INTRODUCTION

Acquisition of multidrug resistance (MDR) by members of the Enterobacteriaceae, especially panaminoglycoside-resistant Klebsiella pneumoniae, has become a global concern. Aminoglycosides are commonly used to treat severe bacterial infections, often in combination with β-lactams (Mingeot-Leclercq et al., 1999). Initially, production of aminoglycoside-modifying enzymes and a decrease in intracellular antibiotic accumulation were considered predominant mechanisms conferring resistance to aminoglycosides. The substitution of ribosomal proteins or mutation of rRNA were less commonly reported. In recent years, the emergence of panaminoglycoside-resistant bacteria producing plasmid-borne 16S rRNA methylases has been increasingly reported (Fritsche et al., 2008). The methylases confer resistance to 4,6-disubstituted deoxystreptamines. This group encompasses the majority of aminoglycosides used in therapy (kanamycin, amikacin, gentamicin, netilmicin, tobramycin). Up to now, six 16S rRNA methylases have been described: ArmA, RmtA, RmtB, RmtC, RmtD and NpmA (Doi et al., 2008). It is noteworthy that NpmA confers resistance not only to 4,6-disubstituted deoxystreptamines but also to 4,5-disubstituted 2-deoxystreptamines, including neomycin and ribostamycin (Wachino et al., 2007).

ArmA (aminoglycoside resistance methyltransferase) was the first 16S RNA methylase reported in a clinical isolate of K. pneumoniae (Galimand et al., 2003). It was found in France in 2003. The armA gene encoding the methylase was detected in a composite transposon, Tn1548, located in a conjugative plasmid of approximately 90 kb (Galimand et al., 2005). Nowadays, ArmA predominates in 16S rRNA methylase-producing members of the Enterobacteriaceae in Europe and is reported worldwide (Fritsche et al., 2008). Although, the highest incidence (1.8%) of ArmA-producing strains in Europe has been reported in Bulgaria (Sabtcheva et al., 2008), the incidence continues to increase in the world. Recently, 3% of clinical isolates of K. pneumoniae in China were reported to carry the armA gene (Wu et al., 2009). The aim of this study was to determine the diversity of ArmA-producing K. pneumoniae in Poland. In addition, we identified a plasmid vector conferring ArmA production, and investigated the genetic environment surrounding the armA gene.

METHODS

Bacterial isolates. We conducted a retrospective screening of the 16S rRNA methylase-producing isolates in our stock of K. pneumoniae from hospitals and sanitary-epidemiological stations in Poland. This stock encompassed nearly 500 isolates of K. pneumoniae from a number of laboratories in the country. No epidemiological link was known for the majority of the isolates. Records of resistance
profiles to aminoglycosides for nearly 300 isolates collected during the last 10 years (from April 2000 to May 2010) were screened in this study. In total, 34 isolates resistant to at least two aminoglycosides of the 4,6-disubstituted deoxystreptamines were found. Isolates resistant to a single agent were ignored. All the 34 isolates of K. pneumoniae were recovered from the stock and were reidentified by classical biochemical tube tests. ArmA-producing Escherichia coli with plasmid pMURO50 (González-Zorn et al., 2005) and E. coli strain Δ32/01 with armA in a large (approx. 90 kb) conjugative plasmid of the pCTX-M-3 family were used as positive controls for 16S rRNA methylase production (Gierczynski et al., 2003; Zacharczuk et al., 2011).

**Screening for 16S rRNA methylase-producing isolates.** All 34 isolates were spread onto Mueller–Hinton agar supplemented with amikacin and kanamycin each at a concentration of 64 μg ml⁻¹.

**Detection of resistance genes and the genetic environment of armA.** Genes armA, rmtA, rmtB, rmtC, rmtD and npmA encoding major 16S rRNA methylases were detected by PCR using primers and conditions described previously (Fritsche et al., 2008). For the 16S rRNA methylase-producing isolates, amplification of extended-spectrum β-lactamase (ESBL)-encoding genes was performed with previously described primers (Dzierżanowska et al., 2010) for blaTEM, blaSHV and blaCTX-M. PCR mapping was conducted as described by Zhang et al. (2008). IncF and IncR primers described by Galimand et al. (2005) were used to detect plasmids of the IncL/M incompatibility group. DNA sequencing of PCR products was conducted for both the DNA strands as previously described (Gierczynski et al., 2004). All the PCR tests were conducted in triplicate.

**Susceptibility testing of 16S rRNA methylase-producing isolates.** The MICs of gentamicin, amikacin, kanamycin, neomycin and streptomycin were determined for the 16S rRNA methylase-producing isolates by a serial double-dilution method in accordance with published recommendations (CLSI, 2008). E. coli with pMURO50 and Salmonella enterica serovar Oranienburg 32/01 were used as aminoglycoside-resistant controls. E. coli ATCC 25922 was used as a susceptible control for susceptibility testing.

**Plasmid extraction and Southern blot hybridization analysis.** A NucleoBond PC20 kit (Machery-Nagel) was used to extract plasmids from isolates producing 16S rRNA methylase. To investigate the armA gene localization, the purified total plasmids were separated on a 1.0% agarose gel (Prona Plus), then subjected to Southern blotting and hybridization with a probe specific for the armA gene. The purified total plasmids were also digested using the CldI endonuclease equivalent BsaI5I (Fermentas Life Science) as recommended by the manufacturer. Restriction fragments were electrophoresed on a 1.5% agarose gel (Prona Plus). The resulting restriction fragment length polymorphism (RFLP) patterns were analysed by Southern blotting with the armA probe.

For Southern blot hybridization, plasmids or the RFLP patterns were transferred from electrophoretic gels to Nylosecond B membrane (SERVA Electrophoresis) and then hybridized with the armA gene specific probe. Probe labelling, hybridization and detection were carried out with a DIG High Prime DNA Labelling and Detection starter kit I (Roche Diagnostics) according to the manufacturer’s instructions. Southern blot hybridization experiments were performed twice.

**PFGE genotyping.** To determine the genetic relatedness of the ArmA-producing K. pneumoniae isolates in Poland, we carried out PFGE. PFGE was conducted as described previously (Dzierżanowska et al., 2010) using the CHEF-DR II system (Bio-Rad Laboratories) and endonuclease XbaI (Fermentas Life Science) with a switching time of 3–30 s for 24 h at 14 °C and voltage gradient of 6.0 V cm⁻¹. PFGE patterns were analysed using the GelCompar II version 5.10 software (Applied Maths). Similarity clustering analyses were performed using UPGMA and the Dice correlation coefficient with a tolerance of 1.2%. PFGE genotyping was performed twice.

**RESULTS**

**Potential 16S rRNA methylase producers**

Among the 34 K. pneumoniae isolates screened, 17 grew on the selective Mueller–Hinton agar with amikacin and kanamycin. This result suggested potential 16S rRNA methylase activity in these 17 isolates. All these isolates were characterized in Table 1.

**Aminoglycoside resistance profiles**

The 17 isolates resistant to amikacin and kanamycin had positive for the armA gene by PCR testing. No PCR products for rmtA, rmtB, rmtC, rmtD and npmA genes were detected in the tested isolates. DNA-sequencing analysis of the PCR products of two randomly selected isolates revealed 100% nucleotide identity to the armA gene of K. pneumoniae strain BM4536 (GenBank accession no. AY220558).

**Screening of 16S rRNA methylase genes**

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**Screening of bla genes for the major β-lactamases**

All the armA-positive isolates also carried the blaCTX-M gene for an ESBL of the CTX-M-type family (Table 1). DNA sequencing of the blaCTX-M gene using primers described previously (Gierczynski et al., 2003) revealed that isolates 474/02 and DM0342 produced a CTX-M-3 ESBL. These isolates also carried the bla gene for a TEM-type β-lactamase. Eleven of the armA-positive isolates were also positive in the PCR with primers for the blagshv gene for an SHV-type enzyme (Table 1). The PCR product for IncL/M plasmids was detected in all ArmA-producing isolates of K. pneumoniae apart from 31/03 (Table 1).

**Exploration of the regions surrounding the armA gene**

PCR mapping experiments revealed that the armA gene and its flanking regions in all 17 K. pneumoniae isolates producing ArmA were in the same order and direction as in transposon Tn1548 found in K. pneumoniae in France
Table 1. Characterization of 17 K. pneumoniae isolates producing 16S rRNA methylase ArmA

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Year of isolation</th>
<th>Origin</th>
<th>MIC (mg l(^{-1})) of*</th>
<th>PCR†</th>
<th>Plasmid profile</th>
<th>PFGE cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>City</td>
<td>Hospital</td>
<td></td>
<td>GEN</td>
<td>AMK</td>
<td>KAN</td>
<td>STR</td>
</tr>
<tr>
<td>468/02</td>
<td>2002</td>
<td>Łódź</td>
<td>H4</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
</tr>
<tr>
<td>473/02</td>
<td>2002</td>
<td>Łódź</td>
<td>H1</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
</tr>
<tr>
<td>474/02</td>
<td>2002</td>
<td>Łódź</td>
<td>H1</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
</tr>
<tr>
<td>475/02</td>
<td>2002</td>
<td>Łódź</td>
<td>H1</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
</tr>
<tr>
<td>477/02</td>
<td>2002</td>
<td>Łódź</td>
<td>H1</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
</tr>
<tr>
<td>478/02</td>
<td>2002</td>
<td>Łódź</td>
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<td>&gt;1024</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
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<tr>
<td>2/03</td>
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<td>512</td>
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<tr>
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<tr>
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<td>H3</td>
<td>&gt;1024</td>
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<td>&gt;1024</td>
</tr>
<tr>
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<td>H2</td>
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<td>&gt;1024</td>
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<tr>
<td>31/03</td>
<td>2003</td>
<td>Łódź</td>
<td>H2</td>
<td>256</td>
<td>&gt;1024</td>
<td>&gt;1024</td>
</tr>
<tr>
<td>177/03</td>
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<td>&gt;1024</td>
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<tr>
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<td>H2</td>
<td>&gt;1024</td>
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</tr>
</tbody>
</table>

NA, Not assigned; ND, no data.*GEN, Gentamicin; AMK, amikacin; KAN, kanamycin; STR, streptomycin; NEO, neomycin.
†Results of PCR test for the bla genes encoding β-lactamases of the CTX-M, TEM and SHV families, and the IncL/M incompatibility group plasmids: +, positive result; −, negative result.

(KGenBank accession no. AY220558) and Citrobacter freundii in Poland (GenBank accession no. AF550415).

Plasmid profiles, RFLP patterns and Southern blots

Fifteen isolates with the armA gene harboured two to six plasmids ranging from 3 to approximately 140 kb. Each of these isolates carried a ~90 kb plasmid. Two other isolates with the armA gene (30/03 and 31/03) possessed a large single plasmid of ~130 kb (Fig. 1a). For all 17 isolates, the armA probe hybridized exclusively to the large plasmid of ~90 or 130 kb. A background hybridization signal was observed for a band of approximately 23 kb corresponding to fragmented chromosome. Plasmid profiles and the corresponding Southern blots with the armA probe for three genetically unrelated isolates, 474/02, 2/03 and DM0265, are shown in Fig. 1(b). The Clal (BsaI) RFLP patterns of the plasmids extracted from each of the three aforementioned isolates are shown in Fig. 1(c). For the three unrelated isolates, the armA probe hybridized exclusively with a restriction fragment of approximately 10 kb in size.

PFGE genotyping

XbaI-PFGE patterns and a dendrogram of the pattern similarity for all 17 isolates of ArmA-producing K. pneumoniae are shown in Fig. 2. Fifteen isolates represented unique PFGE patterns. Two isolates were indistinguishable. Four clonal groups (A, B, C and D) encompassing 14 isolates were distinguished. PFGE profiles of three isolates were distinct and could not be assigned to any of the clusters. Isolates of clusters B, C and D were hospital-specific, while cluster A isolates were specific for the city of Łódź.

DISCUSSION

K. pneumoniae is one of the major nosocomial pathogens. These bacteria effectively colonize patients as well as hospital personnel and occupy a hospital environment. To survive in this niche, K. pneumoniae developed a large variety of resistance traits including production of 16S rRNA methylases with the major representative ArmA (Fritsche et al., 2008). In Europe, this methylase has been reported in K. pneumoniae from patients in France (Galimand et al., 2003), Belgium (Bogaerts et al., 2007) and Bulgaria (Sabtcheva et al., 2008). In this study, we characterized 17 isolates of ArmA-producing K. pneumoniae in Poland. The isolates were collected from patients in six hospitals in the two most populated cities in the country: Warsaw and Łódź. To the best of our knowledge, this is the first report in which 16S rRNA methylase-producing K. pneumoniae isolates from various hospitals in Poland have been characterized. However, the ArmA-producing isolates from Warsaw, which we report herein, were also investigated in our recent study on factors conferring co-production of K. pneumoniae carbapenemase (KPC-2) and ArmA (Zacharczuk et al., 2011).

In the present study, PFGE genotyping distinguished 16 genotypes among 17 ArmA-producing isolates of K.
Fig. 1. Electrophoretic patterns and Southern blots with the armA gene specific probe. (a) Plasmid profiles of 17 K. pneumoniae isolates producing ArmA. Designations of the profiles are shown in capital letters above the patterns. Lane J, profile of E. coli Δ32/01 with pCTX-M plasmid ~90 kb. (b) Plasmid profiles of three epidemiologically unrelated K. pneumoniae isolates and corresponding Southern blots. (c) Bsu15I (ClaI) RFLP patterns of the plasmids shown in (b) together with the corresponding Southern blots. Lanes: 1, 2 and 3, K. pneumoniae isolates 474/02, 2/03 and DM0265M, respectively; M, linear dsDNA ladder – HindIII digest of the lambda phage (Sigma-Aldrich); L, DNA ladder – GeneRuler 1 kb Ladder (Fermentas Life Sciences). DNA size is shown in kb.

Fig. 2. Dendrogram of ArmA-producing K. pneumoniae isolates in Poland. The dendrogram is based on XbaI-PFGE pattern similarity.
pneumoniae collected during the past decade in Poland. The majority of these isolates were grouped to four PFGE clusters. In general, the PFGE clusters were hospital-specific. With a single exception, isolates of the same cluster were collected in the same hospital, and were closely related (similarity above 92%). These findings may reflect the nosocomial nature of K. pneumoniae infections and argue for an effective horizontal transfer of ArmA.

The hybridization experiments revealed that the armA gene was carried on a large ~90 kb plasmid in the majority of the tested K. pneumoniae isolates. However, in two related isolates, armA was detected on a larger plasmid of ~130 kb. All but one of the ArmA-producing K. pneumoniae isolates carried the IncL/M incompatibility group plasmid. The IncL/M plasmids encoding ArmA have been commonly reported for K. pneumoniae in France and in Bulgaria (Galimand et al., 2005). Of note, in all the ArmA-producing K. pneumoniae isolates, the armA gene was found to be flanked by the same elements as reported previously in transposon Tn1548 (Galimand et al., 2005). Since no difference in the elements adjacent to the armA gene was found between the 90 and 130 kb plasmid, this may suggest that armA has disseminated among K. pneumoniae isolates in Poland on the same transposable structure. Further studies are, however, necessary to elucidate the armA gene environment on these two large plasmids.

To characterize the 90 kb plasmid predominating among ArmA-producing K. pneumoniae in Poland, two isolates representative of two main XbaI-PFGE clusters (B and D), together with a third unrelated isolate, were selected for further RFLP and Southern blot experiments. Isolates of cluster B (n=5) were collected in 2002 in Łódź while isolates of cluster D (n=4) were collected in 2009 in Warsaw. The RFLP and Southern blot analysis revealed that the armA gene maps to the same approximately 10 kb Clal (Bsu151I) restriction fragment of the large plasmid in all the three epidemiologically unrelated K. pneumoniae isolates. This finding, together with the results of the transposon mapping, may argue for a relatively conservative structure of the armA gene environment in K. pneumoniae in Poland. It is noteworthy that the presence of the armA gene in Tn1548 on a ~90 kb plasmid (pCTX-M3) was previously reported in C. freundii in Poland (Gołębiewski et al., 2007) and in K. pneumoniae in France (Galimand et al., 2005).

In a recent study, Zacharczuk et al. (2011) transferred by electroporation the 90 kb plasmid from ArmA-producing K. pneumoniae DM0269 to E. coli DH5α. Interestingly, a large plasmid from recipient strain ETarmA belonged to the IncL/M incompatibility group and its Clal-RFLP pattern closely resembled that of a large conjugative plasmid from CTX-M-3-producing E. coli transconjugant A32/01 that was originally found in strain 32/01 of S. Oranienburg isolated in Poland in 2001 (Gierczyński et al., 2003). It is noteworthy that the large plasmids derived from K. pneumoniae DM0269 and S. Oranienburg 32/01 carried the armA gene on a ~10 kb Clal restriction fragment (Zacharczuk et al., 2011). These findings strongly support the results of the present study, and may argue for a conservative nature of the large plasmid on which the armA gene is horizontally transferred amongst K. pneumoniae and other Enterobacteriaceae species in Poland.

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REFERENCES


