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Factors influencing gene expression and resistance for Gram-negative organisms expressing plasmid-encoded *ampC* genes of *Enterobacter* origin

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High-level expression of AmpC β-lactamases results in organisms resistant to multiple β-lactam antibiotics. The mechanism of chromosomally mediated AmpC resistance has been elucidated, however the mechanism(s) driving plasmid-encoded AmpC resistance are unknown. Studies were designed to identify factors which influence expression of plasmid-encoded *ampC* genes and correlate these factors with resistance. As the model system, *ampC* genes of *Enterobacter* origin were used to determine how gene copy number, genetic background and genetic organization influenced resistance phenotypes. To this end, gene expression from the plasmid-encoded inducible bla_{ACT-1} and non-inducible bla_{MIR-1} were compared with chromosomal *ampC* gene expression from both wild-type (WT) and derepressed *Enterobacter cloacae* isolates. RNA levels within the original clinical isolates were examined using primer extension analysis, whereas a new PCR strategy was developed to examine gene copy number. These data revealed that bla_{ACT-1} and bla_{MIR-1} constitutive expression was 33- and 95-fold higher than WT expression, whereas copy numbers of the plasmid-encoded genes were 2 and 12, respectively. Differences in promoters and transcriptional starts for the respective plasmid-encoded genes were noted and contribute to increases observed in overall expression. Finally, β-lactam MICs were increased two- to 16-fold when bla_{ACT-1} was expressed in *Escherichia coli* AmpD$^-$ strains compared with *E. coli* AmpD$^+$ strains. In conclusion, high-level expression of plasmid-encoded *ampC* genes requires interplay between multiple factors including genetic organization, promoter modifications, genetic background, and to some extent gene copy number. In addition, clinical laboratories need to be aware that genetic backgrounds of inducible plasmid-encoded genes can dramatically influence MICs for organisms not normally associated with derepressed phenotypes.

Keywords: AmpC expression, resistance, plasmid-encoded, derepressed, copy number

Introduction

The mechanism of chromosomal AmpC-mediated resistance in Gram-negative organisms is high-level expression of the AmpC β-lactamase. Organisms expressing high levels of AmpC β-lactamases can be resistant to almost all β-lactam drugs except cefepime, cefpirome and the carbapenems.[1] *ampC* genes were first identified on the chromosomes of several genera of Gram-negative organisms including *Enterobacter* spp., *Citrobacter freundii*, *Morganella morganii*, *Hafnia alvei*, *Serratia marcescens* and *Escherichia coli*.[2–6] All these organisms, except *E. coli*, encode an inducible chromosomal *ampC* β-lactamase gene.[3] Induction of the *ampC* gene requires three additional gene products, AmpG, AmpD and AmpR, in addition to an inducing agent, such as cefoxitin or imipenem.[7] AmpG is a permease that allows entry

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of muropeptides into the cytoplasm from the periplasmic space as part of the cell wall recycling pathway.[7–9] In excess, these muropeptides act as cofactors in the induction process in which the amidase, AmpD, fails to process all the muropeptides that enter the cytosol.[7,10,11] AmpR is a DNA binding protein required for regulation of chromosomal *ampC* gene expression.[3,12–14]

The discovery of the AmpC β-lactamase, CMY-1, in 1989 was the first published report of a plasmid-encoded AmpC β-lactamase.[15] Since then, over 20 plasmid-encoded AmpC β-lactamases have been reported in several genera of bacteria (genetic backgrounds) including *Salmonella* spp., *E. coli*, *Proteus mirabilis* and *Klebsiella pneumoniae*.[15–18] Most of these plasmid-encoded genes lack the *ampR* gene and therefore do not display an inducible AmpC phenotype. However, since 1998, three inducible plasmid-encoded AmpC β-lactamases have been reported. Two of these genes, $bla_{\text{DHA-1}}$ and $bla_{\text{DHA-2}}$ are of *M. morganii* origin, whereas the most recently discovered gene, $bla_{\text{ACT-1}}$, is of *Enterobacter* origin.[18–21] These *ampC* genes are linked to an *ampR* gene that is transcribed divergently from *ampC*. This genetic organization is identical to that observed for inducible chromosomal *ampC* genes.

Mutations associated with AmpD lead to constitutive high-level expression (derepression) of the chromosomal *ampC* gene in clinical isolates resulting in high-level expression of the AmpC β-lactamase.[10] Although the general mechanism for high-level expression of chromosomal *ampC* genes is understood, the same cannot be said for the plasmid-encoded *ampC* genes. Several factors could contribute to the high-level expression of these genes. These factors include gene copy number, the genetic background of the organism and the genetic organization or context of the genetic locus from which the genes are expressed. It has been suggested that two factors may contribute to high-level expression from plasmid-encoded *ampC* genes in the absence of AmpR. These factors include expression from high-copy number plasmids[22] and the absence of regulation by AmpR, in which *ampC* gene expression has been observed to increase 2.5- to 5.8-fold.[4,12] However, the contributions of increases in *ampC* transcription and high copy number in the high-level expression of plasmid-encoded *ampC* genes have not been documented. The data presented in this paper correlate the overall gene expression from four *ampC* genes of *Enterobacter* origin in clinical isolates, with gene copy number, genetic organization and genetic background. These genes include the non-inducible plasmid-encoded *ampC* gene $bla_{\text{MIR-1}}$, the inducible plasmid-encoded *ampC* gene $bla_{\text{ACT-1}}$, both expressed in *K. pneumoniae* isolates, and two chromosomal *ampC* genes expressed in *Enterobacter cloacae*, one inducible and one exhibiting high-level constitutive expression (derepressed).

Materials and methods

Bacterial strains

The clinical isolates and bacterial strains used in this study are listed in Table 1.

Primer extension analysis

Total RNA was isolated from Mueller–Hinton broth (MHB) (Oxoid, UK) using modified Trizol (Invitrogen, Carlsbad, CA, USA). Briefly, 6 h 15 mL cultures were diluted 1:20 (100 mL total volume) in fresh pre-warmed MHB and allowed to grow to an OD_{600} of 0.65 at 37°C with shaking at 100 rpm. These cells represent the early log phase of growth. Cells were collected in pre-chilled centrifuge tubes and the cell pellet was suspended in 1 mL of modified Trizol solution. Induction assays using cefoxitin and primer extension analysis were carried out as previously described.[20] Fifty micrograms of total RNA was used for each primer extension reaction for *ampC* transcripts whereas 1 μg was used for the 16S rRNA reactions. The primers for primer extension analysis are listed in Table 2.

β-*Lactamase hydrolysis assays*

Hydrolysis assays for each of the four clinical isolates were carried out as described previously using 100 μM cefalothin as substrate.[20,23] Enzyme was recovered from the same cultures used for RNA isolation.

MIC determinations

The susceptibility profiles were determined by agar dilution according to National Committee for Clinical Laboratory Standards (NCCLS) methods.[24] The antimicrobial agents were obtained from different sources. Imipenem was obtained from Merck (Rahway, NJ, USA); cefotaxime and piperacillin were obtained from Sigma Chemical Co. (St Louis, MO, USA); ceftazidime was obtained from Glaxo-SmithKline (Collegeville, PA, USA); cefpodoxime was from Pharmacia and Upjohn (Kalamazoo, MI, USA); cefepime and aztreonam were from Bristol-Myers Squibb (Princeton, NJ, USA); cefixime was from Wyeth-Ayerst Lederle (Bound Brook, NJ, USA).

Copy number determination

Comparative PCR was used to obtain a ratio of band intensities between a single copy chromosomal gene, in this case *ampD*, and the gene of interest, *ampC*. *ampD* is a single copy gene, as verified by BLAST analysis of the *E. coli* and *K. pneumoniae* genomes.[25] PCRs were carried out as previ-

Table 1. Bacterial strains, clinical isolates and plasmids used in this study

Clinical isolates	*ampC* gene	Abbreviation[a]	Location	Induction phenotype[b]	Induction genotype	Reference
E. cloacae 55	*ampC*	W*tampC*	chromosome	WT	WT	this study
K. pneumoniae 225	bla_{ACT-1}	bla_{ACT-1}	plasmid	WT	WT	20, 26
K. pneumoniae 96D	bla_{MIR-1}	bla_{MIR-1}	plasmid	NI	*ampR*$^-$	31
E. cloacae V204	*ampC*	Dr*ampC*	chromosome	DR	*ampD*$^-$	this study

Plasmids	Characteristics	Reference
pACYC184	*tet*, *cat*	27
pMDR001	pACYC184 w/bla_{ACT-1} and *ampR*; *tet*, Δ*cat*	this study
pMDR002	pACYC184 w/bla_{MIR-1}; *tet*, Δ*cat*	this study

Strain	Genotype	*ampD* genotype	Plasmid-encoded *ampC*[c]	Reference
E. coli Top10 (Invitrogen)	see Reference	*ampD*$^+$	NA	34
E. coli WTpACYC184	*E. coli* Top 10 w/pACYC184	*ampD*$^+$	NA	this study
E. coli WTpACT-1	*E. coli* Top10 w/pMDR001	*ampD*$^+$	bla_{ACT-1} and *ampR*	this study
E. coli WTpMIR-1	*E. coli* Top10 w/pMDR002	*ampD*$^+$	bla_{MIR-1}	this study
E. coli SNO302	(*ampA1*, *ampC8*, *pryB*, *recA*, *rpsL*)(*ampD*E$^-$)	*ampD*$^-$	NA	11
E. coli JRG582	(Δ*nadC-aroP*) (*ampD*$^-$)	*ampD*$^-$	NA	35
E. coli ΔDpACT-1	*E. coli* JRG582 w/pMDR001	*ampD*$^-$	bla_{ACT-1} and *ampR*	this study
E. coli ΔDpMIR-1	*E. coli* SNO302 w/pMDR002	*ampD*$^-$	bla_{MIR-1}	this study

[a]Abbreviation for each *ampC* gene used in the text.
[b]WT, wild-type; NI, non-inducible; DR, derepressed.
[c]NA, not applicable.
[d]*E. coli* WT refers to strains with wild-type *ampD*.

ously described.[26] Primers used for copy number determinations are indicated in Table 2. Ten-fold serial dilutions, ranging from 10^0 to 10^{-5}, of total DNA prepared from each isolate served as templates for PCR. The PCR products were separated on a 0.8% agarose gel, stained with Vistra Green (Amersham Pharmacia, Piscataway, NJ, USA), and visualized with a Storm Molecular Imager (Molecular Dynamics Inc., Sunny, CA, USA). Quantification of the bands was carried out with ImageQuant software (Molecular Dynamics Inc.). A ratio was determined by comparison of band intensities between the PCR products of the target gene, *ampC* and the single copy gene, *ampD*. These ratios were determined between band intensities obtained from PCR products amplified from the same dilution of template (normally the 10^{-3} dilution), which was within the linear range of the PCR cycle. The initial copy number was verified by diluting the PCR product from the diluted template (10^{-3} dilution) by the calculated *ampC* to *ampD* ratio. The diluted PCR product and the undiluted *ampD* PCR product from the original diluted (10^{-3}) template were visualized as described above. Initial copy number was verified when the peak intensities for each band were equivalent between the diluted target *ampC* gene product and the undiluted single copy *ampD* product (see text for further explanation).

Cloning and transformation

bla_{ACT-1} and bla_{MIR-1} were amplified by PCR as described above using Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA). Amplicons were visualized on a 0.8% agarose gel with Crystal Violet staining. DNA was extracted from the gel using a SNAP column (Invitrogen, Carlsbad, CA, USA) and ligated into the pCR-TOPO-XL cloning vector and transformed into *E. coli* Top10 competent cells (Invitrogen). The amplicons were subcloned into pACYC184 as an *Eco*RI fragment.[27] The resulting plasmids were isolated and electroporated into *E. coli* Top10 (Invitrogen), *E. coli* SNO302 and *E. coli* JRG582 (Tables 1 and 3). The plasmid DNA from

Table 2. Primers used in this study

Primer name	Target gene	Sequence[a]	Amplicon size (bp)[b]	Nucleotides[c]	Accession number[d]
Sequencing and cloning primers					
ACT-1 EndR	bla_{ACT-1}	CGGCAATGTTTACTACACAGCG	2263	1169–1185	U58495
K225AMPRR	*ampR*	CCTTTTATTTCTGCATCTTCCC		005–26	AF362955
Ec UP F	*ampC*	GCAACGAATGAATCATTCAGCACCG	sequencing	289–309	this study
ACT-1F	bla_{ACT-1}	CCTTGAACTGCTATTACGG	sequencing	002–20	U58495
AmpRF	*ampR*	CCGTAATAGCGAGTCAAGGG	sequencing	1061–1080	AF362955
MIR-1F	bla_{MIR-1}	GGGAAGCAAACTGGTGTACC	2382	008–27	M37839
MIR-1R	bla_{MIR-1}	GCATCAAAAGGCGTGACGACG		2310–2390	M37839
MIR-1PE	bla_{MIR-1}	GCGAATGCAGAACTGGCGACG	sequencing	966–986	M37839
MIR-1FW	bla_{MIR-1}	CCTTGAACTGCTATTACGG	sequencing	899–917	
Primer extension primers					
ACT-1PE	bla_{ACT-1}	GCCAATACCGAGCAGGAGGTG	NA	69–89	U58495
MIR-1PE	bla_{MIR-1}	GCGAATGCAGAACTGGCGACG	NA	966–986	M37839
ENTB55PE	*ampC*	GGCGAGAGCAGAGCAAGAGATGCC	NA	80–103	X07274
V204PE	*ampC*	GGCGAGAGCAGAGCAAGAAATGCC	NA		this study
KP16SRNA	16*S* rRNA	CCCAGACATTACTCACCCGTCC	NA	82–61	AF390084
EC16SRNA	16*S* rRNA	CCCAGACATTACTCACCCGTCC	NA	70–91	AJ415572
Copy number primers[d]					
Kp-*ampD*F	*ampD*	GGAAACATGCTAACCTGAACG	695	7067–7047	this study[e]
Kp-*ampD*R	*ampD*	CGGCGATAAGCACCAACAGC		6371–6392	this study[e]
Ec-*ampD*F	*ampD*	GCTACTCTGAACCGAGTAACAGC	609	109–131	Z14003
Ec-*ampD*R	*ampD*	TCATGTTATCTCCTTATCTGACG		695–717	Z14003
V204-*ampD*F	*ampD*	GGACTAGGCCTACGCCATAGC	909		this study
Ec-*ampD*R	*ampD*	TCATGTTATCTCCTTATCTGACG			this study
ACT-1CNF	bla_{ACT-1}	GGATGAGGTCAAGGATAACG	546	456–475	U58495
ACT-1CNR	bla_{ACT-1}	GGTGGATTCACTTCTCTCGC		1001–982	U58495
MIR-1CNF	bla_{MIR-1}	GGATGAGGACACGGATACCG	546	1353–1372	M37839
MIR-1CNR	bla_{MIR-1}	GGTGGATTCACTTCTGCCAC		1898–1879	M37839
Ec-CNF	*ampC*	GGATGAGGTCACGGATAACG	546	469–488	X07274
Ec-CNR	*ampC*	GGTGGATTCACTTCTGCCAC		995–1014	X07274

[a]All primers are written 5′ to 3′ as synthesized.
[b]Size of the amplicon generated by the primer pair noted; NA, not applicable.
[c]Nucleotide numbers listed are determined from GenBank sequences from the accession number listed.
[d]GenBank accession number for the sequence used for primer design.
[e]Primers designed from *K. pneumoniae* genome sequence online at http://genome.wustl.edu.

these clones was sequenced as described previously using the primers in Table 2 to verify the insert sequences.[20]

Results

ampC RNA expression

Primer extension and hydrolysis data confirmed the induction phenotypes of *E. cloacae* 55, *K. pneumoniae* 225, *K. pneumoniae* 96D and *E. cloacae* V204. These four isolates represent *ampC* β-lactamase gene expression of *Enterobacter* origin in different genetic backgrounds (Table 1). In addition, primer extension analysis was carried out to determine the relative amounts of steady-state *ampC* RNA expressed from each clinical isolate tested. As determined previously by primer extension analysis, bla_{ACT-1} was induced five-fold over constitutive levels when RNA expression was analysed 15 min post-induction, whereas β-lactamase hydrolysis assays indicated a 1.3-fold induction (Figure 1).[20] β-Lactamase hydrolysis assays showed that wild-type (WT) *E. cloacae*

Table 3. MICs of selected β-lactam antibiotics for clinical isolates and bacterial strains[a,b]

	MIC (mg/L)										
	E. coli	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*K. pneumoniae*	*K. pneumoniae*	*E. cloacae*	*E. cloacae*
β-Lactam antibiotic	WTpACT-1	ΔDpACT-1	WTpMIR-1	ΔDpMIR-1	JRG582	SNO302	pACYC184	225	96D	55	V204
Cefixime	256	2048	256	256	0.06	0.03	0.06	256	512	2	1024
Cefpodoxime	256	1024	256	256	0.25	0.25	0.5	64	512	2	1024
Piperacillin	32	256	32	32	2	1	2	>2048	>2048	2	256
Aztreonam	8	64	8	8	0.06	0.03	0.06	256	32	0.03	16
Ceftazidime	16	256	8	8	0.12	0.06	0.12	128	32	0.25	32
Cefotaxime	8	64	8	8	0.007	0.007	0.015	16	32	0.12	128
Cefepime	0.06	0.5	0.06	0.06	<0.004	<0.004	<0.004	2	0.25	0.25	1
Imipenem	1	2	0.5	1	0.06	0.06	0.12	1	0.5	0.5	0.12

[a]Organisms used for quality control were *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853.
[b]Clinical isolates and bacterial strains are described in Table 1.

Figure 1. Cefalothin hydrolysis assays. AmpC activity from crude protein preparations of each strain was measured spectrophotometrically. Hydrolysis activity from preparations of either untreated (open bars) or cefoxitin (0.25 × MIC of specific strain) treated (shaded bars) cultures are represented relative to AmpC activity from *E. cloacae* 55. Each value represents the mean of three experiments. Error bars represent the standard deviation.

AmpC was also induced, whereas both MIR-1 and *E. cloacae* V204 (derepressed mutant) AmpC were uninducible (Figure 1). These induction phenotypes were also reflected at the RNA level (data not shown). However, when basal (non-induced) level RNA expression between these four strains was examined, there was a 33-, 95- and 75-fold difference between WT*ampC* and bla_{ACT-1}, bla_{MIR-1} and DR*ampC*, respectively (Figure 2a and b).

Gene copy number

In contrast to the predicted 2.5- to 5.8-fold increase in the absence of *ampR* for plasmid-encoded *ampC* genes, differences of 33- (*ampR* present) and 95-fold increases (*ampR* absent) over chromosomal *ampC* expression were observed (Figure 2a and b). However, the constitutive level of RNA expression between the two plasmid-encoded *ampC* genes, bla_{ACT-1} and bla_{MIR-1} was only three-fold. These data indicated a difference in the regulation of constitutive expression between the plasmid-encoded AmpC β-lactamase genes compared with each other and WT*ampC*. One explanation for the large increase in overall RNA expression from the plasmid-encoded genes could be gene copy number. Therefore, the copy number of each of these genes was determined by a comparative analysis using PCR. A ratio was determined between amplified products of the *ampC* gene in question and the single copy chromosomal gene, *ampD* (Figure 3). PCR amplification of each *ampC* gene and the respective *ampD* gene for each organism (*K. pneumoniae* or *E. cloacae*) was carried out using 10-fold serial dilutions of template DNA (10^0–10^{-5}) from each bacterial strain (Figure 3a and c). The band intensities of the *ampC* and *ampD* amplified products from the 10^{-3} template dilutions (Figure 3a and c) are represented by the graphs in Figure 3(b and d). The area under the curve for each peak was used to determine the ratio of ampli-

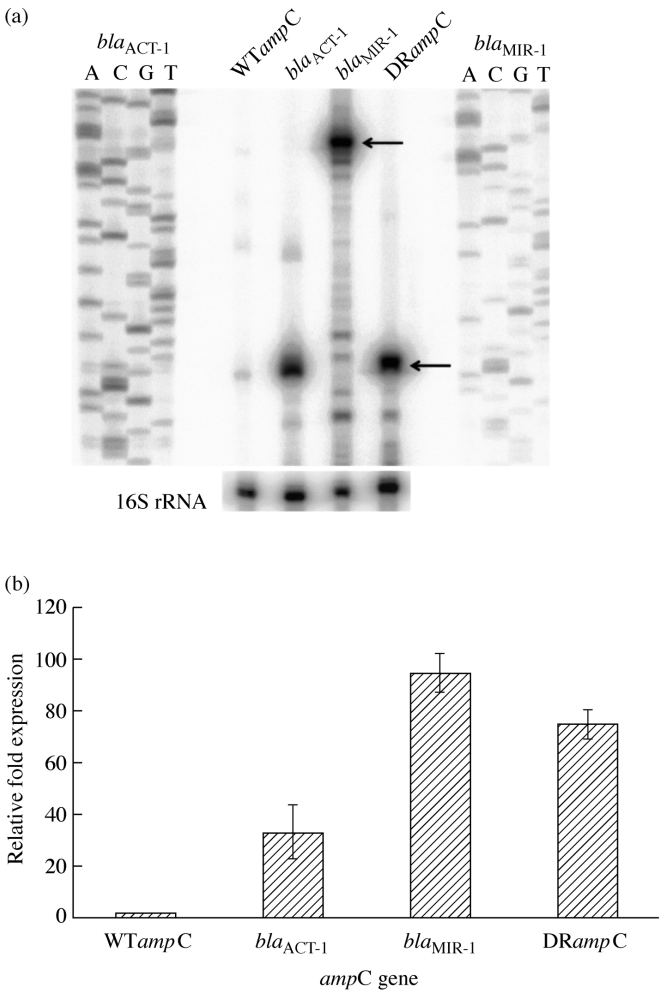

Figure 2. Primer extension analysis. (a) The primary start sites of transcription were mapped using primer extension analysis as indicated by the arrow. The intensities of the bands were quantified using a Storm Molecular Imager with ImageQuant software and normalization of data was obtained using the 16S rRNA bands. The bla_{ACT-1} and bla_{MIR-1} sequencing ladders are represented on the left and right sides, respectively. The bands used to map the start site of transcription represent the amount of *ampC* RNA expression from each strain. These bands were quantified and normalized using the 16S rRNA bands. (b) A bar graph representing *ampC* expression of bla_{ACT-1}, bla_{MIR-1} and DR*ampC* relative to WT*ampC*. Each value represents the mean of three experiments. Error bars represent the standard deviation.

fied products of bla_{ACT-1} to *ampD* (Figure 3b) and bla_{MIR-1} to *ampD* (Figure 3d). The amount of bla_{ACT-1} and bla_{MIR-1} products with respect to the *ampD* genes revealed a ratio of 2 (Figure 3a and b) and 12 (Figure 3c and d), respectively.

Verification of these numbers was obtained by diluting the bla_{ACT-1} and bla_{MIR-1} PCR products from the 10^{-3} reaction (Figure 3a for bla_{ACT-1} and 3c for bla_{MIR-1}) by factors of 2 and 12, respectively, and measuring the amount of the diluted *ampC* products (Figure 3e and f) compared with the undiluted *ampD* products for each strain. The areas under the curve in Figure 3(e and f) represent the intensities of the band obtained from the bla_{ACT-1} PCR amplicons diluted 1:2, and the undiluted *ampD* amplicon. As shown in Figure 3(e), the 1:2 diluted band intensity was now equivalent to the area under the curve detected for the *ampD* product.

Similarly, comparing the band intensities for the 1:12 dilution of the bla_{MIR-1} 10^{-3} PCR product and the undiluted 10^{-3} *ampD* product, the area under the curve was equivalent (Figure 3f). Therefore, the copy number of the inducible plasmid encoded bla_{ACT-1} is 2, whereas the copy number of the non-inducible plasmid encoded bla_{MIR-1} is 12. This method was also used to verify the copy numbers of the chromosomal *ampC* genes from *E. cloacae* 55 and V204, which were both one (data not shown). As a control for the technique, bla_{ACT-1} and bla_{MIR-1} were cloned into the vector pACYC184 which has a copy number of 10 to 12.[27] The copy number of both bla_{ACT-1} and bla_{MIR-1} cloned into pACYC184 was determined to be 11 for each gene (data not shown). These data indicate that the copy number protocol designed in this study is an accurate method for determining relative gene copy number.

Promoter sequence and expression comparisons

Primer extension data were used to map the transcriptional start sites for the mRNA transcript of each gene studied (Figures 2a and 4). As expected, the bla_{ACT-1} mapped to the guanosine at position +1 (Figure 4), 50 bp upstream of the ATG start codon.[20] The start site of transcription for the *E. cloacae* chromosomal *ampC* genes, WT*ampC* and DR*ampC* was a cytosine at the position identical to the bla_{ACT-1} transcriptional start site. Surprisingly, the transcriptional start site of bla_{MIR-1} mapped to the cytosine at position –36 relative to the transcript start sites for bla_{ACT-1} and chromosomal *ampC* genes (Figure 4), 89 bp upstream of the ATG start codon. Putative –10, TAaAtT, and –35, TTGAat, promoter elements having similarity to the *E. coli* σ70 promoter consensus sequences –10, TATAAT and –35, TTGACA were identified. The spacing between these putative sites was 17 bp, the optimal distance between these two elements for maximal expression as described for *E. coli*.[28] The relative level of expression from each promoter was determined by primer extension analysis using clones *E. coli* WTpACT-1 and *E. coli* WTpMIR-1 (Table 1). These clones comprise the native promoter in addition to the structural gene cloned into pACYC184. RNA levels expressed from the same vector were only slightly increased (1.6-fold) for *E. coli* WTpMIR-1 when compared with RNA expression from *E. coli* WTpACT-1 (data not shown).

β-*Lactam MIC levels for AmpD⁻ E. coli*

Constitutive high-level expression (derepression) of the chromosomal *ampC* gene of *E. cloacae* V204 gave a 75-fold increase in expression and significant increases in β-lactam MICs (Figure 2b and Table 3). In contrast, the WT strain,

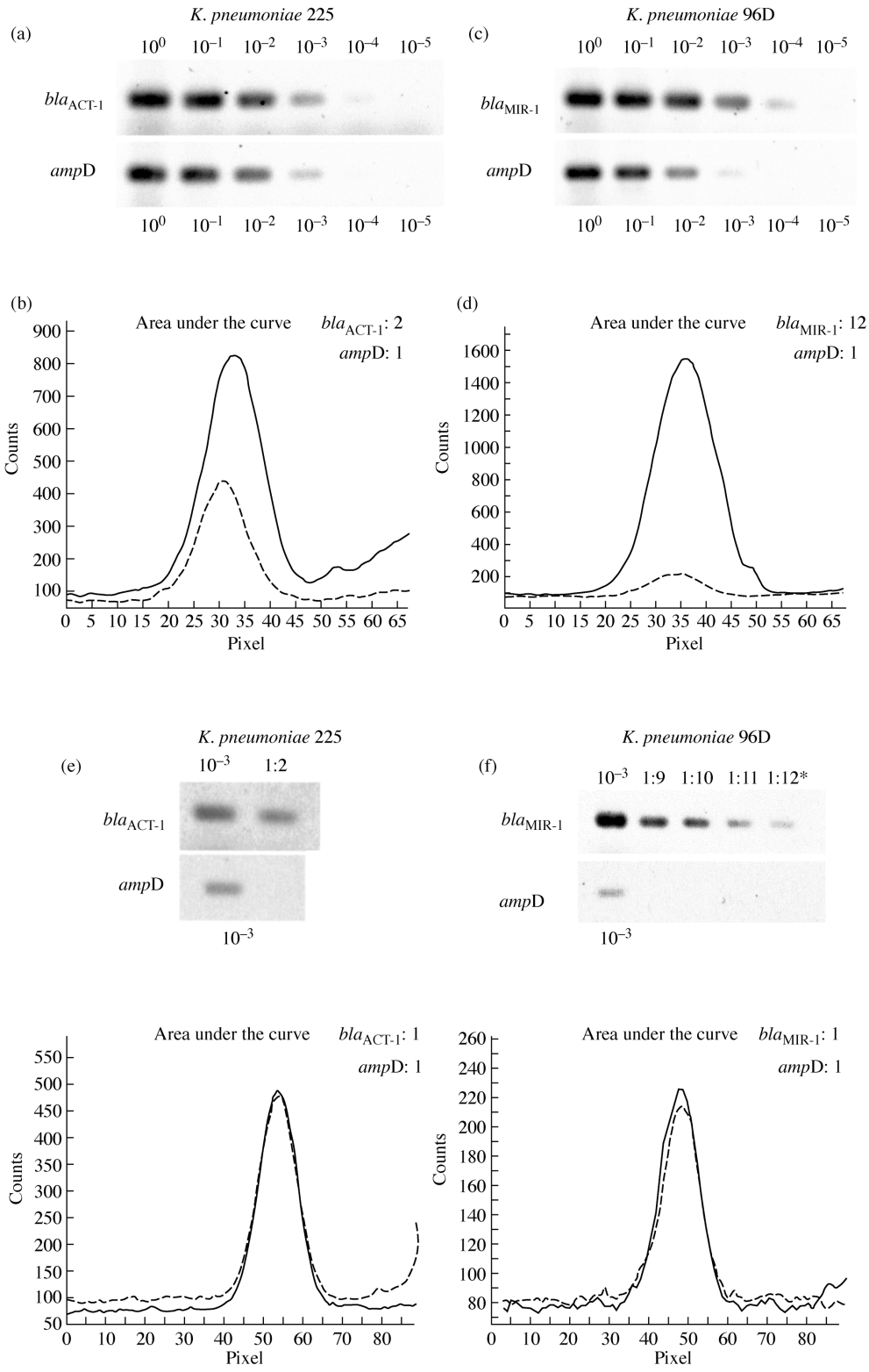

Figure 3. Gene copy number determination of bla_{ACT-1} (a, b and e) and bla_{MIR-1} (c, d and f) by comparative PCR. PCR amplicons from the 10^{-3} dilution in (a), (c), (e) and (f) were quantified using Vistra Green staining and a Storm Molecular Imager with ImageQuant Software (Molecular Dynamics). The area under the curve from the *ampC* and *ampD* amplicons from the 10^{-3} lane of (a) and (c) are represented in graphs (b) and (d) (*ampC*, solid lines; *ampD*, dashed lines). The ratio was confirmed using dilutions of the original PCR products yielding equal areas under the curve (e and f). Graph in (e) represents the comparison of area under the curve determined from lane 1:2 of bla_{ACT-1} (solid line) with the area under the curve determined from the 10^{-3} *ampD* amplicons (dashed line). Graph (f) represents the comparison of the area under the curve determined from the band in lane 1:12 of bla_{MIR-1} (solid line) with the area under the curve determined from the 10^{-3} *ampD* amplicons (dashed line).

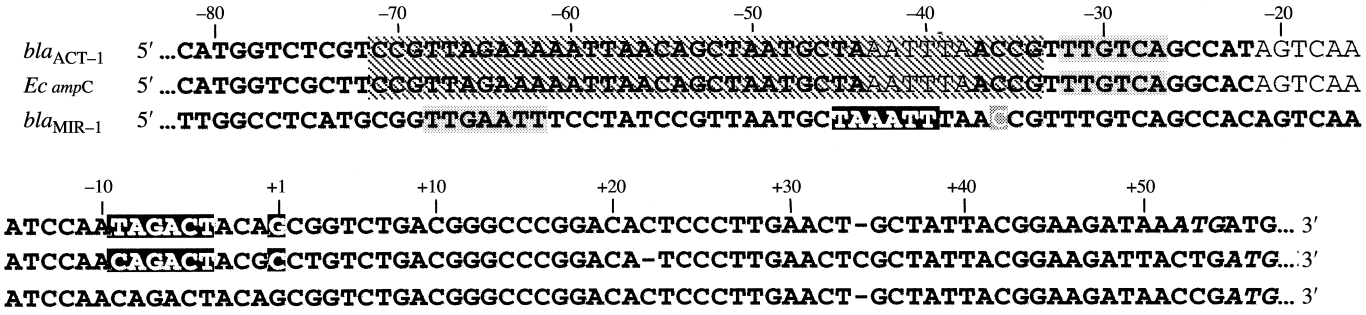

Figure 4. The upstream regions of bla_{ACT-1}, bla_{MIR-1} and both *E. cloacae ampC* (Ec *ampC*) gene start sites. Putative –10 promoter elements are shadowed in black with white letters, –35 promoter elements are shadowed in grey with black letters, and the start sites of transcription are indicated with grey shading and white letters. The AmpR binding site sequence is shaded. The +1 for bla_{MIR-1} is located at position –36 relative to bla_{ACT-1} and the chromosomal *ampC* genes.

E. cloacae 55, expressing an inducible chromosomal *ampC* gene does not exhibit high β-lactam MICs. However, the susceptibility profile of an organism expressing an inducible plasmid-encoded *ampC* gene in an AmpD⁻ background is unknown. Therefore, β-lactam MICs were determined for *E. coli* strains that were either AmpD⁺ or AmpD⁻ expressing either ACT-1 or MIR-1. Under these conditions, the β-lactam MICs increased two- to 16-fold in AmpD⁻ *E. coli* expressing ACT-1 when compared with AmpD⁺ *E. coli* expressing ACT-1, whereas the β-lactam MICs for *E. coli* ΔDpMIR-1 did not change from those for *E. coli* WTpMIR-1 for any drug tested (Table 3). As indicated in Table 3, the β-lactam MICs observed for *E. coli* WTpACT-1 were within one dilution of those for both *E. coli* WTpMIR-1 and *E. coli* ΔDpMIR-1.

Discussion

AmpD mutations have been associated with constitutive high-level *ampC* expression (derepression) and increases in cephalosporin MICs for organisms encoding inducible chromosomal *ampC* β-lactamase genes.[7] These mutations occur spontaneously in every 10^3–10^7 bacterial cells.[29,30] AmpD functions in the cell wall recycling pathway making it a necessary component of other Gram-negative organisms. However, AmpD mutants in clinical isolates of *E. coli*, *K. pneumoniae*, *Salmonella* spp. and other bacterial strains have not been documented because no phenotype can differentiate these mutants from WT cells due to the lack of an inducible chromosomal *ampC* gene. These studies have demonstrated that ACT-1 expressed in an AmpD⁻ background results in an increase in β-lactam MICs of two- to 16-fold. This effect was not observed for the non-inducible plasmid-encoded *ampC* gene, bla_{MIR-1}. Clinical laboratories should be aware of the possibility of phenotypically 'derepressed' *E. coli*, *K. pneumoniae*, *Salmonella* spp. and other Gram-negative strains expressing inducible plasmid-encoded AmpC β-lactamases and the concomitant increase in β-lactam MICs associated with this genotype.

In addition to correlating β-lactam MICs with *ampC* expression, this study aimed to determine what factors influence high-level expression of plasmid-encoded *ampC* genes of *Enterobacter* origin by correlating *in vivo* constitutive *ampC* expression levels with copy number and genetic characteristics. In studies using cloned *ampC*/*ampR* constructs expressed in *E. coli*, in the absence of AmpR, *ampC* expression was shown to increase 2.5- to 5.8-fold.[4,12] Until recently, all plasmid-encoded *ampC* genes described in the literature were not associated with an *ampR* gene. Therefore, it was predicted that expression from a plasmid-encoded *ampC* gene, in the absence of AmpR, would increase 2.5- to 5.8-fold. However, using primer extension, this study demonstrated that bla_{MIR-1}, a plasmid-encoded *ampC* gene expressed in the absence of AmpR, exhibited a 95-fold increase in expression relative to WT*ampC*. These expression levels are 16- to 38-fold higher than the amount of expression previously reported using reporter gene assays in *E. coli* or cloning these genes into vectors such as pACYC184.[12] These data indicate that the level of plasmid-encoded *ampC* expression in clinical isolates lacking AmpR is not due simply to a release from repression by AmpR. Furthermore, for constitutive bla_{ACT-1} expression, a 33-fold increase in expression was observed (Figure 5). This level of expression was in contrast to what might be predicted based on the genetic arrangement of the plasmid and its correlation with chromosomal *ampC* genes.

The differences observed in the relative amounts of RNA expression between bla_{ACT-1} and bla_{MIR-1} suggested that other factors played a role in the overall expression of these genes. It has been suggested that high-level expression of plasmid-encoded *ampC* β-lactamase genes results from the gene being encoded on high copy number plasmids.[22] However, no evidence has been published to substantiate or refute this hypothesis. The copy number of bla_{ACT-1} was 2, and demonstrated that high-level expression of plasmid-encoded *ampC* genes

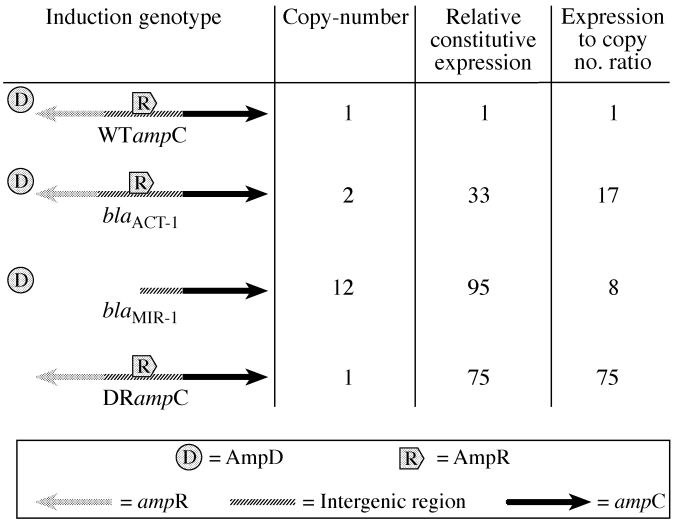

Figure 5. Data summary and expression per copy number ratio. The first column represents the genetic organization of each gene and the genetic make-up of each organism with respect to the inducibility of the *ampC* gene. The presence of AmpD represents a WT amidase in these cases. Column two represents the copy number of the *ampC* gene as described in the text. The third column represents the relative constitutive expression of each *ampC* gene compared with constitutive expression of the WT chromosomal gene. The fourth column represents the expression to copy number ratio when compared with a single-copy WT chromosomal *ampC* gene as described in the text.

does not need to be mediated by high copy number plasmids. However, a moderate copy number (12) of the plasmid encoding bla_{MIR-1} was present in the clinical isolate *K. pneumoniae* 96D.[31] These data indicated that multiple mechanisms are involved in the high-level expression of plasmid-encoded *ampC* genes.

Contribution of copy number to overall gene expression was analysed in the four clinical isolates expressing *ampC* genes of *Enterobacter* origin by comparing the ratio of constitutive expression with gene copy number (Figure 5). The expression per copy number ratio for bla_{MIR-1} is 8, whereas for bla_{ACT-1} it is 17. The similarities between the genetic context of bla_{ACT-1} and the WT*ampC* would predict that the copy number for bla_{ACT-1} would be the same as the relative fold increase in expression, i.e. 33-fold. This was not the case as the copy number of bla_{ACT-1} is two, yielding an expression per copy number ratio of 17. These data indicate that copy number does not account for the difference observed in expression levels between bla_{ACT-1} and WT*ampC*. When the promoter regions of bla_{ACT-1} and WT*ampC* were compared, only three mutations were observed. The most significant mutation occurred in the putative –10 promoter element of bla_{ACT-1}. This mutation was located at position –9 and resulted in a C to T transition, which increased the similarity of the bla_{ACT-1} promoter to the *E. coli* –10 consensus sequence TATAAT (Figure 4).[20] This same substitution in *E. coli ampC* promoters has been shown to increase expression seven- to 20-fold.[32,33] Thus, these data indicate that the point mutation in the bla_{ACT-1} promoter contributes to the increased expression observed for bla_{ACT-1} transcription compared with wild-type expression.

The expression to copy number ratio of 8 for bla_{MIR-1} was surprising. Copy number has been implicated as the major mechanism to explain the increase in AmpC β-lactamase activity when the gene is encoded by a plasmid.[22] This hypothesis would predict that the overall expression of bla_{MIR-1} would be six-fold higher than bla_{ACT-1}. However, the difference in total RNA expression between the two genes was only three-fold. In addition, this same hypothesis would predict that the copy number difference observed between the two genes would be three-fold not six-fold. Furthermore, comparisons between promoter expression of bla_{MIR-1} and bla_{ACT-1} in *E. coli* when the genes were encoded by the same vector indicated that the difference in expression levels was less than two-fold. These data support the expression to copy number ratio for bla_{MIR-1} being less than the bla_{ACT-1} ratio and indicate that not all the plasmid copies of bla_{MIR-1} may participate equally in the overall expression. In addition to the expression to copy number ratio, the location of the start site of bla_{MIR-1} transcription was unique compared with other *Enterobacter ampC* genes. Mapping the bla_{MIR-1} start site to position –36, relative to the transcript start sites for bla_{ACT-1} and chromosomal *ampC* genes, indicated that the putative –10 position correlates to TAAATT (Figure 4). This –10 element also has higher similarity to *E. coli* consensus than the –10 elements of the *E. cloacae* chromosomal *ampC* genes. These data indicate that *ampC* promoter mutations may be necessary to obtain high levels of β-lactamase gene expression. In addition, the creation of new promoters upon insertion of *ampC* genes in the plasmid may be necessary to drive expression levels required for resistance.

In summary, the mechanism of plasmid-encoded AmpC β-lactamase resistance is multifaceted and probably fluctuates depending on the genetic organization and background in which these genes are expressed. This will make 'predicting' the β-lactam MIC values for a particular plasmid-encoded AmpC β-lactamase somewhat 'unpredictable' and incredibly challenging.

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