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CTX-M-1 β-lactamase expression in *Escherichia coli* is dependent on cefotaxime concentration, growth phase and gene location

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Objectives: Knowledge about the regulatory mechanisms of CTX-M β-lactamase-encoding genes in *Escherichia coli* is limited. The objectives of this study were to determine the growth response of CTX-M-1-producing *E. coli* exposed to cefotaxime and to investigate how $bla_{CTX\text{-}M\text{-}1}$ expression at mRNA and protein levels is influenced by cefotaxime concentration, growth phase and gene location (chromosome versus plasmid).

Methods: Two isogenic *E. coli* strains, MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1, containing $bla_{CTX\text{-}M\text{-}1}$ on the chromosome and on a wild-type IncI1 plasmid, respectively, were constructed and the MIC of cefotaxime was determined. Growth of the two strains was studied in the presence of increasing concentrations of cefotaxime ranging from 0 to 512 mg/L. The levels of mRNA and protein in different growth phases and at different cefotaxime concentrations were studied by qPCR and selected-reaction-monitoring MS, respectively.

Results: The MICs of cefotaxime were 168 and 252 mg/L for MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1, respectively. Both strains displayed a prolonged lag phase when exposed to cefotaxime. The mRNA of $bla_{CTX\text{-}M\text{-}1}$ and CTX-M-1 protein levels increased in the presence of high cefotaxime concentrations and varied with growth phase. Higher mRNA expression levels were detected for MG1655/CTX-M-1 compared with MG1655/IncI1/CTX-M-1, but a higher protein level was found for MG1655/IncI1/CTX-M-1 compared with MG1655/CTX-M-1, the latter corresponding well with the higher MIC for this strain.

Conclusions: $bla_{CTX\text{-}M\text{-}1}$ mRNA expression and CTX-M-1 protein levels were dependent on cefotaxime concentration, growth phase and gene location. These results provide insight into the expression of cephalosporin resistance in CTX-M-1-producing *E. coli*, improving our understanding of the relationship between antimicrobial therapy and the expression of resistance mechanisms.

Keywords: antimicrobial resistance, ESBLs, regulation

Introduction

ESBLs are the predominant cephalosporin resistance determinants in Enterobacteriaceae.[1,2] The widespread use of cephalosporins such as ceftriaxone and cefotaxime has been proposed as the major reason for the spread of ESBLs.[3] Among more than 10 ESBL enzyme families recognized in Enterobacteriaceae to date, the CTX-M enzyme family is the most widespread in both hospital and community settings worldwide, especially in *Escherichia coli*.[3–5] In Europe, CTX-M-1 is the most common ESBL type found in livestock[6–8] and the second most frequent variant associated with human clinical isolates in countries such as France and Italy and with commensal *E. coli* in the community in Germany.[9,10] This variant is commonly carried on IncI1 and IncN plasmids.[1,6] However, chromosome-encoded CTX-M genes are also found in clinical isolates.[11–13]

Many studies have investigated the origin, occurrence and diversity of CTX-M in different geographical regions over the last decade.[1,5] However, limited attention has been given to the regulatory mechanism and expression of the CTX-M-encoding

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genes.[14,15] In this study, we investigated (i) the growth response of CTX-M-1-producing *E. coli* exposed to cefotaxime and (ii) how $bla_{CTX\text{-}M\text{-}1}$ expression at the mRNA and protein levels is influenced by cefotaxime concentration, growth phase and chromosomal or plasmid location of the gene. Our hypothesis was that transcription and translation of $bla_{CTX\text{-}M\text{-}1}$ is influenced by drug concentration, growth phase and gene location. To test this hypothesis, mRNA and protein expression profiles of $bla_{CTX\text{-}M\text{-}1}$ were measured in the absence and in the presence of cefotaxime using two isogenic strains carrying $bla_{CTX\text{-}M\text{-}1}$ on the chromosome and on the native IncI1 plasmid, respectively.

Materials and methods

Bacterial strain construction

Bacteria and plasmids used in this study are listed in Table 1. The native IncI1 plasmid carrying $bla_{CTX\text{-}M\text{-}1}$ was isolated from a commensal *E. coli* of cattle origin, had the typical IncI1 plasmid scaffold and did not harbour genes for resistance to additional therapeutic antimicrobials (accession number KJ563250). The plasmid was transformed into *E. coli* DH10β and transferred to *E. coli* MG1655 by conjugation. The native $bla_{CTX\text{-}M\text{-}1}$ gene originating from the IncI1/CTX-M-1 plasmid was cloned into the pseudogene *ybeM*[19] using the Lambda Red recombination system as described previously.[17,20,21] Sequences of oligonucleotides used for Lambda Red-mediated mutagenesis and PCR verifications are listed in Table 2. Insertions and conjugations were confirmed by PCR and sequencing using standard procedures. Strains were maintained in Difco™ lysogeny broth (LB) and Lennox (Becton, Dickinson, Albertslund, Denmark) and on LB agar plates (Becton, Dickinson, Albertslund, Denmark). During strain construction the medium was supplemented with cefotaxime (5 mg/L) (Sigma, Copenhagen, Denmark) when appropriate.

Antimicrobial susceptibility testing

The MIC of cefotaxime was determined using the broth microdilution method following the CLSI guidelines.[22] Mueller–Hinton II (MH-2) (Sigma, Copenhagen, Denmark) broth supplemented with cefotaxime (Sigma, Copenhagen, Denmark) was inoculated with a suspension of 10^5 cfu/mL and incubated aerobically at 37°C for 18–22 h, without shaking. The cefotaxime concentrations tested ranged from 0 to 256 mg/L by 2-fold dilution increase. In addition, MIC determination experiments were performed using serial increases of 4 mg/L cefotaxime to obtain a value as close to the real MIC as possible.

Growth conditions

Growth experiments were performed in triplicate on a BioScreen CTM for 24 h at 37°C. A volume of 200 μL of MH-2 broth (Sigma, Copenhagen, Denmark) was inoculated with cells from blood agar plates [Blood Agar Base (Oxoid, Roskilde, Denmark) supplemented with 5% blood from cattle] to a final cell density of 10^6 cfu/mL, using a Sensititre™ Nephelometer (Thermo Scientific™, Roskilde, Denmark) with a 0.5 McFarland standard ($1-2\times10^8$ cfu/mL). The cultures were supplemented with cefotaxime (ranging from 0 to 512 mg/L by 2-fold dilutions). The OD (recorded with a 600 nm filter) was measured every 5 min with continuous shaking. The Hill coefficient (maximum steepness) of each growth curve was calculated using a non-linear model of the log-transformed OD_{600} values using GraphPad Prism 6. For expression and protein analysis studies, the strains were grown in 250 mL flasks containing 100 mL of MH-2 broth at 37°C and 225 rpm. The medium was supplemented with three different concentrations of cefotaxime representing 1/8, 1/4 and 1/2 MIC for the corresponding strain and inoculated with a preculture grown for 2 h at 37°C and 225 rpm using the method described above. The cefotaxime concentrations represented therapeutic concentrations according to published data.[23,24]

RNA extraction

Samples for RNA extraction were collected at four times during *in vitro* growth: the lag phase ($OD_{600}=0.1-0.2$), the logarithmic phase ($OD_{600}=0.5-0.6$), the late logarithmic phase ($OD_{600}=1-1.3$) and the stationary phase ($OD_{600}=3.3-4.6$). The relation between the OD value and the growth phase was determined in pre-experiments with cfu determinations (data not shown). At each sampling point, 0.5 mL of cell sample was mixed with 1 mL of RNAlater (Ambion®, Naerum, Denmark) according to the manufacturer's instructions and stored for immediate stabilization and protection of the RNA. Total RNA was extracted by mechanical disruption with a FastPrep cell disrupter system (Qbiogene, Illkirch, France) and using an RNeasy Mini Kit (Qiagen, Sollentuna, Sweden). The quantity of extracted RNA was determined by A_{260} measurements and purity by A_{260}/A_{280} ratio measurements using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Hvidovre, Denmark). The total RNA was treated with TURBO™ DNase (2 U/μL) (Ambion®, Naerum, Denmark) and 150 ng of RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Naerum, Denmark) according to the manufacturer's instructions.

Quantitative real-time PCR (qPCR)

qPCR was performed using a LightCycler 96 (Roche, Hvidovre, Denmark), essentially as described by Pfaffl.[25] Primer sequences were designed to generate amplicons of 80–130 bp with a melting temperature of 60°C (Table 2). Reactions were set up in total volumes of 20 μL using 2× FastStart Essential DNA Green Master (Roche, Hvidovre, Denmark). An initial expression study of four genes selected as reference candidates (*gapA*,[25,26] *narH*,[27] *gstA*[25] and *nusG*[28]) was performed using MG1655/CTX-M-1 with (42 mg/L) and without cefotaxime. The resulting Ct values were analysed using the program BestKeeper.[29] The genes *gapA* and *nusG* showed stable expression levels in both conditions and primer efficiencies of 1.90 and 1.95 (data not shown), and were selected. Relative gene expression (fold change) was calculated compared with the

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Reference or source
E. coli K-12 MG1655	wild-type	16
DH10β/IncI1/CTX-M-1	*E. coli* DH10β + IncI1/CTX-M-1 (amp^r)	this work
MG1655/CTX-M-1	*E. coli* MG1655 ΔYbeM::CTX-M-1 (amp^r)	this work
MG1655/IncI1/CTX-M-1	*E. coli* MG1655 + IncI1/CTX-M-1 (amp^r)	this work
pKD46-gm	vector for Lambda Red-mediated mutagenesis: λ-red expression from arabinose-inducible promoter; temperature sensitive (gm^r)	17
E. coli ATCC 25922	reference strain	18

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Table 2. Oligonucleotide sequences for PCR-based amplification and sequencing

Primer	Sequence	Application
CTX-M-1-ybeM	forward: 5′-TGGTGGCACTTCAGGCAGGAAACATCGTCGCCCGTCATTGCAGCAAAGATGAAATCAATG-3′ reverse: 5′-AGGCGGCAGGAAGTACCAGGATTTCAGCTCCCTGTCCGTTTCCGCTATTACAAACCG-3′	recombination
CTX/ybeM	forward: 5′-GAAGCATTGCTGGCGCGCGATG-3′ reverse: 5′-GCCGCCGACGCTAATACATCGC-3′	proof of insertion
CTX-M	forward: 5′-ATGTGCAGYACCAGTAARGTKATGGC-3′ reverse: 5′-TGGGTRAARTARGTSACCAGAAYSAGCGG-3′	proof of insertion + sequencing
CTX-M-1	forward: 5′-TCGGATCCAAGGCGTTTTGACAG-3′ reverse: 5′-GCCAAGCTTCCGTTTCCGCTAT-3′	sequencing
CTX-M-1-qPCR	forward: 5′-GACTATGGCACCACCAACG-3′ reverse: 5′-GCTTTCTGCCTTAGGTTGAGG-3′	qPCR
NusG	forward: 5′-GTCCGTTCGCAGACTTTAAC-3′ reverse: 5′-GCTTTCTCAACCTGACTGAAG-3′	qPCR
GapA	forward: 5′-ACTGACTGGTATGGCGTTCC-3′ reverse: 5′-GTTGCAGCTTTTTCCAGACG-3′	qPCR
NarH	forward: 5′-CCGAACTGGGAAGATGACC-3′ reverse: 5′-GGCTATACATCGCCTTCTGG-3′	qPCR
GstA	forward: 5′-ATATCACCCTGCGTGAGAGC-3′ reverse: 5′-ACGGCAAAGTAATCGTCACC-3′	qPCR

lag-phase sample of strain MG1655/CTX-M-1 without antibiotics. The $2^{-\Delta\Delta Ct}$ method, corrected for different primer efficiencies and multiple reference genes, was used.[25] Two independent biological replicates were performed using two technical replicates.

Quantitative protein analysis by selected-reaction-monitoring MS (SRM-MS)

Cell samples were collected at the same timepoints of the growth curves as for RNA extraction. For relative quantification of CTX-M-1, 3 mL of cell culture was cooled on ice, centrifuged at 4000 **g** for 15 min at 4°C and resuspended in 0.5 mL of PBS (pH 7.4) (Life Technologies, Naerum, Denmark) and kept on ice. Lysates were sonicated seven times for 15 s at 70 W at 1 min intervals (kept on ice the whole time) and cleared by centrifugation at 11000 **g** for 20 min at 4°C. To analyse the culture medium, proteins were concentrated using 3 K Amicon Ultra centrifugal spin filters (Millipore, Hellerup, Denmark). The protein concentrations of the cleared lysates or culture medium samples were determined by the bicinchoninic acid (BCA) method using the Pierce BCA Protein Assay Kit (Thermo Scientific, Hvidovre, Denmark) and BSA as a standard. For each sample, a total of 50 μg of cleared bacterial lysate or culture medium sample was dried in a vacuum centrifuge (Savant SpeedVac SPD2010; Thermo Scientific, Hvidovre, Denmark) and denatured with 30 μL of 50% trifluoroethanol (Sigma, Copenhagen, Denmark) followed by incubation for 1 h at 65°C with agitation. Samples were reduced with 7 mM dithiothreitol (final concentration) (Sigma, Copenhagen, Denmark) for 30 min at 65°C with agitation and subsequently alkylated in 20 mM iodoacetamide (Sigma, Copenhagen, Denmark) in the dark for 30 min at room temperature. Samples were diluted 5-fold with 25 mM ammonium bicarbonate (Sigma, Copenhagen, Denmark) and sequence grade modified trypsin was added (Promega, Roskilde, Denmark) at a protease:protein ratio of 1:20 (w/w). Trypsin digestion was performed for 21 h at 37°C under subtle agitation and stopped by adding 1% formic acid (Sigma, Copenhagen, Denmark). Based on *in silico* analysis of the CTX-M-1 sequence, we selected 14 tryptic peptides for unscheduled SRM-MS analysis of the wild-type (MG1655) and MG1655/CTX-M-1. Using this initial approach, transitions from two peptides (SDLVNYNPIAEK and QLGDETFR) were found specifically in MG1655/CTX-M-1 but absent in the wild-type strain and were used to quantify CTX-M-1 protein levels. These two stable isotope-labelled tryptic peptides (JPT Peptide Technologies, Berlin, Germany), SDLVNYNPIAEK* (0.15 pmol) and QLGDETFR* (0.05 pmol), were added as internal standards prior to solid-phase extraction using Oasis HLB 10 mg cartridges (Waters, Hedehusene, Denmark) and dried. Peptide samples (1 μg) were desalted on a C18 trapping Proxeon Easy-Column (2 cm, ID 100 μm, 5 μm, 120 Å) (Thermo Scientific, Hvidovre, Denmark), and separated on a C18 analytical Proxeon Easy-Column (10 cm, ID 75 μm, 3 μm, 120 Å) (Thermo Scientific, Hvidovre, Denmark) over 20 min with a linear gradient of acetonitrile (5%–35%) (VWR, Herlev, Denmark) in 0.1% formic acid in water at a flow rate of 300 nL/min by use of an Easy-nLC II Nano-LC system (Thermo Scientific, Hvidovre, Denmark). Peptides were ionized with a Nanospray Flex ion source (Thermo Scientific, Hvidovre, Denmark) using an ion spray voltage of 2000 V and a capillary temperature of 270°C. Mass analysis was performed using a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, Hvidovre, Denmark) operated in SRM mode. Five or four charge +2/+1 transitions pairs from each peptide with *m/z* 681.85/557.33, 681.85/671.37, 681.85/834.44, 681.85/948.48, 681.85/1047.55 (SDLVNYNPIAEK) and *m/z* 483.24/423.23, 483.24/552.28, 483.24/667.30, 483.24/724.33 (QLGDETFR) were monitored along with the corresponding transitions for the heavy standard peptides with a mass offset of 8 Da for lysine and 10 Da for arginine. The SRM settings were as follows: collision gas pressure 1.0 mTorr; Q1 peak width 0.70; and cycle time 1.5 s. Collision energy used for peptide fragmentation was 26 eV for peptide SDLVNYNPIAEK and 19 eV for peptide QLGDETFR. The entire SRM workflow was processed in Pinpoint 1.1 (Thermo Scientific, Hvidovre, Denmark). The response of each peptide was calculated as the ratio of the peak area of each endogenous peptide to the peak area of an internal standard. An average fold change for the two peptides was calculated for each biological replication relative to the peptide concentration in MG1655/CTX-M-1 grown without antibiotics. Three independent biological replicates were performed.

Statistical analysis

Observations that differed by >100% from the mean of the triplicates for SRM-MS were excluded. Then, differences in normalized qPCR and SRM-MS measurements between different cefotaxime concentrations within each

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growth phase were compared by differences in least-squares means using analysis of variance stratified by bacterial strain. The analyses were performed using the Mixed procedure in SAS version 9.3 (SAS Institute, Cary, USA) and differences in least-squares mean estimates were assessed with an approximate *t*-test using the LSmeans function in this procedure. The *F*-test was used to determine whether differences existed between concentrations and growth phases, and a P value <0.05 was deemed statistically significant. To achieve residuals that were independent, identically distributed normal (0, σ^2), a log-transformation of the normalized qPCR measurements was deemed necessary. The *F*-test was also used to assess whether there were differences in the total mRNA and protein levels overall between the strains used. The Benjamini–Hockberg 'false discovery rate' approach was used to correct for multiple comparisons of the differences in the least-squares means ($P<0.05$).[30,31] Correlation coefficients of the mRNA and protein levels were calculated within each growth phase using GraphPad Prism 6.

Results

Lag-phase length increases with increasing concentrations of cefotaxime

The two isogenic strains, MG1655/CTX-M-1 carrying $bla_{\text{CTX-M-1}}$ on the chromosome and MG1655/IncI1/CTX-M-1 carrying $bla_{\text{CTX-M-1}}$ on the native IncI1 plasmid, were used to examine whether strain growth was influenced by the presence of different concentrations of cefotaxime. The MICs of cefotaxime were 168 ± 4 and 252 ± 4 mg/L for MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1, respectively.

The two strains displayed similar growth curves and did not grow at the two highest concentrations tested (Figure 1). Using a definition of lag phase as the time necessary to reach an OD_{600} of 0.1, the lag-phase length of the two strains was found to increase as a function of the antibiotic concentration [Figure 1 and Table S1 (available as Supplementary data at *JAC* Online)]. In contrast, the steepness of the growth curve (Hill coefficient) varied only slightly among different cefotaxime concentrations (Table S1). The mean Hill coefficient ($\pm$SD) was 0.177 ± 0.016 for MG1655/CTX-M-1 and 0.181 ± 0.026 for MG1655/IncI1/CTX-M-1.

As a control for antibiotic stability, an *E. coli* reference strain (ATCC 25922) was grown with the same concentrations of cefotaxime as used in the growth study of MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1. The reference strain did not grow at any cefotaxime concentrations other than 0 and 2 mg/L (data not shown). The 2 mg/L cefotaxime culture started to grow after 20 h.

$bla_{\text{CTX-M-1}}$ mRNA levels depend on cefotaxime concentration, growth phase and gene location

To investigate $bla_{\text{CTX-M-1}}$ expression in the presence of cefotaxime, the $bla_{\text{CTX-M-1}}$ mRNA levels were determined at four different cefotaxime concentrations in four growth phases. Analysis of $bla_{\text{CTX-M-1}}$ mRNA levels at different cefotaxime concentrations within each growth phase for each strain demonstrated increased $bla_{\text{CTX-M-1}}$ expression when cefotaxime was present (Figure 2). The mRNA profiles for MG1655/IncI1/CTX-M-1 showed a significant increase in $bla_{\text{CTX-M-1}}$ expression in response to increasing cefotaxime concentrations within all growth phases, except for the mRNA level at 63 mg/L cefotaxime in stationary phase. Similar concentration-dependent significant increases were observed within three growth phases (lag, late logarithmic and stationary) of MG1655/CTX-M-1 at three out of the four cefotaxime concentrations (0, 21 and 42 mg/L). The mRNA levels at the highest cefotaxime concentration (84 mg/L) were an exception to this, as they were almost always lower than the mRNA levels at the second highest cefotaxime concentration (42 mg/L). A lower level was seen with 21 mg/L cefotaxime for MG1655/CTX-M-1 in the log phase compared with the level in the absence of cefotaxime. Significant P values for concentration dependency are listed in Table S2(a). Overall, the expression of $bla_{\text{CTX-M-1}}$ was cefotaxime concentration dependent regardless of growth phase.

Analysis of the $bla_{\text{CTX-M-1}}$ mRNA results at the same cefotaxime concentration but in different growth phases demonstrated growth-phase-dependent $bla_{\text{CTX-M-1}}$ mRNA expression in both strains (Figure 2). Significant differences in $bla_{\text{CTX-M-1}}$ mRNA levels were observed in all four growth phases within all concentration series for MG1656/CTX-M-1; however, significant differences were only observed within the lag and late logarithmic phases at 84 mg/L cefotaxime. The level of $bla_{\text{CTX-M-1}}$ mRNA tended to increase from lag to stationary phase within the same cefotaxime concentration. The only exceptions were the late logarithmic- and stationary-phase samples in the absence of cefotaxime and the stationary-phase sample with the highest cefotaxime concentration. For MG1655/IncI1/CTX-M-1, significant growth-phase

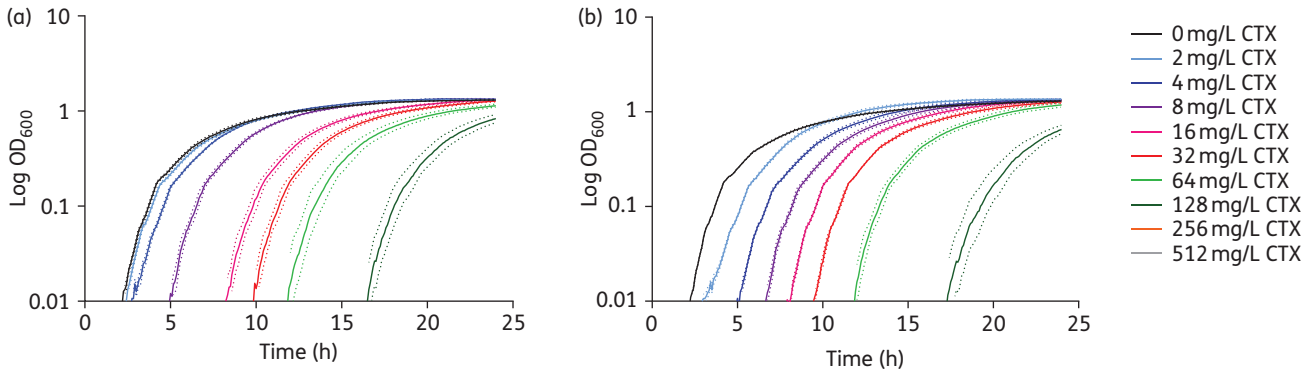

Figure 1. Growth curves of two CTX-M-1-producing *E. coli* strains with different concentrations of cefotaxime. (a) *E. coli* MG1655 containing $bla_{\text{CTX-M-1}}$ on the chromosome (MG1655/CTX-M-1). (b) *E. coli* MG1655 containing $bla_{\text{CTX-M-1}}$ on an IncI1 plasmid (MG1655/IncI1/CTX-M-1). Both strains were grown in MH-2 broth with different concentrations of cefotaxime on a BioScreen CTM. No growth was observed for the two highest concentrations tested. Three independent replicates were performed; the data shown represent the mean and dots represent standard deviations.

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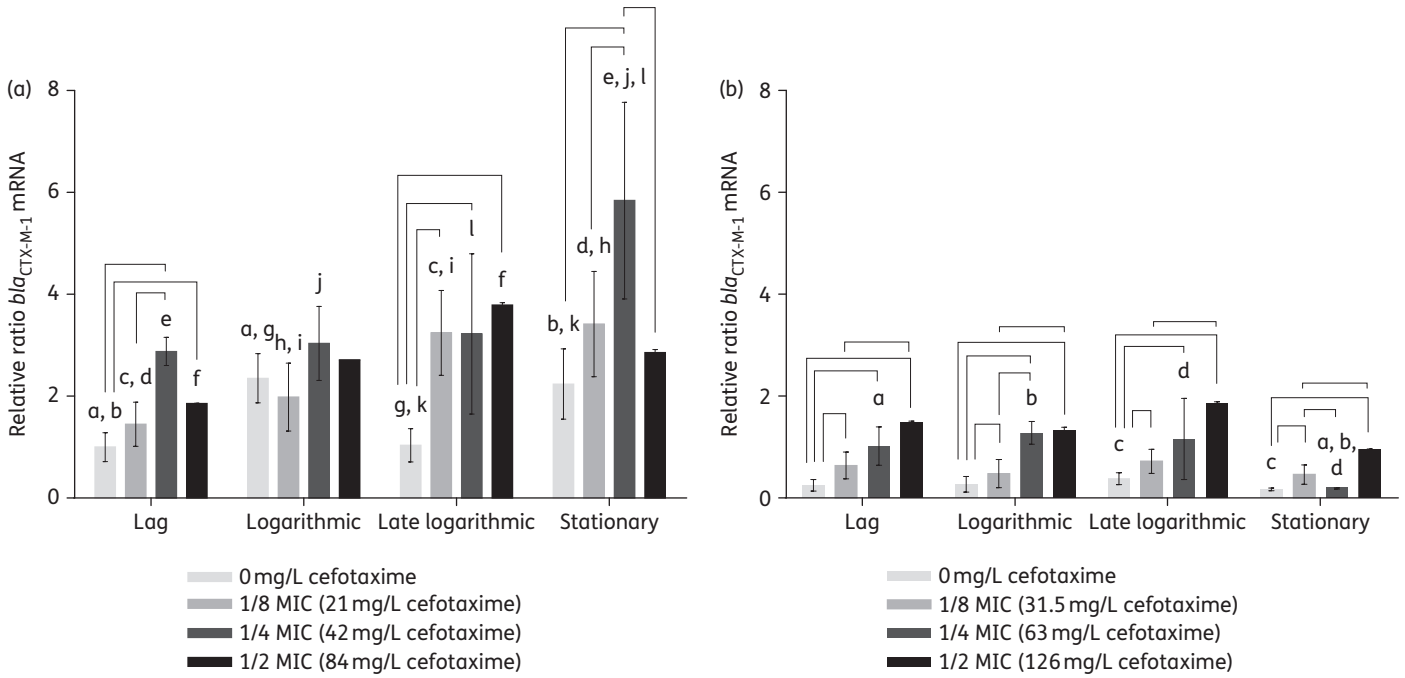

Figure 2. Relative changes in $bla_{CTX-M-1}$ mRNA levels of two $bla_{CTX-M-1}$-encoding *E. coli* strains. (a) $bla_{CTX-M-1}$ mRNA profile from *E. coli* MG1655, containing $bla_{CTX-M-1}$ on the chromosome (MG1655/CTX-M-1). (b) $bla_{CTX-M-1}$ mRNA profile from *E. coli* MG1655, containing $bla_{CTX-M-1}$ on an IncI1 plasmid (MG1655/IncI1/CTX-M-1). Both strains were grown in MH-2 broth without and with cefotaxime at different concentrations (1/8, 1/4 and 1/2 MIC for the corresponding strain). Two independent replicates including two technical replicates each were performed; the data shown represent the mean. The data have been normalized to two validated reference genes, *gapA* and *nusG*, and are relative to the $bla_{CTX-M-1}$ mRNA in the lag phase with no antibiotics for MG1655/CTX-M-1. Error bars represent standard deviations. Identical letters indicate significant growth-phase differences between the two samples ($P<0.05$) and lines between bars indicate significant concentration differences between the two samples ($P<0.05$).

differences in the $bla_{CTX-M-1}$ mRNA levels were only observed in the series with no antibiotics in the late logarithmic and stationary phases and in the series with 63 mg/L cefotaxime within all growth phases. In both series, a decrease in the mRNA level was seen in the stationary phase compared with the lag, logarithmic and late logarithmic phases. Significant *P* values for growth-phase dependency can be found in Table S3(a).

The $bla_{CTX-M-1}$ mRNA results were also analysed for differences in mRNA levels influenced by $bla_{CTX-M-1}$ gene location. The total mRNA level was compared by calculating the mean value of the total normalized mRNA for each strain and showed a significantly higher ($P=0.02$) mRNA level in MG1655/CTX-M-1 compared with MG1655/IncI1/CTX-M-1 (Figure 3a).

Protein levels of CTX-M-1 depend on cefotaxime concentration, growth phase and gene location

The CTX-M-1 protein levels were analysed for significant differences at the different cefotaxime concentrations within each growth phase for each strain (Figure 4). The protein expression profiles for MG1655/CTX-M-1 in the presence of cefotaxime showed no significant increase in CTX-M-1 protein with increasing cefotaxime concentrations within each growth phase by this analysis. However, the general trend of the data indicated increased CTX-M-1 protein levels in the presence of cefotaxime in the logarithmic, late logarithmic and stationary growth phases for MG1655/CTX-M-1. This is supported by the fact that the linear tendencies within each growth phase had increasing slopes between 0.4 and 2.4. Significant changes in the CTX-M-1 protein levels dependent on cefotaxime concentration were observed for MG1655/IncI1/CTX-M-1 in the logarithmic and late logarithmic phases. Significant *P* values for concentration dependency are listed in Table S2(b).

Analysis of the CTX-M-1 levels at the same cefotaxime concentration but different growth phases for each strain demonstrated significant changes dependent on growth phase only for MG1655/IncI1/CTX-M-1 in the series with 63 and 126 mg/L cefotaxime, where the CTX-M-1 level showed a large increase from lag to logarithmic phase, and then declined in stationary phase. The CTX-M-1 levels in the absence of cefotaxime increased from lag to late logarithmic phase and then stabilized in stationary phase for MG1655/CTX-M-1. For MG1655/IncI1/CTX-M-1, the CTX-M-1 levels in the absence of cefotaxime increased from lag to late logarithmic phase and then declined in stationary phase. Significant *P* values for growth-phase dependency can be found in Table S3(b).

The CTX-M-1 protein profiles were also analysed for differences in protein levels influenced by $bla_{CTX-M-1}$ gene location by calculating the mean value of the total protein level for each strain, and this showed a significantly higher ($P=0.009$) CTX-M-1 protein level in MG1655/IncI1/CTX-M-1 compared with MG1655/CTX-M-1 (Figure 3b).

Correlation coefficients for mRNA and proteins levels of $bla_{CTX-M-1}$ were calculated within each growth phase. The mRNA and protein levels of $bla_{CTX-M-1}$ did not correlate in MG1655/CTX-M-1; however, high correlation coefficients ($r>0.940$) were seen for MG1655/IncI1/CTX-M-1 in the lag, logarithmic and late logarithmic phases.

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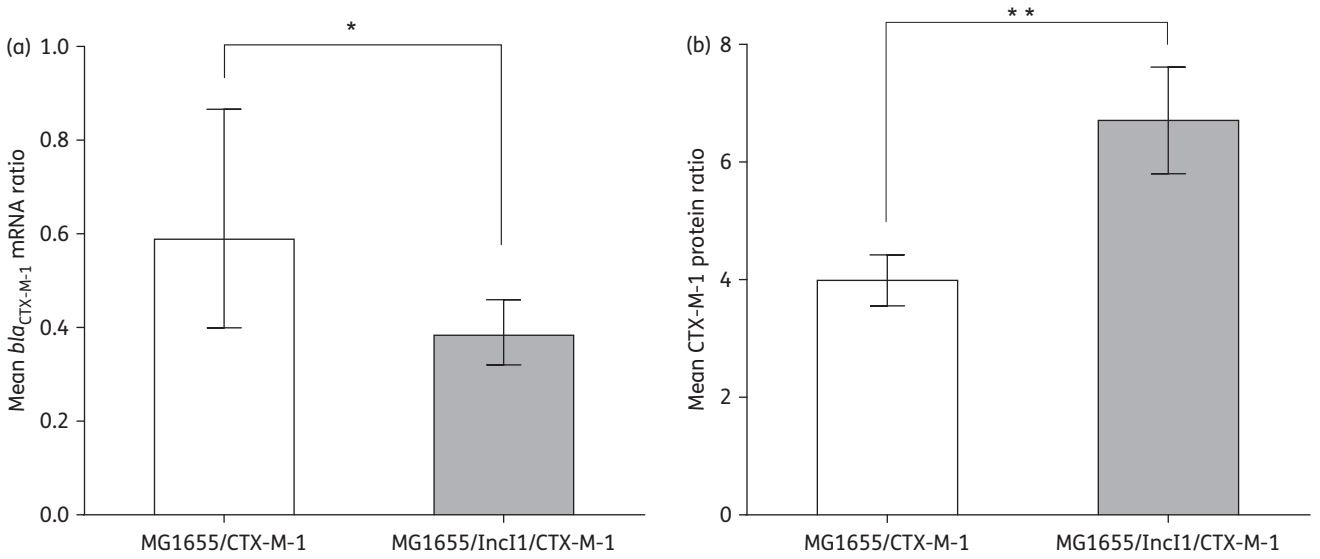

Figure 3. (a) Mean $bla_{CTX-M-1}$ mRNA ratio and (b) mean CTX-M-1 protein ratio in the two $bla_{CTX-M-1}$-encoding *E. coli* strains, MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1. The data represent the mean normalized mRNA ratio and the mean protein ratio, calculated based on the centred normalized ratio values. Error bars represent standard errors. The asterisks indicate statistical significance at different levels: $^{*}P<0.05$ and $^{**}P<0.01$.

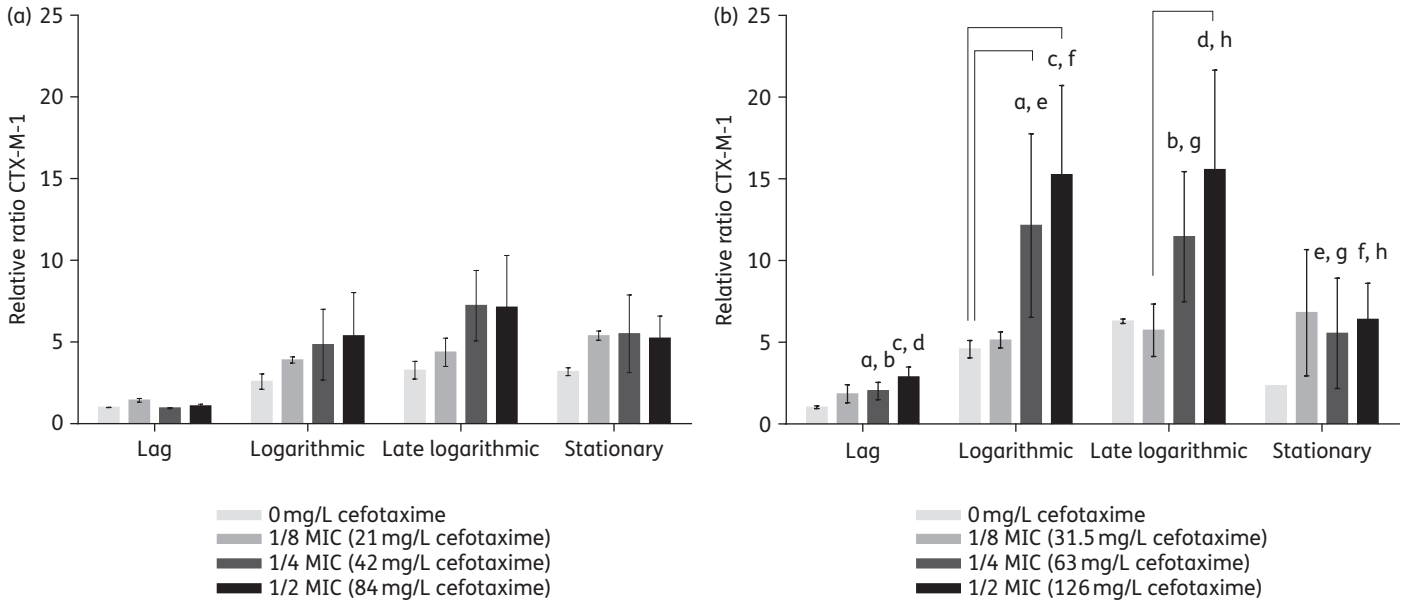

Figure 4. Relative changes in CTX-M-1 protein levels of two CTX-M-1-producing *E. coli* strains. (a) CTX-M-1 protein profile from *E. coli* MG1655 containing $bla_{CTX-M-1}$ on the chromosome (MG1655/CTX-M-1). (b) CTX-M-1 protein profile from *E. coli* MG1655 containing $bla_{CTX-M-1}$ on an IncI1 plasmid (MG1655/IncI1/CTX-M-1). Both strains were grown in MH-2 broth without and with cefotaxime at different concentrations (1/8, 1/4 and 1/2 MIC for the corresponding strain). Three independent replicates were performed and the data represent the mean. The response of each analysed peptide was calculated as the ratio of the peak area of each endogenous peptide to the peak area of an internal standard and normalized to the lag phase sample with no antibiotics for MG1655/CTX-M-1. Error bars represent standard deviations. Identical letters indicate significant growth-phase differences between the two samples ($P<0.05$) and lines between bars indicate significant concentration differences between the two samples ($P<0.05$).

A subset of samples in the late logarithmic phase for MG1655/CTX-M-1 (42 and 84 mg/L cefotaxime) and MG1655/IncI1/CTX-M-1 (31.5, 63 and 126 mg/L cefotaxime) were analysed for the presence of CTX-M-1 in the supernatant. The results showed that the level of CTX-M-1 measured in the medium was proportional to the level measured in the cells (data not shown).

Discussion

In this study, we investigated the growth response of CTX-M-1-producing *E. coli* exposed to cefotaxime and the effects of different concentrations of cefotaxime, growth phase and genomic location (chromosome versus plasmid) of $bla_{CTX-M-1}$ on

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$bla_{CTX\text{-}M\text{-}1}$ expression and translation. We showed that increased concentrations of cefotaxime caused an increase in the lag-phase length of CTX-M-1-producing *E. coli*, independent of chromosomal or plasmid localization of $bla_{CTX\text{-}M\text{-}1}$. We also demonstrated that $bla_{CTX\text{-}M\text{-}1}$ mRNA expression depended on cefotaxime concentration, growth phase and gene location, while CTX-M-1 protein depended on cefotaxime concentration and growth phase only for MG1655/IncI1/CTX-M-1. Gene location dependency was also observed at protein level. Both mRNA and protein levels increased in the presence of high cefotaxime concentrations. These results confirmed our hypothesis that transcription and translation of $bla_{CTX\text{-}M\text{-}1}$ were influenced by drug concentration and growth phase.

The growth curves of MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1 revealed that the localization of $bla_{CTX\text{-}M\text{-}1}$ did not affect the growth of the strains, which suggests no fitness cost related to plasmid carriage in the tested conditions. When exposed to cefotaxime, both strains displayed an increase in lag-phase length. Only limited variation in the Hill coefficients of the growth curves was observed, which indicates that lag-phase length was the only significant difference between the growth curves of strains exposed to different concentrations of cefotaxime. The duration of the lag phase is dependent on many factors, such as strain identity and phenotype, temperature, nutrient availability and pH.[30] The increase in lag-phase length observed in our study in the presence of cefotaxime could have been population related. When a high concentration of cefotaxime is present, a high quantity of antibiotic is available for each cell, which could result in a higher number of non-replicating cells, and growth will only reach measurable levels once non-replicating cells have degraded the antibiotic down to a critical concentration. Further studies are needed to understand this relation between lag phase and antibiotic concentration. Cefotaxime is stable for 4 days at 25°C and 2 days at 45°C according to a colorimetric method by Srinivasa *et al.*,[31] and according to a microbiological assay it is stable for 1 day at 25°C and 4 h at 45°C. In the current study we tested the growth of a sensitive *E. coli* reference strain in the presence of cefotaxime. Growth occurred in the 2 mg/L cefotaxime culture after 20 h, showing that degradation of cefotaxime to some degree takes place at 37°C, but it was not enough to explain the elongated lag phase observed in this study at all measured concentrations.

Several β-lactamase-encoding genes have a strong promoter upstream that increases the MICs of cephalosporins.[3] IS*Ecp*1 is the most frequently found IS upstream of different $bla_{CTX\text{-}M}$ genes[3] and from the deposited sequence of $bla_{CTX\text{-}M\text{-}1}$ it is clear that IS*Ecp*1 is also present upstream of $bla_{CTX\text{-}M\text{-}1}$ used in this study. ISs function as the promoters for many *bla* genes, including $bla_{CTX\text{-}M\text{-}1}$. The proposed promoter regions in IS*Ecp*1[30–32] were kept upstream of $bla_{CTX\text{-}M\text{-}1}$ in both *E. coli* strains used in this study. The spacer sequences between IS*Ecp*1 and $bla_{CTX\text{-}M}$ have been studied and the genetic distance between these sequences has been found to be related to the cephalosporin MIC values.[32] However, the spacer sequences between IS*Ecp*1 and $bla_{CTX\text{-}M\text{-}1}$ in MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1 are identical and cannot explain the MIC differences between the strains. The data obtained in the present study indicate that the IS*Ecp*1 promoter of $bla_{CTX\text{-}M\text{-}1}$ is regulated by cefotaxime. Limited knowledge is available about the regulatory mechanism of ESBLs. It is known that the expression levels of β-lactamases in Gram-negative bacteria are lower compared with those observed in Gram-positive bacteria.[33,34] Nagano *et al.*[14] suggested that β-lactamase expression from the $bla_{CTX\text{-}M\text{-}2}$ gene was inducible by cefotaxime and aztreonam. A transcriptional analysis of $bla_{CTX\text{-}M\text{-}2}$ was performed by Di Conza *et al.*,[15] who found that more than one promoter was involved in the expression of this β-lactamase. In addition, Jacobs *et al.*[35] reported that in many bacteria chromosomally encoded AmpC β-lactamase was induced when the growth medium contained β-lactam antibiotics. However, the molecular basis for this regulation was not clear.[2,35] Recently a study on the regulation of the β-lactamase OXA-61 was published in which a single nucleotide mutation upstream of $bla_{OXA\text{-}61}$ was linked to up-regulation of $bla_{OXA\text{-}61}$ expression in *Campylobacter jejuni*.[34]

Significant changes in mRNA and protein levels were observed depending on antibiotic concentration, but this was limited to one strain with regard to the protein level: MG1655/IncI1/CTX-M-1. This means that $bla_{CTX\text{-}M\text{-}1}$ up-regulation in the presence of cefotaxime was accompanied by a significant increase in the amount of CTX-M-1 protein in MG1655/IncI1/CTX-M-1. However, the trend of the data for MG1655/CTX-M-1 also indicates that more protein was expressed when cefotaxime was present in this strain. Analysis of $bla_{CTX\text{-}M\text{-}1}$ mRNA and CTX-M-1 protein results at the same cefotaxime concentration but different growth phases demonstrated that growth phase significantly influenced $bla_{CTX\text{-}M\text{-}1}$ mRNA expression in MG1655/CTX-M-1 and both $bla_{CTX\text{-}M\text{-}1}$ mRNA and CTX-M-1 protein expression in MG1655/IncI1/CTX-M-1.

Interestingly, higher mRNA expression levels were detected when $bla_{CTX\text{-}M\text{-}1}$ was located on the chromosome compared with the native IncI1 plasmid, even though multiple copies of $bla_{CTX\text{-}M\text{-}1}$ are available for expression due to the copy number of the plasmid. This suggests that gene expression was enhanced by chromosomal location. Currently we can only speculate what the reason for this could be. CTX-M-1 was cloned into the pseudogene *ybeM*, which consists of two (putative) transcriptional units and is predicted not to be transcribed as functional proteins due to a frameshift mutation [accession number G6348 (EcoCyc)]. Whether promoter activity still exists that can induce expression from the cloned gene needs to be established. Even more intriguing, the CTX-M-1 protein level was significantly higher in MG1655/IncI1/CTX-M-1 compared with MG1655/CTX-M-1. While this is in good agreement with the higher MIC observed for this strain, it raises the question of the lack of correlation between mRNA levels in the two strains and the amount of protein produced. Further studies are needed to understand this relationship.

In conclusion, we demonstrated that the lag-phase length of the two CTX-M-1-producing *E. coli* strains under study increased with increasing cefotaxime concentration. Furthermore, we showed that the mRNA expression of $bla_{CTX\text{-}M\text{-}1}$ and the protein levels were significantly dependent on cefotaxime concentration, growth phase and gene location. Higher mRNA expression levels were detected when $bla_{CTX\text{-}M\text{-}1}$ was located on the chromosome compared with the native IncI1 plasmid. However, the CTX-M-1 protein levels were significantly higher in MG1655/IncI1/CTX-M-1 compared with MG1655/CTX-M-1, which corresponds with the differences observed in the resistance phenotype of the two isogenic strains. Our results strengthen our understanding of

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the relationship between antimicrobial therapy and expression of cephalosporin resistance in CTX-M-producing *E. coli*.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 to S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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