Emergence of OXA-carbapenemase- and 16S rRNA methylase-producing international clones of Acinetobacter baumannii in Norway

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This study was designed to investigate the molecular epidemiology and antibiotic-resistance characteristics of 11 carbapenem-resistant clinical isolates of Acinetobacter baumannii obtained in Norway between 2004 and 2009. Interestingly, all the isolates were linked with recent hospitalization outside Norway. The epidemiological status was investigated by multilocus sequence typing (MLST), multiplex PCR assays for major international clones, typing of blaOXA-51-like variants and PFGE. The genotypic-resistance characteristics, including the occurrence of OXA-carbapenemase-encoding and 16S rRNA methylase-encoding genes and class 1 integrons, were investigated by PCR assays and sequencing. Seven isolates were found to harbour blaOXA-66 and belong to MLST clonal complexes (CCs) CC2P (Pasteur Institute scheme) and CC92B (Bartual scheme), and international clone II. One isolate harboured blaOXA-69, and belonged to CC1P, CC109B and international clone I. Two isolates belonged to sequence group 9, probably a subgroup of international clone I, and one isolate belonged to sequence group 4, a proposed novel international clone. All isolates contained an acquired OXA-carbapenemase-encoding gene: blaOXA-23-like (n=9), blaOXA-24-like (n=1) and blaOXA-58-like (n=1). Four isolates with high-level aminoglycoside-resistance contained the 16S rRNA methylase-encoding armA gene. Class 1 integrons with six different variable regions were detected. Sequence analysis of gene cassettes identified four aminoglycoside (aacA4, aac(6')-Im, adaA1 and aacC1), two chloramphenicol (catB8 and cm1A5), one β-lactamase (blaOXA-20) and one rifampicin (arr-2) resistance gene in various combinations. In conclusion, the occurrence of A. baumannii isolates producing OXA carbapenemase and 16S rRNA methylase in Norway was related to the worldwide distribution of international clones I and II, and the emergence of novel international clones.

INTRODUCTION

Acinetobacter baumannii is an important opportunistic pathogen that mainly infects critically ill patients in intensive care units (Dijkshoorn et al., 2007). A. baumannii has several innate resistance mechanisms to a number of antibiotics, such as aminopenicillins, first- and second-generation cephalosporins and chloramphenicol.
(Dijkshoorn et al., 2007). Besides this, it has a considerable capacity to acquire mechanisms conferring resistance to broad-spectrum β-lactams, carbapenems, aminoglycosides and fluoroquinolones (Dijkshoorn et al., 2007; Poirel & Nordmann, 2006a). Resistance to carbapenems in A. baumannii is mainly mediated by the acquisition of class D and class B carbapenemase-encoding genes, with bla_{OXA-23}-like being the most frequently identified carbapenemase-encoding gene (Poirel & Nordmann, 2006a). The occurrence of genes encoding aminoglycoside-modifying enzymes, possibly as gene cassettes in class 1 integrons, is the main mechanism of resistance to aminoglycosides (Nemec et al., 2004). In addition, 16S rRNA methylases conferring high-level pan-aminoglycoside resistance have recently been identified in A. baumannii, including OXA-23-producing isolates (Karhikeyan et al., 2010).

Three epidemic clones of A. baumannii, recently named ‘international clones I, II, and III’, have been found to be responsible for several hospital outbreaks globally (Diancourt et al., 2010). A shift in the current A. baumannii population towards international clone II, rather than international clone I, has been reported (Nemec et al., 2008). Multilocus sequence typing (MLST) has also demonstrated the worldwide predominance of two major clonal complexes (CCs), CC92 and CC109 (http://pubmlst.org/abaumannii/). CC92 and CC109 were correlated with international clones II and I, respectively (Mugnier et al., 2010). However, other clones of A. baumannii have also showed a large-scale distribution in geographically distinct regions in the world (Diancourt et al., 2010; Towner et al., 2008). The association between drug resistance and epidemicity in A. baumannii has been established, probably in a mutual escalating arrangement in which acquisition of drug-resistance determinants has facilitated the spread of specific clones and the epidemic capacity of some clones has contributed in the growing emergence of drug resistance (Diancourt et al., 2010). In the present study, we characterized a set of 11 isolates of carbapenem-resistant A. baumannii imported to Norway from other countries with regard to their molecular epidemiology, and their phenotypic and genetic anti-biostic-resistance features.

**METHODS**

**Bacterial isolates.** The study included all carbapenem-resistant A. baumannii clinical isolates submitted between 2004 and 2009 to the Reference Centre for Detection of Antimicrobial Resistance in Norway (Table 1). The isolates (n=11) were collected by six different diagnostic microbiology laboratories in Norway from different cultures (blood, pus, respiratory secretions, abdominal cavity fluid and spinal fluid) based on carbapenem resistance according to the guidelines of the Reference Centre of Antimicrobial Resistance. Interestingly, all isolates were derived from patients recently hospitalized abroad (Table 1). Species identification of the isolates was genotypically confirmed by partial rpoB (zone 1, 352 bp) and near-complete 16S rRNA gene (1379 bp) sequence analyses (Nemec et al., 2009; Ibrahim et al., 1997).

**Molecular epidemiology.** MLST was performed according to the scheme described on the Pasteur Institute’s MLST website (http://www.pasteur.fr/recherche/genopole/PF8/MLST/) (Nemec et al., 2008), and the scheme developed by Burtual et al. (2005), with minor modifications (http://pubmlst.org/abaumannii/). Primers gpiKres-F and gpiKres-R, and the published primers rpoD-F2 and rpoD-R2, were used for amplification and sequencing of genes gpi and rpoD, respectively (Supplementary Table S1, available with the online journal) (Park et al., 2009). Sequences were compared with those in A. baumannii MLST databases and analysed using eBURST V3 (http://eburst.mlst.net/) under stringent (minimum of six shared alleles) grouping parameters. To differentiate between the two MLST schemes, sequence types (STs) and CCs were designated ST99/CC99 for the Pasteur Institute scheme and ST99/CC99 for the Burtual scheme. Identification of major sequence groups/international clones was done by two multiplex PCRs targeting *ompA*, *cssE* and *bla_{OXA-51-like*} sequences (Turton et al., 2007). Full-length sequencing of *bla_{OXA-51-like*} was performed using primers OXA-69A and OXA-69B, external to the *bla_{OXA-51-like*}-like gene (Herritier et al., 2005). PFGE was performed using Apal-digested genomic DNA, as described by Mugnier et al. (2010). Similarities among the PFGE patterns were calculated by the Dice coefficient method using BioNumerics software (Applied Maths).

**Antimicrobial-susceptibility testing.** MICs were determined by Etest (bioMérieux) according to the manufacturer’s instructions. Results were interpreted using clinical breakpoints as defined by the European Committee on Antimicrobial Susceptibility Testing, except for tigecycline for which the epidemiological cut-off value (non-wild-type > 1 mg L⁻¹) was used (http://www.eucast.org).

**Resistance-gene determination.** PCR assays were performed to detect the presence of five groups of OXA-carbapenemase-encoding genes (*bla_{OXA-23-like*}, *bla_{OXA-4-like*}, *bla_{OXA-51-like*}, *bla_{OXA-58-like*} and *bla_{OXA-143*}) (Woodford et al., 2006; Higgins et al., 2010a), five metallo-β-lactamase-encoding genes (*bla_{VIM*}, *bla_{SPM*}, *bla_{OXA-58-like*}, *bla_{OXA-51-like*} and *bla_{OXA-143*}) (Woodford et al., 2006; Higgins et al., 2010a), OXA-58-like sequences (Turton et al., 2006; Higgins et al., 2008) and the class 1 integrase-encoding gene (*intI1*) (Koeleman et al., 2001) (Supplementary Table S1, available with the online journal). In addition, linkage PCRs between the OXA-carbapenemase-encoding genes and insertion sequence elements (ISAba1, ISAba2 and ISAba3) were performed as described elsewhere (Poirel & Nordmann, 2006b; Corvec et al., 2007). The variable regions (VRs) of class 1 integrons were amplified and sequenced using primers 5’CS and 3’CS, and in-house designed internal primers (Toleman et al., 2007) (Supplementary Table S1, available with the online journal). The occurrence of mutations in the quinolone-resistance determining regions of gyrA and parC genes was detected as described elsewhere (Vila et al., 1995, 1997).

**DNA sequencing.** PCR products were purified directly using ExoSAP-IT (GE Healthcare Bio-Sciences) or from agarose gels using the QIAquick gel extraction kit (Qiagen), according to the manufacturer’s instructions. Sequencing was performed using BigDye 3.1 technology (Applied Biosystems). Sequence analysis and alignments were performed using Lasergene 8 (DNASTAR) and compared to sequences deposited in the GenBank database (www.ncbi.nlm.nih.gov).

**RESULTS**

**Molecular epidemiology**

MLST using the Pasteur Institute scheme revealed four different STs (Table 1). eBURST analysis assigned the isolates into CC2⁷ (n=7), CC1⁷ (n=3) and ST15⁷ (n=1). Using
the Bartual MLST scheme, at least ten different STs were identified (Table 1). An incomplete ST, comprising six of the seven housekeeping loci, was assigned to one isolate (Table 1). The seven isolates of CC2 P/CC92B were assigned to three different STs: ST194B (1-15-4-11-4-58-4), CC104B (1-3-3-2-2-3-3) and ST195 (1-3-3-2-2-96-3) (CC92 (Table 1). PFGE assigned three isolates into one pulsotype (>80% similarity) and two isolates into another pulsotype (100% similarity) (Table 1). The remaining six isolates each revealed a distinct pulsotype.

### Antimicrobial-susceptibility testing

All the isolates were multidrug resistant (Table 2). MICs of imipenem and meropenem ranged from 16 to >32 mg l⁻¹ and from 6 to >32 mg l⁻¹, respectively. All isolates were resistant to at least two aminoglycosides, including four isolates showing high-level resistance to the four aminoglycosides tested. All isolates expressed resistance to ciprofloxacin. In addition, 9/11 isolates were resistant to trimethoprim/sulfamethoxazole and 10/11 isolates had MICs above the epidemiological cut-off value (1 mg l⁻¹) for tigecycline. Colistin was the only antibiotic all isolates were susceptible to.

### Resistance-gene determination

All the isolates contained the naturally occurring blaOXA-51-like gene (Table 1). Acquired OXA-carbapenemase-encoding sequence group 4 contained blaOXA-51 (Table 1). PFGE assigned three isolates into one pulsotype (>80% similarity) and two isolates into another pulsotype (100% similarity) (Table 1). The remaining six isolates each revealed a distinct pulsotype.
genes were also present in all the isolates: *bla*\textsubscript{OXA-23}-like (*n*=9), *bla*\textsubscript{OXA-24}-like (*n*=1) and *bla*\textsubscript{OXA-58}-like (*n*=1). None of the isolates contained *bla*\textsubscript{OXA-143} or metallo-β-lactamase-encoding genes. IS\textsubscript{Abai} was detected upstream and downstream of *bla*\textsubscript{OXA-23}-like in seven isolates whereas it was present only upstream of *bla*\textsubscript{OXA-23}-like in two isolates (Table 2). Sequence analysis of the IS\textsubscript{Abai}-*bla*\textsubscript{OXA-23}-like structure showed a deletion of 7 bp (CTCTTTT) in one of the latter two isolates compared with the corresponding sequences of the other isolate. In the isolate harbouring *bla*\textsubscript{OXA-58}-like, an upstream IS\textsubscript{Abai}-like element and downstream IS\textsubscript{Abai} surrounded the *bla*\textsubscript{OXA-58}-like gene. No linkage was detected between IS\textsubscript{Abai} and the *bla*\textsubscript{OXA-51}-like genes or between IS\textsubscript{Abai} and the *bla*\textsubscript{OXA-24}-like gene.

The 16S rRNA methylase *armA* gene was detected in the four isolates showing high levels of resistance to all aminoglycosides tested (Table 2). Double mutations in the quinolone-resistance determining regions of GyrA (Ser-83 to Leu) and ParC (Ser-80 to Leu) were identified in all isolates.

Seven isolates were positive for the *intI1* gene. However, PCR amplification of the VR of the class 1 integron gave PCR products in only six isolates. No PCR product was obtained from isolate K61–46, presumably due to absence or significant alteration in the 3′CS region. One isolate (K47–42) yielded two amplification products. Sequence analysis of the integrons revealed the presence of six different VRs (Fig. 1).

### DISCUSSION

The worldwide emergence of multidrug- and carbapenem-resistant *A. baumannii* isolates is of great concern (Dijkshoorn et al., 2007; Poirel & Nordmann, 2006a). In Norway, only one minor outbreak of carbapenem-resistant *A. baumannii* has been reported (Onarheim et al., 2000).

### Table 2. Phenotypic and molecular resistance characteristics of the isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Susceptibility pattern (MICs in mg l\textsuperscript{-1})</th>
<th>Genetic structures flanking the OXA genes</th>
<th>16S rRNA methylase</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>IPM</td>
<td>MEM</td>
<td>CIP</td>
</tr>
<tr>
<td>K12–21</td>
<td>32</td>
<td>6</td>
<td>&gt;32</td>
</tr>
<tr>
<td>K44–35</td>
<td>16</td>
<td>32</td>
<td>&gt;32</td>
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<td>K47–42</td>
<td>&gt;32</td>
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<td>K48–42</td>
<td>&gt;32</td>
<td>&gt;32</td>
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<td>&gt;32</td>
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<tr>
<td>K71–71</td>
<td>32</td>
<td>&gt;32</td>
<td>&gt;32</td>
</tr>
</tbody>
</table>

AMK, Amikacin; CIP, ciprofloxacin; CST, colistin; GEN, gentamicin; IPM, imipenem; MEM, meropenem; NET, netilmicin; SXT, trimethoprim/sulfamethoxazole; TGC, tigecycline; TOB, tobramycin.

ARMS of the detected class 1 integrons.

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**Fig. 1.** VRs of the detected class 1 integrons.
The occurrence of clinical isolates producing acquired carbapenemases in Norway has been linked with hospitalization abroad (Samuelsen et al., 2009, 2010). In this study, all the carbapenem-resistant isolates of *A. baumannii* were associated with import to Norway. Thus, the study also provided an international perspective of carbapenem-resistant *A. baumannii*.

The results from this study were consistent with those of other studies regarding the predominance of CC2/CC99/CC89/ international clone I within the *A. baumannii* global population (Mugnier et al., 2010; Hamouda et al., 2010). The association between this group and the bla*OXA-66*-like cluster of variants has also been observed by Evans et al. (2008). Of note, the clone included four isolates with distinct pulsortypes, which was most likely due to a higher genetic diversity indexed by PFGE (Hamouda et al., 2010).

Only one isolate belonged to the bla*OXA-69*-cluster-positive CC1/CC109/ international clone I (Mugnier et al., 2010; Hamouda et al., 2010; Evans et al., 2008). The other two *blaOXA-69*-Positive isolates belonged to CC1/ST194/PCR-based sequence group 9. These two isolates were identical by PFGE and were both obtained in 2009 from two patients who had initially been hospitalized in India. eBURST analysis showed that ST194 was not part of any CC in the PubMLST database (http://pubmlst.org/abaumannii/). However, ST194 (1-15-4-11-4-58-4) was interestingly found to be a single locus variant of an unassigned ST (1-15-4-6-4-58-4) of isolates also obtained from India between 2008 and 2009 (Ko et al., 2010). Nonetheless, the two isolates were both assigned to CC1, suggesting that PCR-based sequence group 9 could represent a subgroup of international clone I.

One isolate, imported from Pakistan, belonged to ST15/CC104/PCR-based sequence group 4. Previous studies have reported the occurrence of isolates from this group in India, Europe and South America (Towner et al., 2008; Higgins et al., 2010b). Furthermore, ST15 has included multidrug-resistant and carbapenem-resistant isolates, supporting the proposal that this group represents an antimicrobial-resistant novel international clone (www.pasteur.fr/recherche/genopole/PF8/mlst/Abbaumannii.html) (Diancourt et al., 2010; Mugnier et al., 2010). Resistance to carbapenems in *A. baumannii* has mainly been related to the presence of OXA carbapenemases and linked to ISAba1 elements (Poirel & Nordmann, 2006a). Seven of our isolates contained the ISAba1-bla*OXA-23*-like-ISAba1 genetic arrangement. The length of PCR products suggested that bla*OXA-23*-like could be part of Tn2006 in these isolates (Corvec et al., 2007). ISAba1-bla*OXA-23*-like-ISAba1 was present in isolates of international clone II (n=3), international clone I (n=1), PCR-based sequence group 4 (n=1) and PCR-based sequence group 9 (n=2). The occurrence of this resistance structure in isolates of different clonal lineages most likely indicates a successful horizontal transfer. The ISAba1-bla*OXA-23*-like arrangement, without a downstream ISAba1, was detected in two isolates. Interestingly, ISAba1-bla*OXA-23*-like showed a 7 bp deletion of CTCTTTT in one of these two isolates, suggesting that bla*OXA-23*-like could be part of Tn2008 in this isolate (Adams-Haduch et al., 2008). In accordance with other studies, the 16S rRNA methylase-encoding armA gene was detected in three isolates belonging to international clone II (Cho et al., 2009). However, the fourth armA-positive isolate in our study belonged to ST15/CC104/PCR-based sequence group 4, adding more data on the significance of this group with regard to antimicrobial resistance in *A. baumannii*.

The ability of integrons to capture and mobilize gene cassettes has considerably contributed to dissemination of resistance genes among bacteria (Koeleman et al., 2001). Our results were similar to the results of other studies on the geographical distribution of class 1 integrons with VR1 (Europe), VR2 (East and South-East Asia), and VR3, VR4, and VR5 (worldwide) (Nemec et al., 2004; Han et al., 2008; Xu et al., 2008; Adams et al., 2008; Post et al., 2010). To our knowledge, the occurrence of the class 1 integron with only arr-2 and cmrA5 as gene cassettes (VR6) has not been reported before. Similar to what other studies have reported, the deduced amino acid sequences of the class 1 integron-located aacA4 genes in our isolates were consistent with AAC(6’)-Ib and not with AAC(6’)-Ib-cr (Xu et al., 2008).

In conclusion, this study demonstrated the major role of the highly successful international clones I and II and the supplementary role of other emerging clones in the worldwide spread of multidrug-resistant *A. baumannii* strains. The emergence of epidemic multidrug-resistant *A. baumannii* clones in Norwegian hospitals points to the necessity of a screening programme for patients after hospitalization abroad, and strict infection control regimes to prevent further antimicrobial-resistance selection and subsequent dissemination.

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