African Journal of Biotechnology Vol. 7 (15), pp. 2547-2550, 4 August, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

The effect of mutations in the AmpC promoter region on β-lactam resistance from an *Escherichia coli* clinical isolate in a public sector hospital in KwaZulu-Natal, South Africa

Chunderika Mocktar[1]*, Usha Govinden[1], A. Willem Sturm[2] and Sabiha Essack[1]

[1]School of Pharmacy and Pharmacology, Faculty of Health Sciences, University of KwaZulu-Natal, South Africa.
[2]Department of Medical Microbiology, Nelson R Mandela Medical School, University of KwaZulu-Natal, South Africa.

Accepted 16 June, 2008

The ampC promoter and attenuator regions of an *Escherichia coli* clinical isolate from a public hospital in KwaZulu-Natal was investigated to detect the presence of mutations in these regions. The isolate was subjected to MIC determinations, IEF analysis, PCR for the presence of β-lactamases and sequencing of the *ampC* gene. Analysis of the *ampC* promoter and attenuator regions of the isolate showed that the isolate had mutations in the promoter region and this included insertions of nucleotides in the spacer region between the -10 and -35 Pribnow boxes. The insertion of an extra nucleotide in the spacer region between the -10 and -35 boxes affects the resistance of bacteria to β-lactam antibiotics.

Key words: *Escherichia coli,* promoter, pribnow box.

INTRODUCTION

All strains of *Escherichia coli* possess a gene that encodes an AmpC β-lactamase (Clark et al., 2003). The AmpC gene in *E. coli* is normally located on the chromosome and is weakly expressed because of a weak promoter and a weak transcriptional attenuator (Siu et al., 2003; Mulvey et al., 2005). Most *E. coli* strains do not produce clinically relevant levels of the chromosomally encoded AmpC β-lactamase (Martinez-Martinez et al., 2000). Wild-type strains produce a basal level of this enzyme which does not result in ampicillin and cephalosporin resistance (Siu et al., 2003; Mulvey et al., 2005). Gene amplification or mutations at either the promoter and/or attenuator regions of the structural β-lactamase gene can result in AmpC hyper-production (Martinez-Martinez et al., 2000). Such hyper-production of AmpC β-lactamase contributes to resistance to ampicillin, extended-spectrum cephalosporins and β-lactam-β-lactamase inhibitor combinations (Martinez-Martinez et al., 2000; Siu et al., 2003). AmpC β- lactamases display similar MIC values to extended-spectrum β-lactamases (ESBLs). However, in contrast to ESBLs, AmpC β-lactamases are poorly inhibited by β-lactam-β-lactamase inhibitor combinations (Siu et al., 2003).

Unlike other members of the family Enterobacteriaceae, in *E. coli, ampC* is not inducible since there is no *ampR* regulatory gene (Caroff et al., 2000) and consequently, in *E. coli* the level of transcription of the *ampC* gene depends mostly on the strength of the *ampC* promoter. *E. coli* harbours two hexamers of conserved sequences, the -35 region and the -10 one, called the Pribnow box, which play an important role in gene transcription. The -35 consensus sequence TTGACA and the -10 consensus sequence TATAAT have been described (Corvec et al., 2002). Genetically, promoter changes, an optimized distance (17 bp) in the Pribnow box (-35 and -10) and the presence of more than one copy of *ampC*, have been described as crucial factors for *ampC* hyper-production (Nelson and Gay Elisha, 1999; Siu et al., 2003). Mutations in the promoter region of *ampC* have been described as a mechanism for AmpC hyper-production. The mutations are thought to generate

*Corresponding author: E-mail: mocktarc@ukzn.ac.za. Tel: +27 31 260 7358; Fax: +27 31 260 7792

Table 1. Primers used in PCR studies.

Primer	Sequence	Purpose of primer	Reference
TEM 1(F)	5'-ATGAGTATTCAACATTTCCGTG-3'	Amp	Essack et al., 2001
TEM 2(R)	5'-TTACCAATGCTTAATCAGTGAG-3'	Amp/Seq	Essack et al., 2001
TEM 3(R)	5'-TTCTGTGACTGGTGAGTACT-3'	Seq	Essack et al., 2001
TEM 4(R)	5'-GAGTAAGTAGTTCGCCAGTT-3'	Seq	Essack et al., 2001
TEM 5(F)	5'-CTGCAGCAATGGCAACAAC-3'	Amp/Seq	Designed for this study
COL A	5'-ACGACGCTCGCGCCTTA-3'	Amp/Seq	Bret et al., 1998
COL B	5'-AAGAATCT GCCAGGCGGC-3'	Amp/Seq	Bret et al., 1998
AmpC R1	5'-GTTTGCTGCGTGACGGGCTG -3'	Amp/Seq	Designed for this study
AmpC F1	5'-ACCACGCGAT GCACGATCTG-3'	Amp/Seq	Designed for this study
AmpC R2	5'- GATGACA GCAAGGAAAAGCGGAG-3'	Amp/Seq	Designed for this study
AmpC F2	5'-GCCGGTAAATCCTGACACCATC-3'	Amp/Seq	Designed for this study
CTX-MF	5'-TTTGCGATGTGCAGTACCAGTAA-3'	Amp/Seq	Edelstein et al., 2003
CTX-MR	5'-CGATATCGTTGGTGGTGCCATA-3'	Amp/Seq	Edelstein et al., 2003

F, forward primer; R, reverse primer; Amp, amplification; Seq, sequencing.

promoters that more closely resemble the *E. coli* consensus, which leads to over-expression of the normally low level constitutively expressed *ampC* (Mulvey et al., 2005; Tracz et al., 2005). The most frequently described *E. coli ampC* 'strong' promoter harbours mutations at positions -88, -82, -42, -18, -1 and +58 (Caroff et al., 2000). Attenuator mutations are thought to destabilise the hairpin structure allowing greater read-through (Mulvey et al., 2005; Tracz et al., 2005).In this study we examined the DNA sequence upstream of the *ampC* gene in an *E. coli* isolate from a patient in a hospital in KwaZulu-Natal, South Africa with increased resistance to cefoxitin to determine mutations occurring in the promoter and attenuator regions.

MATERIALS AND METHODS

Bacterial strains

E. coli NGZ55 was isolated from a patient in a public sector hospital in KwaZulu-Natal, South Africa in 2000. The identity of the isolate as *E. coli* was confirmed using the API20E identification system (bioMérieux sa, Lyon, France). *E. coli* ATCC 25922 was used as the MIC control.

Antibiotic susceptibility testing

Susceptibility testing was undertaken using the disc diffusion test according to CLSI guidelines (2005). MICs values were extrapolated by the BIOMIC an automated reading system and software (Giles Scientific, New York), using the following antibiotics: ampicillin, ampicillin/sulbactam, amoxicillin/clavulanate, ticarcillin, piperacillin, piperacillin/tazobactam, cephalotin, cefuroxime, cefoxitin, ceftriaxone, cefotaxime, ceftazidime, cefepime, meropenem and aztreonam (Mast Diagnostics, Merseyside, UK).

β-lactamase analysis

The β-lactamases of the *E. coli* clinical isolate was extracted from a Nutrient Broth (Biolab, South Africa) culture as described previously (Livermore and Williams, 1996). Analytical isoelectric focusing (IEF) was performed in ampholine polyacrylamide gels (pH 3.5 - 9.5; Amersham Biosciences, Uppsala, Sweden). β-lactamase bands were detected with nitrocefin (Oxoid). An isoelectric point marker pI calibration kit (4.7 to 10.6; BDH, England) was used as the standard.

PCR detection of bla_{TEM}, bla_{CTX}, and bla_{AmpC} genes

Bacterial DNA was prepared by suspending few freshly grown colonies from an overnight Nutrient Agar (Biolab, South Africa) culture of *E. coli* in 50 µl of purified water and heating the cells at 95°C for 5 min. PCR amplifications were then performed in a Gene Amp PCR System (Applied Biosystems, USA). The primers used are described in Table 1. PCR conditions for the amplification of bla_{TEM} was carried out as described by Essack et al. (2001). The amplification mixture for the detection of bla_{CTX} and bla_{AmpC} genes were each made up to 50 µl and contained 25 µl AmpliTaq Gold PCR Master Mix (Applied Biosystems, California, USA), purified water, 10 pmol of each primer (Inqaba Biotechnology, Pretoria, South Africa) and 2 µl of the template DNA. PCR conditions were as follows: AmpC: an initial denaturation for 30 s at 94°C, 30 cycles of 30 s at 94°C, 30 s at 57°C, 1 min at 72°C and a final extension step of 7 min at 72°C; and CTX: an initial denaturation for 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C and a final extension step of 7 min at 72°C. PCR products (5 µl) were analyzed by gel electrophoresis. Gels were stained with ethidium bromide and photographed using UV illumination. PCR products were purified and sequenced (Spectrumedix SCE2410 genetic analysis system, Spectrumedix, Pennsylvania, USA).

RESULTS

The MIC results obtained for *E. coli* NGZ55 and the control strain, *E. coli* ATCC 25922 are shown in Table 2. *E. coli* NGZ55 was resistant to amoxicillin/ clavulanate, ampicillin, cephalotin, cefoxitin and cefuroxime. IEF analysis revealed the presence of β-lactamases with pIs of 6.5 and 6.6. PCR and sequencing data showed that the isolate harboured TEM-1 and CTX-M1. Comparison of

Table 2. MIC (µg/ml) profiles of *E. coli* NGZ55 and *E. coli* ATCC 25922 (control strain).

Isolate	AMP	PIP	AMC	TZP	CEF	CXM	FEP	FOX	CTX	CRO	CAZ	MEM	ATM
NGZ55	6	10	>64	4	>128	8	2	>96	1	2	6	0.5	4
ATCC 25922	4	ND	6	1	24	4	<1	2	<0.5	<1	1	<0.5	<2

AMP, ampicillin; PIP, piperacillin; AMC, amoxicillin/clavulanate; TZP, piperacillin/tazobactam; CEF, cephalotin; CXM, cefuroxime; FEP, cefepime; FOX, cefoxitin; CTX, cefotaxime; CRO, ceftriaxone; CAZ, ceftazidime; MEM, meropenem; ATM, aztreonam.
ND = not determined.

Table 3. Mutations in promoter and attenuator regions[a].

Isolate	Mutation
E. coli NGZ55	Extra G between -26 and -25; A(-10)C; extra A between +8 and +9; extra C between +39 and +38; extra C between +54 and +53

[a]Compared with *E. coli* K-12 (Caroff et al*.,* 2000).

the AmpC sequences using Blast 2.0 (http://www.ncbi.nlm.nih.gov/Blast; last accessed June 2006) showed that the sequences corresponded to the chromosomal AmpC sequences of *E. coli*.

The *E. coli* isolates showed changes in the AmpC promoter region compared to that of *E coli* K-12. The changes observed in the promoter and attenuator regions are shown in Table 3. The optimal distance between the -10 and-35 Pribnow boxes is 17bp. *E. coli* NGZ55 had an additional nucleotide, G, between positions -26 and -25. Mutations in the -10 box included changes at position -10 and this changed the -10 box from TAC**A**AT to TAC**C**AT (changes in boldface). Changes upstream of the -35 box included A(-82)C, T(-47)G, A(-43)C, G(-40)C, A(-39)C and A(-37)T.

Isolate *E. coli* NGZ55 in addition showed a further nucleotide addition, A, between positions +8 and +9. Changes between the attenuator region and the start codon included the following: T(+41)G, T(+43)A and T(+45)G. *E. coli* NGZ55 also had the following extra nucleotides: an additional C between positions +39 and +38 and an additional C between positions +54 and +53.

No SHV-, OXA- and plasmid-mediated AmpC type-β-lactamases were detected (data not shown).

DISCUSSION

E. coli is an important pathogen that commonly causes serious infections (Forward et al., 2001) and therefore, the emergence of *E. coli* strains resistant to the cephamycins should be a cause for concern to clinicians managing infections in both the community and institutional setting. Some of these strains have become resistant by virtue of their hyperproduction of chromosomally encoded AmpC β-lactamases whilst others have acquired plasmidic β-lactamases; most often those derived from *Citrobacter freundii* (Clark et al*.,* 2003).

The chromosomal cephalosporinase gene, *ampC*, of *E. coli* is regulated by a weak promoter and a transcriptional attenuator. Strains carrying the wild-type gene produce low basal amounts of AmpC and are inherently susceptible to ampicillin. Occasionally, this enzyme is overproduced in *E. coli* and these strains are resistant to cefoxitin and have reduced susceptibilities to the newer β-lactams such as the oxyiminocephalosporins. Some hyperproducers also contain more than one copy of *ampC*, while others contain mutations in the regulatory and/or attenuator regions of *ampC*, resulting in more efficient transcription of the structural gene (Nelson and Gay Elisha, 1999).

AmpC β-lactamase production in *E. coli* is controlled by a typical *E. coli* promoter. There is no repressor gene, as is seen in *Enterobacter cloacae* and *C. freundii*. An analysis of the promoter region in *E. coli* revealed the presence of two conserved regions, the -35 box and the -10 box, also called the Pribnow box. The sequence of the -35 and -10 hexamers and the interbox distance are crucial for efficient binding of RNA polymerase, thereby influencing the level of transcription of the gene (Caroff et al., 2000; Forward et al., 2001; Corvec et al., 2002). An interbox distance of 16 bp and the presence of a hairpin attenuator structure contribute to low-level transcription of the gene (Corvec et al*.,* 2002). For most promoters the degree of homology to the -35 box consensus sequence, TTGACA, and the -10 box consensus sequence, TATAAT, is directly related to promoter strength. Variations in the promoter and attenuator regions of *ampC* are a mechanism of hyperproduction of the AmpC protein that results in resistance or reduced susceptibility to cefoxitin (Mulvey et al*.,* 2005). Susceptible strains of *E. coli* have an inefficient *ampC* promoter that differs by several nucleotides from the strong promoter sequence. Consequently, very little AmpC β-lactamase is produced (Forward et al*.,* 2001).

In this study, isolate *E. coli* NGZ55 had an extra nucleotide, G, between positions -26 and -25. This created an optimal distance of 17 bp between the -10 box

and the -35 box and this has been shown in previous studies to play a role in promoter strength (Forward et al*.,* 2001; Siu et al*.,* 2003; Mulvey et al*.,* 2005). This isolate was resistant to cefoxitin with reduced susceptibility to ceftazidime and aztreonam. However, this isolate was susceptible to piperacillin/tazobactam and ampicillin/ sulbactam (results not shown) and this MIC profile is suggestive of an ESBL-producer. However, PCR and DNA sequencing studies have shown the presence of only TEM-1 and CTX-M1 in this isolate. This isolate, in addition to the extra nucleotide between positions -26 and -25, also had a mutation at position -10 to give a new -10 box which changed it from TAC**A**AT to TAC**C**AT (change shown in boldface). It also had nucleotide insertions in the regions just outside the attenuator region and this included an extra A between positions +8 and +9 and an extra C between positions +39 and +38. Sequencing data have also shown an extra nucleotide, C, between positions +54 and +53 which are situated close to the ATG start codon. Previous studies (Forward et al*.,* 2001) have shown that mutations in the attenuator region result in increased transcription of the *ampC* gene.

Conclusion

While chromosomal-mediated resistance might spread more slowly than mobilized β-lactamases, it is likely that with intense antibiotic pressure seen in today's medical practice that chromosomal-mediated resistance will become an increasing problem and may further limit our antibiotic choices (Forward et al*.,* 2001). Routine surveillance of sensitivity to cefoxitin in *E. coli* is thus advocated.

ACKNOWLEDGEMENTS

This study was funded by research grants from the MRC, NRF and the University of KwaZulu-Natal.

REFERENCES

Bret L, Chanal-Claris C, Sirot D, Chaibi EB, Labia R, Sirot J (1998). Chromosomally encoded AmpC-type β-lactamase in a clinical isolate of *Proteus mirabilis*. Antimicrob. Agents Chemother. 42: 1110-1114.

Caroff N, Espaze E, Gautreau D, Richet H, Reynaud A (2000). Analysis of the effects of -42 and -32 *ampC* promoter mutation in clinical isolates of *Escherichia coli* hyper-producing AmpC. J. Antimicrobiol. Chemother. 45: 783-788.

Clark B, Hiltz M, Musgrave H, Forward KR (2003). Cephamycin resistance in clinical isolates and laboratory-derived strains of *Escherichia coli*, Nova Scotia, Canada. Emerg. Infect. Dis. 9: 1254-1256.

Clinical and Laboratory Standards Institute (CLSI) (2005). Performance standards for antimicrobial susceptibility testing: 15th informational supplement. Clinical and Laboratory Standards Institute, Wayne, Pa.

Corvec S, Caroff N, Espaze E, Marraillac J, Reynaud A (2002). -11 mutation in the *ampC* promoter increasing resistance to β-lactams in a clinical *Escherichia coli* strain. Antimicrobiol. Agents Chemother. 46: 3265-3267.

Edelstein M, Pimkin M, Palagin I, Edelstein I, Stratchounski L (2003). Prevalence and molecular epidemiology of CTX-M extended spectrum β-lactamase production in *Escherichia coli* and *Klebsiella pneumoniae* in Russian hospitals. Antimicrobiol. Agents Chemother. 47: 3724-3732.

Essack SY, Hall LMC, Pillay DG, McFadyen ML, Livermore DM (2001). Complexity and diversity of *Klebsiella pneumoniae* strains with extended-spectrum β-lactamases isolated in 1994 and 1996 at a teaching hospital in Durban, South Africa. Antimicrobiol. Agents Chemother. 45: 88-95

Forward KR, Willey BM, Low DE, McGeer A, Kapala MA, Kapala MM, Burrows LL (2001). Molecular mechanisms of cefoxitin resistance in *Escherichia coli* from the Toronto area hospitals. Diag. Microbiol. Infect. Dis. 41: 56-63.

Livermore DM, Williams JD (1996). Mode of action and mechanisms of bacterial resistance. In: Lorian V (ed.) Antibiotics in Laboratory Medicine, 4th ed. Williams and Wilkens, Baltimore.

Martinez-Martinez L, Conejo MC, Pascual A, Hernandez-Alles S, Ballesta S, Ramirez De Arellano-Ramos E, Benedi VJ, Perea EJ (2000). Activities of imipenem and cephalosporins against clonally related strains of *Escherichia coli* hyper-producing chromosomal β-lactamase and showing altered porin profiles. Antimicrobiol. Agents Chemother. 44: 2534-2536.

Mulvey MR, Bryce E, Boyd DA, Ofner-Agostini M, Land AM, Simor AE, Paton S, the Canadian Hospital Committee, the Canadian Nosocomial Infection Surveillance Program, Health Canada (2005). Molecular characterisation of cefoxitin-resistant *Escherichia coli* from Canadian hospitals. Antimicrobiol. Agents Chemother. 49: 358-365.

Nelson EC, Gay Elisha B (1999). Molecular basis of AmpC hyper-production in clinical isolates of *Escherichia coli*. Antimicrobiol. Agents Chemother. 43: 957-959.

Siu LK, Lu P-L, Chen JY, Lin FM, Chang SC (2003). High-level expression of AmpC β-lactamase due to insertion of nucleotides between -10 and -35 promoter sequences in *Escherichia coli* clinical isolates: cases not responsive to extended-spectrum-cephalosporin treatment. Antimicrobiol. Agents Chemother. 47: 2138-2144.

Tracz DM, Boyd DA, Bryden L. Hizon R, Giercke S, Van Caeseele P, Mulvey MR (2005). Increase in *ampC* promoter strength due to mutations and deletion of the attenuator in a clinical isolate of cefoxitin-resistant *Escherichia coli* as determined by RT-PCR. J. Antimicrobiol. Chemother. 55: 768-772.