



25 **ABSTRACT**

26 Carbapenem resistant *Enterobacteriaceae* (CRE) have emerged as a critical public health threat.  
27 Organisms expressing the *Klebsiella pneumoniae* carbapenemase (KPC) were first recognized in the US in  
28 the late 1990s and continue to be the predominant CRE genotype reported in clinical isolates. Strains  
29 harboring *bla*<sub>KPC</sub> alleles have been observed in multiple species of *Enterobacteriaceae*, including the  
30 *Enterobacter cloacae* complex. A major *E. cloacae* clone, *Enterobacter xiangfangensis* ST171, has  
31 emerged as an important cause of hospital associated infections (HAI) and has been shown to carry  
32 different alleles of KPC in the context of Tn4401, residing on plasmids of multiple incompatibility groups.  
33 While CRE are commonly isolated from infected humans, their recovery from animals has been rare,  
34 particularly from companion animals. In the US, only six CRE have been reported from companion  
35 animals, and one from livestock, none of which were *bla*<sub>KPC</sub>. This report describes two *E. xiangfangensis*  
36 sequence type ST171 isolates each with a large IncHI2 plasmid bearing *bla*<sub>KPC-4</sub> recovered from dogs with  
37 infections at the Ohio State University Veterinary Medical Center. Our phylogenetic comparison of these  
38 canine isolates with available sequences from clinical human isolates of KPC-4 identified in ST171  
39 suggest an epidemiologically significant clonal strain.

40

41 **INTRODUCTION**

42 Carbapenem class antimicrobial agents are critically important to human health and are considered  
43 drugs of last-resort. Carbapenem resistant *Enterobacteriaceae* (CRE) are a growing threat to global  
44 public health. In 2013, the CDC classified CRE as an “urgent” public health threat, and estimated that  
45 there were approximately 9300 CRE infections, and 610 related deaths in the US (1).

46

47 Carbapenem resistance among clinically-relevant *Enterobacteriaceae* is often conferred by plasmid-  
48 borne  $\beta$ -lactamase encoding genes that may be categorized in three of the four Ambler  $\beta$ -lactamase

49 classes. KPC and OXA carbapenemases respectively belong to classes A and D and function as serine  
50 proteases whose activity is inhibited by agents such as clavulanate and sulbactam. The NDM, VIM and  
51 IMP enzymes are class B metallo- $\beta$ -lactamases which are inhibited by the presence of EDTA, but not  
52 clavulanate and sulbactam (2). In the US, the *K. pneumoniae* carbapenemase (KPC) emerged in the late  
53 1990s and have become the predominant carbapenemase encoded by clinical CRE isolates primarily due  
54 to clonal dissemination of strains of *K. pneumoniae*, *Enterobacter spp.*, and *Escherichia coli* (3, 4). While  
55 healthcare associated infections (HAI) comprise the majority of cases involving CRE overall, community  
56 associated CRE infections (CAI) comprise up to 10.8% of infections in the US. Active surveillance  
57 performed by hospitals as part of infection control programs indicate that asymptomatic carriage may  
58 be up to 41%; however, the rate of carriage in healthy individuals remains unknown (5).

59

60 Antimicrobial-resistant bacteria and resistance genes are shared among human beings and animals  
61 through either direct or foodborne zoonotic transmission. As a result, veterinary use of antimicrobial  
62 drugs in both food-producing and companion animal species has the potential to impact antibiotic  
63 resistance of human pathogens (6). While carbapenem antimicrobial drugs are illegal to use in food-  
64 producing animals in the US, there is relevant selective pressure favoring CRE in production animal  
65 niches resulting from the use of the third-generation cephalosporin, ceftiofur, and other agents that  
66 may co-select for carbapenemase encoding genes due to co-localization with other resistance-encoding  
67 genes on plasmids (7, 8). To date, there have been two reports of multiple CRE genera expressing  
68 *bla*<sub>IMP-64</sub> located on an IncQ1 plasmid that were recovered from the same US swine farm (9, 10).

69

70 In US companion animals (e.g. dogs and cats), it is permissible for a veterinarian to prescribe  
71 carbapenem-class drugs off-label, as long as there is a valid veterinarian-client-patient relationship  
72 under the Animal Medicinal Drug Use Clarification Act of 1994 (AMDUCA; (11)). While an assessment of

73 the frequency of carbapenem usage in companion animals has not been reported, pharmacokinetic  
74 studies have been performed and companion animal dosage recommendations for imipenem and  
75 meropenem are available in veterinary medical textbooks (12-14). Moreover, ESBL and AmpC  
76  $\beta$ -lactamase mediated resistance among *Enterobacteriaceae* isolated from dogs and cats is commonly  
77 reported, indicating a potential clinical justification for carbapenem administration (15-20).

78  
79 This potential for the veterinary application of carbapenem drugs suggests that the role of companion  
80 animals as reservoirs for the zoonotic transmission of CRE should be considered, particularly for CAIs  
81 (21). There has been only one report of CRE isolated from companion animals in the US, six *E. coli*  
82 expressing *bla*<sub>NDM-1</sub> recovered from a variety of infections in cats and dogs between May 2008- May  
83 2009 (22). Subsequent analysis by pulsed-field gel electrophoresis (PFGE) indicated that the isolates  
84 represented diverse *E. coli* strains, which is consistent with the dissemination of diverse CRE expressing  
85 *bla*<sub>NDM</sub> in human patients.

86  
87 CRE can be challenging to diagnose in clinical laboratories because they frequently exhibit clinical  
88 susceptibility to carbapenem drugs upon routine antimicrobial susceptibility testing via broth  
89 microdilution and Kirby-Bauer disc testing (23). Several strategies, both molecular and phenotypic, have  
90 been used to increase the sensitivity of detecting carbapenemase production by clinical isolates (24, 25).  
91 In this report, we describe two clinical isolates of *E. xiangfengensis* and their mobile genetic elements  
92 recovered from canine patients in central Ohio, US. and belong to a disseminated clone that has been  
93 responsible for major clusters of human CRE infections in the Northeastern and Upper Midwestern  
94 United States (26).

95

96

97 **RESULTS**

98 **Antimicrobial Susceptibility Testing and Detection of KPC** Both isolates, identified by MALDI-TOF as *E.*  
99 *cloacae*, had the same susceptibility pattern (agent, MIC ( $\mu\text{g/ml}$ )): Amikacin,  $\leq 4$ ; Amoxicillin/Clavulanic  
100 acid,  $>8$ ; Ampicillin,  $>8$ ; Cefazolin,  $>32$ ; Cefovecin,  $>32$ ; Cefpodoxime,  $>8$ ; Ceftazidime,  $>16$ ;  
101 Chloramphenicol, 8; Doxycycline  $>8$ ; Enrofloxacin,  $>4$ ; Gentamicin, 0.5; Imipenem,  $\leq 1$ ; Marbofloxacin,  
102  $>4$ ; Piperacillin/Tazobactam, 16; Pradofloxacin,  $>2$ ; Tetracycline,  $>16$ ; and  
103 Trimethoprim/Sulfamethoxazole,  $>4$ . Notably, they were phenotypically susceptible to imipenem. Both  
104 isolates were observed to produce a carbapenemase by a CarbaNP test, and conventional PCR indicated  
105 that both isolates harbored *bla*<sub>KPC</sub>.

106

107 **Whole Genome Sequence and Annotation of Plasmid Containing KPC-4:** Subsequent Kmer analysis of  
108 whole genome sequence identified the isolates as *E. xiangfangensis*, which is included in the *E. cloacae*  
109 complex. Both isolates were identified as ST171 on MLST, and each contained two plasmids belonging to  
110 IncF1B and IncHI2. The IncHI2 plasmids (Figure 1) were 351,806 (pOSUVMCKPC4-1) and 354,256 bps  
111 (pOSUVMCKPC4-2), respectively, and were each identified as ST1 on pMLST. The plasmids both  
112 contained *bla*<sub>KPC-4</sub> in the context of *Tn4401b* (Figure 2), consistent with other reports of  
113 *Enterobacteriaceae* expressing *bla*<sub>KPC-4</sub>. Additional resistance genes in the two genomes were: *strAB*,  
114 *aadA1*, *bla*<sub>OXA-129</sub>, *sul1*, *tet(B)*, and *dfrA21*. Chromosomal analysis also indicated that they both carry  
115 resistance genes *bla*<sub>ACT-16</sub> and *fosA* in the two isolates. In comparison to pOSUVMCKPC4-1,  
116 pOSUVMCKPC4-2 has two additional IS3 at nt 62,536 to 63,760 and nt 180,063 to 181,287, respectively.  
117 In addition, a 21kb region (nt 94,348-115,575 in pOSUVMCKPC4-2), containing the *strA*, *strB*, *aadA1*,  
118 *bla*<sub>OXA-129</sub>, *dfrA21*, *sul1* flanked by two IS4321, is inverted in comparison to pOSUVMCKPC4-1. This region  
119 was identified as In524 (Figure 3), using the INTEGRALL database (27). The *int11* is disrupted by the  
120 Tn5393 insertion sequence, which encodes genes *strA* and *strB* that confer resistance to aminoglycoside

121 antibiotics. Downstream of *strB* is a truncated integrase gene followed by three antimicrobial resistance  
122 gene cassettes, *dfrA21*, *bla*<sub>OXA-129</sub>, and *aadA1*. The 3' region following the integron harbors genes  
123 encoding resistance to quaternary ammonium compounds, *qacEΔ1*, and sulfonamide antibiotics, *sul1*.  
124 This region also contains a putative gene for N-Acyltransferase, Acyl-coA, and the insertion sequence  
125 IS6100, which is a transposase shown to increase the expression of *strA* and *strB* genes. The IncF1B  
126 plasmids in the two isolates were identical in size, 108,403 bp, and contained no identifiable resistance  
127 genes.

128

129 Core SNP phylogenetic analysis placed these two *E. xiangfangensis* strains in the previously described  
130 ST171 cluster II (Figure 4)(26). The two isolates formed a subclade in cluster II, and differ from each  
131 other by 14 core snps. In cluster II, isolates have an average of 75 (10-137) core snp differences  
132 compared with each other, while they showed ~530 core snps difference in comparison to isolates from  
133 cluster I. Of interest, nearly all isolates from cluster II harbor the *bla*<sub>KPC-4</sub> on the Tn4401b element.  
134 Further analysis of the *bla*<sub>KPC-4</sub>-harboring contigs from other clade II isolates revealed that similar to  
135 OSUVMCKPC4-1 and OSUVMCKPC4-2, the majority (except for SMART-264 and BIDMC94) harbor *bla*<sub>KPC-4</sub>  
136 and IncH plasmid backbones.

137

## 138 **DISCUSSION**

139 This first reported isolation of KPC-producing CRE from US companion animals has important  
140 implications for both veterinary medicine and public health, because animals may serve as reservoirs of  
141 significant opportunistic human pathogens. Notably, these two cases represent an epidemiologically  
142 significant clone of *Enterobacter* sp., ST171 that has been described in regional HAI clusters in the US  
143 (28, 29), and which may now be expanding into the community. Analysis of contemporaneous human  
144 clinical *E. cloacae* isolates from The Ohio State University Medical Center collected between 2011-2016

145 with an extended-spectrum  $\beta$ -lactamase phenotype ( $n=8$ ) revealed that two isolates belonged to ST171,  
146 and both contained *bla*<sub>KPC-2</sub> (data not shown), suggesting that these canine strains may have been  
147 uniquely community-associated during that time period. However, our SNP analysis comparing the  
148 canine isolates to other available ST171 whole genome sequence data in GenBank indicated that they  
149 are closely related to historical human clinical isolates from the Northeastern US, the UK, and Colombia.  
150 Together, these observations indicate the diversity regarding the dissemination of ST171 strains  
151 harboring *bla*<sub>KPC</sub>, including transcontinental movement and circulation in the community over multiple  
152 years, with sporadic HAI activity.

153  
154 The role of direct selection pressure by antimicrobial agents associated with the two infected dogs is  
155 unclear. The first dog had a recent history of doxycycline administration for treatment of a chronic  
156 pyoderma, which may have selected for the multidrug resistant *Enterobacter*, as the isolate was also  
157 tetracycline resistant. However, co-selection for carbapenem resistance by tetracycline antibiotics has  
158 not been reported. The second dog had no recent history of antimicrobial drug administration; however,  
159 because the organism and the infection was the result of a dog bite from another dog, it is possible that  
160 the biting dog had undergone antimicrobial selection pressure and inoculated the open wound with the  
161 carbapenem-resistant *Enterobacter*. The dog with the bite wound had not previously been seen at this  
162 facility and the organism was isolated from a specimen acquired on the day of presentation,  
163 approximately two months after the first dog, thus it is reasonable to conclude that there was not a  
164 common nosocomial source. Moreover, medical records indicated that the dogs resided in cities greater  
165 than 240 km apart, suggesting that there is significant regional community dissemination of ST171 in  
166 Ohio.

167

168 Laboratory detection of CRE is a challenge to all clinical diagnostic laboratories, but represents an even a  
169 greater challenge to those in the veterinary setting because of the low prevalence in clinical isolates  
170 from animals. *bla*KPC-4 can be particularly difficult to detect with conventional phenotypic (MIC)  
171 methods because of its low hydrolytic activity (30), which may be compounded by the weak promoter  
172 activity of *TN4401b*, as in these two isolates (31). The isolates described in this study were detected as  
173 part of a surveillance program at a tertiary-referral veterinary hospital that is associated with a  
174 university. Before the implementation of the surveillance program, only conventional AST would have  
175 been performed, and these CRE would not have been detected. Veterinary diagnostic laboratories in the  
176 US typically utilize AST performance standards with susceptibility breakpoints that are based on animal  
177 pharmacokinetics-pharmacodynamics which are published in the CLSI VET01 document (32) and  
178 supplemented with human breakpoints published in the same document when no animal-specific  
179 breakpoints are available. While CLSI VET01 does not mention the need for enhanced detection  
180 methods for carbapenemase activity, its human counterpart, the CLSI M100S27 document, discusses the  
181 need for specific testing (33).

182  
183 As fewer antimicrobial drugs retain their efficacy in the face of the proliferation of multidrug resistant  
184 bacteria, there is a need to take a “One Health” approach that encompasses factors beyond  
185 antimicrobial agent usage and infection control in human medicine. The identification of KPC-encoding  
186 *E. cloacae* complex organisms in a companion animal species is more evidence that there are biological  
187 reservoirs and potentially vectors for transmission to human beings in community circulation. Additional  
188 surveillance work and studies of veterinary antimicrobial usage, as well as increasing the capabilities of  
189 veterinary diagnostic laboratories will be critical to slowing the dissemination of CRE.

190

191



192 **MATERIALS AND METHODS**

193 **Bacterial Isolates** Both isolates, OSUVMCKPC4-1 and OSUVMCKPC4-2, were originally sourced from  
194 clinical veterinary patients (dogs) that were treated at The Ohio State Veterinary Medical Center. The  
195 first dog, seen in July 2016 for acute bacterial cystitis (UTI), was a 13-year old female,  
196 ovariohysterectomized (spayed) Shetland Sheepdog that had a two-month history of chronic kidney  
197 disease and a two-year history of pyoderma. The second dog, seen in September 2016, was a 6-year old  
198 male, castrated, mixed-breed dog that presented for an infected bite wound that resulted from a fight  
199 with another dog. The first dog was an established patient of the hospital for ongoing management of  
200 her chronic health problems and had received doxycycline during the previous year; however, the  
201 second dog had never been seen at the facility prior to presenting with the bite wound (which was  
202 sampled for culture on the day of presentation), and had no recent history of antimicrobial  
203 administration.

204

205 **Identification and Antimicrobial Susceptibility Testing** After routine aerobic cultivation from clinical  
206 specimens, isolates were identified via MALDI-TOF (Biotyper, Bruker Daltonics, Billerica, MA) and  
207 antimicrobial susceptibilities were determined via broth microdilution in accordance with CLSI VET01A4  
208 (COMPGN1F plate, Trek Sensititre, Thermo-Fisher). Carbapenemase production was assessed using a  
209 CarbaNP test (34). Isolates were screened for the presence of the transmissible carbapenemase gene,  
210 *bla<sub>KPC</sub>*, by conventional PCR using previously reported primers (35, 36).

211

212 **Whole Genome Sequencing.** Both isolates were first sequenced using Illumina MiSeq and subsequently  
213 by the PacBio RS II system at the University of Maryland Institute for Genome Sciences (OSUVMCKPC4-  
214 1) and at the University of Delaware Sequencing and Genotyping Center (OSUVMCKPC4-2) using two  
215 SMRT cells per isolate, with genomes assembled using Canu v1.4 (37). Isolate DNA was prepared for

216 PacBio sequencing using a commercial DNA extraction kit (Qiagen Blood & Cell Culture DNA Maxi Kit  
217 (Qiagen, Germantown, MD). Initially, sequences were submitted to the Center for Genomic  
218 Epidemiology website (<https://cge.cbs.dtu.dk>) for Multi Locus Sequence Typing (MLST), plasmid  
219 identification and resistance gene detection using PlasmidFinder and ResFinder (38-40). Additional  
220 antimicrobial resistance databases were used to identify genotypes (41, 42). Identification of insertion  
221 sequences that encode transposases were identified by IS Finder, as well as annotation of the  
222 corresponding IRL, IRR (43). The curated integron database, INTEGRALL (27), identified the class 1  
223 integron. Annotation of plasmid regions of interest were first examined for functional genes with NCBI's  
224 Conserved Domain Database search (44). Regions that contained antimicrobial resistance genes were  
225 then used as query sequences to search the NCBI database with BLAST for similar regions (45).  
226 Sequences are available in GenBank through NCBI (accession numbers CP024908 and CP029246) and  
227 were additionally annotated using the NCBI automated annotation pipeline.

228

229 An additional 16 KPC-producing ST171 genomes from the GenBank Whole Genome Shotgun (WGS)  
230 database were downloaded and compared OSUVMCKPC4-1 and OSUVMCKPC4-2. A core single-  
231 nucleotide polymorphism (SNP) (defined as SNPs shared across all genomes) analysis was conducted  
232 using kSNP3.0 (46), and a core SNP maximum-likelihood tree was produced by RAxML 8.2.4 (47) using  
233 the GTRGAMMA model and 100 bootstrap replicates. Furthermore, BLASTn comparisons between each  
234 isolate's de novo assembly and the reference pOSUVMCKPC4-2 plasmid were conducted using the  
235 method described previously (26), and the phylogenetic tree was annotated using iTOL (48).

236

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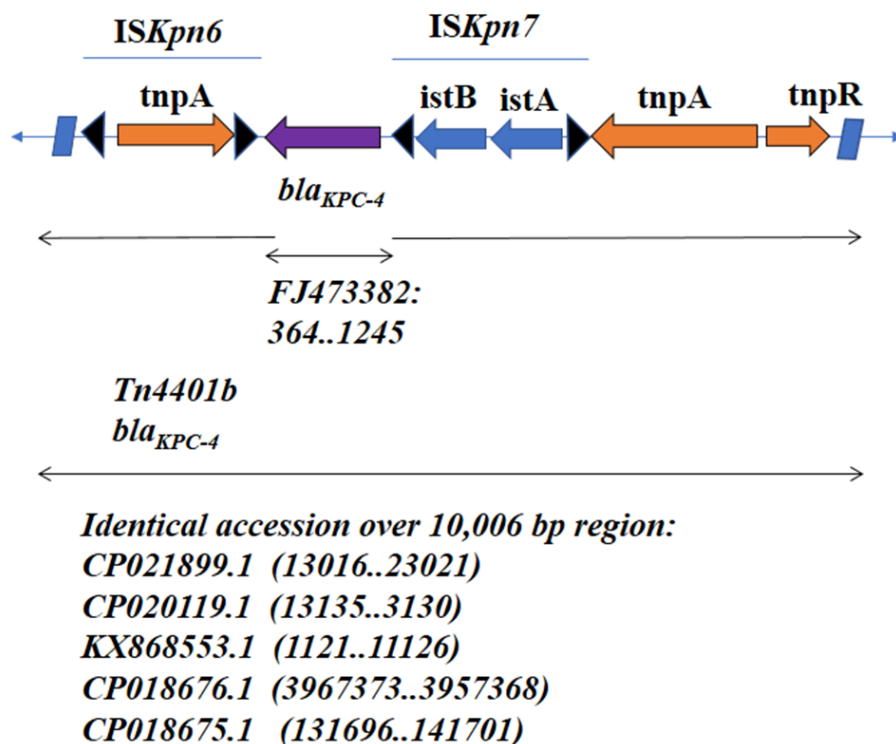
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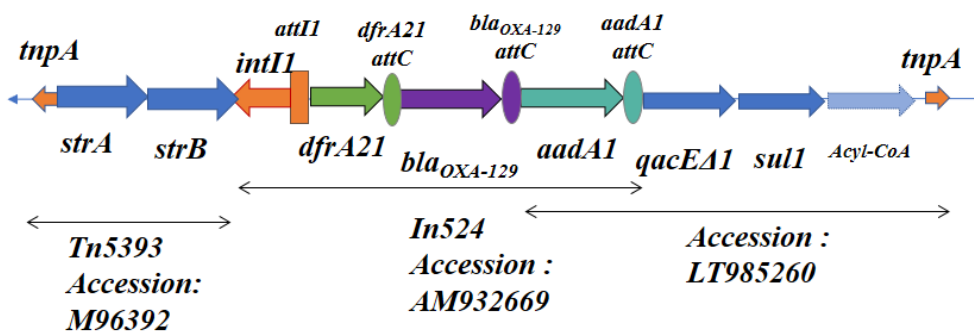


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405 **FIG 2** Schematic structure of Tn4401b containing *bla*<sub>KPC-4</sub> present on large IncH12 plasmids in  
 406 *Enterobacter xiangfangensis* ST171 recovered from two canine patients of The Ohio State University  
 407 Veterinary Medical Center in 2016. Genes and their corresponding transcription orientation are  
 408 indicated by colored horizontal arrows. Corresponding insertion sequences (IS) that encode transposons  
 409 are indicated above the figure with a narrow blue line. Black triangles indicate inverted repeat right and  
 410 left sequences associated with the *ISKpn6* and *ISKpn7*. Flanking parallelograms represent the two  
 411 inverted repeat sequences associated with Tn4401 structures.



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414 **FIG 3** Schematic structure of the In524 class 1 integron and the surrounding region present on large

415 IncH12 plasmids in *Enterobacter xiangfangensis* ST171 recovered from two canine patients of The Ohio

416 State University Veterinary Medical Center in 2016. Genes and their corresponding transcription

417 orientations are indicated by colored horizontal arrows. Homologous alignments to references are

418 indicated with narrow, double sided black arrows with region description and corresponding accession

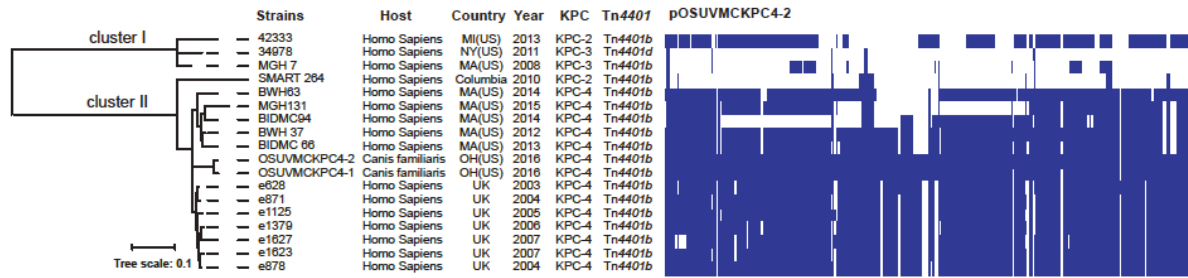
419 number below. Recombination sites, attC, of gene cassettes are illustrated as ovals with color

420 corresponding to the related gene. Integron gene cassettes are incorporated into the rectangular attI1

421 site corresponding to the class 1 integron.

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426 **FIG 4** Heatmap of plasmids among KPC-4 bearing *Enterobacter* ST171 with additional KPC-2 and KPC-3

427 containing ST171 isolates included as references. (Left) Core SNP phylogenetic tree generated by RAxML.

428 (Center) Metadata, including host, isolation location, year, *Tn4401* isoform, and KPC allele. (Right)

429 Plasmid composition is illustrated by showing the BLASTn matches to each *Enterobacter* genome across

430 all of the genes on the pOSUVMCKPC4-2 plasmid.

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432