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MECHANISMS OF RESISTANCE

Enhanced *Klebsiella pneumoniae* Carbapenemase Expression from a Novel Tn*4401* Deletion

Anita Cheruvanky,[a] Nicole Stoesser,[b] Anna E. Sheppard,[b] Derrick W. Crook,[b] Paul S. Hoffman,[a] Erin Weddle,[a,c] Joanne Carroll,[d] Costi D. Sifri,[a] Weidong Chai,[a] Katie Barry,[a] Girija Ramakrishnan,[a] Amy J. Mathers[a,d]

Division of Infectious Diseases and International Health, Department of Medicine, University of Virginia Health System, Charlottesville, Virginia, USA[a]; Modernizing Medical Microbiology Consortium, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Oxford University, Oxford, United Kingdom[b]; Department of Biologic Sciences, Shenandoah University, Winchester, Virginia, USA[c]; Clinical Microbiology, Department of Pathology, University of Virginia Health System, Charlottesville, Virginia, USA[d]

ABSTRACT The *Klebsiella pneumoniae* carbapenemase gene (bla_{KPC}) is typically located within mobile transposon Tn*4401*. Enhanced KPC expression has been associated with deletions in the putative promoter region upstream of bla_{KPC}. Illumina sequences from bla_{KPC}-positive clinical isolates from a single institution were mapped to a Tn*4401*b reference sequence, which carries no deletions. The novel isoform Tn*4401*h (188-bp deletion [between *istB* and bla_{KPC}]) was present in 14% (39/281) of clinical isolates. MICs showed that *Escherichia coli* strains containing plasmids with Tn*4401*a and Tn*4401*h were more resistant to meropenem (≥16 and ≥16, respectively), ertapenem (≥8 and 4, respectively), and cefepime (≥64 and 4, respectively) than *E. coli* strains with Tn*4401*b (0.5, ≤0.5, and ≤1, respectively). Quantitative real-time PCR (qRT-PCR) demonstrated that Tn*4401*a had a 16-fold increase and Tn*4401*h a 4-fold increase in bla_{KPC} mRNA levels compared to the reference Tn*4401*b. A *lacZ* reporter plasmid was used to test the activity of the promoter regions from the different variants, and the results showed that the Tn*4401*a and Tn*4401*h promoter sequences generated higher β-galactosidase activity than the corresponding Tn*4401*b sequence. Further dissection of the promoter region demonstrated that putative promoter P1 was not functional. The activity of the isolated P2 promoter was greatly enhanced by inclusion of the P1-P2 intervening sequence. These studies indicated that gene expression could be an important consideration in understanding resistance phenotypes predicted by genetic signatures in the context of sequencing-based rapid diagnostics.

KEYWORDS KPC, Tn*4401*, carbapenem-resistant *Enterobacteriaceae*, gene expression, meropenem, promoters

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Address correspondence to Amy J. Mathers, ajm5b@virginia.edu.

Carbapenem-resistant *Enterobacteriaceae* (CRE) are an urgent public health threat because of their increasing incidence and the high mortality seen with infection (1). Currently, the most commonly reported mechanism of carbapenem resistance in clinical *Enterobacteriaceae* is *Klebsiella pneumoniae* carbapenemase (KPC), which has disseminated globally over the last decade, with infections becoming endemic in several geographic locations such as Italy, Greece, and the United States (2). KPC is a class A serine beta-lactamase which can hydrolyze penicillins, cephalosporins, aztreonam, and carbapenems, limiting treatment options in infected patients. The KPC gene (bla_{KPC}) is typically located within a 10-kb mobile transposon (Tn*4401*), which is most often situated on conjugative plasmids (3, 4). The association of bla_{KPC} with mobile

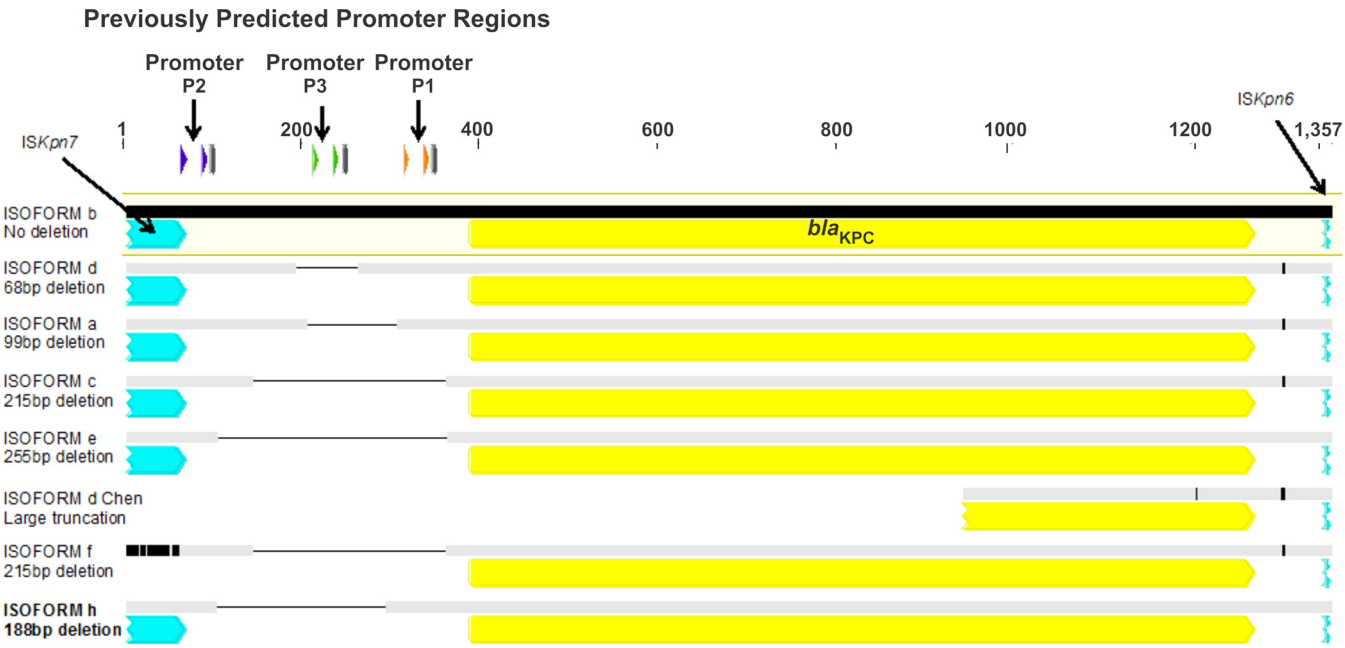

FIG 1 Structural variation in published isoforms of the Tn*4401* transposons. The sequence highlighted in yellow denotes the reference Tn*4401*b sequence (EU176013). Gray sequence bars denote areas of 100% identity to the reference; black sequence bars denote areas of nucleotide variation.

genetic elements such as plasmids and transposons contributes to intraspecies gene transfer and dissemination of carbapenem resistance (2, 5–8).

Tn*4401* is composed of bla_{KPC}, a transposase gene (*tnpA*), a resolvase gene (*tnpR*), and two insertion sequences, IS*Kpn6* and IS*Kpn7*, all flanked by two 32-bp inverted repeat sequences (3). To date, seven unique Tn*4401* isoforms (Tn*4401*a to Tn*4401*g) have been identified (9–12), with Tn*4401*a and Tn*4401*b being the most widespread (13). The original characterization of these isoforms demonstrated deletions in the noncoding region upstream of bla_{KPC} or lacking genes or both as follows: a, −99 bp (14, 15); b, no deletion (3); c, −215 bp (16); d, −68 bp (9) and −5.3 kb (9, 17, 18); e, −255 bp (17); f, truncated *tnpA* and lacking *tnpR*, IS*Kpn7* left, and Tn*4401* IRL-1 (10); g, lacking *tnpA*, *tnpR*, and IS*Kpn7* left, with a −215-bp deletion in the noncoding region (Fig. 1). Most recently, a truncated version of Tn*4401*e, lacking *tnpA* and *tnpR*, was also described (19).

Previously, the noncoding region between IS*Kpn7* and bla_{KPC} was proposed to contain three putative promoter regions (P1, P2, and P3); P1 and P2 were demonstrated to affect expression, while P3 was shown to not be a real promoter (5, 8, 20). Deletions in the noncoding region upstream of bla_{KPC} have demonstrated variable expression of KPC *in vitro*, with the highest levels of expression observed with Tn*4401*a (presence of P1 and P2). However, the relative contributions of P1 and P2 and regions of associated flanking sequences to KPC expression have not been fully characterized.

Here, we describe a novel Tn*4401* transposon variant (Tn*4401*h) which has a unique 188-bp deletion in the noncoding region upstream of bla_{KPC} and, similarly to Tn*4401*a, retains promoters P1 and P2. We explore the effect of this 188-bp deletion on the level of KPC expression and the degree of phenotypic resistance compared to the most frequently seen and best characterized isoforms, Tn*4401*a and Tn*4401*b (4), and further assess promoter activity using fusions to a *lacZ* reporter. Understanding the impact of genetic variability in noncoding and/or promoter regions on resistance gene expression and its correlation with MICs will be important for the use of sequencing-based approaches in clinical diagnostics and antimicrobial susceptibility prediction (8, 21).

RESULTS

The molecular epidemiology of Tn*4401* in the study isolates. The Tn*4401* sequence was identified in 281 isolates across eight genera, mainly represented by *Klebsiella* spp.

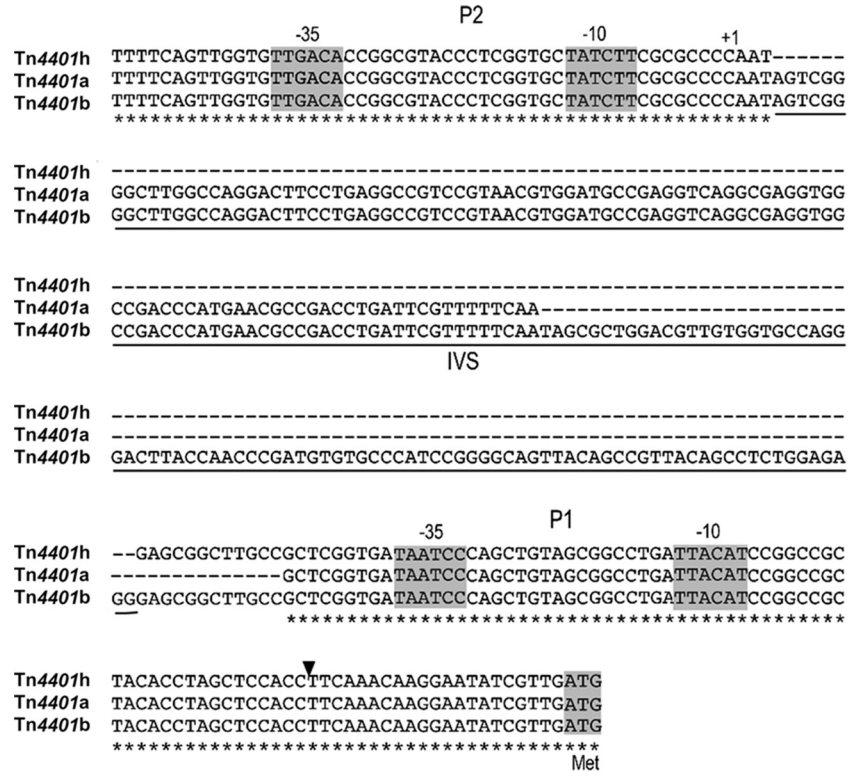

FIG 2 Alignment of the promoter regions of bla_{KPC} isoforms with putative promoters as defined by Naas et al (20). −35 regions and −10 regions are highlighted in gray. +1 indicates the P2 transcription start site previously identified by RACE (rapid amplification of cDNA ends). Isoforms a and h have deletion differences in the intervening sequence between P2 and P1. The extent of the intervening sequence that is deleted in isoform h is underlined and denoted the IVS. The start codon of bla_{KPC} is highlighted in gray. The arrowhead indicates the site of fusions to the *lacZ* reporter.

($n = 129$), *Enterobacter* spp. ($n = 101$), and *Citrobacter* spp. ($n = 32$), with bla_{KPC-2} found in the majority (230/281; 82%) of the isolates as previously characterized (6). The same work also reported that the most common Tn*4401* variant was Tn*4401*b ($n = 230$); Tn*4401*a was seen in 8 isolates (all *K. pneumoniae*), and a novel variant (Tn*4401*h) (all with bla_{KPC-2} [Fig. 1]) with a 188-bp deletion was identified in 39 isolates from 28 patients. Tn*4401*h was first identified in a *K. pneumoniae* strain (February 2009); all other Tn*4401*h variants were identified in highly genetically related *Enterobacter cloacae* isolates from all 28 patients (including the patient with the initial *K. pneumoniae* isolate with Tn*4401*h) over 3 years (May 2009 to December 2012), consistent with the idea of local interspecies and interpatient transmission (6).

Structural variation in published isoforms of Tn*4401* and the novel Tn*4401*h isoform, including variation in IS*Kpn6*, IS*Kpn7*, and the putative promoter (P1, P2, and P3) regions, is shown in Fig. 1 (9). Notably, isoforms a and h do not contain putative promoter P3 and have nucleotide variation in the intervening sequence (IVS) between P2 and P1 (Fig. 2).

Susceptibility testing. Susceptibility results for selected parent strains and *Escherichia coli* transformants from CAV1016, CAV1746, and CAV1438 containing Tn*4401*b, Tn*4401*a, and Tn*4401*h, respectively, are presented in Table 1. Parent strains CAV1746 (Tn*4401*a) and CAV1438 (Tn*4401*h) were resistant to cefepime and meropenem by broth dilution and VITEK2 (Table 1). CAV1016 (Tn*4401*b) was resistant to meropenem by broth dilution and VITEK2 but was susceptible to cefepime by VITEK2.

Testing of susceptibility to cefepime and meropenem of *E. coli* transformants containing Tn*4401*a or Tn*4401*h by VITEK2 and broth dilution demonstrated MICs that were all in the resistant range. For the Tn*4401*b *E. coli* transformant, cefepime results

TABLE 1 MICs for parent strains CAV1016, CAV1746, and CAV1438 and *E. coli* transformants AMGH-1, ACGH, and ACGH2 containing plasmids bearing Tn*4401*b, Tn*4401*a, and Tn*4401*h, respectively, from the parent strains[a]

Strain	Tn*4401* variant	Plasmid size, PCR replicon type, putative plasmid backbone (reference)	Species	MIC (μg/ml)				
				VITEK2			Broth microdilution	
				Meropenem	Ertapenem	Cefepime	Meropenem	Cefepime
CAV1016	Tn*4401*b		*K. pneumoniae*	≥16	4	2	256	64
CAV1746	Tn*4401*a		*K. pneumoniae*	≥16	≥8	≥64	1,024	512
CAV1438	Tn*4401*h		*E. cloacae*	≥16	≥8	≥64	1,024	512
E. coli transformed strain (parent strain)								
AMGH-1 (CAV1016)	Tn*4401*b	43 kb, nontypeable, confirmed pKPC_UVA01 (CP017937.1) (22)	*E. coli*	0.5	≤0.5	≤1	1	8
ACGH (CAV1746)	Tn*4401*a	>60 kb, IncR, putatively resembles pKPC-484 (CP008798.1)	*E. coli*	≥16	≥8	≥64	16	128
ACGH2 (CAV1438)	Tn*4401*h	>70 kb, IncR, putatively resembles pKPC_CAV1176 (CP011661.1) (6)	*E. coli*	≥16	4	4	4	64

[a]MICs were determined using the VITEK2 automated susceptibility testing platform and broth microdilution.

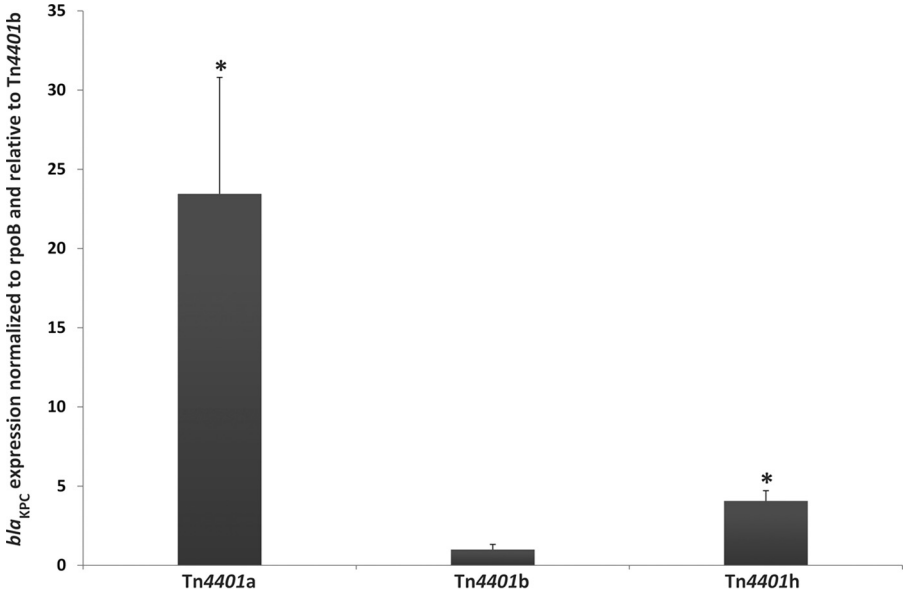

FIG 3 Differences in bla_{KPC} expression normalized to *rpoB* and relative to Tn*4401*b. Tn*4401*a had significantly higher β-galactosidase activity than Tn*4401*b and Tn*4401*h ($P < 0.05$), and the Tn*4401*h putative promoter sequence had significantly higher β-galactosidase activity than Tn*4401*b ($P < 0.05$).

were in the susceptible dose-dependent (SDD) range and meropenem results were in the susceptible range by broth dilution. By VITEK2, both cefepime and meropenem demonstrated MICs which were in the susceptible range (2015 CLSI breakpoints; 23) (Table 1). The plasmid backbones for each transformant were unique, but both Tn*4401*h and Tn*4401*a were on large IncR plasmids whereas Tn*4401*b was on a nontypeable 43-kb plasmid (pKPC_UVA01) (Table 1).

For parent strains CAV1016, CAV1746, and CAV1438 and for the *E. coli* transformants, carbapenemase production was indicated by the results of both the modified Hodge test and the indirect carbapenemase test (24).

Expression assays. Two-step quantitative real-time PCR (qRT-PCR) was performed on all three *E. coli* transformants using primers as noted in Table S1 in the supplemental material. Fold differences in bla_{KPC} expression (normalized to *rpoB*) were quantified relative to Tn*4401*b. Tn*4401*a had 23-fold-greater bla_{KPC} expression than Tn*4401*b (Student *t* test, $P = 0.011$) (Fig. 3). Tn*4401*h had 4-fold-greater bla_{KPC} expression than Tn*4401*b (Student *t* test, $P = 0.03$) (Fig. 3).

Transcription fusions to the *lacZ* reporter in plasmid pRS551 (25) of the putative promoter sequences from plasmids carrying Tn*4401*a, Tn*4401*b, and Tn*4401*h (Table S1) (Fig. 4) were used to quantify β-galactosidase activity associated with each Tn*4401* variant. The promoter sequence of Tn*4401*a had significantly higher β-galactosidase activity than Tn*4401*b (Student *t* test, $P = 0.0001$) and Tn*4401*h (Student *t* test, $P = 0.0002$), and the Tn*4401*h putative promoter sequence had significantly higher β-galactosidase activity than Tn*4401*b (Student *t* test, $P < 0.0001$) (Fig. 4). These results were consistent with the effect that each deletion had on the level of bla_{KPC} mRNA in the transformants (Fig. 3).

Tn*4401*h is missing an intervening sequence (IVS) of 188 nucleotides between P1 and P2, whereas Tn*4401*a carries a smaller deletion in this region (Fig. 2 and 4A). To better discern the contributions of the different sequence domains to gene expression, *lacZ* fusions were constructed of the isolated P1 and P2 promoters as well as of these promoter regions carrying the IVS. Reporter constructs containing P1 or P1 plus IVS (P1+IVS) showed no β-galactosidase activity. Reporter constructs containing P2 showed significantly higher β-galactosidase activity than those containing vector (Student *t* test, $P = 0.00025$) or P1 (Student *t* test, $P = 0.0034$) and P1+IVS (Student-*t* test, $P = 0.0020$). Reporter constructs containing P2+IVS had significantly higher

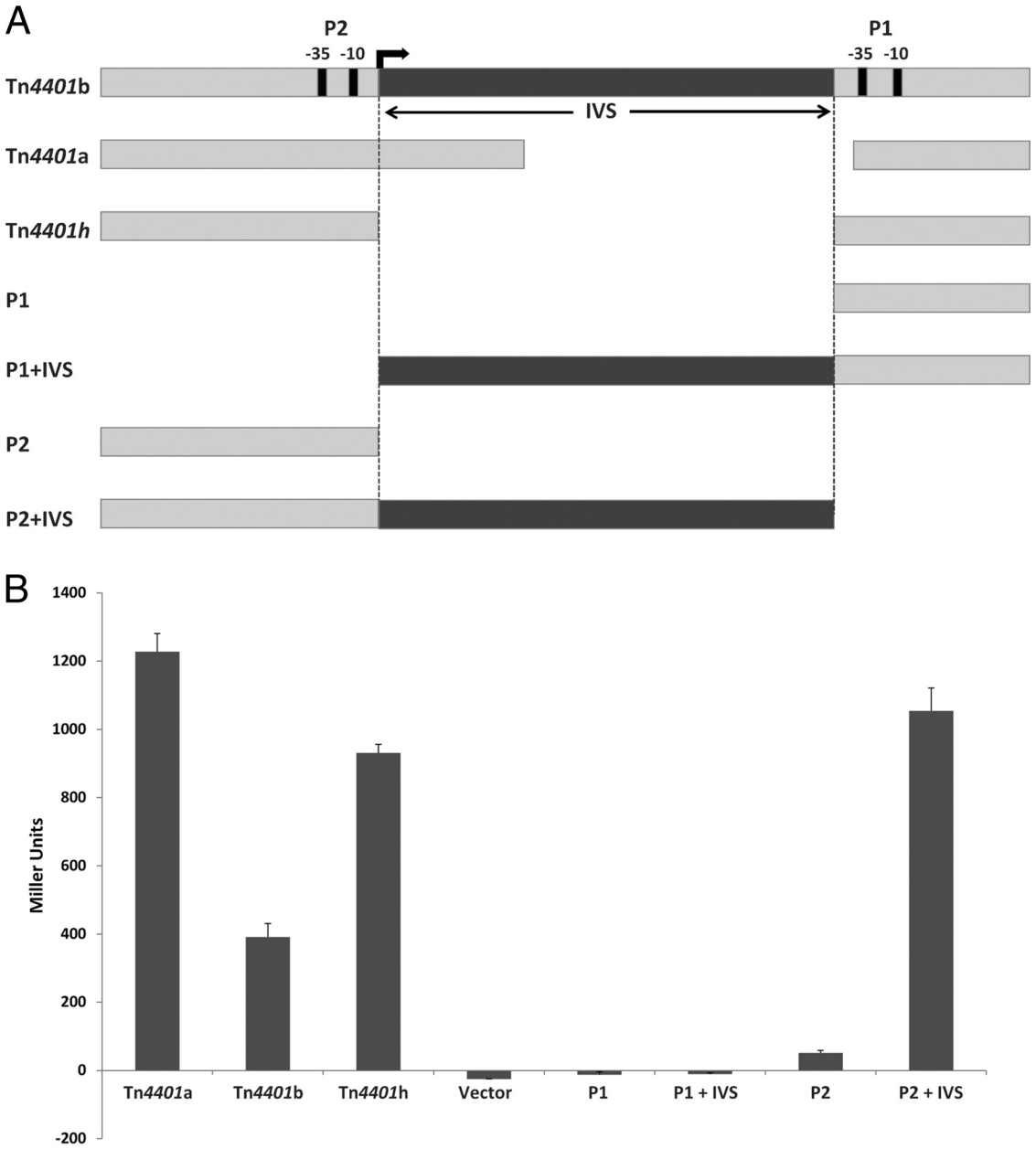

FIG 4 (A) Schematic to explain the constructs, showing the promoter regions cloned in *lacZ* fusions. The arrow indicates the P2 transcription start site. (B) β-Galactosidase activity level associated with putative promoter regions of Tn*4401*a, Tn*4401*b, and Tn*4401*h isoforms (putative promoters P1, P1+IVS, P2, and P+IVS). *lacZ* reporter plasmid pRS551 (vector) served as a negative control. Among the reporter constructs, P2 had significantly higher β-galactosidase activity than vector, P1, and P1+IVS ($P < 0.05$); P2+IVS had significantly higher β-galactosidase activity than P2, P1, and P1+IVS (Student *t* test, $P < 0.05$); and promoter sequence Tn*4401*a had significantly higher β-galactosidase activity than P2+IVS ($P < 0.05$).

β-galactosidase activity than those containing P2 alone (Student *t* test, $P = 0.015$), P1 (Student *t* test, $P < 0.001$), and P1+IVS (Student *t* test, $P < 0.001$) (Fig. 4). Additionally, reporter constructs containing promoter sequence Tn*4401*a had significantly higher β-galactosidase activity than reporter constructs containing P2+IVS (Student *t* test; $P = 0.006$).

DISCUSSION

As KPC-producing *Enterobacteriaceae* are seen in increasing numbers around the globe, understanding expression of bla_{KPC} and its effect on the degree of associated carbapenem resistance could have significant clinical consequences. The Tn*4401* trans-

poson has been the most consistently observed unit of bla_{KPC} transfer, and the noncoding region upstream of bla_{KPC} has been described as a regulatory region (3, 22, 26). We describe a novel isoform of Tn*4401*, Tn*4401*h, which was first identified in a *K. pneumoniae* strain in a patient who also had an *E. cloacae* strain that was subsequently transmitted between several patients over a period of 3 years in our institution.

The 188-bp deletion of Tn*4401*h, encompassing the region between P1 and P2, demonstrated increased bla_{KPC} expression compared with Tn*4401*b (no deletions), although to a lesser degree than Tn*4401*a (99-bp deletion; with a smaller deletion between P1 and P2). To exclude the impact of variability of gene copy number and plasmid background on these results, we directly investigated the impact of promoter regions and intervening sequences on gene expression in a model system by inserting these promoter regions and promoter/intervening sequence combinations into a *lacZ* reporter.

The Tn*4401*h promoter region showed significantly higher β-galactosidase activity than Tn*4401*b, again to a lesser degree than the Tn*4401*a promoter region and consistent with the bla_{KPC} expression results (Tn*4401*a $\rightarrow$ Tn*4401*h $\rightarrow$ Tn*4401*b). Given that both Tn*4401*a and Tn*4401*h have deletions in the intervening sequence between P1 and P2, with P1 and P2 intact, the differential levels of gene expression and promoter activity remain incompletely explained. One possible explanation for this variability is that the differing lengths of the intervening sequences between P2 and P1 for Tn*4401*a (99-bp deletion) and Tn*4401*h (188-bp deletion) may result in a more stable RNA structure for Tn*4401*a.

Previously, Naas et al. reported only P1 and P2 as true promoters involved in bla_{KPC} expression. A review of the -10 and -35 regions of the promoter P1 and P2 sequences shows that the P2 promoter (initially identified by Roth [27]) has a strong -35 consensus sequence (TTGACA) and only two minor differences (indicated with lowercase characters) from the canonical -10 sequence (TATctT), while the P1 promoter has a poor consensus sequence, with 4 differences from the canonical -35 sequence (TaatCc) and 3 differences from the canonical -10 sequence (TtacAT). However, the relative contributions of putative promoter P1 by itself and in relation to the IVS remained previously unknown. Hence, promoter sequences for P1 alone and in combination with IVS were cloned into a *lacZ* reporter plasmid. Surprisingly, the reporter constructs containing P1 and P1+IVS showed no β-galactosidase activity, indicating that this promoter was not active under our assay conditions. We additionally attempted to characterize the relative contributions of promoter P2 alone and in combination with IVS. Higher β-galactosidase activity was observed with the promoter P2 *lacZ* construct than with P1, and this was further enhanced when IVS was included. These results are consistent with findings of Naas et al. (20). The exact role of IVS in the enhanced activity of P2+IVS compared to P2 will require future evaluation, and the data would ideally reflect variations in IVS length.

E. coli transformants containing Tn*4401*a and Tn*4401*h variants on native parent plasmid backgrounds showed meropenem and cefepime resistance MICs compared to *E. coli* transformants containing Tn*4401*b, consistent with the variations in bla_{KPC} expression (i.e., Tn*4401*a $\rightarrow$ Tn*4401*h $\rightarrow$ Tn*4401*b). Previous studies have suggested that another mechanism, in addition to gene expression and copy number, namely, production of porin channel defects, is a primary contributor to the variability in susceptibility in testing KPC-producing isolates (3, 11, 20, 28–30). Here we excluded the impact of porin channel variation on susceptibility by evaluating plasmids bearing the Tn*4401*-bla_{KPC} units in a fixed *E. coli* background. Although we did not exclude gene copy number as a factor contributing to expression, as the different Tn*4401* variants were located on unique plasmid backbones, the differences in the levels of promoter activity seen in the β-galactosidase assay align with the differences seen in the *E. coli* transformants. In addition, Roth et al. previously showed that in bla_{KPC-2} transformants, bla_{KPC} gene copy number did not correlate with expression and increases in β-lactam MICs; similarly, Kitchel et al. demonstrated that factors other than bla_{KPC} copy number likely contribute to elevated KPC production and high-level carbapenem resistance in

certain isolates (26, 27). Taken together, these data suggest that the promoter region upstream of bla_{KPC} appears to have the most substantial effect on expression of carbapenem resistance in the transformants. Future studies utilizing methods such as chromatin immunoprecipitation with sequencing (ChIP-sequencing) could be used to examine the interaction of transcription factors and thus offer further insight into regulation of bla_{KPC} expression.

By characterizing the Tn*4401*h isoform, we have highlighted that the promoter region may be more complex than previously described. Correctly ascertaining the role of promoter regions and 5′ untranslated regions in resistance gene expression is of particular relevance to clinical diagnostic microbiology given the development of an increasing number of genotype-based approaches (e.g., microarray, multiplex, or whole-genome sequencing) to resistance prediction (31–35). There is a trend toward incorporating rapid genotypic methods into routine laboratory diagnostics, and yet there is increasing recognition of the importance of pharmacokinetics/pharmacodynamics in relation to the MIC. Therefore, understanding the predicted impact of promoter regions on the MIC may be of increasing importance to clinical care.

In summary, we have found a novel isoform, Tn*4401*h, with a 188-bp deletion in the upstream noncoding region between IS*Kpn7* and bla_{KPC}. We have shown that this deletion has a differential effect on carbapenem MICs compared with other common Tn*4401* variants. This work increases our understanding of the complexity of bla_{KPC} expression and has implications for predicting the degree of resistance based solely on the presence or absence of a particular resistance gene.

MATERIALS AND METHODS

Sampling. The sampling of bla_{KPC}-positive *Enterobacteriaceae* clinical and surveillance isolates from the University of Virginia Health System (UVaHS) between August 2007 and December 2012 has been previously published (6). Sheppard et al. performed Illumina sequencing on the collected isolates and determined the Tn*4401* isoform of each isolate using BLASTn comparisons between the isolate's *de novo* assembly and the Tn*4401*b reference sequence (EU176013.1) (3, 6). Where available, ertapenem MIC results from a VITEK2 AST GN-70 test kit (bioMérieux, Durham, NC) were obtained retrospectively from laboratory records.

Purified plasmid DNA was electroporated into electrocompetent *E. coli* Genehog cells (Invitrogen, Carlsbad, CA) as previously described (22). To examine the plasmid background in the newly generated transformant incompatibility group, PCR typing was performed (36). For CAV1746 and CAV1438 parent strains (those with unknown bla_{KPC} plasmids), the Illumina contigs containing the corresponding replicon sequences (1,960 bp and 15,241 bp, respectively) and the contigs containing Tn*4401* (18,813 bp and 10,011 bp, respectively) were used as BLASTn queries to determine similarity to previously described plasmids in GenBank. For CAV1016, the bla_{KPC} plasmid background has been previously described (22). For plasmid size estimation, BamHI and EcoRI (New England BioLabs, Ipswich, MA) digested and undigested plasmid extractions were run on a 0.8% agarose gel over 8 h at 70 V with V517 and Hyperladder I (Bioline, Taunton, MA) to estimate plasmid size as previously described (37).

Quantitative real-time PCR (qRT-PCR) experiments. Total RNA was extracted from *E. coli* transformants containing parent plasmid Tn*4401*a, Tn*4401*b, and Tn*4401*h using an RNeasy minikit (Qiagen, GmBH, Hilden, Germany). RNA products underwent column DNase I digestion (Qiagen) and were quantified by measuring the optical density at 260 nm using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized from RNA using a commercial method (qScript cDNA Supermix; Quanta Biosciences, Gaithersburg, MD) in accordance with the manufacturer's instructions.

bla_{KPC} gene expression was examined in *E. coli* transformants utilizing qRT-PCR and the synthesized cDNA (primers are listed in Table S1 in the supplemental material). A 25-ng volume of RNA-equivalent DNA was used in triplicate. qRT-PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA), template DNA, and 500 nM (each) primer RT-KPC-F/RT-KPC-R or EcoliRPOB-F/EcoliRPOB-R (Table S1). Cycling conditions included a 3-min enzyme activation step at 95°C followed by 40 cycles of melting (95°C for 10 s) and annealing/extension (56.3°C for 30 s/72°C for 30 s) followed by a final extension for 72°C for 1 min. Standard curves were generated for both the target (bla_{KPC}) and the endogenous control (*rpoB*) using 4-fold dilutions of template DNA at known concentrations (from 0.39 ng to 100 ng) and by plotting the logarithm of the initial quantity of template (along the *x* axis) versus the respective cycle threshold (C_T) values (along the *y* axis). Thereafter, the amount of bla_{KPC} and *rpoB* was calculated from the appropriate standard curve for each experimental sample. Expression levels of bla_{KPC} were first normalized relative to the *rpoB* gene in *E. coli*, and fold difference in bla_{KPC} expression was calculated as follows: (normalized bla_{KPC} expression in *E. coli* transformant Tn*4401*a or Tn*4401*h)/(normalized bla_{KPC} expression in *E. coli* transformant Tn*4401*b).

PCR and cloning. Online ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was utilized to align promoter regions of isoforms Tn*4401*a, Tn*4401*b, and Tn*4401*h. Primers (F-ISKpn7, R-prom)

containing restriction sites for BamHI and EcoRI (New England BioLabs, Ipswich, MA) were used to amplify putative promoter sequences from purified plasmids carrying Tn*4401*a, Tn*4401*b, and Tn*4401*h (Table S1). Primers were also used to amplify putative promoters P1 (P1Eco-F, R-prom), P1 plus the intervening sequence (IVS; i.e., the sequence intervening between P1 and P2 that is missing in Tn*4401*h) (P1+IVS Eco-F, R-prom), P2 (F-ISKpn7; P2 Bam –R), and P2+IVS (which is in F-ISKpn7; P2+IVS Bam –R), in order to assess the relative activities of putative promoters P1 and P2 alone and in combination with the IVS (Fig. 2 and 4; see also Table S1). All amplified PCR products were ligated into *lacZ* reporter plasmid pRS551 (25) after restriction digestion was performed using BamHI and EcoRI. The ligated constructs and *lacZ* reporter plasmid pRS551 were transformed into *E. coli* DH10B calcium-competent cells and then grown on ampicillin (100 μg/ml)-selective LB plates. Plasmids isolated from transformants were screened by PCR, and all 7 ligated constructs were sequenced using primer (pRS551-R sequencing; Table S1) to confirm the proper orientation and nucleotide sequence of the inserted regions.

β-Galactosidase assay. The protocol for the assay of β-galactosidase activity was adapted from Griffith and Wolf (38). A 5-μl volume of the overnight culture of the DH10B *E. coli* transformants was added to 3 ml LB broth containing ampicillin (50 μg/ml), and the cultures were incubated on a platform shaker at 37°C until the culture reached an optical density at 600 nm of 0.4 to 0.5.

For cell permeabilization to release the enzyme, 1 ml of Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1 mM $MgSO4_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol) was added along with 20 μl of freshly prepared 0.1% SDS and 40 μl of chloroform to100 μl of bacterial culture. Permeabilization was achieved by subjecting test tubes containing the cell-chloroform/SDS mixture to vortex mixing (one at a time) for 30 s followed by a 10-min settling period.

A 100-μl volume of supernatant from each tube was divided into aliquots in quadruplicate and placed into a flat-bottom 96-well plate. At time zero, the assay was initiated by using a multichannel pipette to add 20 μl of o-nitrophenyl-β-D-galactopyranoside (ONPG; 4 mg/ml). Once the yellow chromophore was visualized, the reaction was terminated by the addition of 50 μl of 1 M Na_2CO_3 and then absorbance at A_{420} and A_{550} was measured. β-Galactosidase activity was calculated in Miller units from the absorbance data as follows: Miller units $= 1{,}000 \times [A_{420} - (1.75 \times A_{550})]/(T \times V \times A_{600})$. A_{420} and A_{550} are read from the reaction mixture, A_{600} reflects cell density in the mid-logarithmic phase, T represents the time of the reaction in minutes before addition of 1 M Na_2CO_3, and V represents the volume of the culture used in the assay in milliliters (38).

Statistical analysis. A paired-sample Student *t* test was conducted to compare the levels of bla_{KPC} expression of *E. coli* transformants containing parent plasmids Tn*4401*a, Tn*4401*b, and Tn*4401*h. A paired-sample Student *t* test was also used to compare the levels of β-galactosidase activity of *lacZ* fusion constructs containing Tn*4401*a, Tn*4401*b, Tn*4401*h, P1, P1+IVS, P2, or P2+IVS.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00025-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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