Complete Nucleotide Sequence of *Klebsiella pneumoniae* Multiresistance Plasmid pJHCMW1

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The multiresistance plasmid pJHCMW1, harbored by a clinical *Klebsiella pneumoniae* strain isolated from a neonate with meningitis, was sequenced. A circular sequence of 11,354 bp was generated, of which 7,993 bp make up Tn1331, a transposon including the antibiotic resistance genes *aac(6’)-Ib, aadA1, bla_{OXA-9*}*, and *bla_{TEM-1}*. The gene *aac(6’)-Ib* is included in a gene cassette, and both *aadA1* and *bla_{OXA-9*}*, are included in a single-gene cassette that may have arisen as a consequence of a recombination event involving two integrons. The pJHCMW1 plasmid replicates through a ColE1-like RNA-regulated mechanism, includes a functional *oriT*, and two loci with similarity to XerCD site-specific recombination target sites involved in plasmid stabilization by the resolution of multimers. One of these two loci, mrr, is active and has been the subject of previous studies, and the other, *dxs*, is not functional but binds the recombinase XerD with low affinity. Two additional open reading frames were identified, one with low similarity to two hypothetical membrane proteins from *Mycobacterium tuberculosis* and *Mycobacterium leprae* and the other with low similarity to *psiB*, a gene encoding a function that facilitates the establishment of the transferring plasmid in the recipient bacterial cell during the process of conjugation.

*Klebsiella pneumoniae*, a gram-negative rod, is a known cause of community-acquired bacterial pneumonia and other infectious diseases (7, 18, 38). This bacterium has also been identified as the causative agent of primary liver abscess, an important complication in diabetic patients in some geographical regions (12). *K. pneumoniae* also accounts for a substantial amount of hospital-acquired urinary tract infections, pneumonia, septicemias, meningitis, and soft tissue infections (5, 33, 39). The autoimmune disease ankylosing spondylitis is thought to be a possible sequela of *K. pneumoniae* infection (31). Although the molecular mechanisms of *K. pneumoniae* virulence are still not well understood, it has been proposed that the antiphagocytic capsule of *K. pneumoniae* plays a role in lung infections by preventing phagocytosis and suppressing the host immunological responses (14, 17). A recent study suggested that the capsule may induce production of interleukin 10 at the site of infection, which in turn may down-regulate the expression of other proinflammatory cytokines (40). Other putative virulence factors of pathogenic strains of *K. pneumoniae* could be iron acquisition systems, adhesions, serum resistance, and production of lipopolysaccharides (16). *K. pneumoniae* has been reported to be increasingly resistant to multiple antibiotics (28, 39), and the genetic determinants for resistance are often plasmid mediated (19, 33, 39). The plasmid pJHCMW1 was harbored by a *K. pneumoniae* clinical strain isolated from a neonate with meningitis during an outbreak of hospital infection (39). This plasmid contains the transposon Tn1331 which includes four antibiotic resistance genes: *aac(6’)-Ib, aadA1, bla_{OXA-9*}*, and *bla_{TEM-1}*. Here we report the sequencing and describe the entire pJHCMW1 plasmid. Our analysis indicates that this plasmid can be considered the multiresistance transposon Tn1331 plus a DNA stretch carrying the functions for replication and mobilization.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** Plasmid pJHCMW1 was originally isolated from *Klebsiella pneumoniae* JHCK1 (39). This plasmid was introduced into *Escherichia coli* XLIBlue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacF77K2ZrM15 Tn10) (Stratagene), a strain also used as a host for DNA recombinant methods. Recombinant clones were generated using pUC18 as a cloning vector.

**Bacterial growth medium and general procedures.** Growth of bacteria was in Lennox L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Plasmid DNA was prepared using the Qiagen plasmid mini kit (Qiagen, Inc.). Recombinant clones or pJHCMW1 was sequenced using BigDye (ABI) and DYEnamic ET (Amersham) chemistries on an ABI Prism (model 310 or 3100) instrument and M13 forward and reverse primers or custom-designed primers. Sequences were examined and assembled with Sequencher 4.1.2 software (Gene Codes Corp.). DNA and protein sequence analyses were performed using the Artemis program (http://www.sanger.ac.uk), the CLUSTAL W and BESTFIT programs of the Sequencing Analysis Software Package of the University of Wisconsin Genetics Computer Group (10), the HMMTOP program (http://www.enzim.hu/hmmtop/index.html) (37), and the DAS program (http://www.sbc.usu.edu/~miklos/DAS/) (6).

**DNA binding assays.** The oligonucleotides used had the following sequences: *df*: 5’GATCCCCGATGTCGACGATATCAATCTGGAACTGCGCATACCAAGG

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mwr, 5’GATCCGGCGGTGCACGCAACAGATGTTATGGTAAATACG and 5’/H11032 AATTCGTATTTACCATAACATCTGTTGCGTGCACCGCCG.

Approximately 10 pmol of oligonucleotide was end labeled with 50 Ci of [\(^{32}\)P]ATP and phage T4 polynucleotide kinase (5 U) in kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl\(_2\), 5 mM dithiothreitol, 0.1 mM spermidine) in a final volume of 20 μl. The labeled oligonucleotide was purified by chromatography through a Nuctrap Probe Purification column (Stratagene) and then ethanol precipitation. The radiolabeled oligonucleotide was dissolved in 15 μl of H\(_2\)O and then made double stranded by annealing with 50 pmol of the complementary oligonucleotide. The mixture was heated to 75°C for a few minutes in a water bath, and then the thermostat was turned off to allow the sample to slowly cool to room temperature overnight. The annealed double-stranded radiolabeled oligonucleotides were purified by electrophoresis on an 8% polyacrylamide gel in Tris-borate buffer (100 mM Tris [pH 8], 100 mM boric acid, 2 mM EDTA) as described previously (34). The radiolabeled oligonucleotides were mixed with 0.1 mg of poly(dI-dC) per ml and the appropriate protein(s). The binding reaction was allowed to proceed for 10 min at 37°C, and then the reaction mixtures were immediately transferred to ice. The samples were analyzed by electrophoresis in a polyacrylamide gel as described above. The radioactive complexes were detected by exposure to X-ray film.

**RESULTS AND DISCUSSION**

This project was originally initiated to focus on the resistance to amikacin found in clinical strains of *K. pneumoniae* isolated from diseased neonates (39). A multiresistant clinical *K. pneumoniae* strain isolated in a children’s hospital from a neonate with meningitis was studied further (39). The resistance to amikacin and other aminoglycosides such as tobramycin and kanamycin (but not gentamicin) in this isolate was due to the presence of a plasmid, pJHCMW1, which includes the aac(6’)-Ib gene (39). Studies on the biological properties of pJHCMW1 led to the isolation and characterization of a transposon named Tn1331 (36), which includes aac(6’)-Ib as well as three more resistance genes, aadA, bla\(_{OXA-9}\), and bla\(_{TEM-1}\). Further studies characterized the transposon and the genetic organization and expression of the resistance genes (32, 35). The aac(6’)-Ib, aadA, and bla\(_{OXA-9}\) genes are transcribed as a polycistronic mRNA from a promoter located upstream of aac(6’)-Ib. In addition, bla\(_{OXA-9}\) is expressed from another promoter located immediately upstream of the structural gene (35).

The complete sequence of pJHCMW1 was determined to be 11,534 bp, and analysis of the sequence revealed that 7,993 bp constitute the multiresistance transposon Tn1331. The remaining portion of pJHCMW1 contains sequence coding for the replication, mobilization, and possibly maintenance functions in addition to two hypothetical open reading frames (ORFs)
that encode products that do not have significant matches in the databases. Figure 1 shows the locations and sizes of these genes and genetic structures. The transposon Tn1331 can be considered Tn3 with the addition of a DNA region, which has the structure of the variable portion of the integrons (32, 35) (Fig. 1). The inverted repeats at the Tn1331 ends as well as the tnpR gene are identical to those of Tn3. The Tn1331 tnpA nucleotide sequence is missing 9 nucleotides with respect to the Tn3 tnpA gene. Nucleotides 853 to 870 (coordinates as in GenBank accession number v00613) in the Tn3 tnpA gene are a 9-nucleotide tandem repeat that encodes the amino acid sequence GFHGFH. In contrast, the Tn1331 version of tnpA has only one of the 9-nucleotide repeats encoding the amino acid sequence GFH. The Tn1331 fragment that has the genetic structure of the variable part of the integrons includes two gene cassettes harboring the antibiotic resistance genes aac(6')-Ib, aadA1, and blaOXA-9 (35) and is flanked by 520-bp direct repeats (Fig. 1). Models for the genesis of Tn1331 as an evolutionary product of Tn3 have been described previously, and they involve a duplication of a portion of Tn3 that generated the 520-bp direct repeats (32). To determine whether the gene cassettes or the rest of the DNA

![Diagram](image1)

FIG. 2. Comparison of structures of regions including aac(6')-Ib, aadA1, and blaOXA-9 from pJHCMW1 and aac(6')-Ild and aadA1 from A. baumannii. The black lines represent DNA fragments with high homology. The numbers are the base pair coordinates in the pJHCMW1 nucleotide sequence. The nucleotide sequence of the 59-be element following aadA1 is shown. The homology between A. baumannii and pJHCMW1 DNA ends at the end of the aadA1 structural gene and starts again at coordinate 9727 (final portion of the blaOXA-9 59-be element). At the bottom of the diagram, the positions and lengths of the gene cassettes are shown.

![Diagram](image2)

FIG. 3. Possible mechanism for the generation of the gene cassette containing aadA1 and blaOXA-9. Two putative integrons, one containing blaOXA-9 immediately following attI and one with a gene cassette containing aadA1 crossover (probably an illegitimate recombination event), generating the gene cassette, including both genes found in pJHCMW1. In the process, the sequence of the 59-be element located 3' of blaOXA-9 has been conserved, but the attI site and the 59-be element 3' of aadA1 have been lost, leaving a sequence indicated as attI*. The attI site is located adjacent to the intI gene in the 5' conserved region and is where gene cassettes are inserted in integrase-mediated reactions (22). The nucleotide sequences of the 59-be elements are shown.
FIG. 3. Alignment of the nucleotide sequences at the N terminus of aac(6’)-Ib from pJHCMW1 and aac(6’)-IId from A. baumannii (A. b.). The proteins encoded by these genes have 98% identity [comparison starts at the AAC(6’)-Ib Met at nucleotide 7389]. Identical nucleotides are indicated by the vertical lines between the two sequences. The sequence identical to that of blaTEM-1 in the pJHCMW1 aac(6’)-Ib gene is boxed.

in pJHCMW1 have different origins, we determined the percent G+C along the whole sequence as well as for each ORF. The results indicate that the mean G+C content of this plasmid is 48.96%, with all the ORFs located within TnJ331 displaying values similar to that of the full-length pJHCMW1. In contrast, the hypothetical ORF1 and ORF2, which were mapped outside TnJ331, displayed G+C contents of 43.63 and 42.48%, respectively. These differences in G+C content suggest that the origin of these two ORFs could be different from those contained within the TnJ331 transposable element.

Antibiotic resistance genes are often found as part of gene cassettes, which include a coding region followed by the 5′-be, a site-specific recombination target for integron integrases (30). While aac(6’)-Ib is included in a gene cassette with the regular gene–5′-be structure, the aadA1 and blaOXA-9 genes are unusual because both are included in the same gene cassette (35). Computer analysis demonstrated that the sequence of this gene cassette has very high similarity with the sequence of an integron-borne gene cassette that was recently found in Acinetobacter baumannii that carries only aadA1 (27). As is the case for pJHCMW1, the aadA1 gene cassette found in this A. baumannii integron is preceded by another one containing a gene, aac(6’)-Iid, which codes for a protein that has 98% identity with AAC(6’)-Ib [the A. baumannii gene has Ser instead of Leu, which is characteristic of AAC(6’)-II enzymes (26)]. A diagram summarizing data comparing the nucleotide sequences and the structures of the gene cassettes is shown in Fig. 2. The aac(6’)-IId/aac(6’)-Iid, 5′-be, and aadA1 sequences are highly similar in pJHCMW1 and the A. baumannii integron. However, while as in every regular gene cassette, the A. baumannii aadA1 is followed by a 5′-be element, in pJHCMW1 following aadA1 there is a DNA region inserted that includes what may be considered a remnant of an attI site (attI* [Fig. 2]) and the blaOXA-9 gene followed by its 5′-be (Fig. 2). This arrangement suggests that an integron containing the aadA1 gene cassette underwent an illegitimate recombination event with another integron in which the gene cassette adjacent to the 5′ conserved portion contained blaOXA-9 (Fig. 3) (35). A crossover may have occurred between the attI 5′ of blaOXA-9 and the 5′-be 3′ of aadA1, resulting in the loss of the 5′-be and the generation of attI* (Fig. 3) (35). The diagram in Fig. 3 also shows that after the insertion of blaOXA-9, the 5′-be sequence has not been modified. Acquisition and modification of resistance genes through illegitimate recombination have also been reported in other systems. Analysis of Pasteurella and

FIG. 4. Alignment of the nucleotide sequences at the N terminus of aac(6’)-Ib from pJHCMW1 and aac(6’)-IId from A. baumannii (A. b.). The proteins encoded by these genes have 98% identity [comparison starts at the AAC(6’)-Ib Met at nucleotide 7389]. Identical nucleotides are indicated by the vertical lines between the two sequences. The sequence identical to that of blaTEM-1 in the pJHCMW1 aac(6’)-Ib gene is boxed.

Mannheimia isolates carrying the sulIII and strA genes showed that more than half of the isolates had an insertion of the catIII which occurred by illegitimate recombination (15). Fusions of gene cassettes that may have occurred by other mechanisms have also been described elsewhere (11, 21, 22).

The blaOXA-9 gene cassette has also been found in another integron structure, In40, isolated from Enterobacter aerogenes (GenBank accession number AF034958) (24). The nucleotide sequence of this blaOXA-9 gene cassette is identical to the blaOXA-9–5′-be portion of the pJHCMW1 gene cassette including aadA1 and blaOXA-9 (data not shown). The homology between pJHCMW1 and In40 at the region including blaOXA-9 starts at pJHCMW1 coordinate 8777 and ends at 9848, the beginning of one of the 520-bp direct repeats (data not shown).

Inspection of the N-terminal portion of the aac(6’)-Ib genes from K. pneumoniae and A. baumannii shows that the gene from pJHCMW1 has the potential to encode a longer protein compared to that from A. baumannii due to the fusion to the initial portion of blaTEM-1 (32) (Fig. 4). We do not know whether the actual start codon of the pJHCMW1 AAC(6’)-Ib is at the ATG codon at coordinate 7302, 7389, or 7410. If the actual start codon were at coordinate 7389, both versions of the enzyme would have identical N termini. However, this may not be a critical question, because Casin et al. (3) recently analyzed variants of AAC(6’)-Ib from members of the family Enterobacteriaceae and found that there is a high flexibility in the structural requirements at the N terminus of this enzyme. The
pJHCMW1-encoded AAC(6’)-Ib protein and other proteins in the family of the AAC(6’)-I enzymes have recently been partially characterized by mutagenesis (25, 26, 29).

Maintenance and dissemination functions are included in a region between coordinates 1223 (the first nucleotide of the –35 sequence of the RNAII promoter) and 3342 (the last nucleotide of the mwr recombination site) (Fig. 1). The replication of pJHCMW1 occurs by an RNA-regulated mechanism similar to that described for the CoIE1 plasmid (9), oriV is located at coordinate 1767 (Fig. 1). A sequence with high similarity to the CoIE1 oriT is present with the nick site at coordinate 2003. This oriT has been proven functional in experiments where the CoIE1 mob and RK2 tra genes were supplemented in trans (9). The Xer site-specific recombination site mwr is located at coordinates 3198 to 3342. This site shares homology with other Xer site-specific recombination sites that act as targets of the Xer site-specific recombination system to convert plasmid and chromosome dimers to monomers (1). The mwr core recombination site has 71% identity with the corresponding psi and dif sites and 75% identity with cer, although cer has 8 nucleotides in its central region (Fig. 5a). However, an important difference between mwr and other Xer site-specific recombination target sites is that the efficiency of recombination at mwr in E. coli is strongly dependent on the osmolarity of the growth medium (23). The binding of the mwr core recombination site to the recombinases XerC and/or XerD is shown in Fig. 5b, which shows the cooperative binding of XerC and XerD. At coordinates 2411 to 2442, there is another site that shares a lower degree of similarity with Xer recombination sites; this site was named dxs (for deficient Xer recombination site). Figure 5a shows a comparison of the nucleotide sequences of mwr, cer, psi, and dif with that of dxs. The percentages of identity of the dxs XerD-binding site and the corresponding region of the other sites are 64 (dif) and 54.5 (mwr, cer, and psi). Recombination analysis showed that dxs is not functional under the conditions assayed (data not shown). However, in vitro DNA-protein binding experiments demonstrated that dxs exhibits a low but detectable binding affinity to XerD. Figure 5b shows a comparison of binding of dif, mwr, and dxs to XerC, XerD, and both recombinases. As expected, dif exhibited weak binding to XerC but showed strong binding to XerD and cooperative binding to XerC and XerD (Fig. 5b). As previously known, mwr binding to XerC alone was not detected, but binding to XerD was strong and strong cooperative binding to XerC and XerD was observed (Fig. 5b). Conversely, dxs did not show binding to XerC and showed only very weak binding with XerD. Addition of both, XerC and XerD, did not enhance binding (Fig. 5b). These results suggest that this may be a remnant of a Xer recombination site. To the best of our knowledge, there are no reports of any biological function by a site with the characteristics of dxs.

Flanking the replication and oriT regions, there are two ORFs predicted to encode proteins of 15,705 Da and pf 10.2 (ORF1, encompassing coordinates 973 to 551), and 13,267 Da and pf 5.1 (ORF2, encompassing coordinates 2626 to 2991) (Fig. 1). The predicted ORF1 protein contains two transmembrane helices (aa 28 to 52 and 87 to 106) as determined using the HMMTOP program (http://www.enzim.hu/hmmtop/index.html) (37) and one (aa 28 to 52) as determined using the DAS program (http://www.sbc.su.se/~miklos/DAS/) (6). No transmembrane regions were found in the amino acid sequence of the predicted ORF2 protein. The predicted ORF1 protein shows a low degree of similarity with a hypothetical protein from Mycobacterium tuberculosis (GenBank accession number Z92771, hypothetical protein Rv3278c) (4) (60% similarity and 26% identity in the amino acid sequence from amino acids [aa] 56 to 107) and a Mycobacterium leprae putative membrane protein (GenBank accession number AL583919, hypothetical protein ML0733) (50% similarity and 20% identity in the sequence from aa 56 to 133) (4). The predicted ORF2 protein has a low similarity to the psiB gene from pMK101 (61% similarity and 34% identity in the sequence from aa 8 to 61) (GenBank accession number U72482) (8). This is one of two conserved genes found in several conjugative plasmids thought to encode functions that establish the facilitation of the transferring plasmid in the recipient bacterial cell during conjugation (2, 13). The plasmid Colllb-P9 psiB gene and the ampA and sbb genes have been shown to be expressed via yzyotic induction (13) and to use single-stranded DNA transcription (2). All three genes seem to be transcribed from the same promoter, which shares similarity with that of Fpda, a single-stranded DNA promoter (20) located in the leading region of plasmid F. Plasmid pJHCMW1 carries a functional oriT (9), but no other conjugation-related gene had been identified before. Although the similarity between the ORF2 protein and PsiB is very low, experiments will be performed to determine whether the predicted ORF2 protein plays a role in establishment of pJHCMW1 in the recipient cell upon conjugation and to identify the promoter that drives transcription of this hypothetical protein.

K. pneumoniae has been reported to be increasingly resistant to multiple antibiotics, and the genetic determinants for resistance are often plasmid mediated. The plasmid pJHCMW1 is responsible for resistance to several aminoglycosides and β-lactams in the clinical K. pneumoniae JHCK1 isolate (39). This plasmid seems to have evolved to carry the multidrug resistance transposon Tn1331 and the minimal functions required for replication, stability, and mobilization.

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REFERENCES
