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Characterization of TEM-1 β-Lactamase-Producing *Kingella kingae* Clinical Isolates

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***Kingella kingae* is a human pathogen that causes pediatric osteoarticular infections and infective endocarditis in children and adults. The bacterium is usually susceptible to β-lactam antibiotics, although β-lactam resistance has been reported in rare isolates. This study was conducted to identify β-lactam-resistant strains and to characterize the resistance mechanism. Screening of a set of 90 *K. kingae* clinical isolates obtained from different geographic locations revealed high-level resistance to penicillins among 25% of the strains isolated from Minnesota and Iceland. These strains produced TEM-1 β-lactamase and were shown to contain additional ≥50-kb plasmids. Ion Torrent sequencing of extrachromosomal DNA from a β-lactamase-producing strain confirmed the plasmid location of the bla_{TEM} gene. An identical plasmid pattern was demonstrated by multiplex PCR in all β-lactamase producers. The porin gene's fragments were analyzed to investigate the relatedness of bacterial strains. Phylogenetic analysis revealed 27 single-nucleotide polymorphisms (SNPs) in the *por* gene fragment, resulting in two major clusters with 11 allele types forming bacterial-strain subclusters. β-Lactamase producers were grouped together based on *por* genotyping. Our results suggest that the β-lactamase-producing strains likely originate from a single plasmid-bearing *K. kingae* isolate that traveled from Europe to the United States, or vice versa. This study highlights the prevalence of penicillin resistance among *K. kingae* strains in some regions and emphasizes the importance of surveillance for antibiotic resistance of the pathogen.**

A frequent cause of septic arthritis and osteomyelitis in children younger than 2 years old was recently demonstrated to be infections with *Kingella kingae* (1–8). The bacterium is also a cardiovascular pathogen, causing severe infective endocarditis in children and adults (4, 9–13).

K. kingae, a Gram-negative coccobacillus of the family *Neisseriaceae*, is a normal human oropharynx inhabitant and is transmitted from child to child (14). *K. kingae* osteoarticular infections were underestimated prior to new isolation and PCR identification techniques were developed in the 1990s. Several recent reports describe epidemiological cases of invasive *K. kingae* infections in day care centers, showing the bacterium's ability to cause outbreaks in pediatric communities (15–17).

β-Lactam antibiotics (penicillin and its derivatives) inhibit the formation of bacterial cell wall peptidoglycan cross-linkages and are widely used in the treatment of bacterial infections, including osteoarticular infections (14). Penicillins are essentially first-choice drugs for infective endocarditis prevention in high-risk populations (18). Different mechanisms of β-lactam resistance have been described among Gram-negative bacteria. They include the production of different types of β-lactamases and are also associated with the natural low membrane permeability and with efflux systems (19, 20).

K. kingae is generally characterized as highly susceptible to β-lactams, although β-lactamase production has been reported in rare isolates (21–23). Currently, the mechanisms of β-lactam resistance in *K. kingae* are unknown. We screened isolates from different geographic regions for β-lactam susceptibility and characterized plasmid-encoded β-lactamase produced by the organism. We also developed single-gene sequence typing (SGST) of the isolates based on the major outer membrane protein (porin) gene structure to investigate genetic heterogeneity among the strains.

MATERIALS AND METHODS

Bacterial strains. We acquired a collection of 90 *K. kingae* clinical isolates from different geographic regions. These strains were predominantly obtained from pediatric patients with infective endocarditis ($n = 1$), osteomyelitis ($n = 8$), septic arthritis ($n = 27$), and bacteremia ($n = 28$), as well as from healthy carriers ($n = 26$). Forty-four strains isolated between 1991 and 2010 in Israel were provided by Pablo Yagupsky from the Ben-Gurion University Medical School in southern Israel. Some of these strains were used in previous studies (24–27). Thirty-one strains of U.S. origin, obtained from 2003 to 2012, were contributed by the Minnesota State Health Department. They include strains isolated in an investigation of an outbreak of *K. kingae* infections in 2003 (28). One U.S. strain was donated by Paul Planet (American Museum of Natural History, New York, NY). Fourteen Icelandic isolates, including strains described previously (23), were collected in the period between 1995 and 2010 and were provided by Hjordis Harðardóttir from the Department of Clinical Microbiology, Landspitali University Hospital (Iceland). Strain 23330 (Norway) was obtained from the ATCC. The bacteria were grown on Columbia agar (CA) with 5% sheep blood at 37°C with 10% CO_2 and stored at −80°C in the growth medium with 10% dimethyl sulfoxide (DMSO). The identification of all strains was confirmed by sequence analysis of the 16S rRNA gene. The clinical information on *K. kingae* isolates utilized in this work is presented in Table S1 in the supplemental material. Other strains used in the study were *Kingella oralis* 51147 (ATCC), *Neisseria perflava* 14799

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TABLE 1 Primers designed in the study

Primer	Sequence	Analyzed product size (bp)
bla_{TEM} 550 FW	5′-GAGAATTATGCAGTGCTGCCATAACC-3′	550
bla_{TEM} 550 RV	5′-GTTATCACTCATGGTTATGGCAG-3′	550
por FW	5′-GCACATAAGGAAATGAATAATG-3′	956
por RV	5′-CAAAATTAGAATTTATGGCTCATACCC-3′	956
pAGA1 308-759 FW	5′-GAAGATAAGGCATTAAGCAATTTG-3′	451
pAGA1 308-759 RV	5′-CTCGGCTCATAATCCTACCTG-3′	451
pAGA1 4146-4789 FW	5′-GGCTAAACGAACCACCGAAC-3′	643
pAGA1 4146-4789 RV	5′-CTGCTGCCTGTGTAAATCGTC-3′	643
pAGA1 10146-11114 FW	5′-GCCGAAATTGTCAAAAGTGTAG-3′	968
pAGA1 10146-11114 RV	5′-GCTATCACAAGAAGAAATAC-3′	968
pAGA115085-15294 FW	5′-CCATGAAGATGAAGCATTTC-3′	209
pAGA115085-15294 RV	5′-CTTTCAAGTGGGCTAGGTTG-3′	209

(ATCC), *Escherichia coli* DH5α (Invitrogen), and *Aggregatibacter actinomycetemcomitans* 1704 (29).

Antibiotic sensitivity testing. A Thermo Scientific Remel Nitrocefin Disk was used to identify β-lactamase production. MICs of antibiotics were determined by the agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (1, 30). Growth inhibition was identified after incubation of bacteria on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood and containing various concentrations of antibiotics for 24 h at 37°C with 10% CO_2.

DNA purification. Genomic DNA isolation was accomplished using the Wizard Genomic DNA Purification kit (Promega). Plasmid DNA was purified using a Qiagen Plasmid Midi Kit. The DNA concentration was determined using NanoDrop (NanoDrop Technologies, Wilmington, DE). For sequencing, 200 mg of plasmid DNA stained with ethidium bromide was subjected to ultracentrifugation for 20 h at 45,000 rpm in a 1-g/ml cesium chloride solution. The plasmid bands were collected with the needle positioned just below the band of plasmid DNA.

PCR amplification and typing. We used the primers Bact8F and Bact1391R for the 16S rRNA gene to identify bacterial strains (31). To determine a β-lactamase type, primers for bla_{TEM}, bla_{SHV} (32), and $bla_{\text{CTX-M}}$ (33) were used for PCR amplification. The primers developed in this study are listed in Table 1. For plasmid typing, the PCR utilized the Expand High Fidelity PCR System (Roche) in 25 μl containing 1× buffer solution, 0.5 μM each primer, 0.2 mM deoxynucleoside triphosphate (dNTP), and 50 ng plasmid DNA. The amplification reaction was performed on the automated thermal cycler with an initial step of 10 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C, and a final extension step of 10 min at 72°C. For porin gene typing, the reaction mixture contained bacterial genomic DNA as the template. The amplification reaction was performed with an initial step of 10 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C, and a final extension step of 10 min at 72°C. The amplified products were visualized on a 0.8% agarose gel containing ethidium bromide.

DNA sequencing. The nucleotide sequences of the two strands of PCR products were determined by capillary sequencing using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). For plasmid sequencing, 10 mg of purified plasmid DNA was used for fragment library construction followed by sequencing on an Ion Torrent Platform 314 chip with 200-bp read lengths (Life Technologies). Sequence assembly was performed using Newbler v2.5 software without directed gap filling.

***K. kingae* mutagenesis.** The RTX toxin-deficient strain KKNB100 was obtained using the mariner transposon derivative *Solo*, which carries the *aphA3* kanamycin resistance gene, using a previously described protocol (34) with modifications. Briefly, the *rtxA* gene was PCR amplified from genomic DNA of PYKK081 and cloned into the pUC19 vector (New England BioLabs). Then, the *aphA3* gene, obtained from plasmid pFALCON2, was inserted in *rtxA* using the NsiI restriction site. The *rtxA* gene containing *aphA3* was PCR amplified and used for the transformation of PYKK081. To accomplish this, the bacteria were grown overnight on CA with 5% sheep blood and resuspended to an A_{600} of 0.8 in Trypticase soy broth containing 2% yeast extract. After 30 min of incubation, the cells were resuspended in Trypticase soy broth containing 10 mM $CaCl_2$, 12% horse plasma, and DNA. The transformation mixture was incubated for 1 h at 37°C and plated on CA-5% sheep blood plates containing kanamycin (40 μg/ml). The transconjugants were grown for 48 h at 37°C in a 10% CO_2 incubator.

Mating experiments. Donor and recipient strain cells (10^7) were mixed in a 1:1 ratio and incubated on CA plates with 5% blood for 18 h. The mixed cell culture was washed from the plates with 1 ml saline solution. Aliquots (0.2 ml) of each culture were plated on selective plates containing penicillin (2 μg/ml). Transconjugant colonies were recovered after 24 h and plated on new selective plates. Plasmid presence was confirmed by multiplex PCR.

Phylogenetic analysis. Sequences were aligned with ClustalW (European Bioinformatics Institute), and a tree was constructed by applying the unweighted-pair group method using average linkages (UPGMA) and a Jukes and Cantor DNA distance matrix using Geneious 6.1.4 software. Bootstrap analysis was used to estimate the confidence of each group assignment. The tree was bootstrapped 100 times, and the proportion of data sets supporting each tree topology was recorded as a bootstrap probability value.

Nucleotide sequence accession numbers. The sequence assembly of the *K. kingae* C2005004457 pAGA1 and pAGA2 plasmid DNA was deposited in GenBank (NCBI) under accession number AMPT00000000. The nucleotide sequence for the *K. kingae* TEM-1 gene has been submitted to GenBank under accession number JX855253. The nucleotide sequences for the *K. kingae* porin gene variants have been submitted to GenBank under accession numbers KC142159 to KC142169.

RESULTS

Distribution of β-lactam resistance among *K. kingae* clinical isolates. Forty-four Israeli, 1 Norwegian, 14 Icelandic, and 31 U.S. *K. kingae* isolates were screened for susceptibility to penicillin G (4 μg/ml) and β-lactamase production using the nitrocefin method. Most of the strains, including all the Israeli strains, were negative for β-lactamase production and susceptible to penicillin. Eight U.S. isolates and 4 Icelandic strains were resistant to penicillin G and were β-lactamase producers (see Table S1 in the supplemental material). All penicillin-resistant strains from our collection (n = 12) and 12 random penicillin-susceptible strains were used to determine MICs. Using the agar dilution method, these strains were found to be resistant to penicillin V/G up to ≥250 μg/ml, ampicillin up to ≥200 μg/ml, carbenicillin up to 150 μg/ml, and amoxicillin up to 175 μg/ml, while nonresistant isolates were sus-

TABLE 2 Antibiotic susceptibility testing

Antibiotic	MIC (μg/ml)[a]	
	TEM$^+$ (n = 12)	TEM$^-$ (n = 12)
Penicillin G	≥250	0.20
Penicillin V	≥250	0.20
Ampicillin	≥200	0.15
Carbenicillin	≥150	0.20
Amoxicillin	≥175	0.20
Biapenem	0.35	0.35
Ceftazidime	0.20	0.20
Ceftriaxone	0.20	0.20

[a] MICs were identified by the agar dilution method in β-lactamase-producing (TEM$^+$) and non-β-lactamase-producing (TEM$^-$) strains.

ceptible to these antibiotics at approximately 1,000 times lower concentrations (Table 2). All strains were susceptible to broad-spectrum cephalosporins (ceftazidime and ceftriaxone) and the carbapenem biapenem. MICs were strikingly similar between isolates from each group.

Characterization of *K. kingae* β-lactamase. The β-lactamase-producing strain C2005004457 of U.S. origin was selected for further analysis. To identify a β-lactamase type, primers for the three most common β-lactamase genes in Gram-negative bacteria, bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$, were employed to amplify the product from C2005004457. PCR resulted in amplification of a product of about 1 kb using primers for bla_{TEM} (Fig. 1A). The PCR

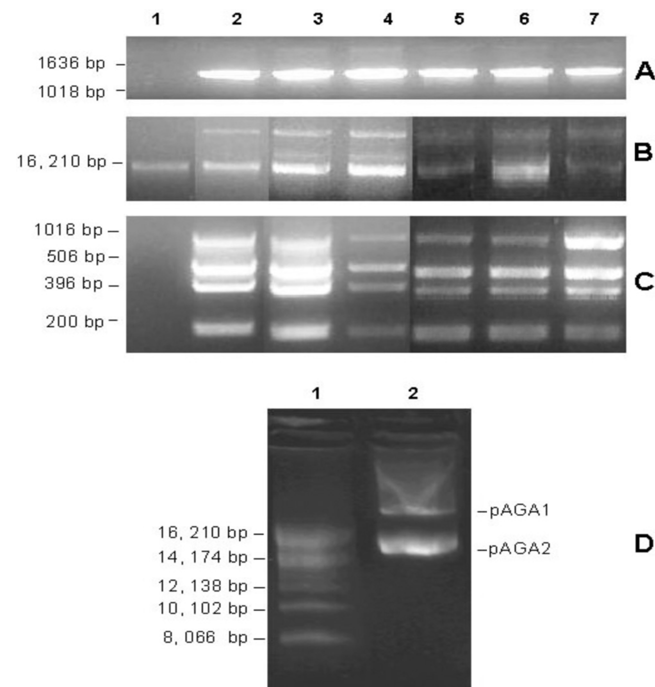

FIG 1 Plasmid DNA analysis in *K. kingae* strains. (A to C) The bla_{TEM} gene amplified from *K. kingae* DNA (A), plasmid DNA identified in *K. kingae* strains (B), and multiplex PCR plasmid typing (C). Lanes: 1, PYKK081 (Israel; 1991); 2, C2005004457 (United States; 2005); 3, C2007000490 (United States; 2007); 4, M2004000037 (United States; 2004); 5, 0303 and 28260 (Iceland; 2003); 6, 0405 and 30002 (Iceland; 2004); 7, 9508 and 31135 (Iceland; 1995). (D) Plasmid DNA purified using a cesium chloride gradient. Lanes: 1, supercoiled DNA ladder (Invitrogen); 2, plasmid DNA purified from strain C2005004457.

TABLE 3 C2005004457 plasmid-encoded products associated with antibiotic resistance

Gene product	Drug
Aminoglycoside/hydroxyurea antibiotic resistance kinase	Aminoglycosides/hydroxyurea
Aminoglycoside phosphotransferase	Aminoglycosides
Beta-lactamase class A; EC 3.5.2.6	Penicillins
Streptomycin 6-kinase	Streptomycin
Streptomycin phosphotransferase	Streptomycin
Dihydropteroate synthase	Sulfonamides
Antitoxin of toxin-antitoxin stability	Antibiotic resistance[a]

[a] This gene product may provide resistance to many antibiotics.

product containing the gene for *K. kingae* TEM was sequenced. The flanking sequences of the bla_{TEM} gene were sequenced using outward primers listed in Table 1. Using BLAST search analysis, the sequence was found to contain an 867-bp β-lactamase gene identical to one encoding *Neisseria gonorrhoeae* TEM-1 (NP_052173.1) (35). The TEM β-lactamase PCR product was obtained from other β-lactamase-positive strains. Comparative sequence analysis showed no variations in the bla_{TEM} sequence between the *K. kingae* β-lactamase producers.

***K. kingae* plasmid analysis.** To identify the locations of genes in the bacterial genome, plasmid DNA was isolated from all β-lactamase-producing strains and 3 random nonproducing strains (PYKK081, C2003003154, and M2003000170) (see Table S1 in the supplemental material). All tested *K. kingae* isolates contained a plasmid band of ~15 kb. β-Lactamase-producing strains also contained a second, larger plasmid. Selected strains are shown in Fig. 1B. The small and large plasmids from strain C2005004457 were designated pAGA1 and pAGA2, respectively.

C2005004457 plasmid DNA sequencing. To purify circular plasmid DNA from linear chromosomal DNA, the plasmid sample from strain C2005004457, obtained by a modified alkaline lysis procedure, was subjected to ultracentrifugal separation in a cesium chloride gradient (Fig. 1D). The pAGA1 and pAGA2 DNA bands were not clearly separated in the gradient, and therefore, total "plasmid bands" of DNA were isolated and used for fragment library preparation, followed by Torrent sequencing. The Torrent run yielded 136,258 individual reads with a mean read length of 209 bases. The total number of bases read was 28,593,075 (ca. 433-fold coverage). The completed extrachromosomal DNA sequence consisted of 65,896 bp with an average G+C content of 39%. The size of pAGA2 was previously identified as approximately 15 kb by using plasmid markers. Therefore, given the total size of the bacterial-plasmid sequence, the size of the other plasmid, pAGA1, was estimated to be approximately 50 kb. BLAST search analysis did not reveal any extensive regions of similarity between the newly generated C2005004457 plasmids' sequences and other known plasmid sequences. In the final assembly, 94.8% of the reads aligned into 127 unique contigs, but only 65 with gene calls and of sufficient length (≥200 bp) were selected for inclusion in GenBank. The contigs could not be assigned to either pAGA1 or pAGA2 in the present sequence state. The sequence was annotated using the NCBI Prokaryotic Genomes Automatic Annotation Pipeline server at NCBI (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html). There were 110 predicted protein-coding genes, and of these, 68 (62%) had functional predictions. Among those with functional predictions, 7 were associated with resistance to antibiotics (they are listed in Table 3) and 16 with phage-

TABLE 4 Mating experiments between C2005004457 and Gram-negative bacteria[a]

Recipient strain	Selective medium[b]	Transconjugants[c]
K. kingae KKNB100	CA-5% blood, KAN 40, PCN 2	+
E. coli DH5α	LA, PCN 2	+
N. perflava 14799	MHA, PCN 2	+
A. actinomycetemcomitans 1704	CA-5% blood, KAN 40, PCN 2	−

[a] C2005004457 and recipient strain cells (10^7) were mixed in a 1:1 ratio and incubated on CA with 5% blood for 18 h. Aliquots of the mixed cell culture were plated on selective plates containing penicillin G (2 μg/ml). Transconjugant colonies were recovered after 24 h.

[b] KAN 40, kanamycin (40 μg/ml); PCN 2, penicillin (2 μg/ml). Luria-Bertani agar (LA) and Mueller-Hinton agar (MHA) media without blood did not support C2005004457 growth.

[c] +, present; −, absent.

associated virulence. The bla_{TEM} gene was localized in the sequence, suggesting that TEM-1 is plasmid encoded.

Plasmid typing. A multiplex PCR approach was developed to identify whether plasmids found in β-lactamase-producing strains are genetically variable. Using the C2005004457 plasmid DNA sequence, we designed a set of primers to amplify four 200- to 1,000-bp PCR products from random parts of the pAGA1 sequence (Table 1). Multiplex PCR resulted in a uniform PCR fragment pattern obtained from all β-lactamase-producing strains. Selected strains are shown in Fig. 1C. This result indicates that all β-lactam-resistant strains contain the same pAGA1 plasmid.

Plasmid transfer. To investigate the possibility of R-plasmid horizontal transfer, mating experiments were carried out using different recipient strains sensitive to penicillin with C2005004457 as a donor (Table 4). We needed a *K. kingae* recipient strain to perform transformation and mating experiments on newly discovered plasmids. Strain KKNB100, carrying the *aphA3* kanamycin resistance gene, was available in our laboratory for such studies. This strain lacks RtxA toxin activity and is not toxic to mammalian leukocytes. This feature was used as an additional phenotypic marker to distinguish between donor and recipient strains in mating experiments. When *K. kingae* KKNB100 was used as a recipient, the transconjugants formed on selective plates at high frequency and contained pAGA1, as demonstrated by multiplex PCR. However, further passages (less than four passages) on both nonselective and selective plates led to the loss of the strains' ability to produce β-lactamase and retain the plasmid. No spontaneous mutants were obtained on selective medium that contained two antibiotics, kanamycin (40 μg/ml) and penicillin (2 μg/ml), when a donor or recipient was plated alone. We conclude that pAGA1 is transmissible by conjugation to other *K. kingae* strains; however, there are special requirements for the cell to support the plasmid. In addition, we performed mating experiments using other bacterial species: *E. coli* DH5α, *N. perflava*, and *A. actinomycetemcomitans* 1704. Similar to KKNB100, transconjugants were formed in *E. coli* DH5α and *N. perflava*; however, the following passage led to the loss of the plasmid. We did not observe the formation of transconjugants in *A. actinomycetemcomitans* 1704.

SGST sequence analysis. To better understand the population structure of circulating *K. kingae* isolates and to identify the relatedness of β-lactamase producers, we classified the isolates based on porin (*por*) gene structure. Porins are essential pore-forming membrane proteins that create channels involved in hydrophilic-molecule transport (36). The *K. kingae* porin gene nucleotide sequence (1,053 bp) shares only 10% identity with *Neisseria meningitidis* and *N. gonorrhoeae* porin sequences. Comparative genome analysis of fully sequenced genomes of ATCC 23330 (GenBank accession number AFHS01000000) and PYKK081 (GenBank accession number AJGB01000000) revealed a number of polymorphic regions in the *K. kingae* porin gene, allowing distinction among strain groups. The genotyping was achieved by comparing 956-bp fragments of the *por* gene obtained from 90 isolates. Sequence analysis of the *por* gene fragment revealed 27 polymorphic nucleotides. Evaluation of sequence variations led to identification of two major *K. kingae* groups of strains with 11 *por* allele types. Seven *por* allele types were found in multiple patients, and 4 types were unique. The most predominant were *por* allele types 1, 9, and 2, which comprised 68% of all isolates. The genetic relatedness of *por* sequences is shown in a dendrogram (Fig. 2). Based on the *por* gene allele typing, β-lactamase production of U.S. and Icelandic isolates is limited to organisms exhibiting the *por* type 1 allele.

DISCUSSION

K. kingae is generally known to be highly susceptible to β-lactams, and infections with the bacterium can be successfully treated by antibiotic therapy with penicillins and cephalosporins (14). The first β-lactamase-producing strain was reported in the United States in 1993 (21), followed by β-lactam-resistant isolates in Iceland in 1997 (23). In our collection, the β-lactamase-producing strains of U.S. origin were isolated in Minnesota from 2004 to 2007, and the Icelandic strains were isolated in different periods from 1995 to 2004. Interestingly, whereas the largest number of strains in our collection were obtained from Israel ($n = 44$), none of the Israeli strains were found to be resistant to β-lactams, which is in good agreement with previous studies (24, 37). These data may suggest the relative prevalence and persistence of such isolates in some regions.

Here, we report for the first time the production of plasmid-mediated TEM-1 in *K. kingae*. TEM-1 is a β-lactamase commonly found in Gram-negative bacteria. The enzyme has been reported in *E. coli* (38), *Haemophilus influenzae* (39), *N. meningitidis* (40), and *N. gonorrhoeae* (41). The native TEM-1 β-lactamase confers high-level resistance to ampicillin, penicillin, and some first-generation cephalosporins. However, mutations within the bla_{TEM} structural gene may allow the enzyme to expand the hydrolysis capabilities to cephalosporins with an oxyimino side chain (42). Infections caused by the extended-spectrum β-lactamase (ESBL) have been associated with severe adverse clinical outcomes that have led to increased mortality, prolonged hospitalization, and additional medical costs (43–45).

Although *K. kingae* is being increasingly recognized as an important human pathogen, progress in understanding *K. kingae* genetics was made only recently, with the genomes of strains ATCC 23330, PYKK081 (46), and 11220434 (47) released. We have demonstrated that *K. kingae* represents a distinct taxonomic group in the family *Neisseriaceae* (46). Here, we report the unique R-plasmid pAGA1 in the organism. The identification and sequencing of new *K. kingae* plasmids will add to our understanding of the organism's genetic system. Plasmid typing, proposed here, can be applied to investigate *Kingella* plasmid diversity.

Pulsed-field gel electrophoresis (PFGE) (26, 27) and multilo-

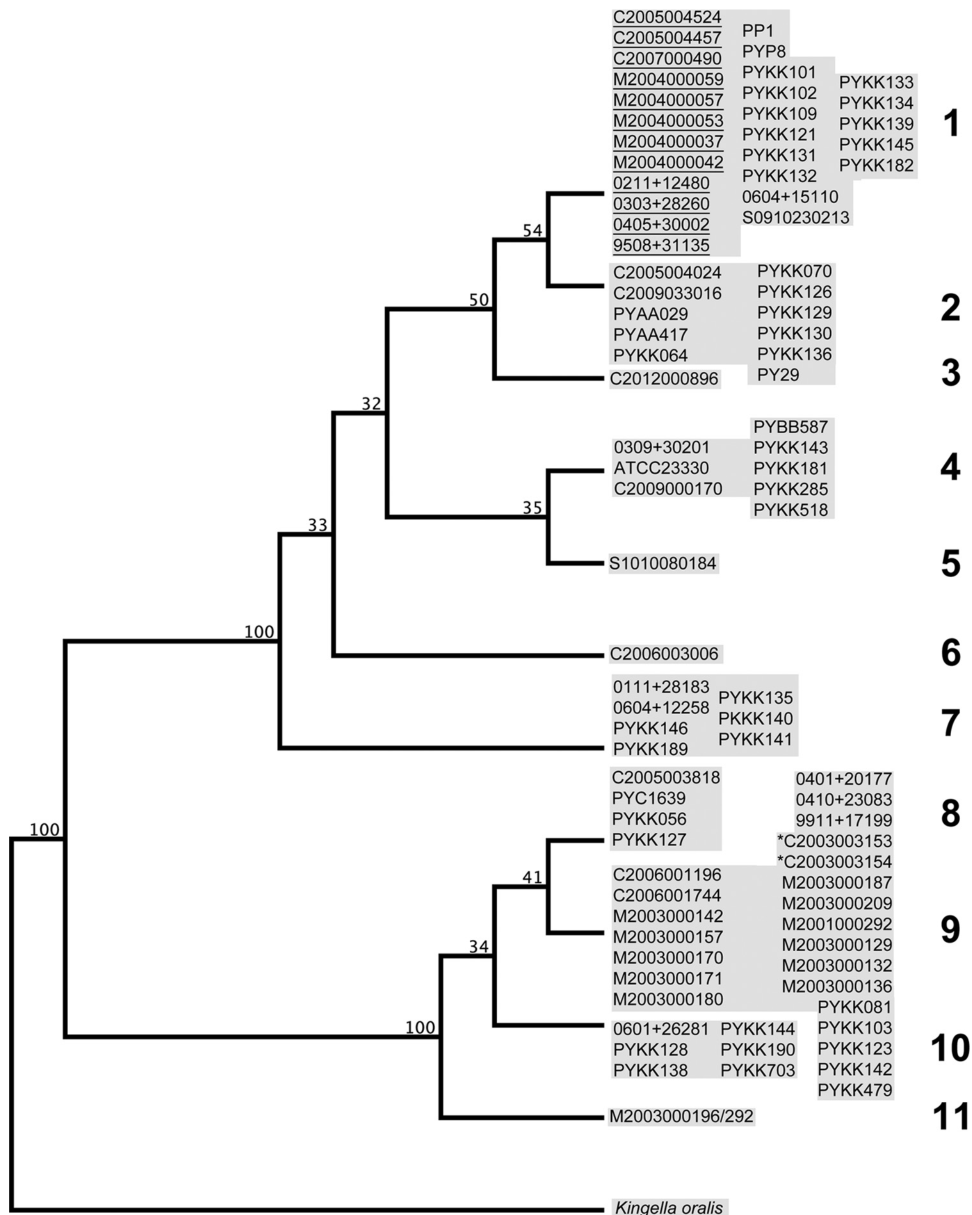

FIG 2 Phylogeny of *Kingella* strains based on the 956-bp *por* gene sequence. Strains that produce β-lactamase are underlined. Strains isolated from outbreak patients in Minnesota are indicated with asterisks. The numbers on the right indicate the 11 allele types discussed in the text. The numbers within the dendrogram indicate the occurrence (percent) of the branching in 100 bootstrapped trees. *K. oralis por* was used as the outgroup.

cus sequence typing (MLST) have been proposed for *K. kingae* genotyping (25). Fragments of the *K. kingae* RTX toxin gene, a putative virulence factor, have been analyzed (25, 26). These methods provide a good foundation to investigate evolutionary relationships among strains; however, each has some limitations. Specifically, PFGE techniques are known to be difficult to reproduce between laboratories. MLST is a laborious and expensive technique for bacterial typing and may not provide sufficient information about small clonal genetic variations. In most strains, the *rtxA* sequencing results were congruent with the allocation to sequence types by MLST and PFGE, but incongruence was observed for several alleles, and the gene was shown to have larger variations in some strains, such as 33-bp duplications (25, 26).

SGST is often used for finer discrimination than MLST, with more variable genes chosen for comparison. Often, these genes correspond to serotyping antigens, for example, *porA* in meningococci (48, 49) and *spa* in *Staphylococcus aureus* (50). Variations in the porin gene structure were used for genotyping other members of the family *Neisseriaceae*: *N. meningitides*, *N. gonorrhoeae*, and *Neisseria lactamica* (48, 51, 52). The porin (PorB) of *N. gonorrhoeae* has been implicated in the species' pathogenesis (53). The *por* gene genotyping proposed here can be used in addition to other typing methods as a molecular epidemiological tool for examination of genetic relationships among circulating *K. kingae* strains. In this study, the utility of this approach was demonstrated, revealing the relatedness of the β-lactam-resistant iso-

lates. Notably, we have identified the *K. kingae* porin as a highly immunogenic protein (54). Future studies may find some correlation between the *por* allele type and the strain serotype. β-Lactamase production was exclusively associated with *por* allele 1 in *K. kingae* isolates from the United States and Iceland, suggesting similarity between the isolates from the two countries, whereas none of the 12 Israeli organisms exhibiting *por* allele 1 was an enzyme producer. Interestingly, 9 of these 12 isolates belonged to PFGE type K and two to the closely related PFGE types ua and E, indicating they belonged to the same subpopulation (26). Clone K is significantly overrepresented among invasive isolates in Israel, causing 28.2% of all invasive *K. kingae* infections (26) among the Jewish population (27), and members are also commonly isolated in French patients (25). Clone K, also characterized by MLST and *rtx*A gene sequencing, shows congruency between the different genotyping results. All clone K organisms belong to MLST sequence type 6 and to the closely related *rtxA* alleles 8, 9, and 10.

In 2003, 2 confirmed cases of *K. kingae* osteomyelitis/septic arthritis occurred among children in the same toddler classroom of a Minnesota child care center (28). According to *por* genotyping, both outbreak strains belong to *por* allele 9 and are not β-lactamase producers. The two strains had indistinguishable PFGE patterns (23), suggesting correlation between different typing techniques. The *por* allele 9 type was the predominant group among mucosal and invasive *K. kingae* isolates from Minnesota. This group was associated with septic arthritis and bacteremia in different geographic regions, indicating that it is truly an international invasive clone.

Since no vaccines for *K. kingae* are available, antimicrobial therapy is the primary measure for infection control. Our results should alert clinicians to the need for β-lactam resistance testing in suspected *K. kingae* infections. In addition, a recent study reported reduced susceptibility to clindamycin and oxacillin in a substantial number of organisms (14, 24). Taken together, these data emphasize the importance of surveillance for antibiotic resistance of the pathogen.

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