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Selection of SHV Extended-Spectrum-β-Lactamase-Dependent Cefotaxime and Ceftazidime Resistance in *Klebsiella pneumoniae* Requires a Plasmid-Borne bla_{SHV} Gene▽

David S. Hammond,[1]† Tegan Harris,[1]† Jan Bell,[2] John Turnidge,[2] and Philip M. Giffard[1]*

Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Queensland,[1] and Women's and Children's Hospital, Adelaide, South Australia,[2] Australia

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In *Klebsiella pneumoniae*, it is common for plasmid-located and chromosome-located bla_{SHV} copies to coexist within single cells. The plasmid-borne genes are mainly derived from two separate IS*26*-mediated mobilizations of bla_{SHV}. The objective of this study was to test the hypothesis that the presence of a non-extended-spectrum β-lactamase (non-ESBL) encoding plasmid-borne form of bla_{SHV} facilitates the cefotaxime (CTX)-mediated selection of ESBL-expressing mutants, even when there is a chromosomal copy of the same gene. Twenty-one diverse ESBL-negative, bla_{TEM}-negative *K. pneumoniae* clinical isolates were tested for the IS*26* insertions characteristic of the two mobilization events. The isolates were then tested for their ability to be selected for ESBL-mediated CTX resistance by serial subculturing with a doubling of the CTX concentration at every subculture. Fourteen isolates possessed neither of the IS*26* insertions. None of these became ESBL positive, and all died during the course of the experiment, despite possessing chromosomal bla_{SHV} copies. The other isolates all became ESBL positive and grew abundantly up to a CTX concentration of 128 μg/ml. Similar results were obtained with ceftazidime. ESBL expression was associated with the appearance of the expected G→A mutation at position 1 of codon 238 and also with bla_{SHV} copy number amplification. It was concluded that plasmid-borne bla_{SHV} greatly facilitates the selection of ESBL expression, even when the same gene is on the chromosome, and that gene dosage effects are likely to contribute to this phenomenon.

Extended-spectrum β-lactamases (ESBLs) confer resistance to expanded-spectrum cephalosporins and monobactams due to their ability to hydrolyze these compounds. Many ESBLs are derived from non-ESBL precursors by point mutation of *bla* genes, and the prevailing assumption is that these variants are selected by exposure to expanded-spectrum cephalosporins and/or monobactams in health care facilities (3, 9). The most intensively studied ESBLs belong to the SHV and TEM families, although other families such as CTX-M are becoming increasingly prominent (3, 9). In the SHV family, ESBL activity is most frequently associated with a Gly-to-Ser substitution at residue 238 (G238S), and activity is frequently increased by a Glu-to-Lys substitution at residue 240 (E240K). The TEM situation is much more complex and less frequently involves Gly238 (3, 9, 16).

The great majority of *Klebsiella pneumoniae* possess a chromosomal copy of either bla_{SHV-1} or bla_{SHV-11} or close relatives (2, 4). bla_{SHV-1} and bla_{SHV-11} both encode non-extended-spectrum enzymes, which differ from each other only at residue 35. There is strong evidence that all extant plasmid-borne bla_{SHV} are descended from one of two *K. pneumoniae* genome-to-plasmid mobilization events. Both mobilizations appear to have been mediated by IS*26* (8). One instance resulted in an IS*26* insertion 2 kbp upstream of the bla_{SHV} coding sequence. The plasmid-borne genes encoding SHV-1, SHV-2, and SHV-5 possess this structure (10). The other instance resulted in IS*26* insertion into the bla_{SHV} promoter, and the plasmid-borne genes encoding SHV-11, SHV-2a, and SHV-12 possess this structure (20). It has been reported by Podbielski et al. (22) that this IS*26* insertion increases promoter strength through the introduction of a different −35 site. In the interests of clarity, we refer to the insertion sequence-free bla_{SHV} promoter sequence as "pr-bla_{SHV}" and to the promoter with the IS*26* insertion as "pr::IS*26*-bla_{SHV}."

In previous work using 21 *K. pneumoniae* isolates (13 ESBL positive) from the Princess Alexandra Hospital (PAH) in Brisbane, Australia (11, 13), it was found that the ESBL-negative isolates possess the non-ESBL-encoding bla_{SHV-11} and that the ESBL-positive isolates possess both bla_{SHV-11} and the ESBL-specifying bla_{SHV-2a}, which encodes the same sequence except for the G→S substitution at residue 238. The ESBL-positive isolates with the highest MICs for expanded-spectrum cephalosporins also possess the bla_{SHV-12} gene, which encodes the same sequence as bla_{SHV-2a} except for the E→K substitution at residue 240 (16). It was of interest that all ESBL-positive isolates possessed mixtures of alleles, with some isolates possessing all three bla_{SHV} variants. In addition, acquisition of high levels of resistance was associated with copy number amplification of the ESBL-encoding bla_{SHV} alleles (11).

The results obtained by Howard et al. (13) and Hammond et al. (11) are not fully consistent with the simple model that the primary mechanism for acquisition of ESBL expression is by acquisition of an ESBL-encoding conjugative plasmid. Rather, the complex mixtures of alleles found within individual iso-

* Corresponding author. Mailing address: Institute of Health and Biomedical Innovation, Queensland University of Technology, Cnr Blamey St. and Musk Ave., Kelvin Grove, Queensland 4059, Australia. Phone: 61 7 31386194. Fax: 61 7 38641534. E-mail: p.giffard@qut.edu.au.

† D.S.H. and T.H. contributed equally to this study.

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lates, including the frequent detection of the non-ESBL-encoding bla_{SHV-11} in *cis* with pr::IS*26*-bla_{SHV}, are indicative of dissemination of a plasmid-borne bla_{SHV-11} and repeated parallel selection of ESBL-encoding bla_{SHV} alleles. Further evidence for this is the close relationships between pr::IS*26*-bla_{SHV}-carrying ESBL-positive and ESBL-negative isolates as determined by pulsed-field gel electrophoresis (11). Given that bla_{SHV} is a normal constituent of the *K. pneumoniae* chromosome, this begs the question as to the capacity of the chromosomal bla_{SHV} copy to contribute to the acquisition of ESBL expression. The observations of Hammond et al. (11) suggest that this is limited; cloning and characterization of PCR products showed that in ESBL-positive isolates, a bla_{SHV} gene in *cis* with pr-bla_{SHV} was much less likely to be mutated than one in *cis* with pr::IS*26*-bla_{SHV}. We have therefore hypothesized that the presence of a plasmid harboring a non-ESBL encoding bla_{SHV} facilitates the acquisition of ESBL-mediated resistance by *K. pneumoniae*, even when there is a chromosomal copy of the same gene. This has clinical significance if correct, since it would mean that the presence of a non-ESBL encoding plasmid-borne bla_{SHV} would increase the risk of selecting ESBL-mediated resistance during treatment.

We have tested here this notion directly by attempting to select ESBL expression in diverse *K. pneumoniae* isolates, while using real-time PCR to monitor the appearance of bla_{SHV} codon 238 and 240 mutations, and the amplification of bla_{SHV}. Unmodified clinical isolates were used rather than recombinant strains because (i) the phenomena being studied are likely to involve gene dosage effects and artificial cloning vectors will differ greatly from wild plasmids in this regard and (ii) the alternative strategy of transferring plasmids by conjugation is not attractive since, because we are using ESBL-negative isolates, the bla_{SHV} gene is not a selective marker that can be used in conjugations into *K. pneumoniae* recipients.

TABLE 1. Primer sequences

Primer name	Target	Sequence (5′→3′)[a]
16sAllBactF	16S rRNA gene	TCCATGAAGTCGGAATCGCTAG
16sAllBactR	16S rRNA gene	CACTCCCATGGTGTGACGG
Pr-F	Wild-type promoter	GATGAAGGAAAAAAGAGGAATTGTGA
SHVquantF	bla_{SHV}	TGCTTGGCCCGAATAACAA
SHVquantR	bla_{SHV}	GCGTATCCCGCAGATAAATCA
Pr::IS26-F	IS*26* promoter	CCGGCCTTTGAATGGGTT
Prquant-R	bla_{SHV}	TAATACACAGGCGAATATAACGCATAAC
Shv238mt	bla_{SHV} codon 238	CGCCGATAAGACCGGAGCTA
Shv238wt	bla_{SHV} codon 238	CGCCGATAAGACCGGAGCTG
Shv238reverse	bla_{SHV}	CGGCGTATCCCGCAGATAA
Shv240mt	bla_{SHV} codon 240	GCGCGCACCCCGCTT
Shv240wt	bla_{SHV} codon 240	GCGCGCACCCCGCTC
Shv240reverse	bla_{SHV}	CCGGCGGGCTGGTTTAT
SHV5-IS26For	IS*26* transposase	TGACGTTGAACACCGACAGATT
SHV5-IS26Rev	p1658/97	AGTATCGCCATATTCAGATTGCC
TEMMGBforward	bla_{TEM}	TGGGTGCACGAGTGGGTTA
TEMMGBreverse	bla_{TEM}	GGGCGAAAACTCTCAAGGATCT

[a] 3′-Terminal bases for allele-specific oligonucleotides are underlined.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. The *K. pneumoniae* clinical isolates used in the present study were selected on the basis of being phenotypically ESBL negative (6), positive for bla_{SHV}, and negative for bla_{TEM}. J4, J5, K2, and M1 are clinical isolates from the PAH, Brisbane, Australia (11, 13). They are described more fully in the introduction above. The remainder of the isolates (i.e. isolates 30, 36, 85, 89, 102, 104, 105, 106, 107, 108, 109, 110, 113, 114, 115, 116, 119, 120, and 121) are derived from the SENTRY collection (12). The relevant gene contents of these isolates are given in Results.

Serial passage and mutation frequency assays were carried out by using Mueller-Hinton (MH) broth (Oxoid, Hampshire, United Kingdom).

ESBL detection and MIC determination. The ESBL status and cefotaxime (CTX) MICs were determined by using Etest ESBL screening strips in accordance with manufacturers instructions (Ab Biodisk, Solna, Sweden).

Selection of resistance using stepwise increases in CTX or CAZ concentration. Following overnight culture at 37°C in MH broth, 100 μl of each culture was used to inoculate fresh MH broth containing 0.25x the previously determined CTX or ceftazidime (CAZ) MIC for that isolate. The cultures were then incubated for 24 h at 37°C with shaking and aeration, after which 10-μl aliquots were removed and streaked onto MH agar. This culture was used for the real-time PCR measurements. A 100-μl aliquot was also used to inoculate the next serial culture, which contained double the CTX or CAZ concentration of the last culture. Serial passage was continued until 128 μg of antibiotic/ml was reached or no growth was observed.

DNA extraction for PCR analysis. DNA was either extracted using a DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions or from 0.5 McFarland unit suspensions as follows. Suspensions were centrifuged for 2 min at room temperature, the supernatant was discarded, and the pellet was resuspended in 100 μl of TE buffer and boiled for 20 min. The lysed cells were centrifuged for 2 min, and the supernatant was removed and stored at −20°C.

Relative quantitation and kinetic PCRs. Real-time PCR was used to determine the interisolate relative copy numbers of the total bla_{SHV} for each isolate. All reactions used SYBR green for product detection. The comparative threshold cycle (C_T) method (1) was used with the16S rRNA-encoding gene as the standard. The C_T is the cycle number at which the fluorescence reaches a predefined threshold value, which is chosen to occur in the logarithmic phase of amplification, and ΔC_T is defined as bla_{SHV} C_T −16S rRNA encoding gene C_T. The procedure was validated by making a series of six twofold dilutions of genomic DNA from a standard isolate (I1 from the PAH collection) (11) and determining that the ΔC_T values at each dilution did not differ significantly. The relative copy number between isolates was calculated as $2^{-\Delta\Delta C_T}$.

The presence and relative copy numbers of bla_{SHV} alleles that vary at codons 238 and 240 was also determined by real-time PCR. Allele-specific primers and PCR conditions were similar to those described by Hammond et al. (11). In order to maintain consistency with the copy number experiments, the estimated copy numbers of the mutant alleles relative to that of the wild-type alleles were depicted as $2^{\Delta C_T}$, where ΔC_T = the C_T of the mutant allele − the C_T of the wild-type allele. The result was recorded as "0" when the number could not be rounded up to 0.1 using two significant figures, i.e., when the result was <0.05.

All primers were designed with the aid of Applied Biosystems Primer Express Version 2. Primer sequences are shown in Table 1.

16S rRNA encoding gene was quantified by using the primers 16SAllBactF and 16SAllBactR, total bla_{SHV} was quantified by using the primers SHVquantF and SHVquantR, pr-bla_{SHV} was detected by using the primers Pr-F and Prquant-R, pr::IS*26*-bla_{SHV} was detected by using Pr:IS*26*-F and Prquant-R, upstream IS*26* was detected by using SHV5-IS26For and SHV5-IS26Rev, and bla_{TEM} was detected by using TEMMGBforward and TEMMGBreverse. The bla_{SHV} codon 238 polymorphism was interrogated by using the allele-specific primers Shv238wt and Shv238mt and the common primer Shv238reverse, as well

TABLE 2. Stepwise selection of CTX resistance[a]

Isolate	IS*26* insertion	MIC/[CTX] reached	ESBL phenotype after selection	Mutation[b]		bla_{SHV} copy no. expansion[c]
				Codon 238	Codon 240	
J4	Promoter	>16/128	Positive	13.6	0	4.9
J5	Promoter	>16/128	Positive	17.6	0	14.8
K2	Promoter	>16/128	Positive	1.8	0	7.8
M1	Neither	4.00/1.00	Negative	0	0	1.0
30	Neither	>16/32	Negative	0.1	0	1.7
85	Neither	>16/16	Negative	0.1	0	1.7
102	Neither	3.00/2.00	Negative	0.1	0	0.6
104	Neither	3.00/8.00	Negative	0.1	0	0.7
105	Neither	>16/64	Negative	0.1	0	0.8
106	Neither	>16/32	Negative	0.2	0	1.3
107	Neither	0.5/0.5	Negative	0.2	0	0.7
108	Neither	6.00/8.00	Negative	0.1	0	1.0
109	Neither	>16/16	Negative	0.1	0	0.9
110	Upstream	>16/128	Positive	1.3	0	19.1
113	Neither	>16/16	Negative	0	0	0.9
114	Neither	>16/32	Negative	0.1	0	0.9
115	Neither	8.00/8.00	Negative	0.1	0	1.1
116	Neither	>16/4.00	Negative	0.1	0	1.1
119	Promoter	>16/128	Positive	1.0	0	19.0
120	Upstream	>16/128	Positive	1.2	0	40.4
121	Upstream	>16/128	Positive	1.0	0	2.5

[a] The MIC and ESBL phenotypes and the real-time PCR measurements were from cells at either the highest CTX concentration at which growth occurred or at a CTX concentration of 128 μg/ml, which is the point at which the experiment was terminated.

[b] Mutant alleles were quantified by using $2^{\Delta CT}$, where ΔC_T = the C_T for the mutant allele − the C_T for the wild-type allele. The result was recorded as "0" when the number could not be rounded up to 0.1 using two significant figures, i.e., when the result was <0.05.

[c] Copy numbers were calculated with reference to the 16S RNA encoding gene and are depicted as an arithmetic ratio of the copy numbers from the starting and the derived cultures.

as the bla_{SHV} codon. Codon 240 was interrogated by using the allele-specific primers Shv240mt and Shv240wt, as well as the common primer Shv240reverse.

Real-time PCR was used for all gene quantification and detection. All real-time PCRs were carried out in 20-μl volumes in either ABI Prism 7000 or 7300 devices using Applied Biosystems SYBR green master mix. Reactions contained 5 pmol of each primer and 1 μl of cell lysate. The thermocycling parameters for all reactions were as follows: initial denaturation at 95°C for 60 s, followed by 40 cycles of 94°C for 15s, 60°C for 30 s, and 72°C for 30s, with the fluorescence read during the 72°C step.

RESULTS

IS*26* insertions in diverse ESBL-negative *K. pneumoniae* isolates. The PAH isolates had previously been screened for the presence of pr::IS*26*-bla_{SHV} (11). Accordingly, the PAH isolates were screened for the presence of the upstream IS*26* insertion, and the SENTRY isolates were screened for the presence of both IS*26* insertions. The combined data are shown in Table 2 (columns 1 and 3). The results were fortuitous in that they provided the optimal range of genotypes. Four isolates possessed pr::IS*26*-bla_{SHV}, three possessed the upstream IS*26* insertion, and fourteen possessed neither of the IS*26* insertions. Thus, the 14 isolates that would be predicted to be unable to evolve to become ESBL expressers were isolate M1 from the PAH collection and isolates 30, 85, 102, 104, 105, 106, 107, 108, 109, 113, 114, 115, and 116 from the SENTRY collection.

Stepwise selection experiments. The isolates were subjected to a stepwise selection regime that was designed to provide the highest probability of an ESBL-positive phenotype being acquired.

All isolates were tested for their ESBL status at every subculture. At the end of the experiment they were tested for the mix of alleles at codons 238 and 240 and the bla_{SHV} copy number. It can be seen in Table 2 that the results are as predicted. None of the 14 isolates that lack both IS*26* insertions became ESBL positive according to the Etest procedure. All of these isolates died before a CTX concentration of 128 μg/ml (the highest concentration used) was reached and were observed to grow extremely poorly in the subcultures leading up to the point at which no growth was detectable. Conversely, the other seven isolates all became become ESBL positive and grew vigorously up to a CTX concentration of 128 μg/ml. All of these isolates became phenotypically ESBL positive at CTX concentrations between 0.25 and 1.0 μg/ml. As expected, the interrogation of codons 238 and 240 by real-time PCR revealed that mutations at codon 238 had occurred in all ESBL-positive derivatives. There was no evidence for mutation at codon 240, although this is to be expected because the codon 240 mutation has little effect on the CTX MIC (14).

Aspects of the stepwise selection experiment were repeated using CAZ and a slightly different set of isolates. It was found that the isolates 36, 89, and 119 (which harbor pr::IS*26*-bla_{SHV}) and isolates 110, 120, and 121 (which harbor the upstream IS*26* insertion) could all be readily selected for phenotypic ESBL expression and grew to the experiment's termination point of 128 μg/ml. Isolates 36 and 89 were not tested in the CTX experiment, and the determination of the IS*26* insertions was carried out as part of this CAZ experiment. In contrast, isolates 102, 106, 113, and 114, which lack these insertions, failed to become ESBL positive and lost viability at 1 μg of CAZ/ml. The experiment was also carried out on isolates 115 and 116, which also lack the IS*26* insertions. Unexpectedly, these became ESBL positive, which would seem to falsify the notion

that the chromosomal bla_{SHV} cannot give rise to phenotypic ESBL expression. However, genetic characterization of these derivatives revealed that the cultures had become contaminated with cells that contained the upstream IS*26* insertion. It was concluded that our principle conjecture had not been falsified and is not specific to CTX.

Amplification of bla_{SHV} copy number has previously been reported to be associated with an ESBL-positive phenotype. This was found in the ESBL-positive isolates from the PAH collection (11). A striking feature of the results in Table 2 is that all ESBL-positive derivative strains possessed an increased bla_{SHV} copy number with respect to the progenitor isolate. The amplification factors ranged from 2.5 to 40.4. Similar measurements with the derivative strains that did not become ESBL positive gave amplification figures from 0.6 to 1.7, with the variation probably reflecting the resolution limits of the real-time PCR method. Therefore, amplification of bla_{SHV} is associated with the acquisition of bla_{SHV}-meditated ESBL expression in diverse *K. pneumoniae* isolates.

DISCUSSION

We were surprised by the extent that the results of the present study were in accord with our conjecture that *K. pneumoniae* isolates that lack a plasmid-borne bla_{SHV} gene and also lack bla_{TEM} are unable to evolve to become phenotypically ESBL positive. The absence of either of the two IS*26* insertions was completely predictive of a lack of ability to be selected for ESBL expression and vice versa. These findings are consistent with the report of Lee et al. (19), who reported an association between plasmid-borne bla_{SHV} and ESBL expression in *K. pneumoniae* clinical isolates.

For several years there has been considerable debate as to whether DNA-based diagnostics could replace phenotypic tests for antibiotic resistance. The current position in the field seems to be that molecular susceptibility determination shows considerable promise (23), although making such procedures immune from false positives for susceptibility is a very significant challenge. However, an alternative way that DNA-based procedures could be used is in conjunction with phenotypic antimicrobial susceptibility tests. Such procedures would not be designed to indicate the actual resistance phenotype but rather would indicate the capacity of the isolate to readily acquire resistance if subjected to selective pressure. This approach has considerable potential to guide antibiotic usage practices. An assay for the IS*26* insertions characteristic of plasmid-borne bla_{SHV} would appear to have considerable value when carried out on isolates that are phenotypically susceptible to expanded-spectrum cephalosporins and also negative for other potential ESBL-encoding genes such as bla_{TEM}. A limitation of this approach, however, is that it does not provide information as to the likelihood that a strain will acquire a new resistance gene.

The results obtained in the present study are largely consistent with current models for the origin and dissemination of bla_{SHV} variants (8, 17). However, they do emphasize that the dissemination of plasmids carrying non-ESBL-encoding bla_{SHV} genes has the potential to be of considerable relevance to the ecology of ESBL-mediated resistance in *K. pneumoniae*, even though bla_{SHV} is a normal component of the *K. pneumoniae* genome. The current literature suggests that there is a pervasive assumption that the de novo acquisition of ESBL-conferring mutations at codons 238 and 240 is a rare event (3, 8, 9), with the dissemination of plasmids carrying ESBL-encoding bla_{SHV} being the ecologically predominant process in the acquisition of resistance. However, the ease with which our research group found both $bla_{SHV\text{-}11}$ (non-ESBL encoding) and $bla_{SHV\text{-}2a}$ (encodes an ESBL that is identical to SHV-11 except for the G→S substitution at codon 238), both in *cis* with IS*26*, and frequently coexisting within a single cell, suggests that this is an oversimplification (11, 13). Rather, it appears that plasmid(s) that harbor non-ESBL-encoding bla_{SHV} are extant and provide a substrate for multiple and parallel instances of selection for the codon 238 substitution. The mutated genes coexist with unmutated genes (both chromosomal and plasmid-borne) in a recombinational equilibrium that is biased by selective pressure. Stochastic segregation and gene amplification events can lead to rapid changes in absolute and relative copy numbers of the different alleles from generation to generation.

The mechanistic basis for the inability of the chromosomal copy of bla_{SHV} to underpin the acquisition of an ESBL-positive phenotype is of interest. The underlying reason is likely to be a lack of any significant effect upon CTX resistance if the appropriate codon 238 mutation occurs in the chromosomal gene. The consequence of this would be that the mutation would not become fixed. This implies that the expression level of the chromosomal bla_{SHV} gene is low. There are two obvious factors that can impact the expression level: the promoter sequence and the gene dosage. Our experimental system did not find any differences between between isolates with the pr::IS*26*-bla_{SHV} promoter and those with the plasmid-borne pr-bla_{SHV} promoter with respect to the ease of selecting ESBL-expressing derivatives. This is despite the pr::IS*26*-bla_{SHV} being known to be of much greater activity than the wild-type promoter (22). While this would seem to rule out promoter effects, the bla_{SHV} promoter is known to contain other polymorphic sites that affect activity (7), so the relative contributions of promoter and copy number effects to our observations are not fully understood and are currently being investigated. Nevertheless, the consistent observation of copy number expansion means that it is likely that gene dosage-related effects are central to the acquisition of bla_{SHV}-mediated ESBL expression in *K. pneumoniae*.

The considerable difference between the plasmid and chromosomally located bla_{SHV} genes is illustrated by our search for mutations in the chromosomal genes. It would be expected that codon 238 mutations would accumulate in the chromosomal bla_{SHV}, even if the strain does not become phenotypically positive. This is because it is highly plausible that the mutation would cause a slight fitness advantage under selective pressure, even if the cell did not become ESBL positive according to the Etest and that such cells would come to dominate the population during the long course of the experiment. Against expectations, this did not occur. There was no sign of any codon 238 mutations in any of the isolates that failed to develop ESBL expression. This emphasizes the minimal impact of the chromosomal bla_{SHV} gene on CTX resistance.

Our findings are in accord those of Xiang et al. (24). These researchers found that selection of higher levels of CTX resis-

tance in *K. pneumoniae* carrying bla_{SHV-5} (which carries both the codon 238 and the codon 240 substitutions but is transcribed by pr-bla_{SHV}) was associated with gene amplification and enzyme hyperproduction. Interestingly, these authors obtained some evidence that the gene amplification was not due to plasmid copy number amplification, and it was speculated that it was due to the formation of tandem repeats. In addition, it was noted by Xiang et al. (24) that the association of resistance gene tandem duplication and antibiotic selective pressure is not a new concept. The phenomenon was extensively studied in the 1970s and 1980s and is sometimes termed gene transition (5, 15, 21). The mechanism was not fully established but is dependent upon repeated sequences flanking the amplifiable region and a functional *recA*. Very recently, a similar mechanism has been shown to underpin reports of "adaptive mutation" in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (18). We have not determined whether bla_{SHV} amplification is due to plasmid copy number relaxation or tandem gene duplication. However, the likely presence of flanking IS*26* sequences and the report by Xiang et al. (24), using a system very similar to ours, that the plasmid copy number does not correlate with bla_{SHV} copy number means that assuming that the copy number increase is plasmid copy number dependent is not justified. Very recently, further evidence for the generation of tandem repeats of bla_{SHV} genes has been reported by Zienkiewicz et al. (25). This phenomenon warrants further investigation.

In conclusion, we have obtained evidence that in *K. pneumoniae*, plasmid-borne non-ESBL-encoding bla_{SHV} is a risk factor for the development of an ESBL-positive phenotype, despite the same gene being present on the chromosome. Copy number measurements suggest that for an ESBL-expressing mutant to arise requires more than just the single codon 238 base change. It is likely to also require bla_{SHV} copy number amplification.

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