ampC gene expression in promoter mutants of cefoxitin-resistant *Escherichia coli* clinical isolates

Dobryan M. Tracz[1], David A. Boyd[1], Romeo Hizon[1], Elizabeth Bryce[2], Allison McGeer[3], Marianna Ofner-Agostini[4], Andrew E. Simor[5], Shirley Paton[6], Michael R. Mulvey[1] & the Canadian Nosocomial Infection Surveillance Program[†]

[1]National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada; [2]The Vancouver General Hospital, Vancouver, BC, Canada; [3]Mount Sinai Hospital, Toronto, ON, Canada; [4]The University of Toronto, Toronto, ON, Canada; [5]The Department of Microbiology, Sunnybrook and Women's College Health Sciences Centre, Toronto, ON, Canada; and [6]Division of Nosocomial and Occupational Infections, Centre for Infectious Disease Prevention and Control, Public Health Agency of Canada, Ottawa, ON, Canada

Correspondence: Michael R. Mulvey, Nosocomial Infections, National Microbiology Laboratory, Public Health Agency of Canada, 1015 Arlington St., Winnipeg, MB, Canada R3E 3R2. Tel.: +204 789 2133; fax: +204 789 5020; e-mail: michael_mulvey@phac-aspc.gc.ca

[†]Members of the Canadian Nosocomial Infection Surveillance Program are listed in the Acknowledgments.

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Abstract

Reverse transcriptase polymerase chain reaction was used to determine the amount of overexpression of the *ampC* gene in 52 cefoxitin-resistant *Escherichia coli* clinical isolates that had previously characterized mutations in their *ampC* promoter/attenuator regions. The results showed that mutations that create a consensus −35 box (TTGACA) are the most important factor in strengthening the *ampC* promoter, followed by base pair insertions that increase the distance between the −35 and −10 boxes to 17 or 18 bp. Mutations in the −10 box are of lesser importance and those in the attenuator region appear to have little effect on *ampC* expression. Three strains overexpress *ampC* due to the effect of insertion elements located in the *ampC* promoter regions. Further, the data show that there is no correlation between *ampC* overexpression and the minimum inhibition concentration of cefoxitin in clinical isolates. Overall, the data indicate that a combination of *ampC* promoter mutations and other strain-specific factors combine to contribute to the magnitude of cefoxitin resistance in *E. coli*.

Introduction

In *Escherichia coli*, chromosomal cephalosporinase gene (*ampC*) is transcribed constitutively from a weak promoter and is subject to attenuation (Jaurin *et al.*, 1981, 1982). Overexpression of the chromosomal *ampC* gene can result in resistance to ampicillin, cefoxitin and expanded-spectrum cephalosporins. The primary mechanism of AmpC hyperproduction is mutation of the *ampC* promoter region to one that more closely resembles the *E. coli* consensus sigma 70 promoter sequence, a TTGACA −35 box separated by 17 bp from a TATAAT −10 box (Hawley & McClure, 1983). Besides mutations that change the wild-type −35 and −10 boxes to ones more closely resembling the *E. coli* consensus boxes, insertions that increase the space between them from 16 to 17 or 18 bp can occur (Jaurin *et al.*, 1982; Olsson *et al.*, 1983; Caroff *et al.*, 1999, 2000; Forward *et al.*, 2001; Corvec *et al.*, 2002; Siu *et al.*, 2003). In addition, mutations can occur that create an alternate displaced promoter with new −35 and −10 boxes separated by 17 bp (Olsson *et al.*, 1982; Caroff *et al.*, 1999, 2000; Nelson & Elisha, 1999; Forward *et al.*, 2001). Mutations also occur in the attenuator region and are thought to result in higher *ampC* expression levels through destabilization of the mRNA hairpin structure (Jaurin *et al.*, 1981).

In a previous report, 52 different *ampC* promoter/attenuator variant types were identified from a collection of cefoxitin-resistant *E. coli* isolated from Canadian hospitals (Mulvey *et al.*, 2005). Here, that study is extended using reverse transcriptase polymerase chain reaction (RT-PCR) to determine the effect of the promoter/attenuator variations on *ampC* expression and whether overexpression levels can be correlated to the magnitude of cefoxitin resistance in these clinical strains.

Materials and methods

Bacterial strains and antimicrobial susceptibility

The *ampC* promoter/attenuator variants examined in this study were from a subset of cefoxitin resistant *E. coli* [minimum inhibition concentration (MIC) of cefoxitin $\geq 32\,mg\,L^{-1}$] that were potential ESBL producers, as described previously (Mulvey *et al.*, 2005). A promoter type has been defined previously as follows: a unique sequence found for a 191 bp region that encompasses the promoter region (-35, -10, and spacer region), the attenuator region ($+17$ to $+37$) and the first 41 bp of the coding sequence of the *ampC* gene (Mulvey *et al.*, 2005). One isolate harbouring each promoter type was chosen for *ampC* expression analysis. In total, 49 *ampC* promoter/attenuator variant isolates plus three isolates with two different types of insertion sequences in the *ampC* promoter region were studied. *Escherichia coli* ATCC 25922 was used as the control for a normally expressed (low level constitutive) *ampC* gene. The MICs for cefoxitin were determined using the Etest (AB Biodisk, Solna, Sweden). The strains used have been characterized previously for the possession of secondary β-lactamases (Mulvey *et al.*, 2005).

RNA isolation and RT-PCR

Clinical isolates were grown in Luria–Bertani broth to the mid-log phase and RNA was isolated with a Qiagen RNEasy RNAprotect Mini Kit (Qiagen Inc., Mississauga, ON). A SuperScript III Platinum Two-step Quantitative RT-PCR kit (Invitrogen Corp., Burlington, ON) was used to assay for expression of *ampC* and glyceraldehyde dehydrogenase (*gapA*) using the AmpC LUX353FL/353FL_361RU and GapA LUX620FL/620FL_671RU primer sets with each reaction performed in triplicate, as described previously (Tracz *et al.*, 2005). Real-time PCR was performed in 96-well plates in an ABI Prism 7900 HT (Applied Biosystems, Foster City, CA), and data were analysed using SEQUENCE DETECTION software 2.0 (Applied Biosystems). The delta–delta cycle threshold method ($2^{-\Delta\Delta C_T}$) was used for the relative quantification of *ampC* expression between isolates (Livak & Schmittgen, 2001). Controls included a no-template negative control, an *E. coli* ATCC 25922 genomic DNA positive control and a PCR control to confirm absence of DNA in RNA samples.

Results and discussion

In a previous study, *ampC* promoter regions were analysed from 183 cefoxitin-resistant *E. coli* isolated from Canadian hospitals and 49 sequence variants were identified when compared with the wild-type *E. coli* K12 sequence (Mulvey *et al.*, 2005). In order to relate the *ampC* promoter structure with gene expression, a real-time RT-PCR assay was used for the relative quantification of *ampC* gene expression as compared with that in *E. coli* ATCC 25922, a strain with a wild-type promoter providing low-level constitutive expression (Tracz *et al.*, 2005). To facilitate data analysis, promoter sequence types were divided into 12 groups based upon a known functional element(s) containing mutations (Fig. 1, Table 1). The functional elements are the -35 and -10 boxes and the spacer region of the wild-type promoter, the -35 and -10 boxes of the alternate displaced promoter, and the attenuator region. Promoter types may also have

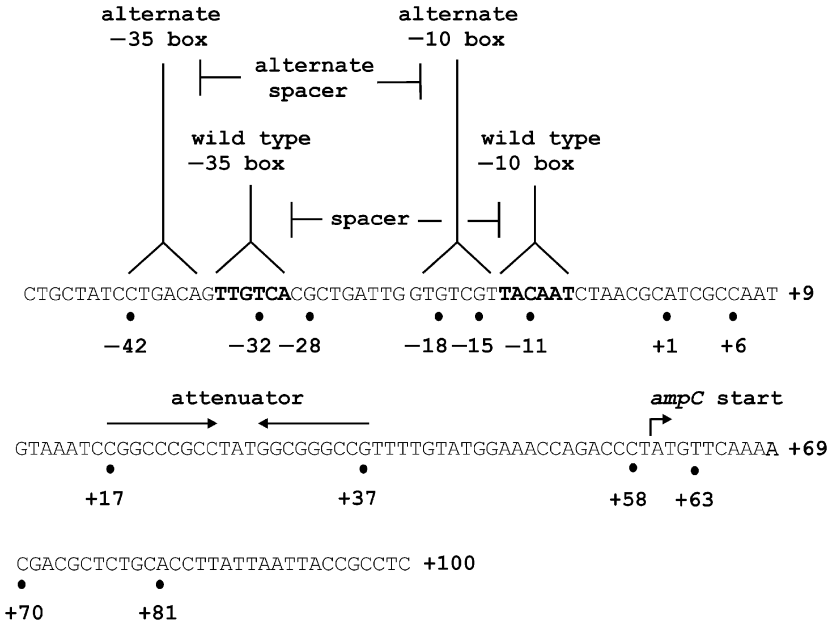

Fig. 1. Sequence of the *Escherichia coli* ATCC 25922 *ampC* promoter region. The sequence elements referred to in the text are indicated. Numbering is according to Jaurin *et al.* (1981).

Table 1. *ampC* promoter groupings and types examined in this study

Group*	Type†	$2^{-\Delta\Delta C_T}$‡	Cefoxitin MIC§	Group*	Type†	$2^{-\Delta\Delta C_T}$‡	Cefoxitin MIC§
Mutations in the wild-type promoter	2	9.8	8	Wild-type			
	9	11.8	192	/alternate −10 box	48‖	123.9	> 256
	23	19.0	24				
	24	45.8	32	Spacer −1 nt	8	24.4	16
	26	43.1	256	Insertion	42	33.6	8
	40	8.3	192		49	61.6	> 256
Mutations in the wild-type promoter	1	63.0	> 256	Spacer −1 nt	10	41.5	64
and the attenuator	13	23.2	> 256	Insertion	27	44.5	48
	20	39.9	24	/attenuator	36	64.3	16
	28	27.1	> 256		37	50.2	48
	39	49.2	24				
	43	23.4	> 256	Spacer −2 nt	25	19.0	24
	44	24.8	16	Insertion			
	46	21.4	> 256				
	51	68.2	24	Spacer −2 nt	6	57.6	32
	35	2.8	24	Insertion	32	82.8	> 256
				/attenuator	38	33.7	24
Mutations in the attenuator	4	1.3	48		50	27.8	24
	7‖	1.0	96		54	46.2	> 256
	14‖	2.0	32				
				Other	5	0.8	64
Mutations that create an alternate	3	22.0	48		11	1.6	24
displaced promoter	56¶	20.4	64		12	1.5	96
	15	1.5	8		17‖	0.5	> 256
	18	2.1	48		45	1.7	64
	55	3.7	16		52‖	2.6	> 256
Mutations that create an alternate displaced	29	59.7	64	IS elements	IS*911*	205.4	32
promoter and in attenuator	31	38.3	256		IS*10*	10.0	> 256
	33	140.6	256		IS*10*	9.0	24
	34	280.5	48				
	22‖	1.2	256				

*Defined by location of mutations in specific promoter elements as indicated in Fig. 1.
†As defined by Mulvey *et al*. (2005). The reader should refer to Table 3 of the given reference to see a complete characterization of each promoter type.
‡Delta–delta cycle threshold, read as fold-expression (Livak & Schmittgen, 2001).
§As determined by Etest in $\mu g\,mL^{-1}$.
‖Isolate harbours bla_{CMY-2} as determined by PCR (Mulvey *et al*., 2005).
¶Isolate harbours bla_{SHV-2a} as determined by PCR (Mulvey *et al*., 2005).

mutations at other positions outside of the functional element, and one group labelled 'Other' had only mutations outside of functional elements. Three strains that had insertion elements located in their *ampC* promoter regions were also analysed.

Mutations in wild type promoter

Promoter types in this group ($n = 6$) contained mutations in the wild-type −35 box (positions −30 to −35) and/or −10 box (positions −8 to −13). All contained the T → A transversion at position −32 that created a −35 box identical to the *E. coli* consensus TTG**A**CA (pertinent nucleotide in bold) (Hawley & McClure, 1983). The results showed that these promoters were overexpressed 8–46-fold. One promoter type, type 26, also had a C → T transition at position −11 that created a −10 box identical to the *E. coli* consensus TA**T**AAT. However, promoter type 26 (43-fold) was expressed similar to type 24 (45-fold), suggesting that the −32 mutation is most responsible for the increase in promoter strength, and that the mutation in the −10 box had a negligible effect on promoter strength. Two promoter types, types 9 and 26, also included a G → A transition at position −28 of the spacer region. Considering that three promoter types in the 'Other' category showed this change and overexpression was only 1–3-fold, there appears to be no or negligible contribution of the −28 change to promoter strength.

Mutations in wild-type promoter and attenuator

Except for a single type, type 35, promoters in this group contained the T → A transversion at −32 and also had mutations in the attenuator region (+17 to +37). Type 35

had the C → T transition at −11 as well as mutations in the attenuator. The results show that the *ampC* gene was overexpressed 21–68-fold, except for promoter type 35, which was overexpressed only about threefold. These results support the above supposition that a consensus −35 box is more important than a consensus −10 box in determining *ampC* promoter strength. In addition, as the results for the majority of types here are similar to the group with mutations in the wild-type promoter, 21–68-fold vs. 8–46-fold, respectively, it would appear that mutations in the attenuator have a negligible role in increasing promoter strength. One promoter type, type 28, contained a C → T transition at position −29 in the spacer region, but as for the change at position −28, it is unlikely that this change had a major impact on promoter strength.

Mutations in the attenuator

Promoter types in this group ($n = 3$) had mutations in the attenuator, positions +17 to +37. The *ampC* gene was expressed at levels similar to the control constitutive level in these promoter types (1–2-fold). Thus, as stated above, it appears that attenuator mutations thought to destabilize the stem loop have only a small role in increasing *ampC* promoter strength.

Mutations that create an alternate displaced promoter

Promoter types in this group ($n = 5$) had mutations that led to an alternate displaced promoter consisting of a −35 box at positions −37 to −42 separated by 17 bp from a −10 box at −14 to −19 (Fig. 1). A C → T transition at position −42 to create the alternate −35 box, **T**TGACA, and/or a G → A transition at position −18 created the alternate −10 box, TATCGT. A single type, type 15, besides the change at −18, had a G → A transition at −15 to create a TATC**A**T −10 box. These results further support the importance of a consensus −35 box to *ampC* overexpression. The two types with the −42 C → T change were overexpressed about 20-fold, whereas the three types without it but with the −18 change were overexpressed only 1.5–3.7-fold.

Mutations in the attenuator and those that create an alternate displaced promoter

The combination of mutations that form an alternate displaced promoter and mutation in the attenuator produced the highest levels of *ampC* expression observed in this study. Two promoter types with the −42 and −18 transitions and a mutated attenuator (types 33 and 34) were found to have 140- and 280-fold *ampC* expression levels, respectively. Two other promoter types with the same transitions at −42 and −18, types 29 and 31, were overexpressed 38- and 60-fold, respectively. In contrast, sequence type 22, which had a −18 transition and a mutated attenuator, showed virtually no increase in *ampC* expression over the wild type. As mentioned above, the data show that attenuator mutations play a small role in promoter overexpression.

Mutations that create a consensus −35 box and that create an alternate −10 box

A single promoter type, type 48, had the −32 T → A transversion that created a consensus −35 box in the wild-type promoter, and the −18 G → A transition in the alternate −10 box. The *ampC* gene was overexpressed about 124-fold most likely due to the consensus −35 box.

Spacer insertions with or without attenuator mutations

We identified three promoter types with a single bp insertion between positions −13 and −14 (position −13.1 in Table 1), either a G or a T, that increased the spacer between the wild-type −35 and −10 boxes from 16 to 17 bp. The results indicated a 24–61-fold increase in *ampC* promoter expression in these strains. The combination of a single nucleotide insertion and an attenuator mutation in four promoter types increased *ampC* expression 41–64-fold, showing that the main contributor to promoter strength is the increased spacer length. A single promoter type, type 25, had a two-nucleotide insertion between −13 and −14 (a TA) and this promoter was overexpressed 19-fold. Five promoter types combined the two-nucleotide insertion (either a GT or TA) with mutations in the attenuator region and these were overexpressed 27–83-fold. Thus, an increase in the spacer region of two nucleotides to 18 bp produces a promoter at least as efficient as one with a 17 bp spacer. Attenuator mutations have only a negligible or a small additive effect in combination with the spacer insertions.

Other mutations

Six promoter types were found to have changes only outside of the promoter elements. These included changes at −1, +6, +58, +63, +70 and +81, with the latter three occurring in the *ampC* coding region. Expression was generally measured at about wild-type levels (0.5–2.6-fold). These changes often occurred with other promoter element changes in other types, but it is likely that their contribution to *ampC* overexpression is negligible. As has been speculated previously, the changes at +63, +70 and +81 lead to amino acid changes in the leader peptide region and may have an effect on transfer of the enzyme precursor into the

periplasmic space, although this awaits further evidence (Mulvey *et al.*, 2005). Except for the two strains that also harboured a bla_{CMY-2} β-lactamase gene, the mechanism of cefoxitin resistance in these strains is unknown.

Insertion sequences

Two sequence types that had insertion elements in their *ampC* promoter region were previously identified (Mulvey *et al.*, 2005). One strain was found to have IS*911* inserted between +24 and +25, which provided a −35 box (TTGACC) separated from the wild-type −10 box by 17 bp. The *ampC* gene in this strain was overexpressed 205-fold. Two strains had an IS*10* element inserted between +35 and +36 near the 3′-end of the attenuator region. It is postulated that *ampC* expression is driven by the strong pOUT promoter of IS*10* resulting in 9–10-fold overexpression in these two strains (Simons *et al.*, 1983). A laboratory mutant of *E. coli* has been described that contained a hybrid *ampC* promoter formed by the insertion of IS2 between positions −14 and −15 that was overexpressed 20-fold (Jaurin & Normark, 1983).

Relationship between MICs of cefoxitin and promoter type

The promoter types in this study were selected from *E. coli* strains from Canadian hospitals that were resistant to cefoxitin ($\geq 32\ mg\ L^{-1}$) as determined by broth microdilution (Mulvey *et al.*, 2005). In that study, the highest concentration of cefoxitin tested was $64\ mg\ L^{-1}$ and hence the highest value that could be assigned to a strain was $\geq 64\ mg\ L^{-1}$. In order to determine whether promoter type (fold overexpression) was directly related to MIC of cefoxitin, Etest strips were used to allow examination of a wider range of MICs (Table 1). Despite being examined by Etest, the six strains that harboured bla_{CMY-2} were not considered in the analysis here as the β-lactamase would be expected to contribute to the overall cefoxitin resistance. It is noted that upon testing by Etest, three strains would be classified as cefoxitin susceptible (MICs $< 8\ mg\ L^{-1}$), and four would be classified as having intermediate resistance to cefoxitin ($16\ mg\ L^{-1}$). These seven strains all had mutations in their *ampC* promoters (Table 1) (Mulvey *et al.*, 2005). The discrepancies could have been due to the different methodologies used and/or different growth characteristics of these strains on solid vs. liquid media. Overall, the data indicate no direct relationship between promoter strength and MIC of cefoxitin. For example, promoter type 34 was overexpressed 280-fold, promoter type 18 was overexpressed twofold, and yet both strains had an MIC of cefoxitin of $48\ mg\ L^{-1}$. The two strains with IS*10* inserted in the promoter were overexpressed about 10-fold; yet, one had an MIC of cefoxitin of $> 256\ mg\ L^{-1}$ and one of $24\ mg\ L^{-1}$. Among the five strains harbouring bla_{CMY-2}, four of them had cefoxitin MICs of $\geq 256\ mg\ L^{-1}$ and one had an MIC of $32\ mg\ L^{-1}$. The lack of a correlation between type of *ampC* promoter mutation and β-lactam resistance has been described previously, as has a lack of correlation between abundance of *ampC* transcript and β-lactam resistance (Caroff *et al.*, 1999; Nelson & Elisha, 1999). In this study, the complete *ampC* gene was not studied and so one cannot rule out variant AmpC proteins with amino acid changes leading to different resistance levels. It has been shown that strains with variant AmpC proteins exhibit altered β-lactam resistance profiles (Morosini *et al.*, 1998; Mammeri *et al.*, 2004). Further, altered porin expression patterns have been shown to contribute to β-lactam resistance (Martínez-Martínez *et al.*, 2000; Ananthan & Subha, 2005) Hence, it is apparent that in the absence of an acquired class C β-lactamase, cefoxitin-resistant *E. coli* often have *ampC* promoter mutations, but other strain-specific factors play a role in determining the MIC of cefoxitin.

Conclusions

The present results showed that 83% of strains with promoter types with mutations in functional elements or that contained an insertion sequence in their *ampC* promoters overexpressed *ampC* 8–280-fold (Table 1). Strains with promoter types with mutations only in the attenuator region or that did not contain mutations creating a consensus −35 box only expressed *ampC* 1–4-fold, similar to strains with promoter types with mutations outside of functional elements (Table 1). This study supports previous studies that showed that it is the creation of a consensus −35 box, whether by a T → A transversion at position −32 or a C → T transition at position −42, that is the most dominant factor in strengthening the *ampC* promoter (Jaurin *et al.*, 1982; Olsson *et al.*, 1982, 1983; Caroff *et al.*, 1999, 2000; Nelson & Elisha, 1999; Forward *et al.*, 2001; Corvec *et al.*, 2003). The results also support previous studies showing that optimal spacing between −35 and −10 boxes is also an important factor contributing to promoter strength (Jaurin *et al.*, 1981; Siu *et al.*, 2003). Mutations in the −10 box or attenuator region by themselves have a negligible effect, although when combined with other more important mutations, they may contribute to overall *ampC* overexpression.

Although mutations that create a consensus −35 box and ones that increase the spacer region can lead to clinical cefoxitin resistance (at least $32\ mg\ L^{-1}$), it appears that an interplay of *ampC* overexpression and other strain-specific factors can affect the degree of resistance (i.e. MIC of cefoxitin) of individual strains.

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