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# Adaptive responses to cefotaxime treatment in ESBL-producing *Escherichia coli* and the possible use of significantly regulated pathways as novel secondary targets

**Thea S. B. Møller[1], Martin H. Rau[2], Charlotte S. Bonde[1], Morten O. A. Sommer[2,3], Luca Guardabassi[1] and John E. Olsen[1]***

[1]*Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, 1870 Frederiksberg C, Denmark;* [2]*Department of Systems Biology, Technical University of Denmark, 2800 Lyngby, Denmark;* [3]*Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2970 Hørsholm, Denmark*

*Corresponding author. Tel: +45-35332784; Fax: +45-35332757; E-mail: jeo@sund.ku.dk

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**Objectives:** The aim of the study was to determine how ESBL-producing *Escherichia coli* change the expression of metabolic and biosynthesis genes when adapting to inhibitory concentrations of cefotaxime. Secondly, it was investigated whether significantly regulated pathways constitute putative secondary targets that can be used to combat the resistant bacteria.

**Methods:** Strains of *E. coli* MG1655 encoding $bla_{CTX\text{-}M\text{-}1}$ from an IncI1 plasmid and from the chromosome were challenged with cefotaxime corresponding to inhibitory concentrations, and transcriptional patterns were compared with growth without or with very low concentrations of cefotaxime by RNA sequencing. Significantly regulated pathways were inhibited with suitable inhibitors, or genes encoding the enzymes of the regulated pathways were knocked out. The ability of the bacteria to grow in the presence of cefotaxime was determined. Chequerboard assays were utilized to confirm synergies between treatments.

**Results:** Genes belonging to 16 different functional gene classes were significantly regulated. Protein and peptidoglycan syntheses were up-regulated and low concentrations of chloramphenicol or D-cycloserine, targeting these systems, strongly reduced the MIC of cefotaxime (>32-fold). Inhibition and/or mutations in other genes that were significantly regulated, belonging to energy synthesis, purine synthesis, proline uptake or potassium uptake, also rendered the resistant bacteria more susceptible to cefotaxime.

**Conclusions:** The results show that ESBL-producing *E. coli* adapt to treatment with cefotaxime by changing their gene expression patterns and furthermore that targeting regulated adaptive pathways may be a suitable way to identify targets for drugs that will specifically inhibit the resistant bacteria.

## Introduction

β-Lactam resistance in Enterobacteriaceae has become a serious health problem during the last decade.[1,2] The most common mechanism for cephalosporin resistance in *Escherichia coli* is ESBLs,[3,4] and among these, the CTX-M enzyme family is the most widespread in both hospital and community settings.[2,5–8]

Microorganisms have complicated regulatory networks, and because of this they are capable of adapting to many different growth conditions.[9] Transcriptomic or proteomic analyses have commonly been used to investigate the adaptive response to antibiotic treatment in antibiotic-susceptible strains.[10–12] In contrast, very few studies have investigated responses to antibiotics in antibiotic-resistant ones. Wang *et al.*[13] obtained proteomic profiles of vancomycin-resistant *Enterococcus faecalis* with and without drug treatment, and showed that proteins known to be involved in vancomycin resistance, as well as in virulence, stress responses, general metabolism, and translation and conjugation were significantly regulated. In a similar study, Liu *et al.*[14] obtained protein expression patterns of *Stenotrophomonas maltophilia* cultures grown with and without imipenem and showed that many proteins were differently expressed compared with cells growing without the drug. Also, the effects of rhodomyrtone on cellular protein expression of methicillin-resistant *Staphylococcus aureus* have been investigated using proteomic approaches.[15] Together, these studies suggest that resistant microorganisms change their metabolic and biosynthesis activity in an effort to adapt to inhibitory concentrations of antibiotics.

The present study aimed to determine how ESBL-producing *E. coli* adapt to treatment with the cephalosporin cefotaxime,

and it further hypothesized that such changes could open a window of opportunity to specifically harm antibiotic-resistant bacteria by targeting the adaptive pathways in the bacteria.

## Methods

### Bacterial strains

Bacteria used in this study are listed in Table 1. The IncI1 plasmid used in the study has the accession number KJ563250, and it does not carry known resistance genes other than $bla_{CTX\text{-}M\text{-}1}$. Strains were maintained in Difco™ lysogeny broth (LB), Lennox (Becton, Dickinson and Company, Denmark) and on LB agar plates (Becton, Dickinson and Company, Denmark) supplemented with cefotaxime (5 mg/L) (Sigma, Copenhagen, Denmark) when appropriate. Mutants were made using the Lambda Red recombination system and plasmids pKD4 and pKD3 as described previously.[16–18] Sequences of oligonucleotides used for Lambda Red-mediated mutagenesis and PCR verifications are listed in Table S1 (available as Supplementary data at *JAC* Online). Primers within the kanamycin gene and chloramphenicol gene were also used for verifications (Kanrev and Camfwdny).[19] Insertions were confirmed by PCR using standard procedures.

### Antimicrobial susceptibility testing

The MICs of cefotaxime, chloramphenicol and D-cycloserine were determined using the broth microdilution method using 0–512 mg/L by 2-fold dilution increases following the CLSI guidelines as previously described.[20]

### Chequerboard assay

Cefotaxime, chloramphenicol (Sigma, Copenhagen, Denmark) and D-cycloserine (Sigma, Copenhagen, Denmark) were used in the ranges 0–512, 0–8 and 0–16 mg/L respectively. The first antibiotic of the combination was serially diluted along the ordinate, while the second antibiotic was serially diluted along the abscissa. Using a McFarland 0.5 standard ($1–2\times10^8$ cfu/mL) and a Sensititre™ Nephelometer (Thermo Scientific™, Roskilde, Denmark) the inoculum was prepared for each strain. All the wells, containing 50 μL of Mueller–Hinton II (MH-II) (Sigma, Copenhagen, Denmark) broth, were inoculated with 50 μL of inoculum to a final cell density of $5\times10^5$ cfu/mL. The panel was incubated aerobically at 37°C for 18–22 h, without shaking. The fractional inhibitory concentration (FIC) index was calculated using the following formula: ∑FIC = FIC A + FIC B, where FIC A is the MIC of drug A in the combination divided by the MIC of drug A alone, and FIC B is the MIC of drug B in the combination divided by the MIC of drug B alone.[21] The combination was interpreted as synergistic when the ∑FIC was ≤0.5, indifferent when the ∑FIC was >0.5–4 and antagonistic when the ∑FIC was >4.[21] Partial chequerboard experiments were performed with enterostatin (Sigma, Copenhagen, Denmark) or quercetin (Sigma, Copenhagen, Denmark) at fixed concentrations (10, 20 and 90 μM for enterostatin and 10, 20 and 40 μM for quercetin) together with cefotaxime in 2-fold dilutions (0–512 mg/L).

### Growth conditions

Growth experiments for RNA sequencing were performed in duplicate and the strains were grown in 250 mL flasks containing 100 mL of MH-II broth at 37°C and 225 rpm. The medium was supplemented with two different concentrations of cefotaxime representing 1/2 MIC for the antibiotic-resistant strains (84 mg/L for MG1655/CTX-M-1 and 126 mg/L for MG1655/IncI1/CTX-M-1) and 1/2 MIC (subinhibitory) for the WT strain (0.016 mg/L, *E. coli* K-12 MG1655[22]). The cultures were inoculated with precultures grown for 2 h at 37°C and 225 rpm to a final cell density of $10^5$ cfu/mL, as determined using the Sensititre™ Nephelometer as stated above. The 1/2 MIC cefotaxime concentrations represent therapeutic concentrations, as previously published, and will therefore be called inhibitory concentrations in this study.[23,24] Growth of the cultures was monitored by measuring $OD_{600}$ until samples were taken for RNA extraction.

For the chloramphenicol assays the strains were grown in 20 mL of MH-II broth at 37°C and 225 rpm for 2 h and then treated with chloramphenicol (2 mg/L), cefotaxime (1/2 MIC for the corresponding strain) and both chloramphenicol and cefotaxime at the same concentrations. The cultures were grown for 1 h at 37°C and 225 rpm, diluted 1:180 with pure MH-II medium to a final volume of 200 μL and grown with cefotaxime at the same concentrations, except the control culture, which had been treated only with chloramphenicol. Growth experiments were performed with three biological and technical replicates on a BioScreen C™ for 24 h at 37°C. $OD_{600}$ was measured every 5 min, keeping bacterial cultures under continuous shaking.

All other growth experiments to determine growth curves were also performed on a BioScreen C™ for 24 h at 37°C. A volume of 200 μL of MH-II broth was inoculated with cells from agar plates (Blood agar Base No. 2, Oxoid, Roskilde, Denmark) supplemented with 5% blood from cattle

**Table 1.** Strains used in the study

| Strain | Genotype | MIC of cefotaxime (mg/L) | Reference |
|---|---|---|---|
| *E. coli* K-12 MG1655 | WT | 0.032 | 23 |
| MG1655/CTX-M-1 | *E. coli* MG1655 ΔYbeM::CTX-M-1 | 256 | 20 |
| MG1655/IncI1/CTX-M-1 | *E. coli* MG1655 + IncI1/CTX-M-1 | 256 | 20 |
| MG1655/CTX-M-1 Δ*PurT* | *E. coli* MG1655 ΔYbeM::CTX-M-1 ΔPurT::Kan | 128 | this study |
| MG1655/IncI1/CTX-M-1 Δ*PurT* | *E. coli* MG1655 + IncI1/CTX-M-1 ΔPurT::Kan | 128 | this study |
| MG1655/CTX-M-1 Δ*PurN* | *E. coli* MG1655 ΔYbeM::CTX-M-1 ΔPurN::Chl | 128 | this study |
| MG1655/IncI1/CTX-M-1 Δ*PurN* | *E. coli* MG1655 + IncI1/CTX-M-1 ΔPurN::Chl | 256 | this study |
| MG1655/CTX-M-1 Δ*PurT*Δ*PurN* | *E. coli* MG1655 ΔYbeM::CTX-M-1 ΔPurT::kan ΔPurN::Chl | 32 | this study |
| MG1655/IncI1/CTX-M-1 Δ*PurT*Δ*PurN* | *E. coli* MG1655 + IncI1/CTX-M-1 ΔPurT::Kan ΔPurN::Chl | 256 | this study |
| MG1655/CTX-M-1 Δ*PutAP* | *E. coli* MG1655 ΔYbeM::CTX-M-1 ΔPutAP::Kan | 64 | this study |
| MG1655/IncI1/CTX-M-1 Δ*PutAP* | *E. coli* MG1655 + IncI1/CTX-M-1 ΔPutAP::Kan | 128 | this study |
| MG1655/CTX-M-1 Δ*KdpFABC* | *E. coli* MG1655 ΔYbeM::CTX-M-1 ΔKdpFABC::Chl | 32 | this study |
| MG1655/IncI1/CTX-M-1 Δ*KdpFABC* | *E. coli* MG1655 + IncI1/CTX-M-1 ΔKdpFABC::Chl | 128 | this study |

to a final cell density of $10^6$ cfu/mL, using the Sensititre™ Nephelometer as stated above. Purine autotroph strains were also grown in M9 minimal medium (2 mM $MgSO_4$/0.1 mM $CaCl_2$/0.4% glucose/8.5 mM NaCl/42 mM $Na_2HPO_4$/22 mM $KH_2PO_4$/18.6 mM $NH_4Cl$). The cultures were supplemented with cefotaxime, enterostatin, quercetin or D-cycloserine (concentrations can be found in the Results section). For all calculations of lag-phase length, a definition of the lag phase as the time necessary to reach an $OD_{600}$ of 0.1 was used.

### RNA extraction

Samples for RNA extraction were collected in the logarithmic phase ($OD_{600}$ = 0.5–0.6) and at each sampling point a volume of 3 mL cell sample was mixed with 6 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 using a FastPrep system (Qbiogene, Illkirch, France) and an RNeasy Mini kit (Qiagen, Sollentuna, Sweden). Quantity of the 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 deoxyribonuclease (DNase) I, nuclease-free (1 U/μL) (Thermo Scientific, Hvidovre, Denmark) according to the manufacturer's instructions. The rRNAs (23S and 16S) were removed from 3 μg of DNase-treated RNA by subtractive hybridization using the MICROBExpress kit (Ambion®, Naerum, Denmark) with one modification: compared with the standard protocol, double the amount of magnetic beads was used. Removal of rRNAs was confirmed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Glostrup, Denmark).

### Library preparation and RNA sequencing

The library was prepared using the TruSeq RNA Sample Preparation Kit (Illumina, Little Chesterford, UK), following the manufacturer's instructions. Transcripts shorter than 100 nt were not retained after using this kit, as purification was performed with Agencourt AMPure XP beads (Beckman Coulter Genomics, Takeley, UK). The cDNA fragments were then ligated with adapter sequences. This was followed by product purification and PCR amplification to generate the final cDNA library. After each step in the TruSeq RNA Sample Preparation Kit the samples were validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Glostrup, Denmark), and the final concentration was measured using a Qubit 2.0 Fluorometer (Invitrogen, Naerum, Denmark). The libraries were sequenced using the Illumina HiSeq2000 platform with a single-end protocol, multiplexing and read lengths of 100 nt.

### Read mapping and differential expression analysis

Reads were trimmed for adaptor sequences using CLC Genomics Workbench (CLC bio, Aarhus, Denmark), with standard settings. These trimmed reads were mapped onto the *Escherichia coli* strain K-12 substrain MG1655 genome (RefSeq accession number NC_000913) using the CLC Genomics Workbench. While mapping, hits of reads to annotated genes were also counted and all rRNA read counts were removed afterwards to obtain a proper normalization. The data were normalized using the conditional quantile normalization method implemented in the statistical programming language R, while also taking into account differences in replicate library sizes and gene GC content using the edgeR bioconductor software package for R.[25] The differential expression of each already annotated mRNA transcript was also calculated using EdgeR. The number of top significant genes was selected based on the false discovery rate (FDR) (Benjamini–Hochberg multiple testing correction).[26] A *P* value threshold of 0.05 was selected. Functional enrichment analysis with regard to gene ontology (GO) categories (biological process and molecular function) was performed using the Cytoscape plugin BINGO.[27] The KEGG and EcoCyC databases were used to further analyse the differentially expressed genes.[28,29] The base condition was defined as the samples without cefotaxime and with subinhibitory concentrations of cefotaxime, as only a few genes (2 genes for MG1655/CTX-M-1 and 26 genes for MG1655/IncI1/CTX-M-1) were significantly regulated between these two conditions. If genes were regulated in both comparisons (without cefotaxime compared with inhibitory concentrations of cefotaxime, and subinhibitory concentrations of cefotaxime compared with inhibitory concentrations of cefotaxime), then the highest fold change was selected.

## Results

### Transcriptional analysis by RNA sequencing

Two strains, MG1655/CTX-M-1 carrying $bla_{CTX\text{-}M\text{-}1}$ on the chromosome and MG1655/IncI1/CTX-M-1 carrying $bla_{CTX\text{-}M\text{-}1}$ on a native IncI1 plasmid, were used to examine the differences in gene expression level between growth under the base condition (no or subinhibitory cefotaxime concentration) and 1/2 MIC of cefotaxime for the two resistant strains (inhibitory concentration). Each library contained 2.5–3 million unique reads of 100 bp, which mapped to unique sites in the *E. coli* K-12 MG1655 reference genome. For MG1655/CTX-M-1 a total of 206 genes were significantly regulated, of which 149 were significantly up-regulated and 57 were significantly down-regulated. For MG1655/IncI1/CTX-M-1 a total of 804 genes were significantly regulated, of which 567 and 237 were significantly up- and down-regulated, respectively. The full data list of differentially regulated genes can be found in Tables S2–S5. The number of genes that were significantly regulated was found to be higher in the strain with the plasmid-encoded resistance gene. A detailed analysis revealed that this was a question of variation between replicates in the two conditions, affecting the cut-off for significance.

### Functional classification of differentially expressed genes

To improve the global analysis of the differentially expressed genes, KEGG clustering was performed by subjecting all regulated genes to the KEGG database.[28] Sixteen functional classes were identified (Figure 1). In general, many genes within the translation/ribosomal structure, nucleotide metabolism, carbohydrate transport/metabolism and amino acid transport/metabolism categories were up-regulated. Many down-regulated genes were observed in the protein transport, carbohydrate transport/metabolism and amino acid transport/metabolism categories.

To support the KEGG analysis, functional enrichment analysis with regard to GO categories (biological process and molecular function) was performed using Cytoscape. Results of this analysis showed that genes that code for protein synthesis, transporters, energy production, purine metabolism, pyrimidine metabolism and the respiratory chain were the largest groups of up-regulated genes in MG1655/CTX-M-1. Genes encoding transporters, anaerobic respiration, pH regulation, polyamine catabolic processes, kinase activities and galactate metabolism were down-regulated in this strain. Probably due to the higher number of regulated genes, the functional enrichment analysis found more functional classes to be regulated in MG1655/IncI1/CTX-M-1; protein synthesis, transporters, energy production, polyamine, purine metabolism, pyrimidine metabolism, respiratory chain, pyruvate metabolism, arginine and proline metabolism were up-regulated while anaerobic respiration, iron sulphur cluster binding, transporters, galactate

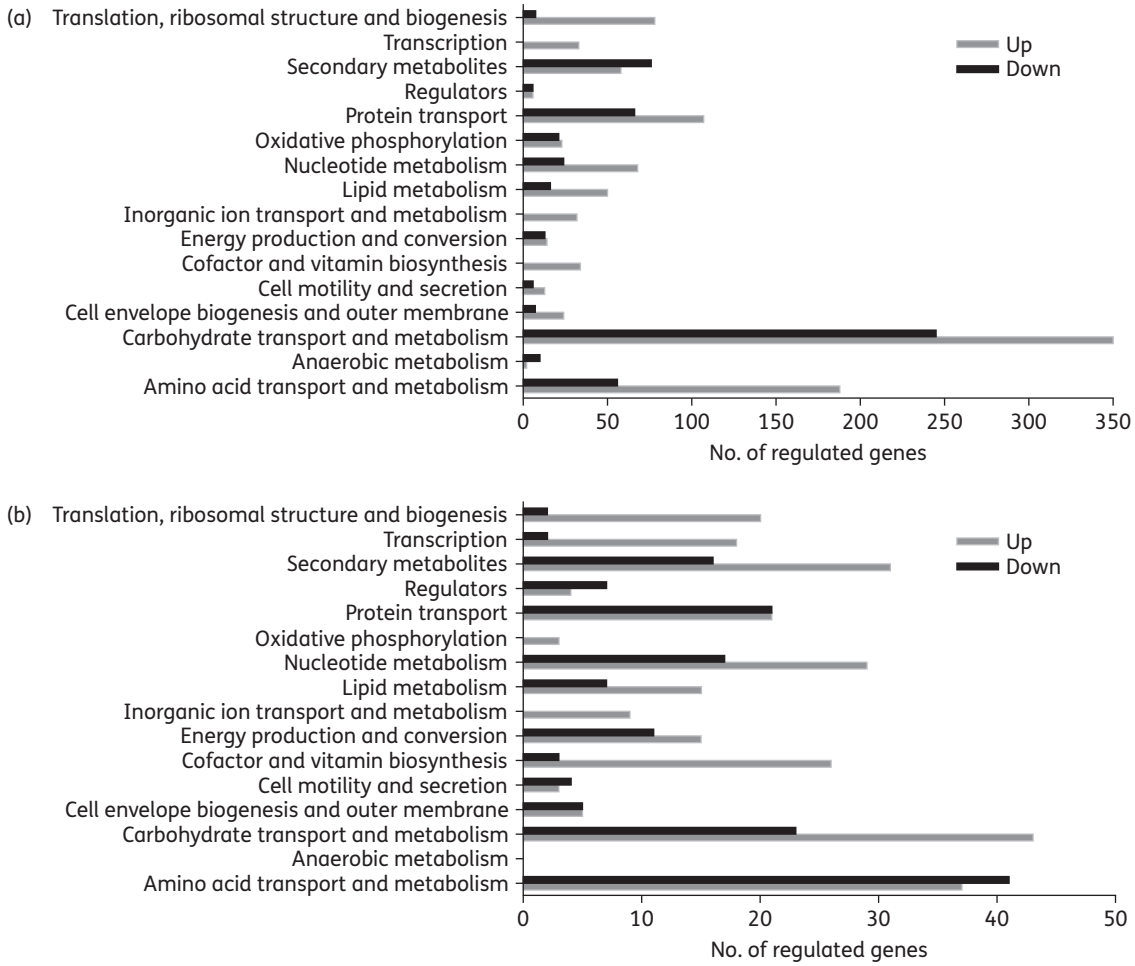



**Figure 1.** KEGG functional classification of differentially expressed genes. Bars represent the number of up- or down-regulated genes in a class. (a) Significantly regulated genes between the base condition and inhibitory concentrations of cefotaxime in MG1655/CTX-M-1. (b) Significantly regulated genes between the base condition and inhibitory concentrations of cefotaxime in MG1655/IncI1/CTX-M-1.

metabolism and pH regulation were down-regulated in the presence of inhibitory concentrations of cefotaxime. Figure S1 shows the figures resulting from these Cytoscape analyses. The significantly regulated genes were found to belong to a high number of pathways. It was considered beyond the scope of the current study to investigate all changes in detail. However, to aid the reader in transferring the transcriptional patterns into putative phenotypes, in Table S6 the functions of the enzymes encoded by the genes are highlighted.

### *Detailed analysis of selected classes of genes regulated in response to cefotaxime*

To test the hypothesis that significantly regulated pathways might be putative new targets that could be used to harm antibiotic-resistant bacteria, subsets of genes/pathways identified by Cytoscape and KEGG analyses were selected for further testing.

### *Protein synthesis*

In total 24 and 30 ribosomal and translational genes were up-regulated ~2- to 2.5-fold in MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1 in the inhibitory condition, suggesting that *de novo* protein synthesis is more important in the presence of cefotaxime than when growing without this drug. Experiments were performed to inhibit the protein synthesis simultaneously with cefotaxime treatment using sub-MIC concentrations of chloramphenicol to inhibit the 50S ribosomal subunit.[30] Combined treatment with chloramphenicol at a concentration corresponding to 1/2 MIC for both strains and cefotaxime for 1 h resulted in a significantly ($P<0.0001$) longer lag phase in MH-II medium compared with sole treatment with either chloramphenicol or cefotaxime (Figure 2). This indicated that the combined inhibition of protein synthesis due to sub-MIC concentrations of chloramphenicol and cefotaxime makes the CTX-M-1-producing *E. coli* strains more susceptible to cefotaxime. In support of this, the MIC of cefotaxime decreased with increasing chloramphenicol concentrations (Figure S2). The MIC of cefotaxime for MG1655/CTX-M-1 decreased from 256 mg/L when no chloramphenicol was present to 1 mg/L when 2 mg/L chloramphenicol was present. For MG1655/IncI1/CTX-M-1, the MIC of cefotaxime decreased from 256 to 0.5 mg/L when 2 mg/L chloramphenicol was present. The MIC of chloramphenicol was 4 mg/L for both strains. Chequerboard assay showed that there was synergy (FIC ≤0.5) between 64 mg/L cefotaxime and 0.25–0.5 mg/L chloramphenicol, 32 mg/L cefotaxime

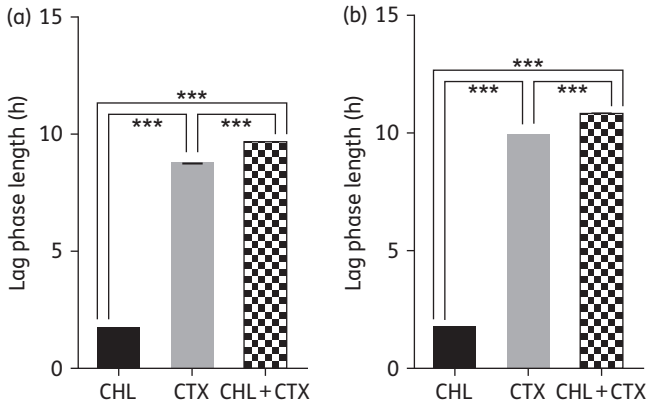



**Figure 2.** Lag phase length of two CTX-M-1-producing *E. coli* strains treated with chloramphenicol (CHL) and/or cefotaxime (CTX). (a) *E. coli* MG1655 containing $bla_{CTX\text{-}M\text{-}1}$ on the chromosome (MG1655/CTX-M-1). (b) *E. coli* MG1655 containing $bla_{CTX\text{-}M\text{-}1}$ on an IncI1 plasmid (MG1655/IncI1/CTX-M-1). All strains were grown in MH-II broth with CTX (1/2 MIC for the corresponding strain) on a BioScreen C™ (except the 'CHL' control culture) after treatment with CHL and/or CTX for 1 h. Three independent replicates were performed; the data shown represent the means and error bars represent standard deviations. The asterisks indicate statistical significance: ***$P < 0.0001$.

and 1 mg/L chloramphenicol, and between 1 mg/L cefotaxime and 2 mg/L chloramphenicol for MG1655/CTX-M-1 (see all FIC indexes in Figure S2). For MG1655/IncI1/CTX-M-1, there was synergy between 32 mg/L cefotaxime and 1 mg/L chloramphenicol, and between 0.5 mg/L cefotaxime and 2 mg/L chloramphenicol (see all FIC indexes in Figure S2). The MIC of cefotaxime did not decrease for the WT MG1655 strain when the cefotaxime and chloramphenicol combinations were used (data not shown).

### *Peptidoglycan biosynthesis*

*ddlA* encodes one of two D-alanine-D-alanine ligases in *E. coli*. Together with alanine racemase, it makes up the D-alanine branch of peptidoglycan biosynthesis.[31] The enzyme synthesizes D-alanine-D-alanine, which is added to the growing cell wall precursor. D-Alanine-D-alanine ligase is an antibacterial drug target of D-cycloserine.[32] *ddlA* was found to be up-regulated in MG1655/IncI1/CTX-M-1. MIC experiments were performed for both strains to test synergy between cefotaxime and D-cycloserine. The MIC of cefotaxime decreased for MG1655/CTX-M-1 from 256 mg/L when no D-cycloserine was present to 8 mg/L when 8 mg/L D-cycloserine was present. For MG1655/IncI1/CTX-M-1, the MIC decreased from 256 mg/L when no D-cycloserine was present to 64 mg/L when 8 mg/L D-cycloserine was present. The chequerboard assay revealed synergy (FIC $\leq$0.5) between 64 mg/L cefotaxime and 0.25–1 mg/L D-cycloserine, and between 32 mg/L cefotaxime and 2–4 mg/L D-cycloserine for MG1655/CTX-M-1 (see all FIC indexes in Figure S2). For MG1655/IncI1/CTX-M-1 no synergy was found (see all FIC indexes in Figure S2).

### *Energy metabolism*

*atpA*, *atpB*, *atpE*, *atpF*, *atpG* and *atpH* were up-regulated 2.3- to 2.9-fold in MG1655/IncI1/CTX-M-1 and *atpF* was up-regulated in MG1655/CTX-M-1. The *atp* genes encode the ATP synthase of *E. coli*, which catalyses the final step in the oxidative phosphorylation of ADP to ATP under aerobic cell growth.[33,34] Experiments were performed to inhibit or impair energy synthesis simultaneously with cefotaxime treatment. The hypothesis was that the strains would have a longer lag phase and a decreased MIC of cefotaxime. Enterostatin is a pentapeptide (Asp-Pro-Gly-Pro-Arg, human) that binds to the β-subunit, encoded by *atpD*, of the F1 complex and inhibits ATP synthesis.[35] Berger *et al.*[36] found that enterostatin caused an ~31% decrease in intracellular ATP after 2 min of incubation of insulinoma cells compared with the control. Quercetin also impairs ATP synthesis by inhibiting the ATPase activities of F1 and F1F0 complexes in e.g. *E. coli*.[35,37] Futai *et al.*[37] showed that 50% inhibition was obtained with 40 μM quercetin. These two compounds were used alone and in combination together with cefotaxime in this study (Figure 3). A significantly longer lag phase was observed with increasing concentrations of enterostatin; in particular, MG1655/IncI1/CTX-M-1 treated with 90 μM enterostatin showed a prolonged lag phase compared with the sample that was only treated with cefotaxime. The growth of the strains was not inhibited when only enterostatin was present (Figure S3). Similar trends were seen when the strains were treated with quercetin and cefotaxime. Here the strains were not able to grow when cefotaxime was combined with the highest quercetin concentration tested (40 μM). A significantly prolonged lag phase was seen with 20 μM quercetin for MG1655/CTX-M-1 and with both 10 and 20 μM quercetin for MG1655/IncI1/CTX-M-1 (Figure 3). Quercetin used alone did affect the growth of the strains as slower growth rates were observed with increasing quercetin concentrations (Figure S3). Quercetin concentrations that did not affect the growth rates to a high extent were therefore chosen for further experiments. When enterostatin and quercetin were used in parallel, the strains were not able to grow at the highest concentrations together with cefotaxime, and prolonged lag phases were seen with all other concentrations tested. Enterostatin and quercetin used without cefotaxime had a small effect on lag-phase length (Figure S3), while the lag-phase extension was more pronounced when cefotaxime was present. The partial chequerboard experiments with these compounds at fixed concentrations and cefotaxime did not, however, show a decreased MIC when tested with the concentrations used in the growth experiments (data not shown).

### *Purine synthesis*

Many purine synthesis genes were up-regulated in the presence of cefotaxime, and this pathway was investigated as a potential helper drug target. In total, 8 and 15 purine synthesis genes were up-regulated in MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1, respectively. Purine autotroph strains were created, and our hypothesis was that these mutants would have impaired growth when treated with cefotaxime. MG1655/CTX-M-1 Δ*purN*Δ*purT* showed increased lag-phase length in the presence of cefotaxime treatment (Figure 4). Furthermore, this strain reached a lower maximum growth compared with MG1655/CTX-M-1 Δ*purN*, MG1655/CTX-M-1 Δ*purT* and MG1655/CTX-M-1, with and without cefotaxime in MH-2 broth. The MG1655/IncI1/CTX-M-1 purine mutants also showed this tendency; however, MG1655/IncI1/CTX-M-1 Δ*purN*Δ*purT* did not show the prolonged lag phase that was observed for MG1655/CTX-M-1 Δ*purN*Δ*purT*. In minimal

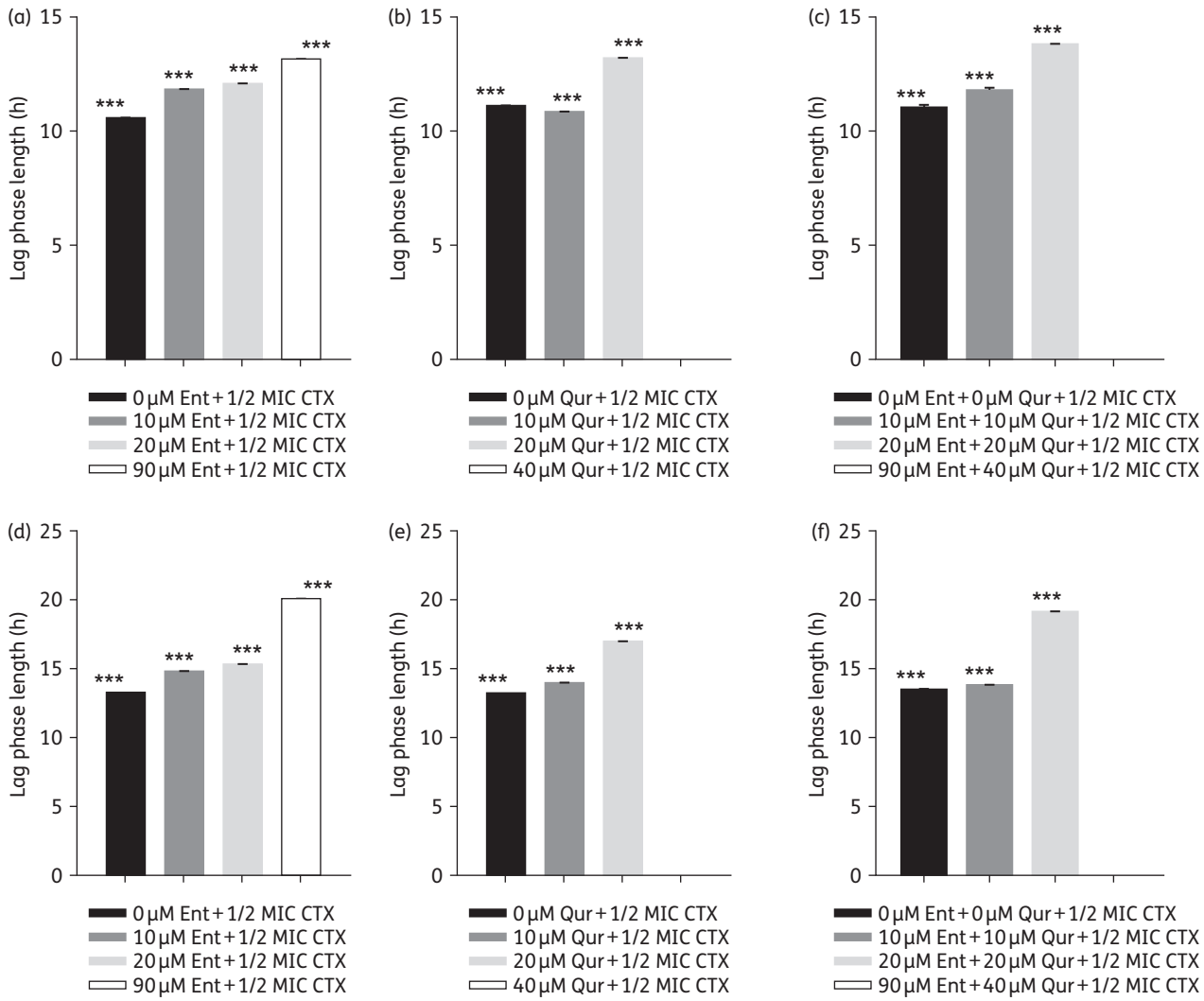
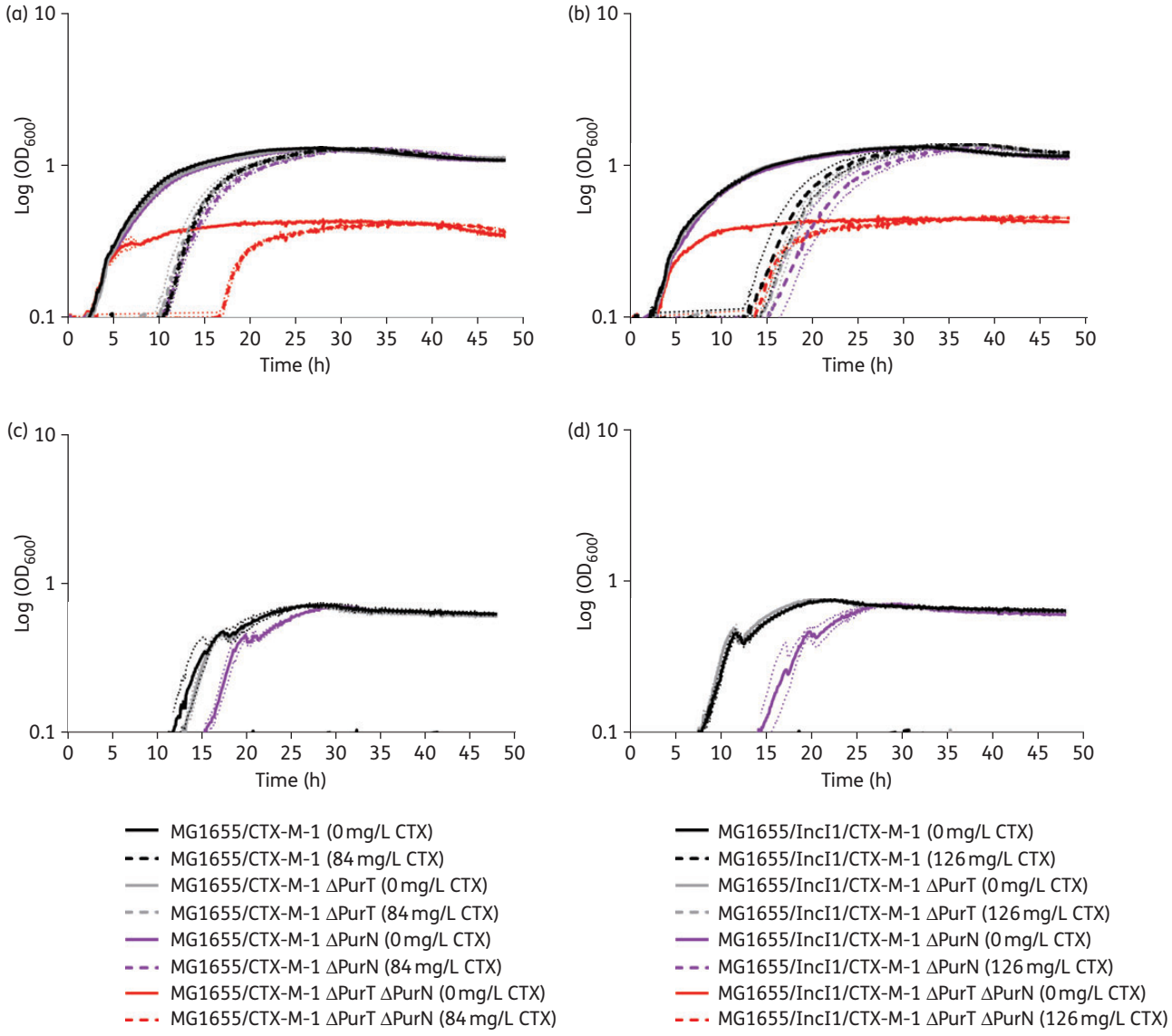



**Figure 3.** Lag-phase length of the two *E. coli* MG1655 containing $bla_{CTX\text{-}M\text{-}1}$ on the chromosome (MG1655/CTX-M-1) and on an IncI1 plasmid (MG1655/IncI1/CTX-M-1) treated with enterostatin (Ent) or/and quercetin (Qur) and cefotaxime (CTX). (a) MG1655/CTX-M-1 with CTX and enterostatin. (b) MG1655/CTX-M-1 with CTX and quercetin. (c) MG1655/CTX-M-1 with CTX, enterostatin and quercetin. (d) MG1655/IncI1/CTX-M-1 with CTX and enterostatin. (e) MG1655/IncI1/CTX-M-1 with CTX and quercetin. (f) MG1655/IncI1/CTX-M-1 with CTX, enterostatin and quercetin. All strains were grown in MH-II broth with CTX (1/2 MIC for the corresponding strain) and different concentrations of enterostatin and quercetin on a BioScreen C™. Three independent replicates were performed; the data shown represent the means and error bars represent standard deviations. The asterisks indicate statistical significance: ***$P < 0.0001$.

medium (M9) the original strains and the single *pur* mutants did not grow when 1/2 MIC of cefotaxime was applied to the culture. The single Δ*purN* mutant showed a prolonged lag phase when no cefotaxime was present. The Δ*purN*Δ*purT* mutants did not display any growth in M9 with or without cefotaxime, as expected, as no purines were available in the medium. MIC experiments with the *pur* mutants showed that the MIC of cefotaxime decreased for both strains (Figure 4e). The MIC of cefotaxime for MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1 single mutants decreased 2-fold from 256 to 128 mg/L for all strains, except MG1655/IncI1/CTX-M-1 Δ*purN*. The MIC of cefotaxime for MG1655/CTX-M-1 Δ*purN*Δ*purT* decreased 8-fold from 256 to 32 mg/L, while the MIC of cefotaxime for MG1655/IncI1/CTX-M-1 Δ*purN*Δ*purT* did not decrease.

### *Proline transport*

The proline transport pump, encoded by the *putP* gene, is a major proline transporter in *E. coli*.[38] The pump is connected to the *putA* gene, encoding a proline dehydrogenase, which converts L-proline to L-glutamate.[38,39] Both genes are induced in the presence of proline in the growth medium and were also up-regulated in the presence of cefotaxime. *putP* was 3.7-fold up-regulated in MG1655/CTX-M-1 and 5.0-fold up-regulated in MG1655/IncI1/CTX-M-1. *putAP* deletion mutants were created for both of the $bla_{CTX\text{-}M\text{-}1}$-encoding strains and the hypothesis was that these mutants would have an increased lag-phase length and a decreased MIC when treated with cefotaxime. MG1655/CTX-M-1 Δ*putAP* showed increased lag-phase length in the presence of cefotaxime treatment compared with MG1655/CTX-M-1 without




(e)

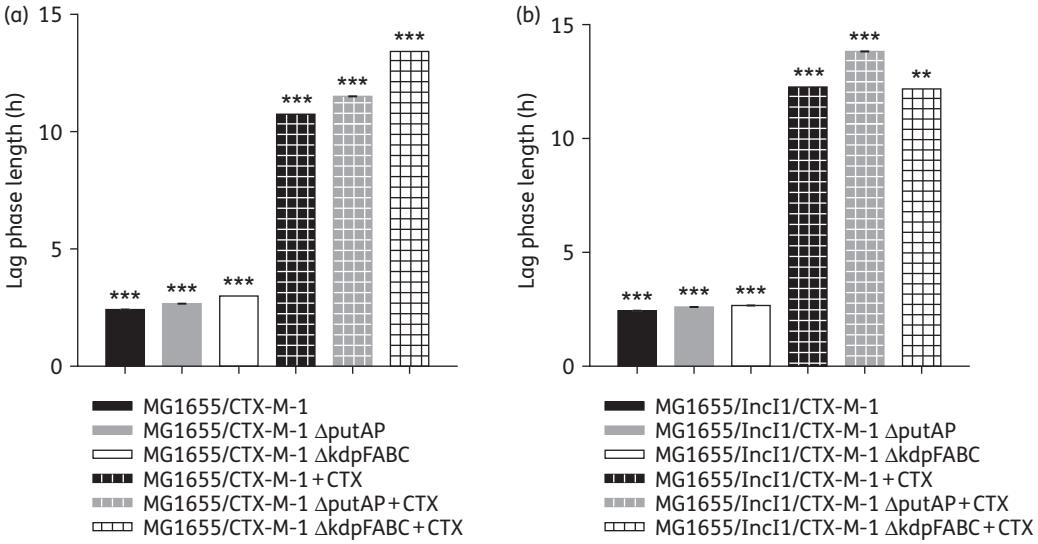
| | WT | ΔPurT | ΔPurN | ΔPurT ΔPurN |
|---|---|---|---|---|
| MG1655/CTX-M-1 | 256 | 128 | 128 | 32 |
| MG1655/IncI1/CTX-M-1 | 256 | 128 | 256 | 256 |

**Figure 4.** Growth curves of the two CTX-M-1-producing *E. coli* strains with *purT* and *purN* single and double knockouts. (a) Growth of *E. coli* MG1655 containing $bla_{CTX\text{-}M\text{-}1}$ on the chromosome (MG1655/CTX-M-1) and its *pur* mutants in MH-2 medium. (b) Growth of *E. coli* MG1655 containing $bla_{CTX\text{-}M\text{-}1}$ on an IncI1 plasmid (MG1655/IncI1/CTX-M-1) and its *pur* mutants in MH-2 medium. (c) Growth of MG1655/CTX-M-1 and its *pur* mutants in M9 medium. (d) Growth of MG1655/IncI1/CTX-M-1 and its *pur* mutants in M9 medium. (e) MIC (mg/L) of cefotaxime (CTX) for MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1 *pur* mutants. All strains were grown in MH-II/M9 broth with and without CTX; 1/2 MIC for the corresponding strain) on a BioScreen C™. WT refers to the strains MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1 without any purine knockouts. Three independent replicates were performed of the growth data; the data shown represent the means and thin dotted lines show standard deviations. The keys for (c) and (d) also apply for (a) and (b), respectively.

any knockouts (Figure 5a). The lag-phase length increased from 10.8 to 11.5 h. MG1655/IncI1/CTX-M-1 Δ*putAP* also showed this significant increase in lag-phase length ($P < 0.0001$) when treated with cefotaxime compared with the strain without knockout (Figure 5b). The lag phase increased by 1.5 h, from 12.3 to 13.8 h. The MIC of cefotaxime decreased 4-fold from 256 to 64 mg/L for MG1655/CTX-M-1 Δ*putAP* and decreased 2-fold from 256 to 128 mg/L for MG1655/IncI1/CTX-M-1 Δ*putAP* (Figure 5c).

### *Potassium transport*

The Kdp complex is a high-affinity ATP-driven potassium uptake system in *E. coli*.[40] It consists of four membrane-bound subunits:




(c)

| | WT | ΔputAP | ΔkdpFABC |
|---|---|---|---|
| MG1655/CTX-M-1 | 256 | 64 | 32 |
| MG1655/IncI1/CTX-M-1 | 256 | 128 | 128 |

**Figure 5.** Lag-phase length of the two CTX-M-1-producing *E. coli* strains with putAP and kdpFABC knockouts treated with and without cefotaxime (CTX). (a) MG1655/CTX-M-1 and its mutants. (b) MG1655/IncI1/CTX-M-1 and its mutants. (c) MIC of CTX (mg/L) for MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1 ΔputAP and ΔkdpFABC mutants. All strains were grown in MH-II broth with CTX (1/2 MIC for the corresponding strain) on a BioScreen C™. WT refers to the strains MG1655/CTX-M-1 and MG1655/IncI1/CTX-M-1 without any knockouts. Two independent biological replicates were performed; the data shown represent the means and error bars represent standard deviations. The asterisks indicate statistical significance: $^{**}P<0.001$ and $^{***}P<0.0001$.

KdpF, KdpA, KdpB and KdpC. Briefly, KdpA is an ion channel that selectively mediates the translocation of potassium, KdpB is an ATPase module, KdpC has catalytic chaperone functions for KdpB in the binding of ATP, and KdpF is responsible for the stability of the complex.[41,42] *kdpA*, *kdpB* and *kdpC* were 5.6-, 7.7- and 5.7-fold up-regulated, respectively, in MG1655/CTX-M-1 and *kdpA* and *kdpB* were 4.7- and 3.4-fold up-regulated, respectively, in MG1655/IncI1/CTX-M-1. *kdpFABC* deletion mutants were created for both of the $bla_{\text{CTX-M-1}}$-encoding strains. MG1655/CTX-M-1 Δ*kdpFABC* showed a significantly increased lag-phase length of 2.6 h (Figure 5a). The MG1655/IncI1/CTX-M-1 Δ*kdpFABC* mutant did not show this change in the lag phase (Figure 5b). The MIC experiments showed that the MIC of cefotaxime decreased for both strains (Figure 5c): an 8-fold decrease from 256 to 32 mg/L for MG1655/CTX-M-1 Δ*kdpFABC* and a 2-fold decrease from 256 to 128 mg/L for MG1655/IncI1/CTX-M-1 Δ*kdpFABC*.

## Discussion

This study demonstrated that the global transcription of CTX-M-1-producing *E. coli* changed dramatically when inhibitory concentrations of cefotaxime were present. Furthermore, the results clearly demonstrated that some regulated genes/gene products could be candidates for helper-drug targets to increase cefotaxime susceptibility in CTX-M-1-producing *E. coli*. The study complements previous studies where responses in antimicrobial-resistant bacteria were analysed by proteomic approaches.[13–15] Contrary to these studies, we have characterized the transcriptional changes, and we have included both chromosome- and plasmid-encoded location of the resistance gene. A comparison between the current study and previous reports reveals overlaps between the responses, although different antibiotics were used. For example, proteins involved in energy production and conversion, amino acid biosynthesis and protein translation were up-regulated in a vancomycin-resistant strain upon vancomycin treatment.[13] These changes correspond closely with the gene responses observed in the current study. This suggests that there may be common elements in the reaction pattern to the treatment with antibiotics in antibiotic-resistant bacteria, and that it should be possible to find secondary targets with broad-spectrum activity.

Cefotaxime was chosen in the current study to investigate changes caused by antibiotic treatment of ESBL *E. coli*. This drug is a third-generation cephalosporin, and has been used extensively in hospitalized neonates and for surgical wound prophylaxis.[43,44] It has an effect against many Gram-positive and Gram-negative organisms and shows good stability towards the most common β-lactamases found in bacteria.[44,45] Among the >130 different CTX-M variants that have been identified, CTX-M-1, encoded by $bla_{\text{CTX-M-1}}$, is the most common type found in livestock in Europe and it is commonly isolated from humans as well.[3,7,46–49] Since this gene can be found on IncI1 and IncN plasmids as well as in the chromosome, both plasmid- and chromosome-encoded $bla_{\text{CTX-M-1}}$ genes were included in the investigation in the form of two strains, both isogenic variants of MG1655, one with $bla_{\text{CTX-M-1}}$ in the chromosome and one with the gene in a naturally occurring resistance plasmid.[3,48,50–52]

The number of genes that were significantly regulated was found to be higher in the strain with the plasmid-encoded

resistance gene. A detailed analysis revealed that this, for the major part, was a question of variation between replicates in the two conditions, affecting the cut-off for significance. The same proportion of up-regulated genes was seen in both strains (~70% of regulated genes), and the difference between the two strains was mostly a matter of a higher number of genes found to be significantly regulated in pathways that were up-regulated in both strains. This was recognized by the functional classification analysis by KEGG, where all 16 classes, except lipid metabolism, were represented in both strains. Only pathways involved in pyruvate metabolism, iron sulphur cluster binding, kinase activities and specific transmembrane activities were found to be significantly regulated in one strain but not in the other (see Table S6 for specific genes). The IncI1 plasmid carrying $bla_{CTX\text{-}M\text{-}1}$ does not harbour genes that can explain these specific differences. However, there are genes with unknown or putative functions on the plasmid. It is thus a possibility that the plasmid backbone may have influenced the response to treatment in the strain where $bla_{CTX\text{-}M\text{-}1}$ was carried on a plasmid, and further studies are indicated.[53] A possible experimental design includes repeating the analysis with the strain with chromosomally located $bla_{CTX\text{-}M\text{-}1}$, but this time harbouring a variant of the native IncI1 plasmid where the $bla_{CTX\text{-}M\text{-}1}$ gene has been knocked out. The results of this analysis could be compared with the expression in the strain with chromosomally located $bla_{CTX\text{-}M\text{-}1}$ without a plasmid.

The results indicated that *de novo* protein synthesis was essential for CTX-M-1-producing *E. coli* to grow in the presence of cefotaxime. The strains became more susceptible to cefotaxime when treated with low concentrations of chloramphenicol and the MIC of cefotaxime deceased dramatically, with values close to the cut-off for cefotaxime resistance (epidemiology cut-off: ≤0.25 mg/L).[54] This demonstrated that it is not only possible to identify helper drug targets by studying adaptive responses in antibiotic-resistant bacteria during treatment, but also that it is possible to reduce MIC values to levels where resistance can be recovered by targeting the adaptive responses. Cefotaxime in combination with metronidazole has previously been indicated to be a highly effective treatment of brain abscesses, and it has also been shown that ampicillin/chloramphenicol is a beneficial combination *in vivo* against *E. coli*.[55,56] Chloramphenicol can cause a rare but potentially fatal aplastic anaemia in humans, and is currently not used for treatment of humans and food animals.[57] Nevertheless, it should be possible to use the principle with other drugs that have inhibitory effects on protein synthesis, e.g. aminoglycosides.

Genes encoding proteins involved in peptidoglycan synthesis were significantly up-regulated, suggesting that the need for these pathways is more important when the organism is growing in the presence of the β-lactam antibiotic than when growing without the drug. In accordance with this, the MIC of cefotaxime for both strains decreased dramatically when D-cycloserine and cefotaxime were used in combination, and synergy was found in most combinations, but only for the strain where the resistance gene was carried in the chromosome. That MIC decreases for both strains indicates that the plasmid-carrying strain cannot circumvent the blockage of D-alanine-D-alanine synthesis due to D-cycloserine treatment, but the interaction between the drugs must be different in the two strains. Further studies are needed to understand why synergy is not observed when the resistance gene is carried on the (specific) plasmid. Another point is that use of this combination requires further investigation to assess *in vivo* efficacy and safety, since D-cycloserine may have neurological effects.[58]

Drugs targeting ATP synthase are considered to be emerging antibacterial drugs, especially to kill mycobacteria.[59] In our study, inhibition of energy metabolism simultaneously with cefotaxime treatment mostly affected the lag phase. A treatment that affects the bacteria before they start dividing may be enough to affect the balance between the invading bacteria and the host; however, it appears that bacteria have redundant systems that can take over once growth is initiated. Targeting proline or potassium transport also showed promising results and unlike when targeting energy synthesis, this also decreased the MIC of cefotaxime. This suggests that these transport systems could be potential helper-drug targets.

Only strains defective in both the redundant phosphoribosylglycinamide transformylases, PurN and PurT, will be purine autotrophs and require an exogenous purine source.[60] The two CTX-M-1-producing *E. coli* strains were not able to grow in M9 medium with cefotaxime. This indicates that initiating growth in the presence of 1/2 MIC of cefotaxime is challenging, even for a resistant strain, when no amino acids and other metabolites are readily available in the environment.

The MIC of cefotaxime for the purine-auxotrophic strains, where the resistance gene was carried in the chromosome, decreased compared with the strain without mutation, whereas the MIC for the corresponding strain with the resistance gene carried on a plasmid did not decrease. A possible explanation can be offered by the fact that some genes of the purine salvage pathway were up-regulated in this plasmid-containing strain (*hpt* and *apt* were up-regulated 2.0- and 2.8-fold; Table S4). No genes on the plasmid have a direct link to this effect. Another plausible reason could be the higher CTX-M-1 protein level in this strain compared with the one with CTX-M-1 encoded in the chromosome; however, the experimental set-up tried to compensate for this difference by standardizing cefotaxime concentration according to MIC.[20]

Treatment with β-lactams has been shown to induce stress (SOS response) when the drug is applied in subinhibitory concentrations to susceptible *E. coli* strains.[61] An important observation in the current study was that the general stress response was not induced, as evaluated by the transcriptional analysis. This indicates that resistant and susceptible strains react differently to treatment. However, we have previously demonstrated that our resistant strains initially react to treatment by elongation, and that this is similar to the susceptible WT MG1655 strain.[62] This suggests an initial stress response, and that the lack of a stress response in the current study is related to the timepoint investigated. It could be interesting to investigate the transcriptional responses in other bacteria and with other cephalosporin drugs, and at other timepoints with the current strains, in order to understand how the transcriptional pattern varies over time, and whether the observations made in the current paper are general to cephalosporin-resistant bacteria.

A weakness of the current study relates to the two strains used to evaluate differentially expressed genes. None of these naturally carries ESBL-mediated resistance. While the experimental set-up allowed comparison of expression levels with a well-characterized strain (MG1655), and comparable results

between cefotaxime treatments in strains with plasmid and chromosomally encoded $bla_{CTX\text{-}M\text{-}1}$, it does not take into account that antibiotic resistance genes and plasmids in general co-evolve with the chromosome in the host where they are expressed. In naturally occurring cefotaxime-resistant strains, this may have led to compensatory mechanisms that may have removed some of the apparent weaknesses we found in the bacteria, when forced to direct their full attention to the combat of inhibitory concentrations of antibiotics. Further studies would therefore be very interesting.

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## Transparency declarations

M. O. A. S. is a founder of AntibioTx, which is developing new antibiotics. AntibioTx was not involved in this study and does not have any conflicts of interest with the study results. All other authors: none to declare.

## Supplementary data

Tables S1 to S6 and Figures S1 to S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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