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pMEX01, a 70 kb plasmid isolated from *Escherichia coli* that confers resistance to multiple β -lactam antibiotics

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ABSTRACT

Multidrug-resistant microorganisms are of great concern to public health. Genetic mobile elements, such as plasmids, are among the most relevant mechanisms by which bacteria achieve this resistance. We obtained an *Escherichia coli* strain CM6, isolated from cattle presenting severe diarrheic symptoms in the State of Querétaro, Mexico. It was found to contain a 70 kb plasmid (pMEX01) with a high similarity to the pHK01-like plasmids that were previously identified and described in Hong Kong. Analysis of the pMEX01 sequence revealed the presence of a *bla*_{CTX-M-14} gene, which is responsible for conferring resistance to multiple β -lactam antibiotics. Several genes putatively involved in the conjugative transfer were also identified on the plasmid. The strain CM6 is of high epidemiological concern because it not only displays resistance to multiple β -lactam antibiotics but also to other kinds of antibiotics.

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Introduction

Plasmids play a crucial role in the dissemination of CTX-M type extended-spectrum β -lactamases (ESBLs), and their

characterization may provide important insight into understanding multidrug-resistant bacterial strains. β -Lactams are among the most widely used antimicrobial agents for the treatment of bacterial infections. However, the intensive exposure of bacteria to β -lactam antibiotics has induced the emergence of several antibiotic resistance strategies, with

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the development of β -lactamases capable of inactivating β -lactams being one of the most relevant. In gram-negative bacteria, the production of ESBLs is the principal cause of resistance to β -lactam antibiotics.^{1,2} Plasmids encoding CTX-M-type cefotaximase were reported in Germany in the late 1980s,^{3,4} and they are currently distributed worldwide, including North America,⁵ Latin America⁶ and Asia.⁷ CTX-M has become so widespread that it is now considered to be the most prevalent β -lactamase among clinical isolates of *Escherichia coli* worldwide.⁸ Diverse studies in Hong Kong,^{9,10} China,¹¹ and South Korea¹² have reported that CTX-M-14 is the most frequently found β -lactamase enzyme in *E. coli*, *Klebsiella pneumoniae*, and *Shigella* isolates. In Mexico, the presence of GES and CTX-M type β -lactamases have been found in *Enterobacteriaceae* clinical isolates.¹³ In a previous study, we sampled multidrug-resistant *E. coli* strains from cattle presenting severe diarrheic symptoms in the State of Querétaro, Mexico, and analyzed their drug resistance spectra, plasmid profiles and conservation using shock waves.¹⁴ In this study, we analyzed the complete nucleotide sequence of the pMEX01 plasmid from *E. coli* strain CM6, which has a high similarity with the previously described pHK01-like plasmids.¹⁵

Materials and methods

Isolation and identification of bacteria

Fecal samples were obtained from cattle presenting severe diarrheic symptoms in the State of Querétaro, Mexico during 2014. Samples from cattle that did not respond to standard antibiotic treatment were selected for further analysis. A total of 10 isolates were identified as *E. coli* by analyzing 16S ribosomal deoxyribonucleic acid (rDNA) sequences, which were amplified using the primers fd1 (CCGAATTCGTCGACAACAGAGTTTGGATCCTGGCTCAG) and rD1 (CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC)¹⁵ and by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry,¹⁶ using a MicroFlex LT mass spectrometer (Bruker Daltonics, Bremen, Germany).

Antibiotic resistance profile

The plasmid pMEX01 was extracted from *E. coli* strain CM6 and transformed by electroporation (250 V, 20 Ω and 250 μ FD) into the *E. coli* strain BL21 STAR (Invitrogen, Carlsbad, CA, U.S.A.). We then determined the antibiotic resistance profiles of the *E. coli* strain CM6 and the *E. coli* strain BL21 STAR containing pMEX01. All antimicrobial testing was performed on agar-solidified LB. The β -lactam antibiotics assayed included carbenicillin, cephalixin, ceftriaxone, cefotaxime and dicloxacillin. Kanamycin, rifampicin, chloramphenicol, polymyxin, spectinomycin and streptomycin were also tested. The minimum inhibitory concentration (MIC) of each *E. coli* strain were evaluated by the criteria of the Clinical and Laboratory Standards Institute.¹⁷ *E. coli* BL21 STAR without the pMEX01 plasmid was used as a control.

Sequencing and bioinformatics

E. coli strain CM6 was selected for plasmid isolation and sequencing. The plasmid was extracted from an overnight culture of the selected strain grown in LB broth (BD DIFCO, Mexico) and purified using a Zyppy Plasmid Miniprep Kit (Zymo Research, CA, U.S.A.). The plasmid was sequenced with an Illumina HiSeq 2000 sequencing platform at the MacroGen Korea Institute (Seoul, Republic of Korea) using a whole-genome shotgun library strategy. The plasmidic DNA sequence was reconstructed using multiple bioinformatic tools. The sequence reads were assembled using the genome assembler program SPAdes v3.1.1.¹⁸ The initial contigs were compared with the Plasmids database from NCBI GenBank (accessed April 2016) using Blast+. The best matches indicated a high similarity with pHK plasmid family, then we use pHK17a as a template to reorder the contigs and reconstruct the full plasmid. Open reading frames (ORFs) were deduced and annotated by using NCBI Glimmer v3.02.^{19–21} ORF sequences were analyzed against protein family databases to obtain information about their functionality.^{22,23}

Nucleotide sequence

The nucleotide sequence for the pMEX01 plasmid has been deposited in GenBank under the accession number KU695535.

Results

Sequence assembly and comparative analysis of the pMEX01 plasmid

The plasmid (pMEX01) from *E. coli* strain CM6 was isolated and sequenced. The sequencing of pMEX01 produced 26,133,370 short-sequences as 2 $\times$ 100 paired-end reads. The initial assembly of the sequence reads produced 1247 contigs, with an average size of 4879bp, an expected 2119 $\times$ average coverage and a calculated N50 value of 20,000. The fine assembly using pHK17a sequence as a reference showed the presence of a circular replicon consisting of 70,093 nucleotides, with an average GC content of 52.27% (Fig. 1).

The pMEX01 plasmid is comparable in size and is highly similar to other plasmids isolated and sequenced from *E. coli* in Asia, including pHK17a (99%), pHK01 (99%), pHK08 (99%), and pHK09 (99%); pKF3-70 (98%) from *K. pneumoniae*; pEG0356 (99%) (except for the *bla*_{CTX-M-14} allele) from *S. sonnei*; and pO26-L (98%) a plasmid from a Canadian *E. coli* isolate that carries a tetracycline resistance gene cluster (Fig. 2). Our assembly covers 88.71% of the reference sequence (pHK17a) with an average coverage of 7547 $\times$. A sequence analysis of pMEX01 showed that it belongs to the incompatibility group IncFII. Analyses using several algorithms and approaches showed that the pMEX01 plasmid had 98 potential ORFs, with only 8 showing similarities to unknown proteins from the NCBI non-redundant microbial protein database (Supplementary Table S1). pMEX01 contains a number of primary structural features that are highly similar to pHK01-like plasmids. These include a DNA transfer region (*tra* genes), required for efficient

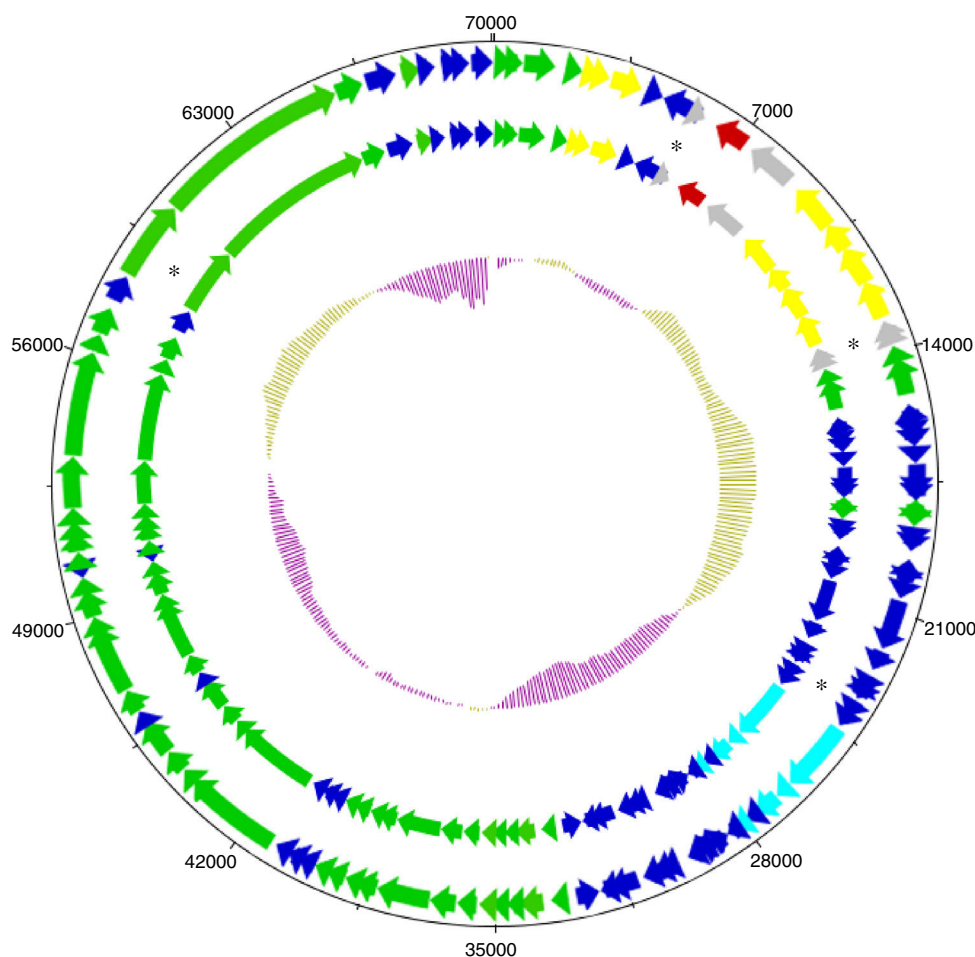


Fig. 1 – Circular map of pMEX01 (inner map) compared to pHK17a (outer map). The length of pMEX01 is 70,093 bp. Dark green, ORF related to plasmid replication; light green, ORF related to plasmid stabilization; red, CTX-M-14 β -lactamase; gray, ORF related to transposases, insertion sequences; yellow, ORF related ABC transport system; light blue, ORF related to plasmid conjugation, transfer; dark blue, miscellaneous ORF. Inner circle indicates a GC plot, with purple and brown indicating below and above average GC contents, respectively. Asterisks indicate ORF with significant differences.

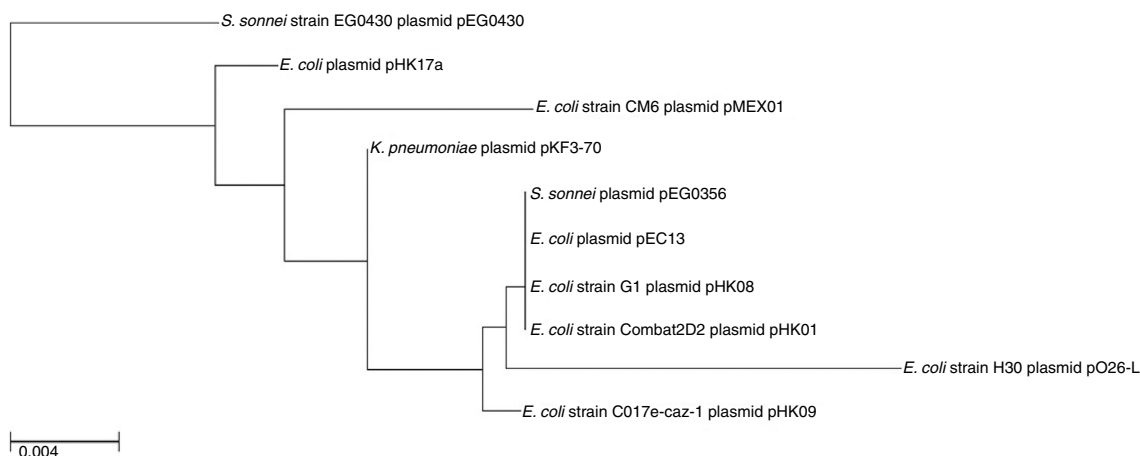


Fig. 2 – Phylogenetic dendrogram for pMEX01. The assembled sequence was compared with the NCBI Plasmid database (accessed April 2016) with Blastn (megablast) to identify highly similar full plasmid sequences with E -value $< 10^{-10}$. Plasmid sequences were aligned and a dendrogram was generated using the NJ algorithm in ClustalW v2.1 with the default parameters.

conjugative transfer; the *trb* genes, which are involved in pilus assembly, the putative plasmid mobility gene (*mob*); the conjugation gene *trwB*; and the *traT* gene, which appears to be responsible for maintaining the regulation of transfer efficiencies. The *finO* gene is involved in inhibiting the expression of the regulatory gene *traJ* in a process known as fertility inhibition. In addition, pMEX01 carries genetic information for the control of replication, including a structural gene (*rep*) that encodes an initiation protein (Rep), as well as a protein involved in the toxin–antitoxin system mechanism that participates in plasmid maintenance (*relE/parE*). Genes encoding an iron ABC transport system (*eit* genes) are also present, including a permease, a substrate-binding protein, and an export-associated protein. The plasmid harbors a well-defined potential transposon consisting of IS903, a *bla*_{CTX-M-14} gene, and an ISEcp1 element (*tnpA* gene), which according to the sequence analysis and distance that separates these genetic elements, corresponds to a type II transposon (Fig. 1).

Since strain CM6 was isolated from the feces of cattle that did not respond to the standard treatment, we assessed the antibiotic resistance profile of strain CM6 to gain insight into its potential multidrug-resistance. We found that it has a resistance not only to high concentrations of β -lactam antibiotics (Table 1), but also to several other antibiotics, including kanamycin, streptomycin and to spectinomycin, but not rifampicin and polymyxin, to which CM6 is susceptible. To correlate these phenotypic traits with the presence of the pMEX01 plasmid, the *E. coli* BL21 STAR transformant carrying the plasmid was evaluated. The results showed that the presence of pMEX01 provides the *E. coli* BL21 STAR strain with resistance to all β -lactam antibiotics analyzed (Table 1). Taken together, our findings suggest that pMEX01 is highly similar to other pHK01-like plasmids disseminated worldwide with an extended-spectrum β -lactamase.

Discussion

This study demonstrated the presence of a pHK01-like plasmid (pMEX01) in *E. coli* strain CM6 that was isolated from fecal samples of cattle in the State of Querétaro, Mexico. Although pMEX01 is highly similar to pHK17a (99%), several open reading frames significantly differ between the two plasmids. For

instance, CDS10 and 17, which are involved in transposition, have identities of 40.4% and 81.5%, respectively, when compared with their corresponding open reading frames in pHK17a. CDS37, which codes for a putative *ydaA*, has 62.2% identity and has a premature stop codon. Interestingly, CDS88, which encodes the Type IV conjugative transfer system coupling protein TraD, is 85.5% identical to the corresponding TraD in pHK17a and has a premature stop codon. These changes in this open reading frame may have a significant impact on the fitness of pMEX01 since the last 37 amino acid residues are involved in plasmid specificity and transfer efficiency,²⁴ although this has to be verified experimentally.

In the pMEX01 plasmid, *bla*_{CTX-M-14} was the only β -lactam resistant determinant found. We compared the antibiotic resistance profile of CM6 against the *E. coli* BL21 STAR transformant carrying the pMEX01 plasmid. Although both CM6 and the *E. coli* BL21 STAR transformant exhibited resistance to a wide range of β -lactams, both strains did not exhibit similar resistance to kanamycin, spectinomycin, streptomycin or chloramphenicol, indicating that these resistance traits might be encoded on a different extra-chromosomal element present in the CM6 isolate. Indeed, pMEX01 was not the only plasmid detected when total CM6 DNA was analyzed by PFGE (data not shown). The aminoglycoside and chloramphenicol resistances displayed by CM6, along with the resistance to different generations of β -lactams, may explain why the afflicted cattle were not responsive to standard treatment.

The CTX-M enzymes are a subfamily of the extended-spectrum β -lactamases (ESBLs) that confer resistance to β -lactam antibiotics. More than one hundred types of CTX-M enzymes have been reported and are classified into five classes (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25); these enzymes are associated with ISEcp1-like insertion sequences (IS) for mobilization and are the most broadly distributed enzymes among a wide range of bacteria, particularly the *Enterobacteriaceae* family.^{25,26} The CTX-M-14 enzyme identified in the *E. coli* strain CM6 pMEX01 plasmid has been sub-classified into the CTX-M-9 group.²⁷ Although *E. coli* is recognized as the major source of ESBLs,²⁸ it is accepted that the CTX-M group has a chromosomal origin from *Kluyvera georgiana* and is the likely source of CTX-M-9 family, of which the pMEX01 CTX-M-14 enzyme is a member.²⁹ Members of the CTX-M-5 family have been reported in North America,²⁵ but to the best of our knowledge, this is the first report of a member of the CTX-M-9 family in this region. This finding suggests that multiple genetic processes are involved in the evolution and persistence of the pHK01-like plasmids in diverse countries.¹² The β -lactam resistance gene present in pMEX01 likely emerged from a bacterial lineage with multidrug resistance and maintenance of a pHK01-like plasmid. The pMEX01 plasmid has a potential transposon comprised of an IS903, the *bla*_{CTX-M-14} gene and an ISEcp1 element, which has important epidemiological implications for the dissemination of this β -lactamase.³⁰ In fact, diverse studies have demonstrated the wide dissemination of pHK01 plasmid among *E. coli* isolates obtained from patients with urinary tract infections in Hong Kong.³¹ Plasmid variants closely related to pHK01 have also been isolated among *E. coli* isolates from pigs (pHK17a) and human fecal samples (pHK09) in Hong Kong.³² The dissemination of pHK01-like plasmids has also been detected among

Table 1 – Minimum inhibitory concentration (μ g/mL) of *E. coli* strain CM6 and *E. coli* BL21 STAR transformants carrying pMEX01 plasmid.

Antibiotic	CM6	BL21 STAR pMEX01
Carbenicillin	>400	>400
Dicloxacillin	>400	>400
Cephalexin	>400	>400
Ceftriaxone	>400	>400
Cefotaxime	>400	>400
Kanamycin	>30	30
Rifampicin	10	10
Chloramphenicol	>30	30
Polymyxin	2	2
Spectinomycin	>100	100
Streptomycin	>50	50

K. pneumonia isolates from human-fecal samples (pKF3-70) in China²⁶ and among *S. sonnei* isolates from human fecal samples (pEG356) circulating in southern Vietnam.³³ Previous studies have shown that Shiga toxin-producing *E. coli* (STEC) are medically important foodborne pathogens responsible for diverse outbreaks of hemorrhagic colitis and hemolytic uremic syndrome. These bacteria often carry a variety of plasmids that encode virulence factors and antibiotic-resistance genes, such as the pO26-L plasmid, a closely related variant of pHK01 that has a tetracycline resistance operon; this plasmid was isolated from *E. coli* O26:H11 strain H30, which was isolated from a child presenting diarrheic symptoms in Canada.³⁴ Considering these studies and the fact that the pMEX01 plasmid has a similar structure as several other plasmids isolated from diverse parts of the world, we can infer that not only are cattle an important reservoir, but also that farm workers could be important for the dissemination of *E. coli* strains with multidrug resistance and for the persistence of pHK01-like plasmids.

The economic importance of cattle in the State of Querétaro, Mexico, as well as the diverse federal regulations for their transport has forced public health authorities to create diverse strategies to reduce the transfer of these multi-drug-resistant bacteria in human and animal populations, and understand the role of these *E. coli* strains and its plasmids in pathogenesis. Therefore, a detailed molecular characterization of this plasmid from diverse farms and geographical origins in the State of Querétaro, Mexico should be investigated to identify the epidemiologic pattern of pMEX01 plasmid sequences and changes over time.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bjm.2017.11.002](https://doi.org/10.1016/j.bjm.2017.11.002).

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