MODELING THE EFFECT OF COPY NUMBER OF PLASMIDS CARRYING ESBL GENES ON RESISTANCE LEVELS TO β-LACTAM ANTIBIOTICS

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INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) constitute one of the most important group of β-lactamases due to their ability to hydrolyse oxyimino-β-lactams. The levels of resistance to oxyimino-β-lactams conferred by ESBLs in bacterial strains may differ depending on kinetics and quantity of enzymes and the interplay of additional resistance determinants, such as decreased outer membrane permeability. This leads to serious difficulties in interpreting the susceptibility testing results of ESBL-producing strains exhibiting low-level resistance to β-lactams. Studies on clinical isolates and isogenic laboratory strains have demonstrated that the association of ESBL genes with more efficient promoters results in significant elevation of resistance levels to oxyimino-β-lactams even if some of them are weakly hydrolysed by particular ESBLs [3, 5]. It has been also shown that the copy number of ESBL genes or their hosting plasmids in clinical isolates correlates with the MICs of β-lactams [4]. In this study we further explored the effect of copy number of plasmids carrying ESBL genes on resistance levels to β-lactams in the isogenic *E. coli* strains. For this purpose we used a CopyControl [2] cloning system consisting of a dual origin of replication pCC1 vector and an EPI300 *E. coli* strain which allows maintaining of the vector at single copy number per cell upon its replication from F-factor origin of replication or at multiple copy number per cell upon arabinose-dependent induction of replication from oriV origin of replication.

MATERIALS AND METHODS

Amplification and cloning of ESBL genes. The gene for SHV-3 β-lactamase was amplified from the *E. coli* J53 strain carrying the natural plasmid pUD18 [1] using the primers: SHV-Upstr1: 5'-GCG GGC GTG GTG ATC GGC AAA CAG-3' and SHV-R: 5'-TCT TTC CGA TGC CGC CGC CAG TCA-3'. The genes for CTX-M-3 and its P167T-mutation variant, CTX-M-42, were amplified from the *E. coli* clinical isolates [6] using the primers ISEcp1: 5'-TGT CTG GTA TAA TAA GAA TAT CAT C-3'and CTX-M-R-stop: 5'-CTA TTA CAA ACC GTC GGT GAC-3'. The PCR products containing the ESBL structural genes with the 5'-adjacent natural promoters were cloned in the pCC1 vector using the blunt-end cloning procedure and introduced into the *E. coli* EPI300 [2].

Susceptibility testing. The MICs of ampicillin (AMP), ceftazidime (CAZ), cefotaxime (CTX), cefepime (FEP) and aztreonam (AZT) were determined for the strains carrying recombinant plasmids or the pCC1 vector without insertion (β-lactamase negative control) using the broth microdilution method as recommended by NCCLS except that LB broth was used as testing medium instead of MH broth. This was done to avoid inhibition of vector replication from oriV by residual sugars contained in the MH broth [7]. All MICs were determined in duplicate. In order to assess susceptibilities at different copy number of plasmid per cell, each strain was tested in two parallel sets of experiments with or without addition of L-arabinose in a final concentration of 0.02% in LB medium. The *E. coli* ATCC 25922 and *P. aeruginosa* ATCC® 27853 strains were used for quality control of susceptibility testing.

Determination of plasmid copy number per cell. The plasmid copy number was determined by real-time quantitative PCR with SYBR Green I and primers targeting the internal sequences of bla_{SHV} and bla_{CTX-M} genes or the vector-specific primers. Amplifications were carried out in a Rotor-Gene 2000 System operated by software v.5.0 (Corbett Research, Australia). Total DNA was extracted from 20µl aliquots of each culture grown overnight in LB medium (with or without addition of arabinose) using the InstaGene Matrix (BioRad, USA) and 5 µl of each extract was used for PCR. At the same time, the number of bacterial cells was determined by diluting the aliquots of the same cultures and plating them on agar plates containing 12.5 mg/L of chloramphenicol . The DNA-target copy number from PCR experiments was then related to the total number of cells to yield the plasmid copy number per cell. Calibration standards were prepared from serial dilutions of each culture grown in the sugar-free LB broth. The number of plasmid copies in the calibration standards was considered to be equal to the number of cells assuming that the plasmids were maintained at a single-copy state under the absence of inducer.

RESULTS AND DISCUSSION

According to the results of real-time quantitative PCR, the copy number of plasmids carrying ESBL genes was increased approximately 10 times (from 1 to 10±1.1) upon induction with arabinose. Figure 1 shows an example of determination of copy number of pCC vector carrying the $bla_{CTX-M-3}$ gene by real-time PCR.

As expected, all the strains carrying ESBL genes were resistant to AMP and had elevated MICs of oxyimino-β-lactams (as compared to the β-lactamase-negative strain) even when the plasmids were maintained at a single-copy state (Tab. 1). In these conditions, however, the SHV-3-producing strain had the MICs of all oxyimino-β-lactams below the CLSI breakpoints for nonsusceptibility while the strains producing CTX-M-3 or CTX-M-42 were resistant only to CTX or CAZ, respectively.

A multiplication of the ESBL-coding plasmids was paralleled by proportional increase in resistance of the host strains to all the antibiotics including those known to be weak substrates for particular ESBLs (e.g. CAZ for SHV-3 and CTX-M-3 or CTX for CTX-M-42). Resistance levels to oxyimino-β-lactams raised by 4 to 5 log2 MIC dilutions for the SHV-3-producing strain, and by 1 to 4 log2 MIC dilutions for the CTX-M producers. At the same time, induction with arabinose did not affect the MICs for the β-lactamase negative strain.

It is interesting to note that the clinical *E. coli* strains producing the CTX-M-3 and CTX-M-42 [6] carried the same copy number of bla_{CTX-M} genes per cell and displayed the same MICs of β-lactams as the respective recombinant strains in which the plasmid multiplication was induced by arabinose (data not shown). These data further support the correlation between the ESBL gene copy number and the MICs of oxyimino-β-lactams.

Table 1. Susceptibilities of *E. coli* EPI300 strains containing recombinant plasmids and pCC1 at different copy numbers.

Plasmid	ESBL	Copy number per cell	MICs, mg/L AMP	CTX	CAZ	FEP	AZT
pCC-bla_{SHV-3}	SHV-3	1	1024	2	1	0.25	0.5
		10	≥2048	64	16	4	8
pCC-$bla_{CTX-M-3}$	CTX-M-3	1	512	16	1	1	2
		10	≥2048	256	4	4	16
pCC-$bla_{CTX-M-42}$	CTX-M-42	1	256	1	32	0.5	1
		10	≥2048	4	128	1	4
pCC1 (*bla* negative)	-	1	2	0.25	0.5	≤0.125	0.25
		>10	2	0.25	0.5	≤0.125	0.25

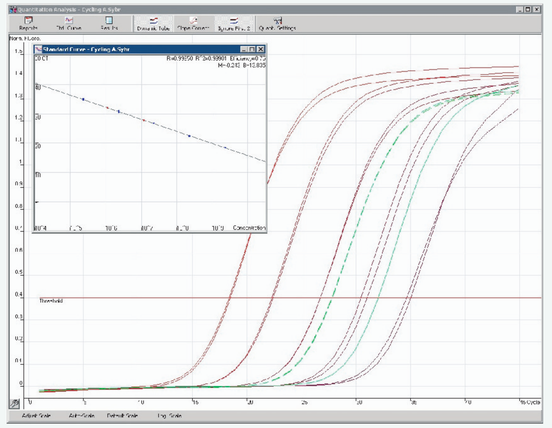

Figure 1. Determination of copy number of pCC-$bla_{CTX-M-3}$ plasmid by real-time PCR.

CONCLUSIONS

We have found that the CopyControl cloning system provides an effective means for quantifying the role of β-lactamases in resistance.

Our model experiments demonstrated a strong correlation between the copy number of ESBL-coding plasmids and MICs of oxyimino-β-lactams.

The results presented above, together with the fact that the copy number of ESBL genes may vary in clinical strains, raises the importance of accurate detection of ESBL-mediated resistance.

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