Analysis of the effects of −42 and −32 ampC promoter mutations in clinical isolates of *Escherichia coli* hyperproducing AmpC

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*Escherichia coli* usually produces only very small amounts of a constitutive AmpC β-lactamase, but clinical strains overproducing this enzyme have been isolated. Three different ampC promoters of *E. coli* clinical strains were cloned upstream of the chloramphenicol acetyltransferase (CAT) gene in the pKK232-8 reporter plasmid and their relative strengths were compared by two different methods. The strength of the promoters from AmpC hyperproducers was 70- to 120-fold higher than those from a low-level AmpC producer. One of the strong promoters, which differs from strain K12 at bases −88, −82, −42, −18, −1 and +58, was mutated to abolish the −42 mutation. This change resulted in a 43-fold decrease in CAT concentration. In another promoter, with eight different mutations at positions −88, −82, −32, −18, −1, +5, +24 and +58, the −32T→A transversion, which created perfect homology with the −35 consensus sequence, was reverted; this led to a 13-fold decrease in CAT concentration. The −42 and −32 mutations play an important role in *E. coli* resistance to β-lactams by increasing ampC transcription.

**Introduction**

*Escherichia coli* AmpC cephalosporinase differs from other class C chromosomal β-lactamases of Enterobacteriaceae in that its production is not inducible but constitutive.\textsuperscript{1} As a consequence, AmpC production depends mostly on the strength of the ampC promoter, although other factors—such as gene amplification or insertion of an IS2 sequence—can cause AmpC hyperproduction \textit{in vitro}.\textsuperscript{2,3}

Numerous mutations have been described in the ampC promoter of clinical strains hyperproducing a chromosomal cephalosporinase,\textsuperscript{4–7} but the precise role of these mutations has not been determined. Some of them may be simple polymorphisms while others are certainly responsible for an increased transcription rate.

The most frequently described *E. coli* ampC ‘strong’ promoter harbours mutations at positions −88, −82, −42, −18, −1 and +58.\textsuperscript{5} The −42C→T transition creates a new displaced −35 perfect consensus sequence, TTGACA, separated by 17 bp from a new −10 sequence, caused by the −18G→A transition.\textsuperscript{7} Less frequent mutations have been described in clinical strains, particularly in the attenuator and in the fourth base of the −35 box. This last mutation has also been described in mutants selected \textit{in vitro}.\textsuperscript{8}

In this paper, different ampC promoters from *E. coli* clinical strains were cloned upstream of the chloramphenicol acetyltransferase (*cat*) gene in the reporter plasmid pKK232-8.\textsuperscript{9} By site-directed mutagenesis, we have investigated the role of the −32 and −42 mutations that create a consensus TTGACA sequence.

**Materials and methods**

**Bacterial strains and plasmids**

All plasmids were obtained by transforming highly competent JM109 cells (Promega, Charbonnières, France). pGEM-T Easy vector (Promega) was used for post-PCR cloning experiments, pKK232-8 (Amersham Pharmacia Biotech, Uppsala, Sweden) as a promoterless reporter gene and pALTER-Ex2 (Promega) for site-directed mutagenesis experiments. Strain 96006296 is a susceptible *E. coli* clinical isolate. *E. coli* strains 96004153 and 96010266 are AmpC hyperproducers.\textsuperscript{8} Details of the plasmids used in this study are given in Table I.

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PCR amplification of the E. coli ampC promoter

Primers AB1 (5’GATCGTTCTGCCTGCTGTG134) (5’ to 3’) and ampC2 (120GGGCAGCAAATGTGGAGCAA101) (5’ to 3’) were used to amplify a 271 bp fragment containing the –35 box, the –10 box and the attenuator from the E. coli ampC promoter.10 PCR amplification was performed in a Perkin–Elmer 480 DNA thermal cycler (Perkin–Elmer Applied Biosystems, Cergy-Pontoise, France). PCR reactions were performed in a final volume of 50 μL containing 10 mM Tris–HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 200 μM of each nucleotide, 0.5 μM of each primer, 2.5 units of Taq DNA polymerase (Gibco BRL Life Technologies SARL, Cergy Pontoise, France) and 10 ng of target DNA. After 90 s denaturation at 94°C, 30 PCR cycles were performed, each consisting of 90 s denaturation at 94°C, 30 s annealing at 57°C and 60 s extension at 72°C; a final extension step of 10 min at 72°C was performed.

Construction of reporter plasmids

The 271 bp PCR fragment was cloned in the post-PCR cloning vector, pGEM-T Easy. This plasmid was then digested with EcoRI and the fragment containing the ampC promoter was recovered from a low-melting-point agarose gel, blunt ended with the Klenow fragment of E. coli DNA polymerase I, and ligated into the SmaI restriction site of pKK232-8, previously treated with calf intestinal phosphatase (Promega). After overnight incubation at 16°C, these ligation products were used to transform highly competent JM109 cells (Promega).

Preparation of mutant promoters by site-directed mutagenesis

Site-directed mutagenesis was performed using the Altered Sites II Ex2 in vitro mutagenesis system (Promega). The

Table I. Plasmids used and constructed in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Resistance</th>
<th>Characteristics</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-T Easy</td>
<td>ampicillin</td>
<td>post-PCR cloning vector</td>
<td>Promega</td>
</tr>
<tr>
<td>pKK232-8</td>
<td>ampicillin</td>
<td>reporter vector</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pGEM-T Easy (96006296)</td>
<td>ampicillin</td>
<td>pGEM-T Easy containing the promoter from strain 96006296</td>
<td>Promega</td>
</tr>
<tr>
<td>pGEM-T Easy (96004153)</td>
<td>ampicillin</td>
<td>pGEM-T Easy containing the promoter from strain 96004153</td>
<td>constructed by cloning</td>
</tr>
<tr>
<td>pGEM-T Easy (96010266)</td>
<td>ampicillin</td>
<td>pGEM-T Easy containing the promoter from strain 96010266</td>
<td>constructed by cloning</td>
</tr>
<tr>
<td>pKK232-8 (96006296)</td>
<td>ampicillin</td>
<td>pKK232-8 containing the promoter from strain 96006296</td>
<td>constructed by cloning</td>
</tr>
<tr>
<td>pKK232-8 (96004153)</td>
<td>ampicillin</td>
<td>pKK232-8 containing the promoter from strain 96004153</td>
<td>constructed by cloning</td>
</tr>
<tr>
<td>pKK232-8 (96010266)</td>
<td>ampicillin</td>
<td>pKK232-8 containing the promoter from strain 96010266</td>
<td>constructed by cloning</td>
</tr>
<tr>
<td>pALTER-Ex2 (96004153)</td>
<td>tetracycline</td>
<td>pALTER-Ex2 containing the promoter from strain 96004153</td>
<td>constructed by cloning</td>
</tr>
<tr>
<td>pALTER-Ex2 (96004153M)</td>
<td>chloramphenicol</td>
<td>pALTER-Ex2 containing the promoter from strain 96004153 mutated at base –32</td>
<td>constructed by cloning and mutagenesis</td>
</tr>
<tr>
<td>pALTER-Ex2 (96010266)</td>
<td>tetracycline</td>
<td>pALTER-Ex2 containing the promoter from strain 96010266</td>
<td>constructed by cloning</td>
</tr>
<tr>
<td>pALTER-Ex2 (96010266M)</td>
<td>chloramphenicol</td>
<td>pALTER-Ex2 containing the promoter from strain 96010266 mutated at base –42</td>
<td>constructed by cloning and mutagenesis</td>
</tr>
<tr>
<td>pKK232-8 (96004153M)</td>
<td>ampicillin</td>
<td>pKK232-8 containing the promoter from strain 96004153 mutated at base –32</td>
<td>constructed by cloning</td>
</tr>
<tr>
<td>pKK232-8 (96010266M)</td>
<td>ampicillin</td>
<td>pKK232-8 containing the promoter from strain 96010266 mutated at base –42</td>
<td>constructed by cloning</td>
</tr>
</tbody>
</table>
EcoRI-digested fragment from pGEM-T Easy was subcloned in the EcoRI site of the mutagenesis vector pALTER-Ex2. This vector contains genes for tetracycline and chloramphenicol resistance, but the latter has been inactivated. During the mutagenesis reaction, a chloramphenicol repair oligonucleotide was annealed to alkali-denatured DNA at the same time as the mutagenesis oligonucleotide, and restored resistance to chloramphenicol. The mutagenesis reaction was carried out according to the manufacturer’s recommendations; mutants were selected on LB agar containing chloramphenicol 20 mg/L. The mutagenic oligonucleotides, Mut32 (41P-TGACA-GTGTTTCAGCTGAT33) (5’ to 3’) for strain 96004153 and Mut42 (51P-GCTGCTATCCTGACAGTTG) (5’ to 3’) for strain 96010266 were centred on the desired mutations and 5’-phosphorylated (Genosys Biotechnologies, London, UK). The EcoRI fragments from the mutated promoters were then subcloned in pKK232-8, as previously described.

Verification of the constructions

In order to control the sequence of the inserted fragments, two primers from pKK232-8, pKK1 (12GGAATTC-CAACGGCCCGGCGA) and pKK2 (312AAGCTTGCTT-GCGAGGTGAAG40) (5’ to 3’) were synthesized to amplify a 389 bp fragment containing the 271 bp from the ampC gene. The PCR products were then purified on a Bio-Gel P100 (Bio-Rad SA, Ivry Sur Seine, France) and sequenced in both strands with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer). Sequence analysis was performed on a ABI 373 DNA sequencer (Perkin–Elmer).

CAT assays

Bacteria containing each plasmid were cultured overnight in 20 mL of Luria broth. The bacterial pellet was obtained by centrifugation of the previous culture for 30 min at 10000g. After washing and resuspension in distilled water, bacteria were sonicated in a Branson Sonifier 250 (intermittent exposure: 5 × 30 s). After centrifugation, the supernatant was used for CAT determination. Total protein concentration was determined by the ECL protein assay (Boehringer Mannheim, Meylan, France) and the volume of extract used for CAT determination was adjusted to equalize the protein concentration. CAT enzyme was measured by a sandwich ELISA test performed in a microtitre plate (Boehringer Mannheim). This test, which quantifies CAT in transfected eukaryotic cells, was used according to the manufacturer’s recommendation except that five dilutions of total protein (250, 25, 2.5, 0.25 and 0.025 μg/mL) were tested for each sample to be in the linear range of the test. Each determination was performed in triplicate. Untransfected JM109 cells were used as a control.

MIC determinations

The chloramphenicol MIC was determined by serial two-fold dilution in Mueller–Hinton agar (Difco Laboratories, Detroit, MI, USA). Inocula of 10⁶ cfu per spot from an 18 h culture in Mueller–Hinton broth were applied with a Steers multiple-inoculum replicator. After 18 h incubation at 37°C, the chloramphenicol MIC was defined as the lowest concentration that prevented visible growth of the spot on the plate.

**Results**

**Strength of the different ampC promoters**

A 271 bp fragment containing the promoter of the gene for three different AmpC β-lactamases from three *E. coli* clinical strains was cloned upstream of the *cat* gene of the reporter plasmid pKK232-8. The sequences of the cloned fragments are shown in the Figure. One of the promoters came from a susceptible *E. coli* strain (96006296); the other two were from strains hyperproducing a chromosomal cephalosporinase. These constructs were used to transform *E. coli* JM109 competent cells. CAT determination was then performed in two ways: (i) indirectly, by measuring the chloramphenicol MIC; and (ii) directly, using an immunoenzymatic method (Table II).

With untransformed JM109 cells, the CAT concentration was low. In the same cells transformed with pKK232-8, neither the CAT concentration nor the MIC of chloramphenicol increased, confirming the absence of promoter activity for the *cat* gene in this plasmid. For plasmids containing a promoter from *E. coli* *ampC* β-lactamase, the CAT concentration was always greater. A significant difference was observed between the plasmid containing the promoter from the susceptible isolate and those containing

**Table II. Comparison of the strength of the ampC promoters from the *E. coli* clinical isolates**

<table>
<thead>
<tr>
<th>Plasmid used to transform JM109 cells</th>
<th>Chloramphenicol MIC (mg/L)</th>
<th>[CAT] (pg/μg)³</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>pKK232-8</td>
<td>8</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td>pKK232-8 (96006296)⁴</td>
<td>32</td>
<td>60.7 ± 6.4</td>
</tr>
<tr>
<td>pKK232-8 (96004153)⁴</td>
<td>2048</td>
<td>7408 ± 809</td>
</tr>
<tr>
<td>pKK232-8 (96010266)⁴</td>
<td>2048</td>
<td>4118 ± 482</td>
</tr>
</tbody>
</table>

³Mean ± s.d. of three determinations of CAT concentration.
⁴pKK232-8 without any insert.
⁵pKK232-8 containing the *ampC* promoter from strain 96006296 (low-level AmpC producer).
⁶pKK232-8 containing the *ampC* promoter from strain 96004153 (AmpC hyperproducer).
⁷pKK232-8 containing the *ampC* promoter from strain 96010266 (AmpC hyperproducer).
promoters from AmpC hyperproducers. In the presence of a strong promoter, the chloramphenicol MIC was 64-fold higher while CAT concentrations increased 70- to 120-fold, according to the type of promoter inserted.

Role of the –42 and –32 point mutations
Using site-directed mutagenesis, the promoter from strain 96010266 was modified by reverting the C→T transition at position –42. This single change in the promoter sequence resulted in the CAT concentration and chloramphenicol MIC decreasing to levels found with the promoter of a susceptible isolate (Table III).

Using the same method, the promoter from strain 96004153 was modified, causing reversion of the T→A transversion at position –32. This change resulted in a 13-fold decrease in the CAT concentration, and a decrease in chloramphenicol MIC from 2048 to 256 mg/L (Table III).

Table III. Effect of two different mutations on the strength of the promoters

<table>
<thead>
<tr>
<th>Plasmid used to transform JM109 cells</th>
<th>Mutations in the promoter(^d)</th>
<th>Chloramphenicol MIC(mg/L)</th>
<th>CAT (pg/μg)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKK232-8 (96010266)(^c)</td>
<td>–88, –82, (-42), –18, –1, +58</td>
<td>2048</td>
<td>4118 ± 482</td>
</tr>
<tr>
<td>pKK232-8 (96010266M)(^d)</td>
<td>–88, –82, –18, –1, +58</td>
<td>32</td>
<td>95 ± 8.3</td>
</tr>
<tr>
<td>pKK232-8 (96004153)(^c)</td>
<td>–88, –82, –32, –18, –1, +5, +24, +58</td>
<td>2048</td>
<td>7408 ± 809</td>
</tr>
<tr>
<td>pKK232-8 (96004153M)(^d)</td>
<td>–88, –82, –18, –1, +5, +24, +58</td>
<td>256</td>
<td>589 ± 109</td>
</tr>
</tbody>
</table>

\(^a\)Compared with \(ampC\) sequence from \(E. coli\) K12.10
\(^b\)Mean ± s.d. of three determinations of CAT concentration.
\(^c\)These constructs were based on pKK232-8 containing the \(ampC\) promoters from \(c\) strain 96010266 (AmpC hyperproducer), \(d\) strain 96010266 reverted at base –42, \(e\) strain 96004153 (AmpC hyperproducer) and \(f\) strain 96004153 reverted at base –32.
**Escherichia coli AmpC hyperproduction**

**Discussion**

Reporter genes have been widely used for studying gene regulation and comparing the strength of various promoters in eukaryotes and prokaryotes. The first protein to be used as a reporter was CAT, but numerous other systems have been developed since then, including $\beta$-galactosidase, luciferase and alkaline phosphatase. In this study, we used the plasmid pKK 232-8, containing a promoterless $cat$ gene, to study transcriptional regulation of the $ampC$ gene from *E. coli*. This method has been widely used for studying prokaryotic gene expression, including $\beta$-lactamase gene expression. Recently, using a luciferase reporter gene method, Nelson & Elisha detected an eight- to 18-fold increase in promoter strength from AmpC hyperproduced.7

With this method, CAT concentration was 70- to 120-fold higher when a strong promoter was cloned than when a weak promoter from a sensitive isolate was used. At the same time, the chloramphenicol MIC increased by 64-fold. Aramori & Kojo showed a 23- to 70-fold increase in CAT activity for $ampC$ promoters from *E. coli* hyperproducing a chromosomal cephalosporinase, but the sequence of these promoters was unknown. Recently, using a luciferase reporter gene method, Nelson & Elisha detected an eight- to 18-fold increase in promoter strength from AmpC hyperproduced.7

In 1983, by analysis of 168 promoter regions of *E. coli* genes, Hawley & McClure confirmed the presence of two conserved regions, the $-35$ hexamer and the $-10$ hexamer, also called the Pribnow box. It is now known that these elements are crucial for fixation of the $\sigma$ subunit of RNA polymerase. For most promoters, there is a good correlation between promoter strength and the degree of homology to the consensus sequence for the $-35$ box, TTGACA, and for the $-10$ box, TATAAT. The distance between these two sequences also plays an important role in the strength of the promoter, the optimal distance being 17 bp. In *E. coli* wild-type $ampC$ promoter, a $-35$ TTGTA sequence is separated by 16 bp from a $-10$ TACAAT sequence. The most frequently described strong $ampC$ *E. coli* promoter presents a C$\rightarrow$T transition at base $-42$ that creates a perfect TTGACA box upstream of the normal $-35$ box, modifying the transcription initiation site. By site-directed mutagenesis, we confirmed that the abolition of this single mutation in the promoter from strain 96010266 decreased its strength to the level of a sensitive isolate. This does not mean that other mutations present in this promoter do not play any role. The $-18$ mutation, by creating a new $-10$ box, certainly plays an important role.

In the $ampC$ promoter from strain 96004153, the T$\rightarrow$A transversion at position $-32$ gave perfect homology to the $-35$ sequence and was suspected to increase the transcription rate. This hypothesis was confirmed by reverting this mutation using site-directed mutagenesis; the strength of the mutated promoter was decreased by about 13-fold. However, it was still stronger than the weak promoters (96006296), suggesting that at least one of the other mutations present in this strong promoter must be implicated in the increased transcription rate. Among the other mutations, one ($+24$ mutation) is localized in the attenuator. This structure has been identified in the *E. coli* $ampC$ promoter and is involved in low-level transcription of the gene. A mutation in this part of the gene could destabilize the hairpin structure and increase transcription. We tried to revert the mutation at position $+24$, but were not successful, probably because of the hairpin structure.

With this system, it could be interesting to determine the role played by mutations in other parts of the promoter, particularly in the Pribnow box, since such mutations have been described in laboratory mutants but, as yet, not in clinical strains.8

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**References**


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