 214124 | 81 | 530386 |

The strain was therefore sequenced on the MinION, as detailed in Section 2.2.5.3 with a total of 6232 two-direction reads that passed Metrichor analysis obtained. Hybrid *de novo* assembly, using the MinION long reads together with the Illumina reads, was carried out with SPAdes, as described in Section 4.2.5, resulting in an assembly of 82 contigs with an N50 of 214 kb (table 4.4); this placed the $bla_{VIM-2}$ gene on the edge of a 125774-bp contig (designated node 21).

The MinION reads were mapped to the contigs from the hybrid assembly using Map with LAST and parsed as described in Section 4.2.5. A total of 225 MinION reads

were identified that mapped to the *bla*$_{VIM-2}$-containing contig (node 21) but, there was a ~10-kb region of node 21 (nucleotides 58985-68829) to which no MinION reads mapped, indicating that this contig was misassembled.

Based on this experience, the MinION reads were re-mapped to the SPAdes assembly obtained from the Illumina data only, using Map with LAST, and parsed as before. Nineteen MinION reads mapped to the *bla*$_{VIM-2}$-containing contig (node 84, 4661 bp) linking it to the full length of nodes 53, 93 and parts of node 73, indicating that this latter contig was again misassembled. MinION reads additionally linked node 93 and parts of node 73 to node 20, and overlapped with reads that mapped to node 84, the *bla*$_{VIM-2}$-containing contig. The relative orientation of these contigs, as inferred from the overlapping MinION reads, is shown in figure 4.15.

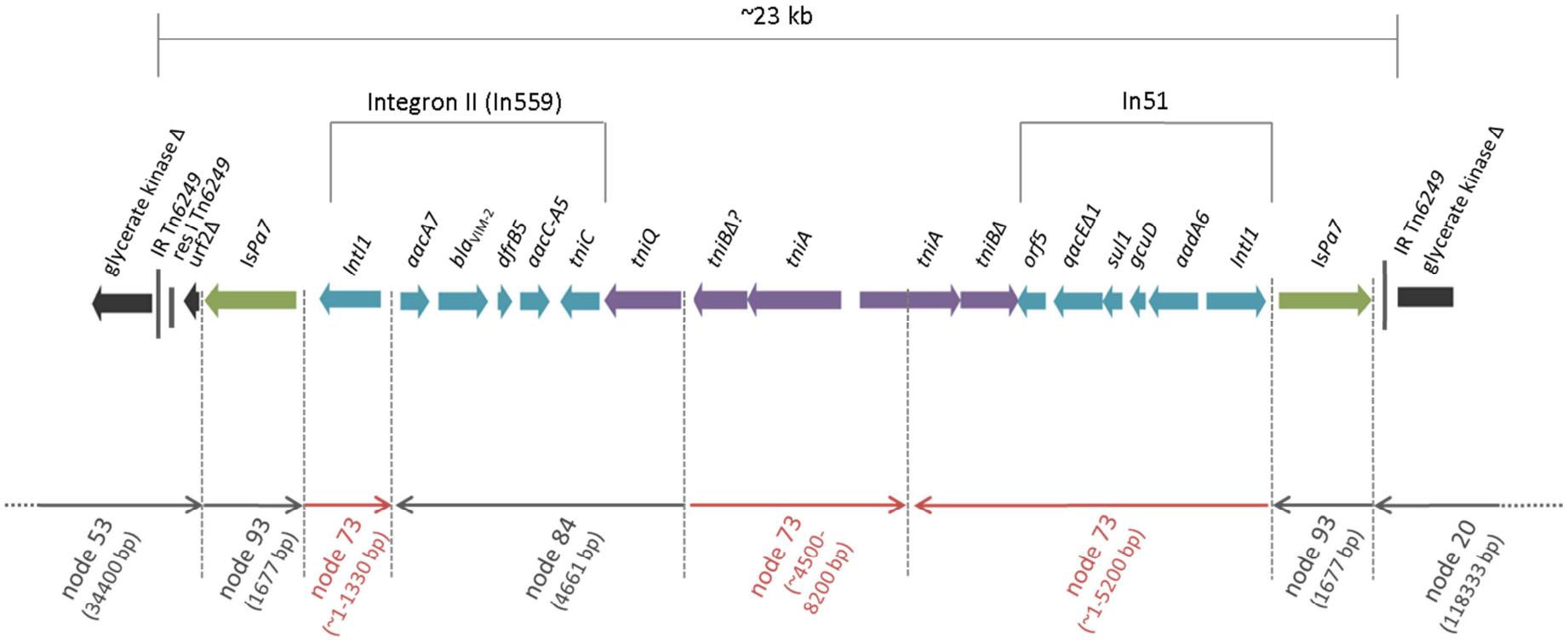



**Figure 4.15:** Deduced structure of the~23-kb genomic island found in the ST235 representative (Paer_LW_13) carrying integron II (In559). The relative positions of the contigs from the SPAdes Illumina-only assembly (nodes 53, 93, 73, 84 and 20) as determined by scaffolding with the MinION reads are shown. Positions of the inverted repeats (IRs) (GGGGNNATAGAGAAAACGGAAAAAATCGTACGNTAAG) and the putative resolution (*res*) sites (TGACGCCTAGTTAGGGCATGCCTCAGTCTGACAGTGATGAGTCGCAGGCGTTCGGATCGGCATCGGTTTCGCTCCCGGTCTTGGCGC GCGCCTGGAAGCGCTGATAGCACTCCAGCCCGCAGAAGTGCTCGACGTAT), as seen in the previously reported Tn*6249* structure (GenBank accession LK054503),[182] are indicated. The *tniB* gene downstream of the In559 integron spanned the junction of node 84 and a fragment of node 73 and so it is unclear whether this gene is complete.

Annotation of these contigs indicated that the *bla*$_{VIM}$-containing integron II (In559) was part of an approximately 23-kb genomic island itself inserted within the glycerate kinase gene of another PACS171b-like genomic island (GenBank accession number EU595750), which was in turn situated within the disrupted *endA* chromosomal gene. The genomic island contained two integrons in a tail-to-tail organisation: these were integron II (In559) as previously identified here, and another integron with 100% nucleotide identity to In51 (GenBank Accession AF140629). Integron II (In559) had the Tn*5090*-like *tniC*, *tniQ, tniB* and *tniA* genes in its 3' region; the *tniB* gene spanned the junction of nodes 84 and part of node 73, so it is unclear whether this gene is complete. In51 contained the genes *aadA6* and *gcuD*, with a classical 3'CS structure; it was also part of a Tn*5090*-like structure, which had a *tniA* and a disrupted *tniB* gene. The double integron structure was flanked by the transposase genes of Is*Pa7*. Tn*6249*-like inverted repeat (IR) sequences were present at both junctions of the VIM-2-encoding genomic island, as in a previously reported *bla*$_{VIM-1}$ containing genomic island sequence (figure 4.4, GenBank accession LK054503), but the *tnpA* gene of Tn*6249* and the adjacent *tnpR* and *res* sequences of this previous sequence were not present.

Long-range PCR with primers glykin_F and VIM2004B (as described in Section 4.2.7) confirmed that *bla*$_{VIM}$ was linked to the disrupted glycerate kinase, with an amplicon of approximately 4.6 kb obtained, (figure 4.16) as expected based on the inferred structure illustrated in figure 4.15.

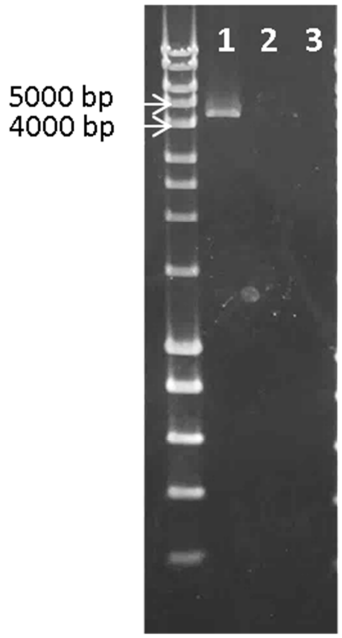



**Figure 4.16:** Long-range PCR indicating that the *bla*$_{VIM}$ gene of the ST235 representative (Paer_LW_13) was linked to the glycerate kinase gene of the PACS171b-like genomic island (genomic island C) using primers glykin_F and VIM2004B for isolates carrying integron II (In559) of ST235 (lane 1), and ST233 (lane 2) and a no template control (lane 3)**.**

This double integron arrangement is similar to that seen in a previously published[182] genomic island containing *bla*$_{VIM-1}$, shown in figure 4.4, which was also situated within the disrupted glycerate kinase gene of a PACS171b-like genomic island. On this basis, and in parallel to the WGS work, PCR investigations were carried out on ST235 isolates seeking evidence of this previously-described structure.

Two multiplex PCRs were designed, as described in Section 4.2.6 and shown in figure 4.6c.

The first PCR targeted the insertion site of the PACS171b genomic island (genomic island C) in the *endA* gene of the *P. aeruginosa* chromosome (PCR 3), using primers targeting the chromosomal *endA* gene (endA_F and endA_R) together with primers

targeting the left and right hand junctions of the PACS171b-like genomic island (PACS171b_J1_R and PACS171b_J2_F).

The second PCR targeted a Tn*6249*-like insertion in the glycerate kinase gene of the PACS171b genomic island (PCR 4), using primers targeting the glycerate kinase gene of the PACS171b-like genomic island (primers glykin_F and glykin_R) together with those targeting the left- and right-hand junctions of the Tn*6249*-like transposon (primers urf2_R and TnpATn6249_F).

A panel of 54 isolates was subjected to PCR. This included 44/52 MBL-positive complex B (ST235) isolates that were still viable in the archives. These came from 25 different laboratories, spanning ten years. Also included were two MBL-negative and largely antibiotic susceptible ST235 representatives, collected in 2012. Three integron II-carrying isolates not belonging to the main VNTR complexes and single representatives of each of the other main complexes A and C-F (STs 111, 233, 654, 773 and 357) were also analysed. A summary of amplicons obtained from these isolates for the two PCRs is shown in tables 4.5 and 4.6, and figure 4.17a and b, respectively.

**Table 4.5** Amplicons obtained by multiplex PCR (PCR 3) targeting the insertion of a PACS171b-like genomic island in the chromosomal *endA* gene for isolates of ST235 and other types (n=54)

| Amplicons[a] | MBL-positive ST235 (n=44) | MBL-negative ST235 (n=2) | Other types | Implication |
|---|---|---|---|---|
| **377 bp and 600 bp** | 40 | 1 | 0 | PACS171b-like insertion present in *endA* gene and both junctions present |
| **377 bp only** | 2 | 0 | 0 | PACS171b-like insertion present in *endA* gene but only single junction amplified |
| **283 bp only** | 2 | 1 | 8 | Intact *endA* gene, no insertion of a PACS171b island |

[a] expected amplicon sizes for the respective primer pairs included in the multiplex were 283 bp (endA_F and endA_R), 377 bp (endA_F and PACS171b_J1_R), and 600 bp (PACS171b_J2_F and endA_R); also see figure 4.6c

**Table 4.6:** Amplicons obtained by multiplex PCR (PCR 4) targeting the insertion of a Tn*6249*-like structure in the glycerate kinase gene of the PACS171b-like genomic island, for the ST235 isolates positive for the PACS171b-like island (n=43)

| Amplicons[a] | MBL-positive (n=42) | MBL-negative (n=1) | Implication |
|---|---|---|---|
| **591 bp and 373 bp** | 19 | 1 | A Tn*6249*-like insertion in glycerate kinase gene, with both junctions amplified |
| **373 bp only** | 16 | 0 | Tn*6249*-like insertion present, but with only the left-hand junction amplified suggesting differences in sequence at the right-hand junction of the Tn*6249* gene with the PACS171b-like genomic island |
| **591 bp only** | 6[b] | 0 | Tn*6249*-like insertion present, but only the right-hand junction amplified suggesting differences in sequence at the left-hand junction of the Tn*6249* gene with the PACS171b-like genomic island |
| **No amplicons** | 1 | 0 | A different insertion was present in the glycerate kinase gene without sequence similarity to Tn*6249* or no insertion was present |

[a] expected amplicon sizes for the respective primer pairs included in the multiplex were 271 bp (glykin_F and glykin_R), 373 bp (glykin_F and urf2_R), and 591 bp (TnpATn6249_R and glykin_R); also see figure 4.6c

[b] three of these six isolates additionally had a 2kb amplicon

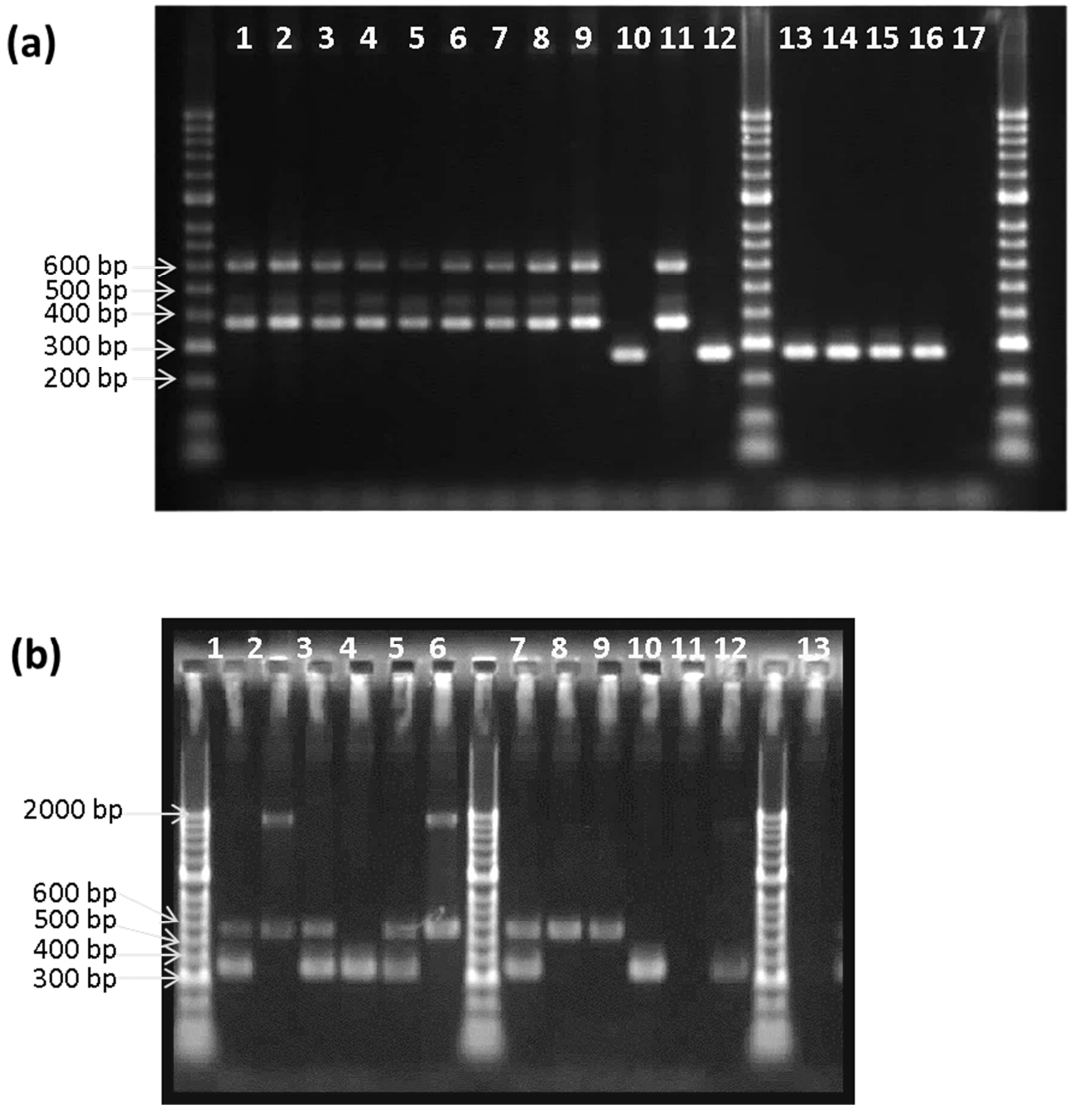



**Figure 4.17: (a)** Example of amplicons obtained using primers targeting the insertion site of the PACS171b-like island in the chromosomal *endA* gene (PCR 3) for MBL-positive ST235 isolates (lanes 1-9), MBL-negative ST235 isolates (lanes 10 and 11), isolates belonging to STs 111, 233, 357, 654 and 773 (lanes 12-16) and the no template control (lane 17). These variously yielded either two amplicons (377 and 600 bp) corresponding to the junctions of the chromosome with the PACS171b-like genomic island and indicating an insertion within the *endA* gene, or a single amplicon (283 bp) corresponding that expected for the intact *endA* gene. **(b)** Example of amplicons obtained using primers targeting the insertion of a Tn*4661*-like structure in the glycerate kinase gene of the PACS171b-like genomic island (PCR 4). MBL-positive ST235 (lanes 1-11) and the MBL-neg ST235 isolate (lane 12); all had products indicating an insertion in the glycerate kinase gene except for the representative in lane 11). The no template control is shown in lane 13.

The first of these multiplex PCRs (PCR 3; table 4.5 and figure 4.17a), targeting the insertion of a PACS171b-like genomic island in the chromosomal *endA* gene, suggested that such an insertion was present in 40/44 MBL-positive ST235 *P. aeruginosa* isolates and one of the two MBL-negative ST235 isolates. For each of these isolates two amplicons were obtained corresponding to the expected sizes (377 and 600 bp) for primer pairs targeting the two junctions of the PACS171b-like genomic island with the chromosome (endA_F with PACS171b_J1_R, and PACS171b_J2_F with endA_R, respectively). Two of the four remaining MBL-positive ST235 isolates yielded only a 377-bp PCR product, indicating that the left-hand junction of the PACS171b genomic island was present (primers endA_F and PACS171b_J1_R), but that the right-hand junction was modified. The remaining two MBL-positive and one MBL-negative ST235 isolates yielded a 283-bp product corresponding to that expected for an intact *endA* gene (primers endA_F and endA_R), as did the three isolates carrying integron II that did not belong to the main VNTR complexes, and the five representatives of other complexes.

PCR 4 (table 4.6 and figure 4.17b) was carried out on the 42 MBL-positive representatives and one MBL-negative (and fully antibiotic susceptible) ST235 isolate that produced an amplicon with PCR 3. Nineteen of the 42 MBL-positive isolates, and the MBL-negative isolate, yielded two PCR products with expected size (373 and 591 bp) using primers targeting the glycerate kinase gene of the PACS171b-like genomic island (primers glykin_F and glykin_R) together with those targeting the left- and right-hand junctions of the Tn*6249*-like transposon (primers urf2_R and TnpATn6249_F); this indicated that a similar structure was inserted within the PACS171b genomic island in these 19 isolates (table 4.6, figure 4.17b).

Twenty-two of the remaining 23 MBL-positive isolates yielded PCR products for one of the two junctions indicating that the glycerate kinase was also disrupted. Sixteen of these 22 isolates, including the 13 isolates carrying integron II, yielded a 373-bp amplicon of the expected size for primer pair glykin_F and urf2_R only. Six further isolates yielded 591-bp amplicons corresponding to the expected size for primers glykin_R and TnpATn6249_F, with three of these additionally yielding an approximately 2-kb PCR product. The final MBL-positive ST235 isolate gave no amplicons with any primer combination.

## 4.4 Discussion

Diverse MBL-encoding class 1 integrons were seen amongst the 218 MBL-producing *P. aeruginosa* from UK hospital laboratories, with at least three different 3' regions observed. The most common arrangements were the classical 3'CS (55%) and Tn*5090*-like regions (30%). In four of the six major sequence types (STs 111, 233, 654/964 and 773), single integrons or integron-like elements (I, II, IV and III respectively) predominated, with the same or similar integrons also having previously been reported internationally, as outlined below, in isolates belonging to the same STs. In contrast, a high diversity of integron types was seen in ST235 (complex B) and ST357 (complex E) isolates, with 17 and nine profiles seen, respectively.

The majority of isolates belonging to ST111 (complex A) carried integron I, a variant of In59; this organisation has also been reported in several European countries, again usually associated with ST111. In59 itself was first reported in 2001 in France.[197] Subsequently, an In59 variant identical to that seen here was reported in ST111 isolates from Norway and Sweden, including from a patient previously hospitalised in Greece,[124] and in three hospitals in central Greece.[122] ST111 isolates harbouring In59-like integrons have also been reported in Austria[200] and, most recently, in Colombia.[128]

Integron II, which corresponds to In559, was seen here in all MBL-producing ST233 isolates, 30% of ST235 isolates and three isolates not belonging to the main VNTR complexes. This integron was first described in isolates of unknown ST from a hospital outbreak in the USA in 2005,[87] and was subsequently reported in a single

ST233 isolate recovered from a patient in Norway (thought to be imported from Ghana),[124] and in an outbreak of ST233 *P. aeruginosa* in a US hospital.[147] The integron was also reported in isolates of ST1488, which is closely related to ST233, from the Ivory Coast.[201] An ST235 *P. aeruginosa* clone carrying In559 is widely disseminated across Russia, Belarus and Kazakhstan;[136] In559 was also associated with sporadic isolates of STs 234 and 244 in these countries. Additionally, In559 was detected in ST235 isolates from Malaysia and Sri Lanka,[137] ST244 and ST640 isolates in Tanzania,[179] and isolates of unknown STs in Taiwan[202] and India (GenBank: HQ005291).

All isolates belonging to ST773 (complex F) carried integron III-like elements. The partial sequence obtained had an identical cassette array to an integron previously reported in ST773 isolates in India,[137] although we were not able to amplify the 3' region reported in the Indian isolates. Of the 5/12 patients for whom travel history was available, four had travelled to India. These four were admitted to different UK hospitals; the remaining patient was treated on the same hospital ward as one of the patients who had travelled and may reflect local cross infection.

Integron IV predominated amongst the isolates belonging to ST654/ST964 (complex D). A similar integron, with the same unusual 3' region seen in our isolates, but with an additional *aadB* cassette, was previously reported in an ST654 isolate from Sweden and was thought to have been imported from Tunisia.[124] This previously-described integron was located upstream of Tn*5501*-like and Tn*5393*-like transposon genes, along with s*trA* and s*trB* cassettes, in this previous study.[124] PCR analyses showed that these cassettes were also linked to the $bla_{VIM}$-containing

integron in our isolates, suggesting a similar genetic environment to the Swedish isolate.

Integron V was observed from six out of 16 isolates belonging to ST357 (complex E). This cassette arrangement has not been reported previously and sequencing of an amplicon obtained for the $bla_{VIM}$-to-3'CS region consistently resulted in mixed reads, possibly due to two co-migrating amplicons, as a result of two copies of the $bla_{VIM}$ gene being present in different integrons.

Isolates of diverse types harboured integron VI, including members of ST235 and five other VNTR types. This integron is identical to In496, and was previously reported in *P. aeruginosa* isolates from India and the Philippines.[198]

Although some hospitals had persistent problems, including the ST111 outbreak that was possibly associated with the colonisation of the wastewater systems at London_17,[157] many isolates with the prevalent VNTR complex/integron combinations did not cluster in terms of time or place, being referred from between six and 21 laboratories over seven- to nine-year periods, suggesting multiple introductions into different UK hospitals. A major exception was the case of ST357 (complex E) isolates carrying integron V, where all six representatives were from the same laboratory (North West_15) and were part of the previously-reported outbreak.[156]

In some cases, integron data provided further discrimination between isolates that otherwise were identical or very similar by VNTR typing. For example, isolates of ST235 from eight patients were received from hospital laboratory London_7 over a seven-year period; three isolates were received in a three-month period with the

remaining five being received between six and 33 months apart from each other. Although they comprised just two VNTR profiles the organisms variously carried six different integrons of diverse sizes, with both 3'CS and Tn*5090*-like 3' regions represented. This observation, together with their temporal scatter, suggests that ST235 organisms were likely imported to the hospital on multiple occasions rather than persisting in the hospital. In contrast, the large (n=21) cluster of ST111 isolates referred from London_17 consistently carried integron I despite being collected over a nine-year period. This fits with the persistence of the strain in the environment, possibly the wastewater system,[157] with repeated infection from this source.

While four of the six main integron structures (I, III, IV and V) were only seen in single VNTR complexes (corresponding to STs 111, 773, 654/964 and 357, respectively) integrons II and VI were seen in more diverse STs. Identical integron II (In559) structures were found in isolates of different VNTR types, including ST235 and ST233 (complexes B and C). Wider analysis of their genetic environments indicated that these integrons were located in lineage-specific chromosomally-located genomic islands in these ST235 and ST233 isolates. Neither of these were detected in isolates of other VNTR types carrying integron II, indicating that horizontal transfer of an entire genomic island was not responsible for the integrons dissemination. In both the ST233 and ST235 genomic islands the integrons were part of a Tn*5090*-like transposon that included all the genes necessary for transposition, although the *tniC* gene of this transposon was disrupted by IS*Pa*54 in the ST233 isolate, and it was not known whether *tniB* was intact in the ST235 strain, due to low sequence coverage of the region. Nevertheless, it is possible that an

ancestor of this Tn*5090*-like transposon containing integron II, with the intact transposition genes, was transferred independently to representatives of ST235, ST233 and other lineages, with the *tniC* gene subsequently becoming disrupted in an ancestor of the ST233 strains. Alternatively this gene cassette arrangement may have been assembled independently in the different lineages.

Isolates of different types carrying integron VI were referred from six different laboratories spread over a seven-year period. These submissions included two isolates of different VNTR types referred over a ten-week period from two patients treated on the same hospital ward at South East_6. Analysis of a representative carrying integron VI from this study, and a previous report[198] showed that the integron was located on a plasmid. This, together with the epidemiological information, suggests that plasmid-mediated spread of integron VI may have occurred between *P. aeruginosa* of different VNTR types.

Various *Pseudomonas* spp. besides *P. aeruginosa* with $bla_{IMP}$ or $bla_{VIM}$ genes are sometimes isolated from patients or clinical environments,[203–206] and it is suggested they may act as an environmental reservoir for MBL-containing integrons.[205] Of the 16 isolates of other *Pseudomonas* spp. studied here, four had integron profiles matching those of minor types seen amongst *P. aeruginosa* (none corresponded to major integrons I-VI) but only one of these isolates (a *P. mosselii*) was found at the same hospital as *P. aeruginosa* isolates carrying the same integron. As the *P. mosselii* was recovered from the hospital environment after known cases of *P. aeruginosa* patient infections, it is not clear whether the integron was transferred to or from a *P. aeruginosa* strain.

Integrons I, II and III were previously reported to be located on the *P. aeruginosa* chromosome in isolates from Scandinavia, USA, and India respectively,[124,137,147] whereas integron VI (also associated with transmission among VNTR types here) was found on a 35-kb plasmid in Indian isolates.[198] Attempts to transform any VIM-carbapenemase-encoding elements from isolates by electroporation were consistently unsuccessful. S1 nuclease and I-*ceu* I digests confirmed that integrons III, IV and V were located on the chromosome in representatives of STs 773, 654 and 357, respectively, while integron VI was located on a plasmid in an ST235 isolate. Further analysis by PCR and WGS data confirmed that integron I, in a representative of ST111, and integron II, in representatives of STs 233 and 235 were also located chromosomally.

In559, which is identical to integron II, was previously found to be located in a 35-kb genomic island inserted in the chromosomal *PA5101* gene in ST233 *P. aeruginosa* isolates from a recent outbreak in the USA.[147] We found evidence of a similar organisation, with *PA5101* disrupted, in all the ST233 (complex C) isolates studied here, supporting the view that ST233 *P. aeruginosa* with this arrangement may have spread internationally. However, the *PA5101* gene was not disrupted in ST235 (complex B) or other VNTR types with integron II, and these were not linked in time and place to the ST233 isolates, suggesting a different genetic environment.

A representative of ST235 carrying integron II (In559) was therefore investigated further using WGS, and the integron was located in an approximately 23-kb genomic island, as part of a double integron structure within a defective Tn*6249*-like transposon. This genomic island was inserted within the glycerate kinase gene

of a PACS171b-like genomic island, which was in turn inserted within the *endA* chromosomal gene. This is similar to the genetic context of previously-reported transposons Tn*6249* and Tn*6160*, both containing $bla_{VIM-1}$ as part of a double integron structure, reported in *P. aeruginosa* isolates of ST235 and an unknown ST from Italy[182] and Australia,[207] respectively. Tn*6162,* containing a single In51 integron, was also located in the same PACS171b-like genomic island in isolates from Australia and Uruguay.[208] PCR analyses detected PACS171b-genomic-island-like insertions in the chromosomal *endA* of 42 of the 44 MBL-positive ST235 isolates in our collection and insertions within the glycerate kinase gene of the PACS171b-like genomic island were present in all but one of these 42. This suggests that transposons inserted within PACS171b-like genomic islands may represent a common platform, shared by many ST235 isolates, by which they can acquire new resistance genes. Nevertheless, MBL genes were reported in genomic islands inserted elsewhere in the genomes of ST235 isolates, for example $bla_{IMP-1}$-containing IS*26*-composite transposons were found inserted into the chromosomal *hmgB* or *oprD* genes,[209,210] and a $bla_{GES-5}$-containing integron, as part of transposon Tn*6163*, inserted into a different genomic island with a chromosomal insertion point close to the *PA2583* gene.[208]

A representative of ST111 carrying integron I (In59) was investigated by WGS, and again the integron was located on a genomic island, this time inserted within the *PA2229* chromosomal gene and including a region with 99% identity to Tn*4661*, a Tn*4561*-like replicative transposon. Sequences identical to Tn*4661* have also been reported in the *P. aeruginosa* plasmid Rms148[199] and on the PAGI-4 genomic island of *P. aeruginosa* strain C.[211] In our ST111 representative the genomic island,

including the Tn21-like transposon with integron I (In59), was flanked by the Tn*4661* inverted repeats, suggesting that the entire structure could be moved by the action of the transposon. However we only saw integron I (In59) in ST111 strains and it is only rarely reported in strains of other types (nevertheless including those belonging to STs 277, 244, 253 and 773 in several Greek hospitals, where In59-encoding ST111 representatives were more prevalent[122]) suggesting that Tn*4661*-mediated transposition of this integron, if it occurs, is uncommon. A PCR designed to amplify the junctions of the genomic island and link to it with the chromosome revealed that all but one of our 65 ST111 isolates had a similar Tn*4661*-like insertion in the *PA2229* gene; amplicons were obtained for one (8 isolates) or both (56 isolates) junctions of the identified genomic island with the chromosome, even in isolates carrying diverse integrons. This implies that integrons contained within Tn*4661*-like transposon structures inserted at this location in ST111 genomes could act as a lineage-specific platform for the acquisition of diverse resistance genes including $bla_{VIM}$ or $bla_{IMP}$.

In summary, 'high-risk clones' carrying particular integron types, for example ST111 with In59 and ST233 with In559, were prevalent MBL-producing *P. aeruginosa* in the UK and appear to have become widespread internationally. Despite this, a variety of integron structures, including those with different 3' structures, were seen amongst most of the 'high-risk clones' in our collection (STs 111, 654, 235 and 357) and were reported elsewhere.[68,122,137] Genomic islands were identified and these contained integrons In59 (integron I) and In559 (integron II) in ST111 and ST235 representatives, respectively. Lineage-specific genomic island insertions were seen in most isolates belonging to ST111 and ST235 strains, and were reported

elsewhere, although carrying different integron structures to those found here. Carriage of these shared island structures may predispose the lineages to the acquisition of diverse resistance genes. WGS methods, which are becoming increasingly available have the potential to provide increased discrimination between members of 'high-risk clones', including analysis of their resistance gene content and this will be explored in the following chapter.

# 5 Whole genome sequencing (WGS) for the analysis of 'high-risk clones'

## 5.1 Introduction

Members of 'high-risk clones', such as *P. aeruginosa* ST111 and ST235, are often very similar or identical when analysed with conventional typing methods, including VNTR, even when isolated at diverse locations and times. It can therefore be difficult to determine whether repeated isolation of these types represent an outbreak or not, although this is critical to inform epidemiological investigations and to target effective interventions to limit their spread. Whole genome sequencing (WGS) is increasingly used in clinical microbiology and can provide the higher resolution when it is needed.

### 5.1.1 WGS for investigation of outbreaks

WGS, usually based on analysis of single nucleotide polymorphisms (SNPs) has been used to track outbreaks of numerous bacterial species, for example *Mycobacterium tuberculosis*,[212] *Staphylococcus aureus*,[213] *Klebsiella pneumoniae*,[214] *Salmonella enterica*[215] and *P. aeruginosa*.[216–218] These studies give insights into transmission events and potential sources and reservoirs of the bacteria. For example, retrospective WGS analysis was used to track an outbreak of KPC-producing *K. pneumoniae* belonging to the ST258 clone to presumptively identify likely transmission events.[214] WGS was also used in near-real-time to identify the likely source of an ST253 *P. aeruginosa* outbreak in an Australian hospital as a sink in the neo-natal intensive care unit, thus informing infection control interventions.[218] In addition, WGS has been used to study international members of 'high-risk clones', for example the *E. coli* ST131 clone,[219] to understand better their global spread.

### 5.1.2 Other applications of WGS

In addition to molecular epidemiology, WGS data can be used to investigate other aspects of the biology of the organism. For example, WGS studies have detected antibiotic resistance determinants in *P. aeruginosa,*[30] including acquired resistance genes. Similar studies have also been carried out on other bacterial species, including *S. aureus*[220] and Enterobacteriaceae.[221]

WGS data may also be used to seek specific features that may account for the apparent increased ability of some 'high-risk clones' to acquire and/or maintain resistance genes. For example, Kos and colleagues recently reported that, among a collection of diverse *P. aeruginosa* genomes (including those belonging to STs 111 and 235), those that commonly carried varied β-lactamase genes also lacked known clustered regularly interspersed short palindromic repeats (CRISPR), and did not encode any CRISPR-associated proteins (Cas).[30] These CRISPR-Cas systems are bacterial adaptive immunity systems that defend bacteria against mobile genetic elements, including bacteriophage, plasmids and other horizontally-acquired DNA.[222] Multi-drug resistant strains of enterococci have also been found to lack such defence systems.[223] It is suggested that this lack of CRISPR-Cas systems, described more fully in the following section, may facilitate acquisition of new resistance genes via mobile genetic elements.

### 5.1.3 CRISPR–Cas adaptive immunity systems

CRISPR-Cas bacterial adaptive immunity systems are composed of CRISPR repeat arrays together with *cas* genes (figure 5.1). The CRISPR arrays consist of conserved repeat sequences, interspersed with unique spacer sequences, usually derived from

a mobile genetic element; the *cas* genes encode proteins involved in the action of the defence system.

CRISPR-Cas function can be divided into three distinct stages: adaptation, expression and interference (figure 5.1).[222,224] The first stage, adaptation, involves the introduction of new spacer sequences into the CRISPR array from a DNA element such as a bacteriophage (where the corresponding sequence is called a protospacer). During the expression stage a long primary transcript of the CRISPR repeat array is generated and processed into short CRISPR RNAs (crRNAs; also known as guide RNAs), each containing a single spacer sequence along with a portion of one or both of the flanking CRISPR repeats. In the final stage, interference, the crRNA together with Cas proteins form a complex that targets the foreign DNA containing the corresponding protospacer sequence (e.g. phage) and cleaves the DNA.

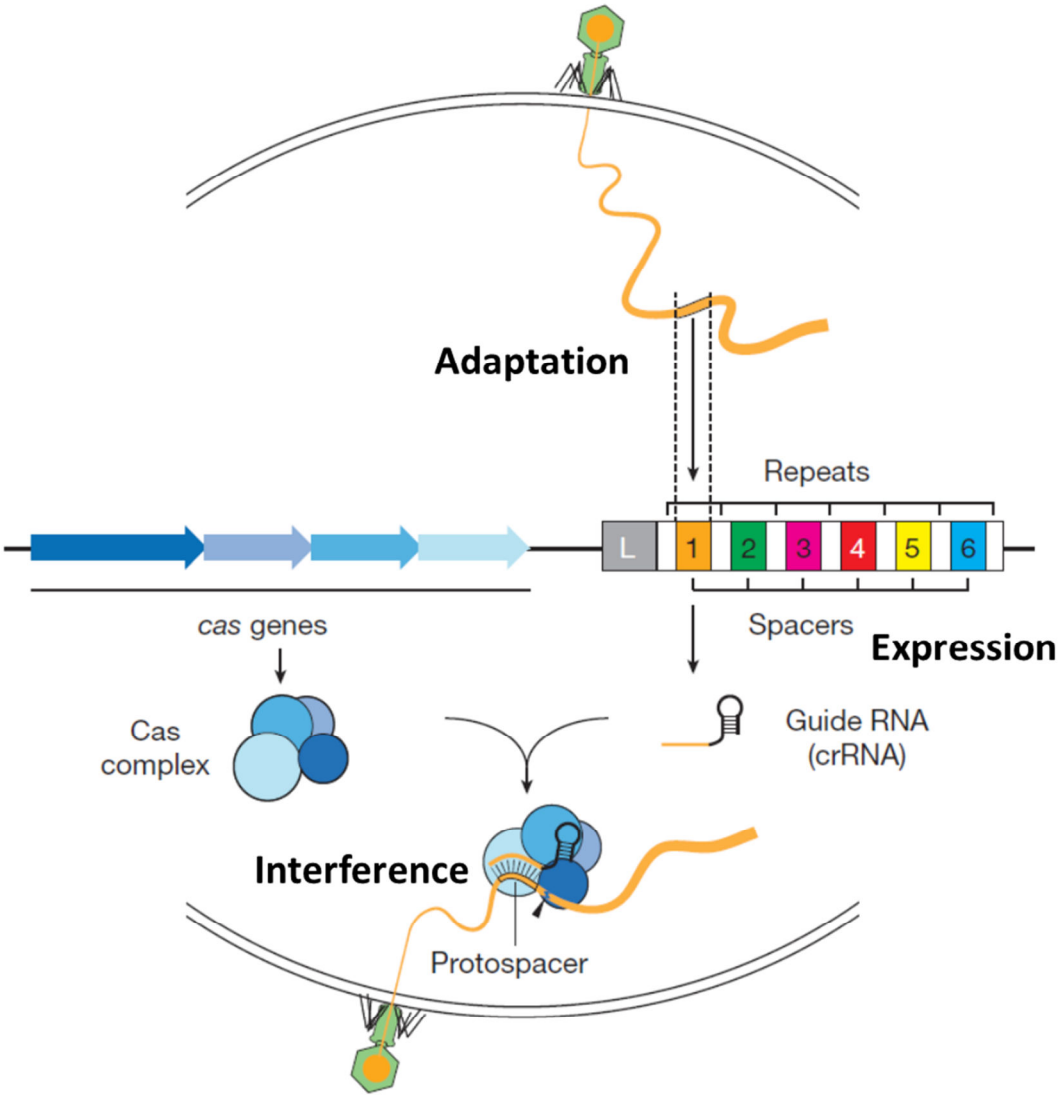
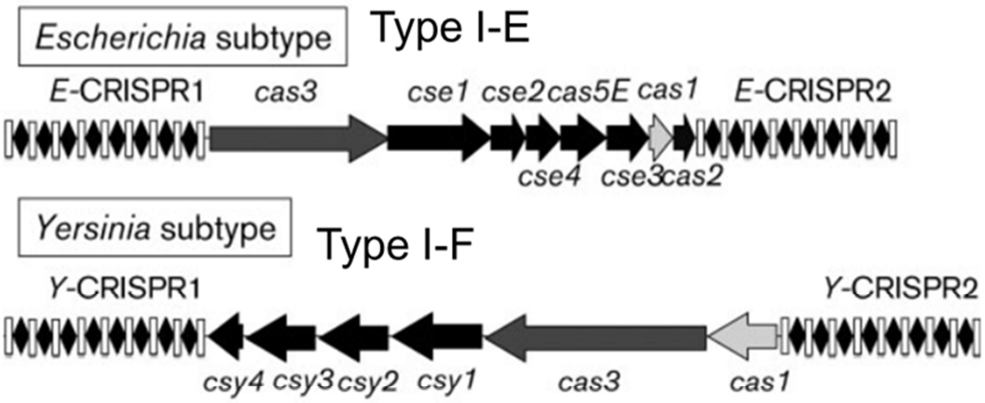



**Figure 5.1:** The three stages of CRISPR-Cas action. The CRISPR repeat region consists of a series of conserved repeat sequences (white boxes) interspersed with unique spacer sequences (coloured boxes labelled 1-6) and preceded by a leader sequence (grey box labelled L; includes a promoter sequence). CRISPR-associated (*cas*) gene arrays (blue arrows) are usually located adjacent to the CRISPR repeat region, and encode genes involved in the action of the defence system. CRISPR-Cas system action has three stages. During the first stage, adaptation, new spacer sequences are incorporated into the CRISPR array from the bacteriophage or other DNA element. During the second stage, expression, a long primary transcript is generated and processed into short CRISPR-RNAs (crRNAs) also known as guide RNAs, each containing a single spacer sequence. In the final interference stage a complex is formed of the crRNA and Cas proteins which specifically targets the corresponding protospacer sequence (from which the spacer sequence present in the crRNA was derived) and cleaves the foreign DNA. Figure adapted from Marraffini et al.[222]

Estimates based on available genome sequences suggest that CRISPR-Cas systems are present in around 40% of all bacterial genomes, with their prevalence varying from species to species.[224] They can be classified into three main types (I-III), which differ in their *cas* gene content and the mechanisms by which they generate crRNA and target foreign DNA. While most characterised CRISPR-cas systems target DNA elements, as described above, a recent study suggests that a member of sub-type III-A found in *Streptococcus thermophilus* may target RNA bacteriophage.[222,225] All types include *cas1* and *cas2* in their gene array while unique type-specific genes, *cas3*, *cas9* and *cas6,* respectively, are associated with members of each of the three main types (I-III). The only CRISPR-Cas subtypes identified in *P. aeruginosa* genomes belong to the type I group,[169] specifically subtypes I-E (previously known as *Escherichia* subtype) and I-F (previously known as *Yersinia* subtype), and these are described more fully in figure 5.2.

Estimates suggest that CRISPR-Cas systems are present in 39-54% of *P. aeruginosa* strains with type I-F being more prevalent than type I-E. This conclusion is based on studies of clinical isolates from the USA, Lebanon and India where 39% of representatives from all three countries carried at least one CRISPR-Cas system (n=112, 6% type I-E, 33% type I-F)[169] whilst 54% of isolates from cystic fibrosis patients in France carried at least one system (n=50, 12% type I-E, 48% type I-F).[226] However, as these isolates were from specific patient groups, or included countries where the prevalence of multi-drug resistant strains is extremely high (e.g. India), it is unclear how representative they are of the diversity generally seen in clinical settings.

(a)




(b)

| CRISPR | Repeat sequence |
|---|---|
| *E*-CRISPR1 | GTGTTCCCCACGGGTGTGGGGATGAACCG |
| *E*-CRISPR2 | GTGTTCCCCACATGCGTGGGGATGAACCG |
| *Y*-CRISPR1 | TTTCTTAGCTGCCTATACGGCAGTGAAC |
| *Y*-CRISPR2 | TTTCTTAGCTGCCTACACGGCAGTGAAC |

**Figure 5.2:** (a) Structures of Type I-E (*Escherichia* subtype) and Type I-F (*Yersinia* subtype) CRISPR-Cas systems found in *P. aeruginosa* isolates. (b) Repeat sequences of the CRISPR arrays seen in the type I-E (*E*-CRISPR1/2*)* and type I-F (*Y*-CRISPR1/2) CRISPR-Cas systems in *P. aeruginosa.* Figure adapted from Cady et al.[169]

### 5.1.4 **Aims**

The overall aim of this part of the study was to use WGS to investigate members of 'high-risk clones' in our collection of MBL-producing *P. aeruginosa*. The study focused on isolates belonging to STs 111 and 235 (VNTR complexes A and B), as they are the two most-commonly-seen MBL-associated 'high-risk clones', both in this study and worldwide.

Specific aims were (1) to investigate whether SNP analysis could be used to distinguish between closely-related *P. aeruginosa* isolates belonging to each of these 'high-risk clones' (2) to identify the resistance determinants present in these isolates and (3) to investigate whether members of these 'high-risk clones' carried CRISPR-Cas systems more or less often than isolates in our comparator collection from the BSAC Bacteraemia Surveillance Programme.

## 5.2 Methods

### 5.2.1 SNP analysis

SNP analysis was carried out by Anthony Underwood and Ali Al-Shahib (Applied Laboratory and Bioinformatics Unit, PHE) for the ST111 data or Michel Doumith (AMRHAI, PHE) for the ST235 data.

Illumina sequencing was performed as described in Section 2.2.5. Fully finished reference genomes were chosen that were closely related to the clones studied: these comprised the ST146 LESB58 chromosome (GenBank accession number NC_011770.1) for the ST111 isolates, or the ST235 NCGM2.S1 chromosome (GenBank accession number AP012280) for the ST235 representatives.[210] Trimmed fastq reads were mapped to the chosen reference strain using bwa-mem 0.7.5a, and variants were determined with GATK 2.6.5.[227] In order to ensure that only high quality SNPs were retained, variants were parsed according to the following conditions:

Depth of coverage (DP) ≥5,

AD ratio (ratio between variant base and alternative bases) ≥ 0.8,

Mapping Quality (MQ) ≥ 30,

Ratio of reads with MQ=0 to total number of reads ≤ 0.05,

Distance to nearest SNP >10

All SNP positions that fulfilled these criteria in more than 90% of the samples were included in a multi-fasta file, where the sequence for each strain consisted of the

concatenated SNPs. The multi-fasta file was used as an input to generate a maximum likelihood (ML) tree using RAxML[228] on the CIPRES portal[229] with the following parameters:

–m (substitutionModel) GTRCAT

–b (bootstrapRandomNumberSeed) 12345

–# (numberOfRuns) 100

–c (numberOfCategories) 25

SNP tree figures were generated using the EvolView online tool (http://www.evolgenius.info).[230]

### 5.2.2 Prediction of resistance determinants

Identification of resistance determinants was carried out by Michel Doumith (AMRHAI, PHE), using a database of reference sequences assembled to include acquired resistance genes, as well as chromosomal genes in which mutation is known to affect fluoroquinolone resistance (*gyrA*, *gyrB*, *parC* and *parE*). Acquired resistance gene sequences used were obtained from the Comprehensive Antibiotic Resistance Database (http://arpcard.mcmaster.ca) and the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) using the accession numbers described by Zankari and colleagues.[231] The *gyrA*, *gyrB*, *parC* and *parE* gene sequences were obtained from the *P. aeruginosa* PA01 complete genome sequence (GenBank accession number NC_002516).

Trimmed Illumina reads (fastq files) were mapped against this database using bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2), and Binary Alignment Map (BAM) and Variant Calling Format (VCF) files were produced with SAMtools (http://samtools.sourceforge.net). VCF files were parsed using a simple script that extracts, for each reference sequence with aligned reads, the number of sites covered by more than five reads and nucleotide alterations with respect to the reference sequence to calculate nucleotide identities. The presence of acquired resistance genes was determined with a stringent threshold of inclusion of >90% identity over the length of the reference sequence; except for β-lactamase variants where 100% identity was required, so as to identify specific alleles (e.g. $bla_{VIM}$ variants). For the *gyrA*, *gyrB*, *parC* and *parE* genes mutations were detected in the quinolone resistance determining regions (QRDR) as described by Kos et al;[30] corresponding to amino acid positions 83-87 (GyrA), 429-585 (GyrB), 82-84 (ParC) and 357-503 (ParE).

### 5.2.3 **Detection of CRISPR-*cas* genes**

Detection of CRISPR-*cas* genes from the WGS data was carried out by Michel Doumith (AMRHAI, PHE). Illumina reads were mapped, as described above for the resistance determinants, against sequences of sub-type I-E CRISPR-*cas* genes (*cas2*, *cas1*, *cas6e*/*cse3*, *cas5*, *cas7*, *cse2* and *cas3*) from the RP73 *P. aeruginosa* complete genome sequence (GenBank accession: NC_021577) and sub-type I-F CRISPR-*cas* genes (*cas1*, *cas3*, *csy1*, *csy2*, *csy3*, *csy4*) from the PA14 *P. aeruginosa* complete genome sequence (GenBank accession: NC_008463).

CRISPR-*cas* genes specific to type I-E or type I-F systems were sought by PCR in BSAC Bacteraemia Surveillance Programme isolates from 2011 (see Section 2.1.2), using previously-described primers.[169] Primers cse3_For and cse3_Rev were used to seek the *cse3* gene (also known as *cas6e*) of type I-E CRISPR-Cas systems and csy1_FOR with csy1_REV to amplify the *csy1* gene of type I-F CRISPR-Cas systems. Primer sequences are shown in table 2.1.

## 5.3 Results

### 5.3.1 WGS analysis of ST111 isolates

As described in Chapter 3, ST111 isolates were referred to AMRHAI from 15 London-region laboratories between 2003 and 2012. We continued to receive MBL-producing ST111 representatives from several London hospitals in 2013-2014 and there were concerns that this might reflect spread between hospital sites. Thus, a WGS investigation of this lineage was instigated in a collaboration between AMRHAI, the PHE Genomic Services and Development Unit, the PHE Applied Laboratory and Bioinformatics Unit, the PHE London regional laboratory and St George's, University of London.[232] A total of 120 ST111 *P. aeruginosa* isolates, submitted to AMRHAI between 2003 and 2014, were subjected to Illumina sequencing, as described in Section 2.2.5. Of these 120, 87 gave a sufficient mean depth of coverage (15x or greater) to be included in further analyses.

Seventy-five of the 87 isolates were from patients. They included 51 of the 75 ST111 representatives in the non-duplicate-patient MBL-producing isolate collection (see Section 2.1.1). The remaining 12/87 isolates were derived from environmental samples, collected by laboratories as part of their epidemiological investigations of outbreaks. The 87 isolates included those carrying $bla_{VIM}$-(n=73), $bla_{IMP}$-(n=5), $bla_{NDM}$-(n=1), or both $bla_{VIM}$- and $bla_{IMP}$- (n=1) type MBLs and seven MBL-negative ST111 comparator strains, received by AMRHAI in 2011-2014.

#### 5.3.1.1 SNP analysis of the ST111 isolates

SNP analysis was carried out as described in Section 5.2.1 using the ST146 LESB58 reference strain (GenBank accession number NC011770), which is one of the most-

closely-related organisms to ST111 for which a fully finished genome sequence is available. On analysis of the SNP data it was clear that the one *bla*$_{NDM}$-carrying isolate was an outlier, having an average of 1,377 SNPs difference from any other isolate in the tree, so it was removed from a further SNP analysis. Among the remaining 86 isolates, regions of the reference that could be aligned against all sequenced genomes yielded 21,278 SNPs, with individual genomes differing at 0-548 of these positions. The maximum likelihood tree resulting from this analysis is shown in figure 5.3.

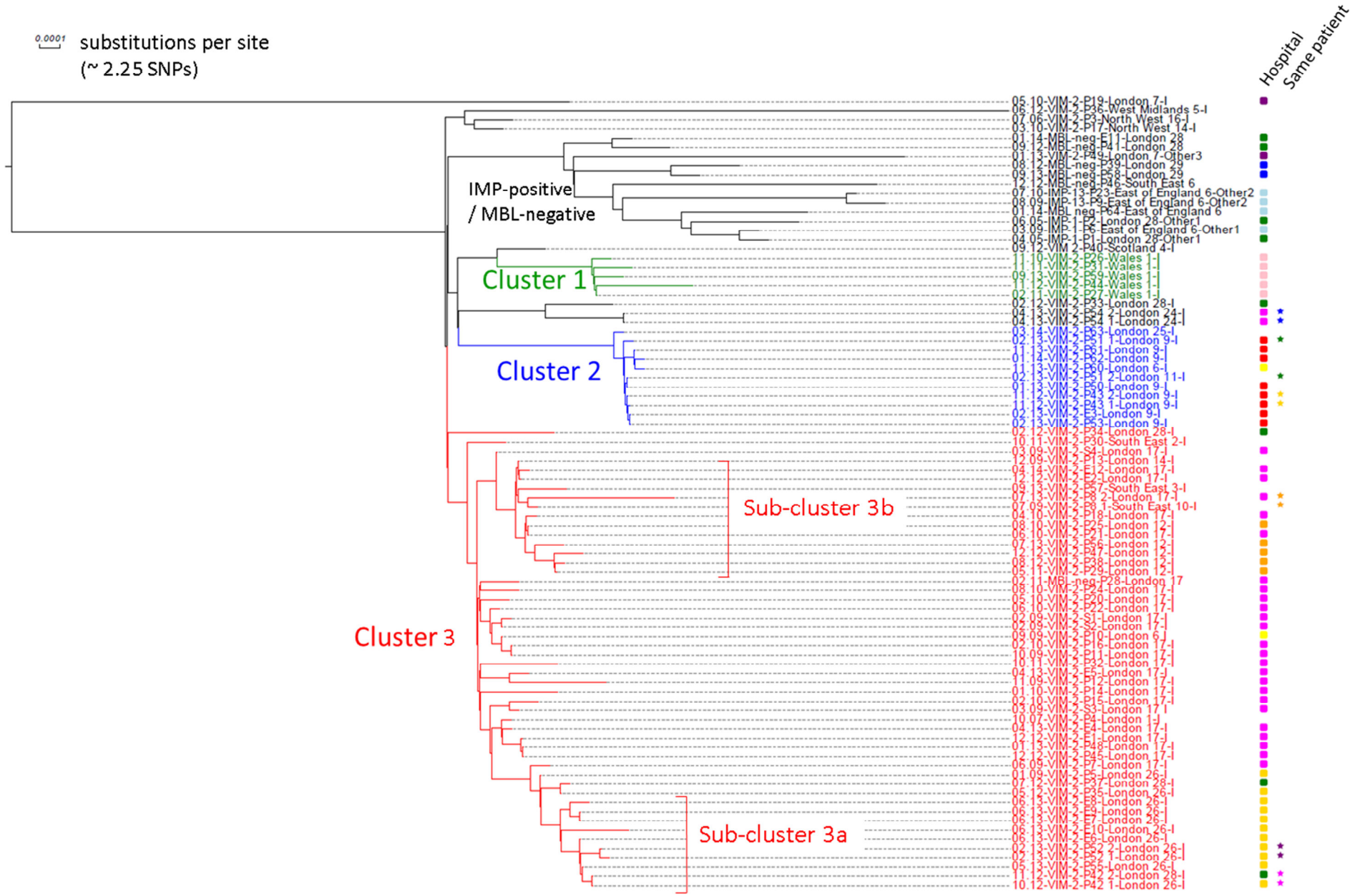

0.0001
substitutions per site
(~ 2.25 SNPs)
Hospital
Same patient
IMP-positive
/ MBL-negative
Cluster 1
Cluster 2
Cluster 3
Sub-cluster 3b
Sub-cluster 3a
05.10-VIM-2-P19-London 7-I
06.12-VIM-2-P36-West Midlands 5-I
07.06-VIM-2-P3-North West 16-I
03.10-VIM-2-P17-North West 14-I
01.14-MBL-neg-E11-London 28
09.12-MBL-neg-P41-London 28
01.13-VIM-2-P49-London 7-Other3
08.12-MBL-neg-P39-London 29
09.13-MBL-neg-P58-London 29
12.12-MBL-neg-P46-South East 6
07.10-IMP-13-P23-East of England 6-Other2
08.09-IMP-13-P9-East of England 6-Other2
01.14-MBL neg-P64-East of England 6
06.05-IMP-1-P2-London 28-Other1
03.09-IMP-1-P6-East of England 6-Other1
04.05-IMP-1-P1-London 28-Other1
09.12-VIM-2-P40-Scotland 4-I
11.10-VIM-2-P26-Wales 1-I
11.11-VIM-2-P31-Wales 1-I
09.13-VIM-2-P59-Wales 1-I
11.12-VIM-2-P44-Wales 1-I
02.11-VIM-2-P27-Wales 1-I
02.12-VIM-2-P33-London 28-I
04.13-VIM-2-P54 2-London 24-I
04.13-VIM-2-P54 1-London 24-I
03.14-VIM-2-P63-London 25-I
02.13-VIM-2-P51 1-London 9-I
11.13-VIM-2-P61-London 9-I
01.14-VIM-2-P62-London 9-I
11.13-VIM-2-P60-London 6-I
02.13-VIM-2-P51 2-London 11-I
01.13-VIM-2-P50-London 9-I
11.12-VIM-2-P43 2-London 9-I
11.12-VIM-2-P43 1-London 9-I
02.13-VIM-2-E3-London 9-I
02.13-VIM-2-P53-London 9-I
02.12-VIM-2-P34-London 28-I
10.11-VIM-2-P30-South East 2-I
03.09-VIM-2-S4-London 17-I
12.09-VIM-2-P13-London 14-I
04.14-VIM-2-E12-London 17-I
12.12-VIM-2-P47-London 17-I
09.13-VIM-2-P57-South East 3-I
07.13-VIM-2-P58 2-London 17-I
07.09-VIM-2-P8 1-South East 10-I
04.10-VIM-2-P18-London 17-I
08.10-VIM-2-P25-London 12-I
06.10-VIM-2-P21-London 17-I
07.13-VIM-2-P56-London 12-I
12.12-VIM-2-P47-London 12-I
08.12-VIM-2-P38-London 12-I
05.11-VIM-2-P29-London 12-I
02.11-MBL-neg-P28-London 17
08.10-VIM-2-P24-London 17-I
05.10-VIM-2-P20-London 17-I
06.10-VIM-2-P22-London 17-I
02.09-VIM-2-S1-London 17-I
02.09-VIM-2-S2-London 17-I
09.09-VIM-2-P10-London 6-I
02.10-VIM-2-P16-London 17-I
10.09-VIM-2-P11-London 17-I
10.11-VIM-2-P32-London 17-I
04.13-VIM-2-E5-London 17-I
11.09-VIM-2-P12-London 17-I
01.10-VIM-2-P14-London 17-I
02.10-VIM-2-P15-London 17-I
03.09-VIM-2-S3-London 17 I
10.07-VIM-2-P4-London 1-I
04.13-VIM-2-E4-London 17-I
12.-VIM-2-E1-London 17-I
01.15-VIM-2-P48-Lon 17-I
12.12-VIM-2-P45-London 17-I
06.09-VIM-2-P7-London 17-I
01.09-VIM-2-P5-London 26-I
07.12-VIM-2-P37-London 28-I
05.12-VIM-2-P35-London 26-I
06.13-VIM-2-E8-London 26-I
06.13-VIM-2-E9-London 26-I
06.13-VIM-2-E7-London 26-I
06.13-VIM-2-E10-London 26-I
06.13-VIM-2-E6-London 26-I
02.13-VIM-2-P52 2-London 26-I
02.13-VIM-2-P52 1-London 26-I
05.13-VIM-2-P55-London 26-I
11.12-VIM-2-P42 2-London 28-I
10.12-VIM-2-P42 1-London 26-I

**Figure 5.3 (Previous page):** Maximum likelihood tree based on SNP analysis of the 86 ST111 representatives compared with the reference strain (ST146 LESB58); one outlying ST111 representative with *bla*$_{NDM}$ was excluded. Labels for individual nodes indicate information about each isolate in the format: date of isolation (month.year), VIM or IMP allele identified, patient (P), screening (S) or environmental (E) isolate number (followed by _1, _2, etc. for patients for which we received multiple isolates), referring hospital, and integron detected (integron I, or others (1-3) seen among ST111 isolates, as detailed in Chapter 4). Coloured branches and node labels represent the identified clusters (1-3); sub-clusters are also labelled. Coloured squares represent hospitals submitting more than one isolate, and coloured stars represent patients from whom multiple isolates were submitted.

#### 5.3.1.1.1 Main clusters identified within ST111

Several clusters and sub-clusters were identified among the 86 ST111 isolates (figure 5.3); these included cluster 1 (n=5, one laboratory), cluster 2 (n=11, four laboratories) and cluster 3 (n=50, ten laboratories). Variation within cluster 1, 2 and 3 corresponded to 19, 6 and 181 SNPs, respectively. The remaining isolates were more diverse and included all five *bla*$_{IMP}$-positive isolates and 6/7 of the MBL-negative ST111 representatives, which were from four different hospitals (figure 5.3).

All five cluster 1 representatives were from Wales_1 and were referred between 2010 and 2013. Isolates in cluster 2 (n=11) that differed by only six SNPs were referred over a 17-month period (2012-2014) and were mostly from London_9 (8 isolates); single isolates were also submitted from three further London hospitals; One of these three isolates was collected at London_11 from a patient (P51) who had also attended London_9, but there were no known epidemiological links between London_9 and the other two patients, who attended London_25 or London_6.

More than half (50/86) of the isolates belonged to cluster 3: these 50 comprised 40 patient isolates and ten environmental isolates received between 2007 and 2014. Half (20 patient and 5 environmental) were from London_17 while the remaining 25 isolates were referred by a further six London laboratories and three in South-East England. Two sub-clusters could be seen among these cluster 3 isolates.

Sub-cluster 3a (n=13, 22 SNPs) included representatives referred mainly from London_26 (six patient and five environmental) but with an additional two isolates

from London_28, both from patients who were also hospitalised at London_26; these were mostly submitted in 2012-2013 but one London_26 isolate dated from 2009.

Sub-cluster 3b (n= 14, 20 SNPs) isolates were referred over a five-year period (2009-2014); six were from London_17 and five from London_12, with single isolates from three other London or South-East England laboratories. This included a patient (P8) for which we had received two isolates from different laboratories (South East_10 and London_17), but there were no known epidemiological links between London_17 and the isolates from other sites (South East_3, London_14 and London_12).

#### 5.3.1.1.2 Variation among ST111 isolates from single laboratories

Where multiple ST111 isolates were received from the same laboratory they generally belonged to the same cluster or sub-cluster, for example those received from Wales_1 (n=5, cluster1), London_9 (n=7, cluster 2), London_17 (n=25, cluster 3), London_26 (n=11, cluster 3a) and London_12 (n= 5, cluster 3b). However, there were exceptions where isolates from the same hospital were distant by SNP analysis, indicating independent acquisition of ST111 strains. These included representatives referred from London_28 (from 7 patients and 1 environmental) between 2005 and 2014, which were dispersed across the tree. Other examples are two isolates from London_7 (P19, 2010 and P49, 2013), which were on different long branches, and two representatives from London_6 (P60, 2013 and P10, 2009), which fell into clusters 2 and 3, respectively.

#### 5.3.1.1.3 Variation among ST111 isolates from single patients

Six patients had two MBL-producing representatives submitted to AMRHAI and isolates from the same patient consistently clustered together on the tree (figure 5.3, table 5.1). For five of these six patients the two isolates were collected over short periods (up to 3 weeks), from different body sites, and differed by 0-1 SNPs. However, for one further patient (P8) two isolates were submitted four years apart and differed by 18 SNPs. The SNP tree indicated that the two isolates had a common ancestor not shared with any of the other ST111 isolates indicating that the strain had probably evolved within the patient.

**Table 5.1:** Details of patients (n=6) from whom multiple ST111 *P. aeruginosa* isolates were submitted

| Patient | Cluster | No. of isolates | Time period separating isolates | Isolation sites | Number of SNPs different |
|---|---|---|---|---|---|
| **P8** | 3b | 2 | 4 years | sputum, urine | 18 |
| **P42** | 3a | 2 | 3 weeks | sputum, unknown | 0 |
| **P43** | 2 | 2 | 3 days | urine, blood | 0 |
| **P51** | 2 | 2 | 2 weeks | unknown, sputum | 0 |
| **P52** | 3a | 2 | 2 weeks | urine, TIP | 1 |
| **P54** | other | 2 | 3 weeks | urine, faeces | 0 |

TIP; trans-jugular intrahepatic portosystemic stent

#### 5.3.1.1.4 Comparison of sequence with VNTR data for ST111 isolates

The most common VNTR type among the ST111 isolates was 11,3,4,3,2,2,5,4,7, seen in 57 of the 86 representatives; these 57 included most of the isolates that fell into SNP clusters 1, 2 and 3, and some of the MBL-negative strains. All of the $bla_{IMP}$-carrying representatives, spread over five years, had the same VNTR type (11,3,4,3,2,2,5,4,6) as did several MBL-negative isolates as well as two cluster 3a representatives from London_12. Those (n=5) with the profile 11,3,4,3,2,2,5,4,8

were from three different hospitals and all fell into cluster 3. Ten of the 13 isolates in cluster 3a had a consistent VNTR profile (11,3,4,3,2,2,6,4,7) and were all from patients hospitalised at London_26. The *bla*$_{VIM-2}$-carrying isolate from patient P19, which was clearly different from the remaining 85/86 isolates included in the SNP tree had a unique VNTR profile (11,3,4,3,2,2,4,4,7) as did the NDM-producing outlier (11,3,4,3,2,2,6,4,13).

#### 5.3.1.2 Sequencing-based detection of resistance determinants in ST111 isolates

Resistance determinants were detected from the sequence data as described in Section 5.2.2. The intrinsic resistance genes *bla*$_{OXA-50}$, *bla*$_{PAO}$ (*ampC*), *aph(3')-IIb* and *catB7* were identified in 85/87 isolates; the two remaining isolates had all these genes except *catB7*. *gyrA, gyrB*, *parC* and *parE* genes were detected too, but mutations in the QRDR of these genes could not be determined for most (69/87) ST111 genomes owing to insufficient depth of coverage of the region by the Illumina reads. A mutation affecting GyrA (83:T-I) was seen in all 18 isolates that could be analysed; this was also the most common mutation seen among fluoroquinolone-resistant *P. aeruginosa* isolates of diverse origin in a previous study.[30]

The acquired resistance genes seen among the ST111 isolates are shown in figure 5.4 and variously included MBL genes *bla*$_{VIM-2}$, *bla*$_{IMP-1}$, *bla*$_{IMP-13}$ and *bla*$_{IMP-18}$, in addition to those encoding other β-lactamases (*bla*$_{PSE-1}$ and *bla*$_{OXA-2}$), aminoglycoside-modifying enzymes (*aacA4*, *aacA29*, *aac(3)-Ic* and *aadA2*) and the class-1-integron-associated sulphonamide resistance cassette *sul1*.

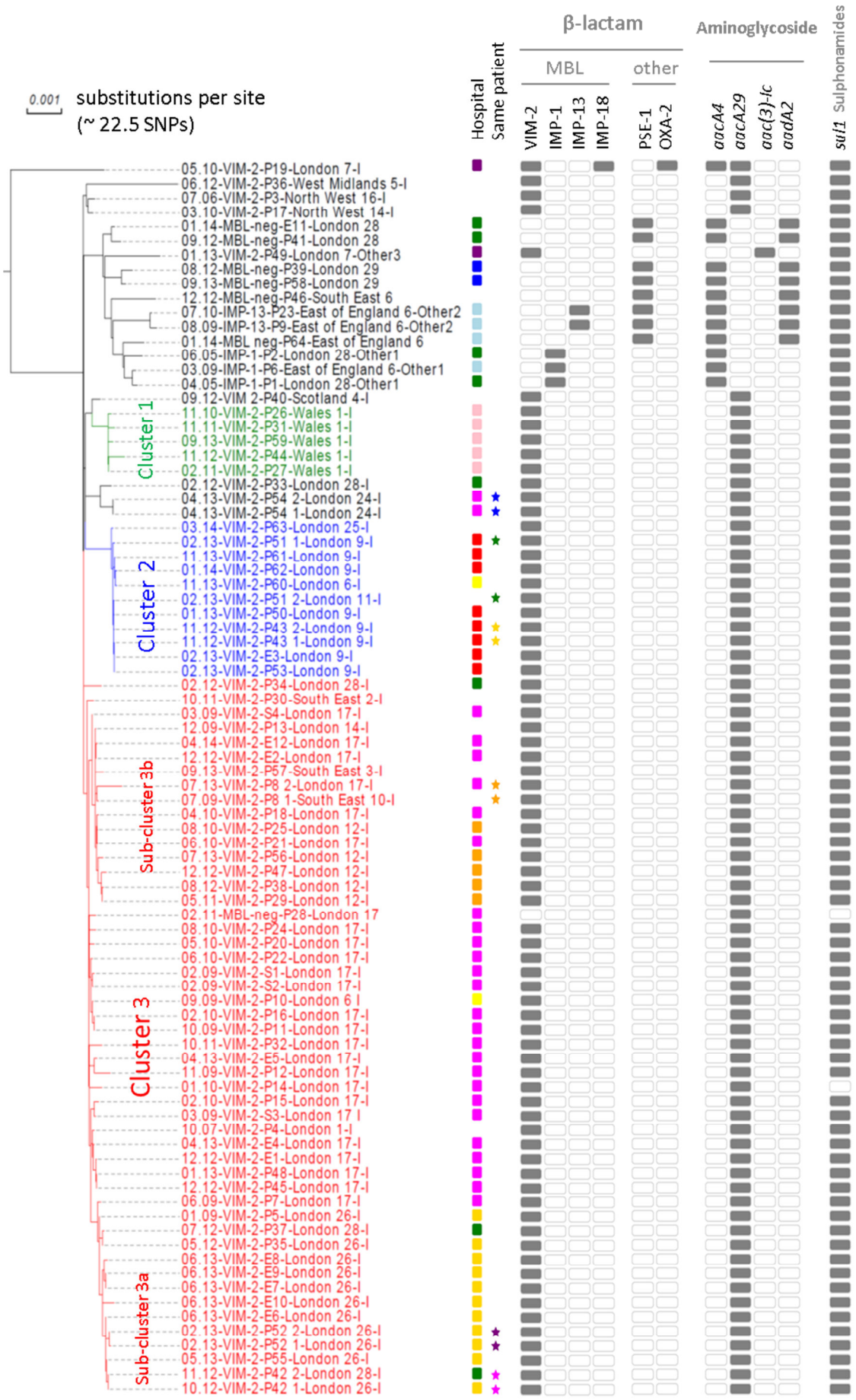

0.001 substitutions per site
(~ 22.5 SNPs)
Hospital
Same patient
β-lactam
MBL
other
Aminoglycoside
VIM-2
IMP-1
IMP-13
IMP-18
PSE-1
OXA-2
aacA4
aacA29
aac(3)-Ic
aadA2
sul1 Sulphonamides
05.10-VIM-2-P19-London 7-I
06.12-VIM-2-P36-West Midlands 5-I
07.06-VIM-2-P3-North West 16-I
03.10-VIM-2-P17-North West 14-I
01.14-MBL-neg-E11-London 28
09.12-MBL-neg-P41-London 28
01.13-VIM-2-P49-London 7-Other3
08.12-MBL-neg-P39-London 29
09.13-MBL-neg-P58-London 29
12.12-MBL-neg-P46-South East 6
07.10-IMP-13-P23-East of England 6-Other2
08.09-IMP-13-P9-East of England 6-Other2
01.14-MBL neg-P64-East of England 6
06.05-IMP-1-P2-London 28-Other1
03.09-IMP-1-P6-East of England 6-Other1
04.05-IMP-1-P1-London 28-Other1
09.12-VIM 2-P40-Scotland 4-I
Cluster 1
11.10-VIM-2-P26-Wales 1-I
11.11-VIM-2-P31-Wales 1-I
09.13-VIM-2-P59-Wales 1-I
11.12-VIM-2-P44-Wales 1-I
02.11-VIM-2-P27-Wales 1-I
02.12-VIM-2-P33-London 28-I
04.13-VIM-2-P54 2-London 24-I
04.13-VIM-2-P54 1-London 24-I
Cluster 2
03.14-VIM-2-P63-London 25-I
02.13-VIM-2-P51 1-London 9-I
11.13-VIM-2-P61-London 9-I
01.14-VIM-2-P62-London 9-I
11.13-VIM-2-P60-London 6-I
02.13-VIM-2-P51 2-London 11-I
01.13-VIM-2-P50-London 9-I
11.12-VIM-2-P43 2-London 9-I
11.12-VIM-2-P43 1-London 9-I
02.13-VIM-2-E3-London 9-I
02.13-VIM-2-P53-London 9-I
02.12-VIM-2-P34-London 28-I
10.11-VIM-2-P30-South East 2-I
03.09-VIM-2-S4-London 17-I
12.09-VIM-2-P13-London 14-I
04.14-VIM-2-E12-London 17-I
12.12-VIM-2-E2-London 17-I
09.13-VIM-2-P57-South East 3-I
Sub-cluster 3b
07.13-VIM-2-P8 2-London 17-I
07.09-VIM-2-P8 1-South East 10-I
04.10-VIM-2-P18-London 17-I
08.10-VIM-2-P25-London 12-I
06.10-VIM-2-P21-London 17-I
07.13-VIM-2-P56-London 12-I
12.12-VIM-2-P47-London 12-I
08.12-VIM-2-P38-London 12-I
05.11-VIM-2-P29-London 12-I
02.11-MBL-neg-P28-London 17
08.10-VIM-2-P24-London 17-I
05.10-VIM-2-P20-London 17-I
06.10-VIM-2-P22-London 17-I
02.09-VIM-2-S1-London 17-I
02.09-VIM-2-S2-London 17-I
09.09-VIM-2-P10-London 6 I
02.10-VIM-2-P16-London 17-I
10.09-VIM-2-P11-London 17-I
10.11-VIM-2-P32-London 17-I
04.13-VIM-2-E5-London 17-I
11.09-VIM-2-P12-London 17-I
01.10-VIM-2-P14-London 17-I
02.10-VIM-2-P15-London 17-I
03.09-VIM-2-S3-London 17 I
10.07-VIM-2-P4-London 1-I
04.13-VIM-2-E4-London 17-I
12.12-VIM-2-E1-London 17-I
01.13-VIM-2-P48-London 17-I
12.12-VIM-2-P45-London 17-I
Cluster 3
06.09-VIM-2-P7-London 17-I
01.09-VIM-2-P5-London 26-I
07.12-VIM-2-P37-London 28-I
05.12-VIM-2-P35-London 26-I
06.13-VIM-2-E8-London 26-I
06.13-VIM-2-E9-London 26-I
06.13-VIM-2-E7-London 26-I
06.13-VIM-2-E10-London 26-I
06.13-VIM-2-E6-London 26-I
02.13-VIM-2-P52 2-London 26-I
02.13-VIM-2-P52 1-London 26-I
05.13-VIM-2-P55-London 26-I
11.12-VIM-2-P42 2-London 28-I
10.12-VIM-2-P42 1-London 26-I
Sub-cluster 3a

**Figure 5.4 (previous page):** Resistance determinants seen among the 86 ST111 representatives. The maximum likelihood tree based on SNP analysis of the ST111 isolates, as shown in figure 5.3. Labels for individual nodes indicate information about each isolate in the format: date of isolation (month.year), VIM or IMP allele identified, patient (P), screening (S) or environmental (E) isolate number (followed by _1, _2, etc. for patients for which we received multiple isolates), referring hospital, and integron detected (Integron I or others (1-3) seen among ST111 isolates, as detailed in Chapter 4). Coloured branches and node labels represent the identified clusters (1-3); sub-clusters are also indicated. Coloured squares represent hospitals submitting more than one isolate, and coloured stars represent patients from whom multiple isolates were submitted. Filled grey rectangles represent acquired resistance genes detected among the isolates while empty rectangles indicate that this resistance determinant was not detected in a particular isolate.

Most (74/87) isolates were found to harbour *bla*$_{VIM-2}$, 72 of these 74 also had *aacA29* (*aacA29a* and *aacA29b* differ by only four nucleotides and were not distinguished by the mapping script) and *sul1.* This result is consistent with the gene content of integron I (In59-like), which were amplified from all representatives of these isolates (Section 4.3.1). For a further isolate in cluster 3 *bla*$_{VIM-2}$ and *aacA29* were detected but not *sul1*, despite the presence of integron I being confirmed by PCR. One integron I-carrying isolate (from patient P19, 2010, London_7) additionally had the *aac(6')-Ib*, *bla*$_{IMP-18}$ and *bla*$_{OXA-2}$ genes (the *bla*$_{IMP-18}$ gene had not initially been detected by PCR, but both the *bla*$_{VIM}$ and *bla*$_{IMP}$ genes were subsequently confirmed by PCR from a single colony pick). The representative was also the most distant, based on SNP analysis, from the other integron I-carrying isolates, none of which were referred from London_7. The final *bla*$_{VIM-2}$-positive isolate (P49), among these 74 was most closely related to the five *bla*$_{IMP}$-positive and six of the MBL-negative strains had a different integron profile, designated other3 (determined by PCR as described in Section 4.2.1); an *aac(3)-Ic* gene was detected in this isolate in addition to the *bla*$_{VIM-2}$ and *sul1* genes.

The 13 isolates that did not carry *bla*$_{VIM-2}$ included seven that lacked MBLs, five with IMP-MBLs and one with NDM. One MBL-negative isolate belonged to cluster 3 but had only the *aacA29* and *sul1* genes; this isolate was also negative by PCR both for *bla*$_{VIM}$ and for any *bla*$_{VIM}$-containing integron; it may have lost the *bla*$_{VIM-2}$ gene cassette, consistently seen in all other cluster 3 isolates. Six further MBL-negative strains, from four different laboratories, with no known links between them, had the same acquired resistance gene content as each other, including aminoglycoside resistance genes *aacA4*, *aadA2,* the penicillinase *bla*$_{PSE-1}$ and *sul1*; this is similar to

the gene content of an integron detected in a *P. aeruginosa* from an outbreak at a Spanish hospital,[233] and it is possible that they are representatives of the PSE-1-associated O12 strain commonly seen in Europe in the 1980s.[119] The two IMP-13-positive representatives, both from South_East_6 had similar resistance gene content to these six MBL-negative isolates, with which they shared a common ancestor, but with the addition of $bla_{\text{IMP-13}}$. The remaining three IMP-positive representatives however carried *aacA4* and *sul1* genes in addition to $bla_{\text{IMP-1}}$. The NDM-positive outlier (not included in figure 5.4) had $bla_{\text{NDM-1}}$, $bla_{\text{VEB-1,}}$ *aadA2*, *aph(3')-VIb*, *ant(2')-Ia* and *sul1* genes.

#### 5.3.1.2.1 Comparison of genotypes with MIC data for ST111 isolates

The 60/80 MBL-positive isolates for which MICs were available were resistant to the majority of tested antibiotics (table 5.2), including imipenem, meropenem and ciprofloxacin, but not colistin, to which all but one isolate remained susceptible, and aztreonam, to which most isolates were intermediate, based on EUCAST criteria. Surprisingly seven isolates (12%) remained susceptible to piperacillin-tazobactam, six of these carried $bla_{VIM-2}$ and one $bla_{IMP-1}$ and they were dispersed across the SNP tree.

Greatest isolate-to-isolate variation in susceptibility was seen for the aminoglycoside antibiotics amikacin (23% susceptible or intermediate) and gentamicin (30% susceptible). All five IMP-producing isolates were susceptible or intermediate to amikacin despite carrying *aacA4,* which has been previously associated with resistance to amikacin.[25] While most of the tested isolates carrying *aacA29* variants (associated with resistance to amikacin and tobramycin, but not gentamicin)[25,197] were resistant to all aminoglycosides, 17/62 remained susceptible or intermediate to amikacin and/or gentamicin. Notably this included all five London_12 isolates in cluster 3b, which were susceptible or intermediate to both amikacin and gentamicin (n=4) or amikacin only (n=1), and all tested isolates (9/11) in cluster 2 that remained susceptible to gentamicin.

MIC data were available for three of the seven MBL-negative ST111 isolates, all carrying $bla_{PSE-1}$, *aacA4* and *aadA2.* All three remained sensitive to amikacin, ceftazidime and colistin, and were resistant to gentamicin, tobramycin and piperacillin-tazobactam, consistent with the previous report of an outbreak of a *P.*

*aeruginosa* strain with the same resistance gene content ($bla_{PSE-1}$, *aacA4* and *aadA2*).[233] They were also consistently resistant to imipenem but had variable resistance to meropenem and ciprofloxacin, with resistance to these agents likely to be due to mutations rather than acquired resistance genes.

**Table 5.2:** Antibiotic susceptibility of ST111 representatives subjected to WGS analysis (n=87)

| | MBL-positive (n=80) | | | | MBL-negative (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| **Antibiotic** | **S** | **I** | **R** | **ND** | **S** | **I** | **R** | **ND** |
| Amikacin | 8 | 6 | 46 | 20 | 2 | 1 | 0 | 4 |
| Gentamicin | 18 | - | 42 | 20 | 0 | - | 3 | 4 |
| Tobramycin | 3 | - | 57 | 20 | 0 | - | 3 | 4 |
| Piperacillin-tazobactam | 7 | - | 53 | 20 | 0 | - | 3 | 4 |
| Ceftazidime | 3 | - | 57 | 20 | 3 | - | 0 | 4 |
| Aztreonam | 1 | 53 | 5 | 21 | 0 | 3 | 0 | 4 |
| Imipenem | 0 | 0 | 60 | 20 | 0 | 0 | 3 | 4 |
| Meropenem | 0 | 3 | 57 | 20 | 0 | 1 | 2 | 4 |
| Ciprofloxacin | 1 | 0 | 59 | 20 | 0 | 1 | 2 | 4 |
| Colistin | 59 | - | 1 | 20 | 3 | - | 0 | 4 |

S, susceptible; I, intermediate; R; resistant, ND; not done

### 5.3.2 WGS analysis of ST235 isolates

In order to assess diversity among MBL-producing ST235 isolates, 57 representatives from 46 patients and 27 hospital laboratories were subjected to WGS. These isolates were received by AMRHAI between 2003 and 2012 and included 44 of the 52 MBL-producing ST235 *P. aeruginosa* isolates from the non-duplicate-patient isolate set (see Section 2.1.1) along with 11 further MBL-producing isolates from the same patients and two predominantly antibiotic susceptible MBL-negative comparators submitted to AMRHAI in 2012.We received no ST235 environmental isolates linked in time and place to any of the MBL-producing isolates.

Illumina sequencing was carried out as described in Section 2.2.5. As a result of the problems with low coverage that led to some of the ST111 isolates being excluded from later analyses (above), all ST235 isolates were sequenced twice and the resulting reads concatenated. This ensured that sufficient average genome coverage (>15x) was achieved for all isolates.

#### 5.3.2.1 SNP analysis of ST235 isolates

SNP analysis was carried out as described in Section 5.2.1 using the ST235 reference strain NGCM2.S1 (GenBank accession number AP012280).[210] Regions of the reference that could be aligned against all sequenced genomes identified 953 SNPs, with individual genomes differing at 0-548 of these positions. The SNP tree generated is shown in figure 5.5.

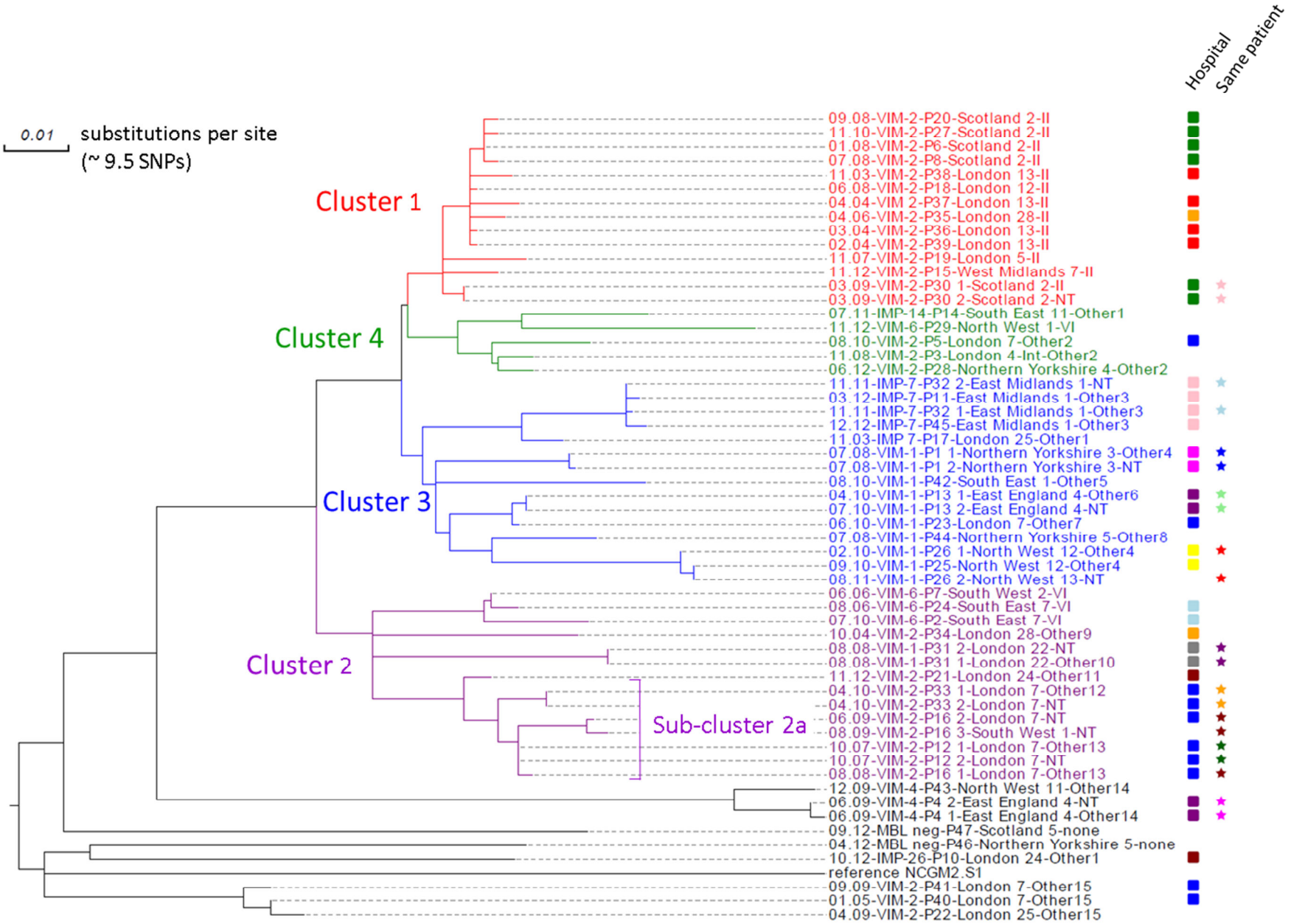

0.01 substitutions per site
(~ 9.5 SNPs)
Cluster 1
Cluster 4
Cluster 3
Cluster 2
Sub-cluster 2a
Hospital
Same patient
09.08-VIM-2-P20-Scotland 2-II
11.10-VIM-2-P27-Scotland 2-II
01.08-VIM-2-P6-Scotland 2-II
07.08-VIM-2-P8-Scotland 2-II
11.03-VIM-2-P38-London 13-II
06.08-VIM-2-P18-London 12-II
04.04-VIM 2-P37-London 13-II
04.06-VIM-2-P35-London 28-II
03.04-VIM-2-P36-London 13-II
02.04-VIM-2-P39-London 13-II
11.07-VIM-2-P19-London 5-II
11.12-VIM-2-P15-West Midlands 7-II
03.09-VIM-2-P30 1-Scotland 2-II
03.09-VIM-2-P30 2-Scotland 2-NT
07.11-IMP-14-P14-South East 11-Other1
11.12-VIM-6-P29-North West 1-VI
08.10-VIM-2-P5-London 7-Other2
11.08-VIM-2-P3-London 4-Int-Other2
06.12-VIM-2-P28-Northern Yorkshire 4-Other2
11.11-IMP-7-P32 2-East Midlands 1-NT
03.12-IMP-7-P11-East Midlands 1-Other3
11.11-IMP-7-P32 1-East Midlands 1-Other3
12.12-IMP-7-P45-East Midlands 1-Other3
11.03-IMP 7-P17-London 25-Other1
07.08-VIM-1-P1 1-Northern Yorkshire 3-Other4
07.08-VIM-1-P1 2-Northern Yorkshire 3-NT
08.10-VIM-1-P42-South East 1-Other5
04.10-VIM-1-P13 1-East England 4-Other6
07.10-VIM-1-P13 2-East England 4-NT
06.10-VIM-1-P23-London 7-Other7
07.08-VIM-1-P44-Northern Yorkshire 5-Other8
02.10-VIM-1-P26 1-North West 12-Other4
09.10-VIM-1-P25-North West 12-Other4
08.11-VIM-1-P26 2-North West 13-NT
06.06-VIM-6-P7-South West 2-VI
08.06-VIM-6-P24-South East 7-VI
07.10-VIM-6-P2-South East 7-VI
10.04-VIM-2-P34-London 28-Other9
08.08-VIM-1-P31 2-London 22-NT
08.08-VIM-1-P31 1-London 22-Other10
11.12-VIM-2-P21-London 24-Other11
04.10-VIM-2-P33 1-London 7-Other12
04.10-VIM-2-P33 2-London 7-NT
06.09-VIM-2-P16 2-London 7-NT
08.09-VIM-2-P16 3-South West 1-NT
10.07-VIM-2-P12 1-London 7-Other13
10.07-VIM-2-P12 2-London 7-NT
08.08-VIM-2-P16 1-London 7-Other13
12.09-VIM-4-P43-North West 11-Other14
06.09-VIM-4-P4 2-East England 4-NT
06.09-VIM-4-P4 1-East England 4-Other14
09.12-MBL neg-P47-Scotland 5-none
04.12-MBL neg-P46-Northern Yorkshire 5-none
10.12-IMP-26-P10-London 24-Other1
reference NCGM2.S1
09.09-VIM-2-P41-London 7-Other15
01.05-VIM-2-P40-London 7-Other15
04.09-VIM-2-P22-London 25-Other15

**Figure 5.5 (previous page):** Maximum likelihood tree based on SNP analysis of the 55 MBL-producing and 2 MBL-negative ST235 representatives compared with the reference strain (NCGM.S1). Labels for individual nodes indicate information about each isolate in the format: date of isolation (month.year), VIM or IMP allele identified, patient number (followed by 1, 2, etc. for patients where we received multiple isolates), referring hospital, and integron detected (major integrons I-VI as detailed in Chapter 4, or others (1-15) seen among ST235 isolates). Coloured branches and node labels represent the identified clusters (1-4). Coloured squares represent hospitals submitting more than one isolate, and coloured stars represent patients from whom multiple isolates were submitted, respectively.

#### 5.3.2.1.1 Main clusters identified within ST235

Four distinct clusters (designated clusters 1 - 4) were identified (figure 5.5). Isolates belonging to cluster 1 (n=14, 6 laboratories) were most tightly grouped, differing by 56 SNPs, while those belonging to clusters 2 (n=14, 7 laboratories), 3 (n=15, 9 laboratories) and 4 (n=5, 5 laboratories) were more diverse, varying by 146, 156 and 90 SNPs, respectively. Seven MBL-producing isolates did not belong to any of these four identified clusters; these were from five patients and six laboratories and were diverse in terms of the SNP analysis, as were the reference (NCGM2.S1) and two MBL-negative ST235 strains.

Cluster 1 isolates (n=14) were epidemiologically diverse, being received from six hospital laboratories across the UK between 2004 and 2012. The biggest contributors were Scotland_2 (6 isolates, 2008-2010) and London_13 (4 isolates, 2004); single isolates were submitted from three further London laboratories and one in the West Midlands, each in a different year between 2006 and 2012.

The 14 isolates belonging to cluster 2 were submitted from seven different laboratories in London and the south of England between 2004 and 2012. London_7 was a major contributor, referring six isolates from three patients between 2008 and 2010; all belonged to the same sub-cluster (2a), which also included an isolate referred from South West_1 in 2009 from the same patient (P16) as one of the London_7 isolates and a further isolate referred from London_24 in 2012. The remaining six cluster 2 isolates were submitted from five patients and four different laboratories between 2006 and 2010.

Cluster 3 isolates (n=15) were submitted from nine laboratories across the UK between 2003 and 2012, with some small sub-clusters, comprising isolates from two to three patients each, generally referred from the same laboratory. The five representatives in cluster 4 were also each referred from a different laboratory, in four different UK regions, between 2008 and 2012, each separated by at least five months.

#### 5.3.2.1.2 Variation among ST235 isolates from single laboratories

In general, ST235 isolates submitted from the same hospital laboratory clustered together on the tree. Major exceptions were ten isolates submitted from London_7 in 2005 to 2010, which variously belonged to cluster 2 (six isolates from three patients, 2007-2010), cluster 3 (one isolate, 2010), cluster 4 (one isolate, 2010) or did not fall into any of the main SNP clusters (2 isolates from different patients, 2005 and 2009). Other exceptions included two isolates submitted from East_England_4 in 2009 and 2010, which belonged to cluster 3 or did not fall into any major SNP cluster, respectively, and two isolates from London_28 collected in 2006 and 2004, which belonged to clusters 1 or 2, respectively.

#### 5.3.2.1.3 Variation among ST235 isolates from single patients

Multiple isolates were submitted for ten patients. All these single-patient sets clustered on the SNP tree (figure 5.5; table 5.3). For most patients (8/10) two isolates were submitted 0-12 weeks apart and differed by 0-2 SNPs. For the two remaining patients, isolates were received over 12-18 month periods. Two isolates from one patient (P26) were received 18 months apart and differed by 2 SNPs. Three representatives were received from patient P16; the first was derived from a

tissue sample submitted by London_7 in 2008, while two further representatives, both from urine samples, but collected at two different hospitals (London_7 and South West_1) were received seven weeks apart approximately one year after the initial representative. These two later isolates differed by 4 SNPs from each other, and 13 and 15 SNPs, respectively, from the initial isolate from the same patient (table 5.4).

**Table 5.3:** Details of patients from whom multiple ST235 *P. aeruginosa* isolates were submitted (n=10)

| Patient | Cluster | No. of isolates | Time period separating isolates | Isolation sites | Number of SNPs different |
|---|---|---|---|---|---|
| **P30** | 1 | 2 | 0 weeks | unknown | 0 |
| **P31** | 2 | 2 | 0 weeks | both blood | 0 |
| **P33** | 2 | 2 | 1 week | both blood | 0 |
| **P16** | 2 | 3 | 1 year (P16_1 to P16_2) and 7 weeks (P16_2 to P16_3) | tissue, urine, urine | 13 (P16_1 and P16_2), 4 (P16_2 and P16_3) and 15 (P16_1 and P16_3) |
| **P12** | 2 | 2 | 1 week | both blood | 0 |
| **P32** | 3 | 2 | 0 weeks | PEG site, tracheostomy site | 1 |
| **P1** | 3 | 2 | 12 weeks | both sputum | 1 |
| **P13** | 3 | 2 | 12 weeks | both urine | 0 |
| **P26** | 3 | 2 | 18 months | sputum, wound | 2 |
| **P4** | Others | 2 | 0 weeks | both urine | 2 |

#### 5.3.2.1.4 Comparison of sequence with VNTR data for ST235 isolates

The most common VNTR type seen among the 57 ST235 isolates was 13,3,6,4,5,1,9,2,8 (n=21, 38%) with representatives of this VNTR type dispersed across the SNP tree. Fourteen different VNTR types were seen among the remaining 34 isolates, differing at the 7th (locus 207; 7, 8 or 10 repeats) and/or 9$^{th}$ loci (locus 61; 9, 10, 11, 12 or 15 repeats), with one to seven isolates having each VNTR type. Representatives referred from the same hospital generally shared the same VNTR type in addition to clustering together on the SNP tree. The main exceptions were

the ten isolates received from London_7, which encompassed two different VNTR profiles, specifically 13,3,6,4,5,1,7,2,9 (n=7, SNP clusters 2 or 4), or 13,3,6,4,5,1,8,2,10 (n=3, SNP cluster 3 or not belonging to main SNP clusters).

#### 5.3.2.2 Sequencing-based detection of resistance determinants in ST235 isolates

The intrinsic resistance genes *bla*$_{OXA-50}$, *bla*$_{PAO}$ (*ampC*), *aph(3')-IIb* and *catB7* were identified in all 57 ST235 isolates. All 55 MBL-positive isolates also had a mutation in the QRDR of GyrA (83:T-I), as seen in ST111 representatives, while sub-clusters of the MBL-positive isolates had mutations in the QRDR of ParE; these were 457:S-G (all isolates in cluster 1), 419:D-N (two isolates from P1 in cluster 3) and 457:S-R (the isolate from patient P10, not in any of the main clusters). Neither of the MBL-negative isolates had the GyrA (83:T-I) mutation seen in the MBL-producers, but one isolate did have a mutation in GyrB (466:S-F), also previously associated with fluoroquinolone resistance in *P. aeruginosa*.[30]

Acquired resistance gene content varied greatly among the 55 MBL-positive isolates, consistent with the diverse integron contents seen in these representatives (Section 4.3.1; figure 5.6). Some clusters and sub-clusters shared similar gene content, as outlined below. Acquired genes detected included those conferring resistance to β-lactam antibiotics (including multiple MBL genes *bla*$_{VIM-1}$, $_{-2}$, $_{-4}$, or $_{-6}$ or *bla*$_{IMP-7}$, $_{-14}$ or $_{-26}$), aminoglycosides, tetracycline, trimethoprim, rifampicin and chloramphenicol. The *sul1* gene, which is found in the classical 3'CS region of class 1 integrons, was seen in all MBL-positive isolates but not in the two MBL-negative isolates.

0.01 substitutions per site
~ 9.5 SNPs
β-lactam
MBL
other
Aminoglycoside
Trimethoprim
Tetracycline
Chloramphenicol
Rifampicin
Sulphonamides
VIM-1
VIM-2
VIM-4
VIM-6
IMP-7
IMP-14
IMP-26
OXA-2
OXA-10
OXA-35
PER-1
VEB-1[v]
VEB-9
PSE-1
LCR-1
aacCA5
aac(3)-Ic
aac(6')-31[v]
aacA4[v]
aadA1b
aadA2
aadA6
ant(2'')-Ia
aph(3')-VIb[v]
aph(3')-XV
aac(6')-v1
strB
dfrB5
dfrA1
tet(G)
catB3[v]
catB10[v]
cmlA1[v]
arr7
sul1
Cluster 1
Cluster 4
Cluster 3
Cluster 2
2a
09.08-VIM-2-P20-Scotland 2-II
11.10-VIM-2-P27-Scotland 2-II
01.08-VIM-2-P6-Scotland 2-II
07.08-VIM-2-P8-Scotland 2-II
11.03-VIM-2-P38-London 13-II
06.08-VIM-2-P18-London 12-II
04.04-VIM 2-P37-London 13-II
04.06-VIM-2-P35-London 28-II
03.04-VIM-2-P36-London 13-II
02.04-VIM-2-P39-London 13-II
11.07-VIM-2-P19-London 5-II
11.12-VIM-2-P15-West Midlands 7-II
03.09-VIM-2-P30 1-Scotland 2-II
03.09-VIM-2-P30 2-Scotland 2-NT
07.11-IMP-14-P14-South East 11-Other1
11.12-VIM-6-P29-North West 1-VI
08.10-VIM-2-P5-London 7-Other2
11.08-VIM-2-P3-London 4-Other2
06.12-VIM-2-P28-Northern Yorkshire 4-Other2
11.11-IMP-7-P32 2-East Midlands 1-NT
03.12-IMP-7-P11-East Midlands 1-Other3
11.11-IMP-7-P32 1-East Midlands 1-Other3
12.12-IMP-7-P45-East Midlands 1-Other3
11.03-IMP 7-P17-London 25-Other1
07.08-VIM-1-P1 1-Northern Yorkshire 3-Other4
07.08-VIM-1-P1 2-Northern Yorkshire 3-NT
08.10-VIM-1-P42-South East 1-Other5
04.10-VIM-1-P13 1-East England 4-Other6
07.10-VIM-1-P13 2-East England 4-NT
06.10-VIM-1-P23-London 7-Other7
07.08-VIM-1-P44-Northern Yorkshire 5-Other8
02.10-VIM-1-P26 1-North West 12-Other4
09.10-VIM-1-P25-North West 12-Other4
08.11-VIM-1-P26 2-North West 13-NT
06.06-VIM-6-P7-South West 2-VI
08.06-VIM-6-P24-South East 7-VI
07.10-VIM-6-P2-South East 7-VI
10.04-VIM-2-P34-London 28-Other9
08.08-VIM-1-P31 2-London 22-NT
08.08-VIM-1-P31 1-London 22-Other10
11.12-VIM-2-P21-London 24-Other11
04.10-VIM-2-P33 1-London 7-Other12
04.10-VIM-2-P33 2-London 7-NT
06.09-VIM-2-P16 2-London 7-NT
08.09-VIM-2-P16 3-South West 1-NT
10.07-VIM-2-P12 1-London 7-Other13
10.07-VIM-2-P12 2-London 7-NT
08.08-VIM-2-P16 1-London 7-Other13
12.09-VIM-4-P43-North West 11-Other14
06.09-VIM-4-P4 2-East England 4-NT
06.09-VIM-4-P4 1-East England 4-Other14
09.12-MBL neg-P47-Scotland 5-none
04.12-MBL neg-P46-Northern Yorkshire 5-none
10.12-IMP-26-P10-London 24-Other1
reference NCGM2.S1
09.09-VIM-2-P41-London 7-Other15
01.05-VIM-2-P40-London 7-Other15
04.09-VIM-2-P22-London 25-Other15

**Figure 5.6 (previous page):** Maximum likelihood tree showing SNP analysis and resistance determinants for the 55 MBL-producing and 2 MBL-negative ST235 isolates against the reference strain (NCGM.S1). Coloured branches represent the four clusters identified, as indicated. Labels for the individual nodes indicate information about the isolate in the format: date of isolation (month.year), VIM or IMP allele, patient number (followed by 1, 2, etc. for patients where we received multiple isolates), referring hospital, and integron detected (major integrons I-VI as detailed in Chapter 4, or others (1-15) seen among ST235 isolates). Coloured squares represent hospitals submitting more than one isolate, and coloured stars represent patients from whom multiple isolates were submitted, respectively. Filled grey rectangles represent acquired resistance genes detected among the isolates while empty rectangles indicate that this resistance determinant was not detected in a particular isolate.

All 14 cluster 1 isolates harboured *bla*$_{VIM-2}$, *aacCA5*, *aadA6* and *dfrB5;* these 14 included all of the integron II (In559)-carrying ST235 isolates described in Section 4.3.1. Four isolates in the cluster additionally carried the *aac(3)-Ic* gene and three had a variant of *cmlA1*.

Isolates in clusters 2-4 had more varied acquired resistance gene content than those in cluster 1. Those belonging to cluster 2 variously carried *bla*$_{VIM-1}$ (n=2), *bla*$_{VIM-2}$ (n=9), or *bla*$_{VIM-6}$ (n=3) MBL genes and all also had the acquired resistance genes *bla*$_{OXA-2}$ and *aadA6.* Sub-clusters shared additional genes including the β-lactamase determinants *bla*$_{LCR-1,}$ *bla*$_{OXA-10}$ or *bla*$_{VEB-9,}$ and aminoglycoside resistance determinants, *strB*, *ant(2")-Ia* or variants of *aac(6')-31* or *aph(3')-Vib*. Cluster 3 (n=15) representatives carried either *bla*$_{VIM-1}$ (n=10) or *bla*$_{IMP-7}$ (n=5). Sub-clusters carried various additional resistance genes including *bla*$_{PER-1}$, *aac(3)-Ic*, *an aacA4* variant, *aadA2*, *aadA1, aadA6*, *aph(3')-XV, strB,* and/or variants of *catB10* or *cmlA1.* Representatives in cluster 4 (n=5) carried the MBL genes *bla*$_{VIM-2}$ (n=3), *bla*$_{VIM-6}$ (n=1) or *bla*$_{IMP-14}$ (n=1) with variable additional resistance genes including *bla*$_{OXA-10}$, a *bla*$_{VEB-1}$ variant, *bla*$_{VEB-9}$, *aadA6*, *aacCA5*, *ant(2')-Ia*, a *aph(3')-Vib* variant, *tet*(G) and/or *dfrB5*. In the remaining seven MBL-producing isolates diverse resistance genes were variously seen including *bla*$_{VIM-2}$, *bla*$_{VIM-4}$, *bla*$_{IMP-26}$, *bla*$_{PSE-1}$, *bla*$_{OXA-35}$, *aadA6, aac(6')-Ib* variant, *aph(3')-VIb* variant, *arr7*, *aac(6')-v1* and/or *dfrB5*.

#### 5.3.2.2.1 Comparison of genotypes with MIC data for ST235 isolates

All 55 MBL-producing ST235 *P. aeruginosa* isolates were resistant to most of the tested antibiotics (table 5.4); major exceptions being aztreonam, to which 64% of tested isolates were intermediate, and colistin, to which all isolates remained

susceptible. All 55 isolates were resistant to imipenem and meropenem, as well as ceftazidime, consistent with the presence of an MBL gene. Ciprofloxacin resistance was also seen in all these MBL-positive isolates, consistent with the GyrA (83:T-I) mutation. Five isolates (9%) remained susceptible to piperacillin-tazobactam; these carried $bla_{\text{IMP-7}}$, $bla_{\text{IMP-14}}$, $bla_{\text{IMP-26}}$ or $bla_{\text{VIM-2}}$ MBL genes. All isolates were resistant to the aminoglycosides gentamicin and tobramycin, but variable resistance was seen to amikacin to which 16/55 (29%) remained susceptible (n=5) or intermediate (n=11); this is despite 11 of these 16 isolates carrying variants of *aacA4* or *aac(6')-31* genes, which (if strongly expressed) are usually associated with amikacin resistance.[25]

The two MBL-negative ST235 isolates were widely susceptible to tested antibiotics, exceptions being aztreonam (both isolates intermediate), meropenem (one isolate intermediate), and ciprofloxacin (one isolate resistant); the ciprofloxacin-resistant isolate had a mutation in *gyrB* (466:S-F), while the elevated meropenem MIC was likely due to mutations leading to reduced efflux and/or overproduction of the chromosomally-encoded AmpC β-lactamase.

**Table 5.4:** Antibiotic susceptibility of ST235 representatives subjected to WGS analysis

| | MBL-positive (n=55) | | | | MBL-negative (n=2) | | | |
|---|---|---|---|---|---|---|---|---|
| **Antibiotic** | **S** | **I** | **R** | **NT** | **S** | **I** | **R** | **NT** |
| Amikacin | 5 | 11 | 39 | 0 | 2 | 0 | 0 | 0 |
| Gentamicin | 0 | - | 55 | 0 | 2 | - | 0 | 0 |
| Tobramycin | 0 | - | 54 | 1 | 2 | - | 0 | 0 |
| Piperacillin-tazobactam | 5 | - | 50 | 1 | 2 | - | 0 | 0 |
| Ceftazidime | 0 | - | 54 | 1 | 2 | - | 0 | 0 |
| Aztreonam | 0 | 34 | 19 | 2 | 0 | 2 | 0 | 0 |
| Imipenem | 0 | 0 | 55 | 0 | 2 | 0 | 0 | 0 |
| Meropenem | 0 | 0 | 55 | 0 | 1 | 1 | 0 | 0 |
| Ciprofloxacin | 0 | 0 | 55 | 0 | 1 | 0 | 1 | 0 |
| Colistin | 54 | - | 0 | 1 | 2 | - | 0 | 0 |

S, susceptible; I, intermediate; R; resistant, NT; not tested

### 5.3.3 **WGS analysis of representatives of other 'high-risk clones'**

Single representatives of ST233 (Paer_LW_109), ST357 (Paer_LW_197), ST654 (Paer_LW_121) and ST773 (Paer_LW_167) were also sequenced. All carried the intrinsic resistance genes *bla*$_{OXA-50}$, *bla*$_{PAO}$ (*ampC*), *aph(3')-IIb* and *catB7*. Their acquired resistance gene content, shown in table 5.5, was variable; all representatives had *bla*$_{VIM-2}$ and carried additional acquired resistance genes including those encoding resistance to β-lactams and aminoglycosides. All four representatives also had the GyrA (83:T-I) chromosomal mutation seen in ST111 and ST235 isolates, with the ST773 representative having an additional mutation (457:S-T) in the QRDR of ParE (table 5.5). All four isolates were resistant to all tested antibiotics except for colistin, to which all remained susceptible, and aztreonam to which the ST773 isolate was intermediate.

**Table 5.5:** Resistance determinants seen in single sequenced isolates belonging to ST233, ST357, ST654 and ST773

| Antibiotic | ST233 | ST357 | ST654 | ST773 |
|---|---|---|---|---|
| **β-lactams** | *bla*$_{OXA-1}$<br>*bla*$_{VIM-2}$ | *bla*$_{OXA-10}$<br>*bla*$_{VIM-2}$<br>*bla*$_{VEB-1}$[v] | *bla*$_{VIM-2}$<br>*bla*$_{PME-1}$ | *bla*$_{OXA-10}$<br>*bla*$_{VIM-2}$ |
| **aminoglycosides** | *aac(3)-Id*[v]<br>*aadA2*<br>*aadA3*[v] | *aadA1**<br>*ant(2'')-Ia* | *aph(3')-VIb*[v]<br>*strB* | *aac(6')-Ib*[v]<br>*aadA1** |
| **trimethoprim** | *dfrB5* | *dfrB2*[v] | - | *dfrB2*[v] |
| **tetracycline** | *tet(G)* | *tet(A)* | *tet(G)*<br>*tet(A)*[v] | |
| **chloramphenicol** | *cmlA1*[v] | - | - | *cmlA1*[v] |
| **sulphonamides** | *sul1* | *sul1* | *sul1* | *sul1* |
| **rifampicin** | - | - | - | *arr-2* |
| **fluoroquinolones** | GyrA[83:T-I] | GyrA[83:T-I] | GyrA[83:T-I] | GyrA[83:T-I]<br>ParE[457:S-T] |

[v] indicates a variant of the given resistance gene
* indicates that only fragment of the gene was detected

### 5.3.4 **Detection of CRISPR-*cas* genes**

Illumina reads were mapped against type I-E and type I-F CRISPR-*cas* genes, as described in Section 5.2.3. For the 87 ST111 isolates, and 57 ST235 isolates (including MBL-positive and MBL-negative representatives) subjected to Illumina sequencing none of the reads mapped to any of the type I-E or type I-F CRISPR-*cas* genes.

The single MBL-producing representatives of ST233 and ST357 had all seven type I-F CRISPR-*cas* genes (*cas1*, *cas3*, *csy1*, *csy2*, *csy3*, *csy4*); the ST773 representative had all six type I-E genes (*cas2*, *cas1*, *cas6e*/*cse3*, *cas5*, *cas7*, *cse2* and *cas3*), and the representative of ST654 had all genes of both type I-E and type I-F CRISPR-Cas systems. The identified CRISPR-*cas* genes shared 99-100% nucleotide identity with the type I-E and type I-F genes from the RP73 (GenBank accession NC_021577) and PA14 (GenBank accession NC_008563) *P. aeruginosa* genomes, respectively, except for the *cse1*, *cse2* and *cas3* genes of the type I-E CRISPR-Cas system in the ST773 representative, which shared 93% nucleotide identity with the reference sequences.

To estimate the prevalence of these CRISPR-Cas systems in clinical *P. aeruginosa*, we screened 50 representatives randomly selected from the 209 isolates submitted as part of the BSAC Bacteraemia Surveillance Programme, 2011 (see also Sections 2.1.2 and 3.3.3). These isolates were largely antibiotic susceptible and were diverse by VNTR typing, with few belonging to 'high-risk clones' (ST233, n=1 or ST235, n=2). Two PCRs were undertaken, targeting either the *cse3* gene of type I-E, or the *csy1* gene of type I-F CRISPR-Cas systems, respectively, as described in section 5.2.3. Figure 5.7 shows the distribution of the detected genes among the 50 isolates. Ten

isolates (20%) yielded an amplicon using primers targeting the *cse3* gene, indicating that they harboured a type I-E CRISPR-Cas system. A further 14 isolates (28%) were positive using primers targeting the *csy1* gene, indicating that they carried a type I-F CRISPR-Cas system. One further isolate was positive for both PCRs, indicating that it had both type I-E and type I-F CRISPR-Cas systems. The remaining 25 isolates were negative by both PCRs.

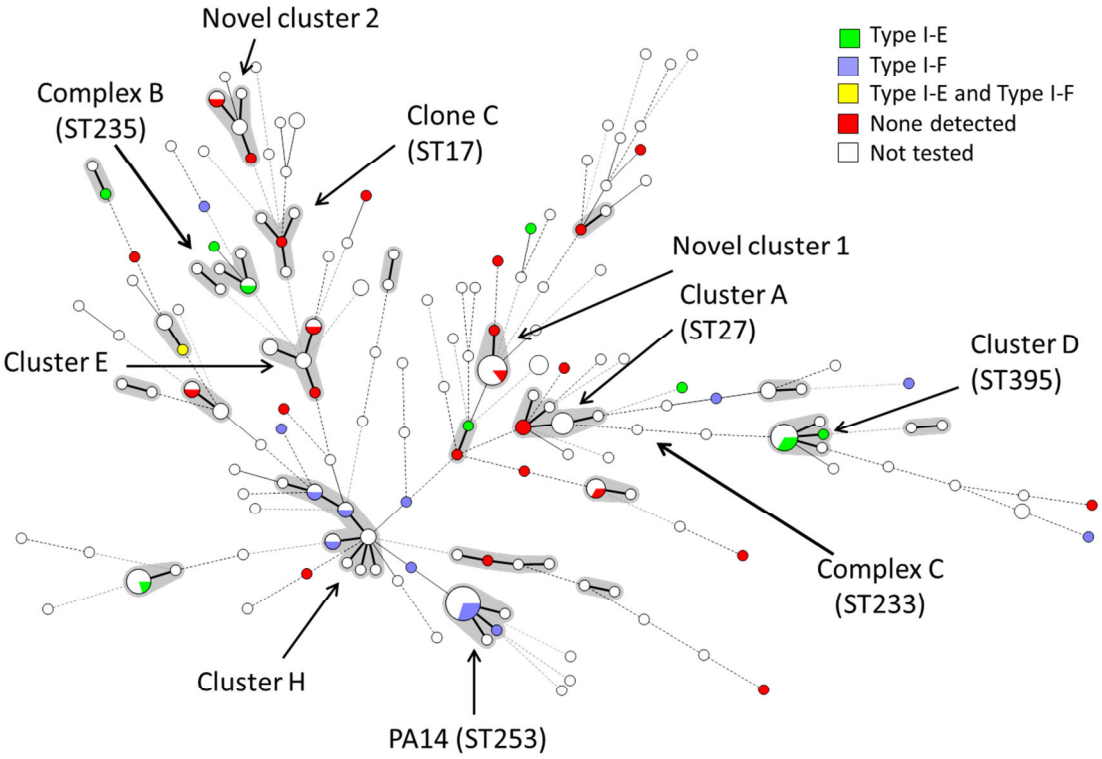



**Figure 5.7:** CRISPR-Cas systems detected among *P .aeruginosa* isolates from the BSAC Bacteraemia Resistance Surveillance Programme for 2011. The minimum spanning tree is based on clustering at the first eight VNTR loci for *P. aeruginosa* isolates. Coloured circles indicate whether genes representative of the CRISPR-Cas systems were detected by PCR for the 50 tested isolates, white circles indicate isolates that were not tested. The diameter of the circle is relative to the number of isolates with that VNTR profile. Grey shading indicates complexes. Thick solid lines represent single locus variants while thin solid lines and dotted lines represent multi-locus variants. Isolates corresponding to previously reported clones as described by Martin et al.[115] are indicated and had the following VNTR profiles; Clusters A (8,3,4,5,2,3,5,2,x), D (10,3,5,5,4,1,3,x,x,), E (11,4,5,2,2,1,x,2,x) and H (12,5,1,5,2,2,x,x,x), Clone C (11,6,2,2,1,3,7/8,2/3,x), and PA14 (12,2,1,5,5,2,x,5,x). Two novel clusters are indicated with VNTR profiles 12,5,5,5,4,3,7,6,x and 12,8,2,2,4,3,5,1,x respectively. See also figure 3.6.

## 5.4 Discussion

SNP analysis was able to discriminate among members of the ST111 and ST235 lineages, and revealed several different clusters and sub-clusters among representatives of both STs. These data, together with the epidemiological information indicate that separate introductions of these 'high-risk clones' had occurred in UK hospitals. However there was also evidence of spread within and between hospitals, indicated by closely related SNP clusters. The SNP analysis was consistent with VNTR and available epidemiological data, but provided discrimination between isolates that were identical by VNTR.

Previous work (Chapter 3) had shown that the ST111 isolates shared the same or very similar VNTR types, dominated by large numbers of isolates from a few hospitals including London_17, London_9 and London_26, and with the majority carrying integron I (In59-like integrons), with $bla_{VIM-2}$ and *aacA29a* and *aacA29b* genes. Some probable sub-clusters had already been identified based on a combination of the epidemiological and VNTR data, for example, ST111 isolates referred from London_26 (cluster 3a) all had a distinct VNTR type (11,3,4,3,2,2,6,4,7) and this was confirmed as a tight cluster by the SNP analysis. The WGS data however also allowed further subdivision of many isolates with identical VNTR types. In particular it confirmed that $bla_{IMP}$-carrying and MBL-negative ST111 representatives were only distantly related to those carrying $bla_{VIM-2}$ as part of integron I.

The ST235 *P. aeruginosa* strains were more diverse than ST111 isolates in terms of VNTR typing; they also (unlike the vast majority of the ST111 isolates) had MBL-

containing integrons of different sizes, carried various *bla*$_{VIM}$ and *bla*$_{IMP}$ MBL alleles, and were isolated at many different times and places, with representatives received from no more than seven patients at a single site. This diversity was confirmed by the large variation seen between most isolates by SNP analysis and the huge range of acquired resistance genes present, suggesting that ST235 strains have repeatedly acquired different MBL genes.

*P. aeruginosa* strains can cause prolonged outbreaks, suggesting that there are hidden environmental reservoirs, acting as sources of infection.[18,218] A notable example here is the previously discussed (Sections 3.4 and 4.4) nine-year persistence of a ST111 strain at London_17, which was attributed to contamination of the waste-water system.[157] There was considerable sequence diversity among isolates received from London_17 (up to 35 SNPs between isolates) consistent with repeated patient infections from a wider environmental source, with a diverse ST111 population. Environmental isolates were also studied here from outbreaks of ST111 organisms at London_9 and London_26 and these clustered closely with patient representatives from the same hospital. However, as with the London_17 environmental isolates, they were generally cultured from hospital sinks and sink drains, and so it is unclear whether these represent sources of infection or environmental contamination from the patients.

Aside from environmental reservoirs, it is also possible that long-term asymptomatic colonisation of patients could be a hidden reservoir of these organisms. *P. aeruginosa* is not thought to be a member of the normal human flora, with any colonisation being only transient (with the exception of patients with

chronic respiratory colonisations, as is seen in cystic fibrosis). However, in this study, three patients appeared to carry the same ST235 or ST111 strain, closely related by SNP analysis, over periods of one to four years, although it also remains possible that these patients were re-infected with a very similar strain. Assuming that these three patients had become colonised, the inferred mutation rates were approximately 1.3, 4.5 or 13 SNPs/year; two of these were higher than a previous estimate of the mutation rate of *P. aeruginosa* (2.6 SNPs/year),[234] but it still seems plausible that they resulted from a single colonisation event when considering the selection pressures that may be acting on bacteria adapting to the human host, and that patients could be initially colonised with a mixed population. Any such long-term colonisation could confound attempts to find epidemiological links between patients as a strain could be acquired and carried asymptomatically for a long time before it was detected. For example, in a three-year outbreak of IMP-8-producing ST308 *P. aeruginosa* at a German hospital,[235] two closely-related strains, differing by only one SNP, were isolated from different patients in 2009 and 2011, respectively. The patients had consecutively stayed in the same room in 2009 and the authors suspected that the transfer of the strain had occurred in 2009, with the second patient remaining colonised over the following two years, only detected when the patient was re-admitted to the hospital in 2011.

In addition to spread of ST111 and ST235 strains within a hospital, there was evidence of spread occurring between different hospital sites. For example, ST111 cluster 3 isolates were received from multiple London and South East England hospitals. Although there were known patient transfers between some of these hospitals, for example between London_26 and London_28, and between London_9

and London_11, in many cases no epidemiological links were identified, albeit based on incomplete information about patient transfers, which are often not notified for isolates sent to the reference laboratory.

In general, isolates that were referred from the same hospital clustered together by SNP analysis and shared similar resistance determinants, supporting the view that they were closely related. However, in a few cases the SNP analysis clearly indicated that representatives from the same hospital, which might otherwise have been considered part of the same outbreak, were genetically distinct. For example, four ST111 *bla*$_{VIM-2}$-positive isolates received from London_28 during 2012 shared the same VNTR type or differed by one repeat at a single VNTR locus, but were dispersed across the SNP tree indicating that the infections were acquired from different sources. Another example concerned ten *bla*$_{VIM}$-positive ST235 isolates submitted from seven patients at London_7 between 2007 and 2010. As discussed in Section 4.4, two different VNTR types and five different *bla*$_{VIM}$-containing integron profiles were seen among these ten isolates leading us to suspect that they were likely from different sources. SNP analysis confirmed that these isolates were diverse, with four different sub-clusters, each from one to three patients spread across the SNP tree, and with each sub-cluster harbouring a distinct set of acquired resistance genes. They included three isolates received from different patients over a four-month period in 2010, with these belonging to clusters 2, 3 and 4; SNP analysis thus confirmed that these infections likely resulted from independent acquisitions of different ST235 strains rather than spread within the hospital.

The MBL-producing ST111 and ST235 isolates were resistant to the majority of antibiotics tested and the susceptibility patterns generally correlated with the presence of acquired genes for the β-lactam antibiotics, or (where these data were available) mutations affecting the GyrA, GyrB or ParE proteins, for ciprofloxacin. Resistance to aminoglycosides, particularly to amikacin and gentamicin, was more variable and was not completely explained by the presence of acquired resistance genes, as isolates carrying the same set of resistance determinants often had differing susceptibility patterns. For example ST111 isolates carrying integron I had variable susceptibility to gentamicin and amikacin. Where aminoglycoside resistance was present in the absence of any acquired genes known to confer such resistance it is likely that other factors are contributing to this variable aminoglycoside resistance, such as mutations affecting efflux, or cytoplasmic membrane transport. On the other hand, isolates that retained susceptibility despite carrying genes known to confer resistance to a particular aminoglycoside (e.g. the 17/62 ST111 isolates carrying aacA29a/b as part of integron I that remained susceptible to amikacin), the acquired resistance genes may be poorly expressed.

PCR analyses showed that 50% of tested isolates from the BSAC Bacteraemia Surveillance Programme (n=50) harboured at least one CRISPR-Cas system, which was in the same range as previous studies (39-54%).[169,226] By contrast, type I-E or type I-F *cas* genes were not present in any of the 87 ST111 or 57 ST235 genome sequences, consistent with a previous study.[30] It is possible that the lack of CRISPR-Cas systems may contribute to the ability of ST111 and ST235 'high-risk clones' to acquire new genetic material. However, as these CRISPR-Cas systems are only

present in around 50% of *P. aeruginosa* isolates generally, and since representatives of other 'high-risk clones' (STs 233, 357, 654 and 773) did carry them, additional factors must be involved in their clonal success and accumulation of resistance. CRISPR-Cas is just one of several known bacterial defence systems that may interact with each other to protect against exogenous DNA elements; these include restriction-modification, toxin-antitoxin, abortive-infection and phage-exclusion systems.[236–238] More research is needed into the action of these systems, how they interact with mobile DNA elements, and whether they are less prevalent or act differently in 'high-risk clones'.

In summary, WGS analyses of isolates belonging to the 'high-risk clones' of ST111 or ST235 provided increased resolution between representatives of these lineages compared with VNTR and MLST. The analysis additionally allowed aspects of the bacterial biology to be explored, including the large diversity of resistance determinants seen among the isolates. Using WGS analysis in real-time will be useful to inform epidemiological investigations, particularly in helping to identify probable sources of infection so that hospitals and public health bodies can better target effective interventions to limit their spread.

# 6 Conclusions and future work

Increasing numbers of MBL-producing *P. aeruginosa* isolates were referred to AMRHAI from UK hospitals over recent years (figure 3.1). The aim of this work was to investigate the molecular epidemiology of these organisms and to elucidate to what extent their MBL genes may be transferred between strains. It is important to have a better understanding of these multi-drug resistant organisms in order to identify effective infection prevention and control strategies to limit their future spread. The overall findings and implications of the research presented in this thesis will be discussed in this chapter, along with suggestions for future work.

## 6.1 Main conclusions

We found that internationally-recognised 'high-risk clones' (namely STs 111, 235, 233, 357, 654 and 773) were important in the accumulation of MBL-producing *P. aeruginosa* in the UK, accounting for 86% of all such isolates referred to AMRHAI. This included isolates from 71 hospital laboratories, most of which did not have substantial outbreaks. Diverse ST and VNTR types were represented among the remaining 14% of MBL producers, indicating independent acquisition of MBLs by sporadic types. Whilst MBL-producing *P. aeruginosa* were rare at most of the referring laboratories, a few hospitals did have more persistent problems lasting up to nine years. These included long-term persistence of single strains at some hospitals, such as the nine-year 'outbreak' of an ST111 organism at London_17. Other laboratories referred isolates of diverse types, for example, London_7 submitted 19 MBL-positive isolates over seven years belonging to STs 111, 235, 233 and 654, indicating that independent introductions of MBL-producing strains had probably occurred.

Comparison with a collection of largely antibiotic-susceptible isolates from the BSAC Bacteraemia Surveillance Programme in 2011 indicated that these 'high-risk clones', though strongly represented among MBL producers, were not prevalent generally among *P. aeruginosa* isolates from serious infections in the UK and Ireland. Rather, these lineages seem to represent a distinct subset of *P. aeruginosa* that are better able to acquire and/or maintain whatever resistance genes they may encounter. This view is supported by (1) the presence of various MBL genes, including different $bla_{VIM}$ and $bla_{IMP}$ alleles, in representatives of each high-risk lineage in this study and/or internationally, (2) the range of integron structures reported among most of the 'high-risk clones' in this study and/or internationally and (3) the varied acquired resistance genes detected among ST111 and particularly ST235 isolates by WGS.

To better understand potential reasons underlying this increased ability to acquire and/or maintain resistance genes, the genetic context of the MBL genes were studied. The $bla_{VIM}$ and $bla_{IMP}$ genes were located within class 1 integrons (as is typical for these genes) and, in representatives of most of the common lineages, these integrons were chromosomally located. The genetic environment of $bla_{VIM-2}$-containing integrons was characterised fully for representatives of STs 111, 233 and 235, and, in all cases, they were found within large chromosomally-located genomic islands. PCR indicated that most or all members of each lineage (STs 111, 233 or 235) had insertions of a similar genomic island structure at the same genome position, even when the organisms carried different integrons. This observation suggests that members of the 'high-risk clones' may share stable lineage-specific chromosomally-located genetic platforms that can acquire varied resistance genes,

via the class 1 integrons that each genomic island encodes. This feature is likely to contribute to the success of these lineages in acquiring diverse resistance genes.

Another feature suggested to contribute to the success of 'high-risk clones'[30,223] is the absence of bacterial defence systems against exogenous DNA, such as CRISPR-Cas, leading to increased invasion by foreign DNA elements such as plasmids and bacteriophage. This theory was explored here, and the CRISPR-Cas systems previously described in *P. aeruginosa* were found to be absent from ST111 and ST235 representatives, indicating that this could be a factor in the success of these particular clones. However, CRISPR-Cas systems were present in only around 50% of *P. aeruginosa* in our BSAC Bacteraemia Surveillance Programme comparator collection from 2011, and were present in the other 'high-risk clones' (STs 233, 357, 654 and 773). These observations suggest that the lack of CRISPR-Cas is unlikely to be a major determinant of all 'high-risk clones'.

Finally, the use of WGS to discriminate among otherwise-indistinguishable members of 'high-risk clones', was explored. Increased discrimination was seen between members of the same 'high-risk clone' using WGS analyses and varied resistance determinants were identified. This method, together with existing epidemiological data, helped to identify cases of probable cross-transmission between hospitals, as well as strains that were likely acquired from independent sources at the same hospital. This kind of investigation applied in real-time is likely to aid outbreak investigations as well as long-term surveillance of multi-drug resistant organisms. For example, recent studies have used WGS to identify probable sources of *P. aeruginosa* infections in real-time in a newly-opened hospital,[217] and to identify the

likely source of an Australian ST253 *P. aeruginosa* outbreak in a neo-natal intensive care unit as a sink,[218] informing infection control interventions in near real-time.

Better understanding of the way in which these organisms spread within and between hospitals, and how they acquire resistance genes, has implications for their control in hospitals. Where there is an environmental source, interventions to decontaminate, and prevent recolonisation of such reservoirs are necessary. This could include regular flushing of hospital taps, or the use of basins with back outlets, rather than the conventional bottom outlet, to minimise the creation of bacterial aerosols from the drain.[239] On the other hand, repeated import of diverse strains would require early detection of such organisms, possibly by screening of patients considered to be at-risk; this could include patients known to have been previously colonised with MBL-producing *P. aeruginosa* or hospitalised where there are known problems, either in the UK, or abroad (e.g. in Russia, or India).

## 6.2 Future work

This work strongly suggests that 'high-risk clones' have an increased ability to acquire and/or maintain resistance genes. Future studies should be conducted to test this observation, for example by determining whether 'high-risk clones' have increased transformation of resistance genes, by conjugation or electroporation, compared to other *P. aeruginosa* strains, and/or whether they have higher levels of retention of such genes over time.

In addition, the biological reasons for the 'high-risk clones' prevalence among multi-drug resistant clones should be elucidated. This work suggests that 'high-risk clones' such as ST111, ST235 and ST233 may carry lineage-specific chromosomal platforms,

known as genomic islands, that enable them to capture diverse resistance elements, and this could be part of the reason for their success. Studies, including the work presented here, are only recently beginning to elucidate the genetic context of integrons within transposons, genomic islands and mobile genetic elements, either as part of plasmids or in the bacterial chromosome.[89,147,181,182] Long-read sequencing technologies such as MinION or PacBio should facilitate characterisation of resistance-associated genomic islands and their association with 'high-risk clones'.

It remains possible that absence, modification or downregulation of systems that defend bacteria against exogenous DNA may contribute to the ability of 'high-risk clones' to acquire and maintain resistance genes. Although the absence of CRISPR-Cas systems does not seem to be a common feature of all 'high-risk clones' in our collection, other bacterial defence systems against exogenous DNA, such as restriction-modification, toxin-antitoxin, abortive-infection, and phage-exclusion systems could be involved.[236–238] Further study of the function, regulation, and interactions of these anti-DNA bacterial defence systems, as well as of their prevalence and distribution in *P. aeruginosa* (and specifically in 'high-risk clones') is needed to better understand the contribution they may make to the success of high-risk lineages.

While previous work has identified phenotypic features including reduced motility, increased biofilm formation and reduced *in vitro* fitness in which 'high-risk clones' (STs 111, 175, and 235) may differ[240] it is difficult to understand how these features might lead to increased acquired resistance. Further work is thus required to

elucidate the precise reasons why 'high-risk clones' are successful, which may differ from one lineage to another. Comparative genomics studies should help to identify particular genes that are specific to or absent in these 'high-risk clones' compared with other *P. aeruginosa* strains and should be facilitated by the increasing availability of WGS sequencing data for diverse *P. aeruginosa*. Transcriptomics methods such as RNASeq could also be used to determine whether there are any differences in gene expression in 'high-risk clones' compared with other lineages that do not commonly harbour resistance genes. One recent study using this approach revealed varied gene expression profiles among diverse *K. pneumoniae* isolates, including a specific transcription profile associated with the ST258 'high-risk clone'.[241]

Rapid tests, such as PCRs, to detect 'high-risk clones' may be useful in hospitals to quickly identify these potentially multi-drug resistant organisms in clinical and surveillance specimens, thereby allowing rapid implementation of appropriate infection control measures. This may be particularly useful in hospitals and regions where a particular clone is widespread, for example the VIM-2-producing ST111 clone that persisted at London_17 over a nine years, in this work, or the ST235 clone producing VIM-2 that is prevalent in Russia, Belarus and Kazakhstan.[136] MALDI-TOF was recently shown to have potential in distinguishing between different clones, including STs 111 and 235.[242] In addition, WGS methods should facilitate the identification of specific genes associated with particular 'high-risk clones', and these could be used as PCR targets, facilitating detection.

Finally, more work is needed to better understand where the reservoirs of multi-resistant *P. aeruginosa* lie in hospitals. These may include environmental contamination of plumbing systems and/or medical equipment (e.g. ventilators), or long-term colonisation of patients themselves. Real-time investigation of outbreaks using the increased resolution that is offered by WGS should help us to better understand these reservoirs, as well as the routes of transmission of *P. aeruginosa* between patients and environmental sources. This should enable early implementation of appropriate infection control measures in an outbreak situation.

## 6.3 Summary

Overall, this research has shown that 'high-risk clones' are important in the spread of MBL-producing *P. aeruginosa* in the UK. The success of these clones is likely to be due to multiple factors, which may include the association with particular genomic islands or the absence of host defence mechanisms against exogenous DNA elements. Future work should focus on further elucidating the reasons for the success of these clones, to better understand how to limit the spread of these multi-drug resistant organisms.

# 7 Abbreviations

AAC; aminoglycoside acetyltransferase

AIM; Adelaide Imipenemase

AME; aminoglycoside-modifying enzyme

AMRHAI; Antimicrobial Resistance and Healthcare-Associated Infections Reference Unit

ANT; aminoglycoside nucleotidyltransferase

APH; aminoglycoside phosphotransferase

Arr; rifampicin ADP-ribosyltransferase

BAM; binary alignment map

BSAC; British Society for Antimicrobial Chemotherapy

Cas; CRISPR-associated proteins

*cas*; CRISPR-associated genes

cat; chloramphenicol acetyltransferase

cml; chromamphenicol efflux protein

CRISPR; clustered regularly interspaced short palindromic repeats

Dfr: dihydrofolate reductase

ECDC; European Centre for Disease Prevention and Control

ESBL; extended-spectrum β-lactamase

FIM; Florence imipenemase

GES; Guiana extended-spectrum (β-lactamase)

GIM; German imipenemase

ICE; integrative and conjugative elements

IME; integrative and mobilisable elements

IMP; imipenemase

IS; insertion sequence

ISCR; insertion sequence common regions

KPC; *Klebsiella pneumoniae* carbapenemase

LPS; lipopolysaccharide

MBL; metallo-β-lactamase

MIC; minimum inhibitory concentration

MLST; multi-locus sequence typing

NDM; New Delhi metallo-β-lactamase

OXA; oxacillin-hydrolysing (β-lactamase)

PER; *Pseudomonas* extended resistance (β-lactamase)

PFGE; pulsed-field gel electrophoresis

PHE; Public Health England

PSE; *Pseudomonas*-specific enzyme (β-lactamase)

QRDR; quinolone resistance determining region

SIM; Seoul imipenemase

SNP; single nucleotide polymorphism

SPM; Sao Paulo metallo-β-lactamase

ST; sequence type

TEM; Temoneira (β-lactamase)

VCF; variant calling format

VEB; Vietnam extended-spectrum β-lactamase

VIM; Verona integron-encoded metallo-β-lactamase

VNTR; variable number tandem repeat

WGS; whole genome sequencing

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# Appendix A: publications relating to this work