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Global protein responses of multidrug resistance plasmid-containing *Escherichia coli* to ampicillin, cefotaxime, imipenem and ciprofloxacin

Anatte Margalit, James C. Carolan, Fiona Walsh*

Department of Biology, Maynooth University, Co. Kildare, Ireland

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ABSTRACT

Objectives: This study compared the proteomics of *Escherichia coli* containing the multidrug resistance plasmid pEK499 under antimicrobial stress and with no antimicrobial.
Methods: We utilised mass spectrometry-based proteomics to compare the proteomes of the bacteria and plasmid under antimicrobial stress and no antimicrobial.
Results: Our analysis identified statistically significant differentially abundant (SSDA) proteins common to groups exposed to the β-lactam antimicrobials but not ciprofloxacin, indicating a β-lactam stress response to exposure from this class of drugs, irrespective of β-lactam resistance or susceptibility. Data arising from comparisons of the proteomes of ciprofloxacin-treated *E. coli* and controls detected an increase in the relative abundance of proteins associated with ribosomes, translation, the TCA cycle and several proteins associated with detoxification, and a decrease in the relative abundance of proteins associated with the stress response, including oxidative stress. We identified changes in proteins associated with persister formation in the presence of ciprofloxacin but not the β-lactams. The plasmid proteome differed across each treatment and did not follow the pattern of antimicrobial–antimicrobial resistance (AMR) protein associations: a relative increase was observed in the amount of CTX-M-15 in the presence of cefotaxime and ciprofloxacin, but not the other β-lactams, suggesting regulation of CTX-M-15 protein production.
Conclusion: The proteomic data from this study provided novel insights into the proteins produced from the chromosome and plasmid under different antimicrobial stresses. These data also identified novel proteins not previously associated with AMR or antimicrobial responses in pathogens, which may well represent potential targets of AMR inhibition.
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1. Introduction

The World Health Organization (WHO) has recognised extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* on their critical list of priority pathogens in relation to human health [1], which are frequently disseminated via horizontal transfer of plasmids. The pathogen response to an antimicrobial is measured in relation to its classification as resistant or susceptible and the specific antimicrobial resistance (AMR) gene present. AMR and multidrug resistance (MDR) are frequently mediated by plasmids. One such pathogen–plasmid combination is the internationally prevalent *E. coli* O25:H4-ST131 clone containing the MDR plasmid pEK499. The pEK499 plasmid harbours ten AMR genes conferring resistance to β-lactams, macrolides, chloramphenicol, tetracycline, trimethoprim, streptomycin, spectinomycin and sulfonamide and reduced susceptibility to ciprofloxacin [2]. The clonal expansion of *E. coli* ST131 has significantly increased global dissemination of the $bla_{\text{CTX-M-15}}$ gene [3].

Although genomic analysis of pathogens is an increasing approach to AMR analysis, the bacterial genotype does not necessarily directly dictate the phenotype as numerous regulatory mechanisms are also involved. Interactions between plasmids and the bacterial host clearly exist, but studies of plasmid-mediated AMR focus mainly on the plasmid-mediated genes or phenotypic response of the host pathogen. A recent study provided an insight into how proteomics may be used in an unbiased fashion for the detection of AMR in pathogenic bacteria cultured in the absence of antimicrobial [4]. The authors followed a workflow that resulted in 98% sensitivity and 100% specificity across

* Corresponding author.
E-mail address: fiona.walsh@mu.ie (F. Walsh).

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seven pathogens and 11 AMR determinants, thus demonstrating the applicability of such a proteomics workflow in clinical microbiology. Few studies currently exist on the proteomic response of pathogens containing AMR plasmids to antimicrobials. These include adaptation of $bla_{CTX\text{-}M\text{-}1}$-containing *E. coli* to cefotaxime [5] and the global response of tetracycline-resistant *E. coli* to oxytetracycline [6]. We have not identified any publication analysing the proteomic changes of MDR plasmid-mediated resistance under different antimicrobial stresses, such as the study presented here.

The aim of this study was to understand specifically how the plasmid and bacterial host proteins were influenced by different antimicrobial stresses. By analysing these factors systematically, we aimed to identify the pathways and antimicrobial-specific responses of the bacterium. Using proteomics, we provide an unbiased protein map of a pathogen with a MDR plasmid under antimicrobial stress.

2. Materials and methods

2.1. Preparation of Escherichia coli proteins for mass spectrometry

Escherichia coli NCTC 13400, containing the plasmid pEK499, was used in all experiments [2]. The pEK499 plasmid confers resistance to eight antibiotic classes via the following genes: $bla_{CTX\text{-}M\text{-}15}$; $bla_{OXA\text{-}1}$; $bla_{TEM\text{-}1}$; *aac(6′)-Ib*-cr; *mph(A); catB4; tet(A)*; and the integron-borne *dfrA7 aadA5* and *sulI* genes. It is a 117 536-bp plasmid with a fusion of type FII and FIA replicons. The *E. coli* strain was isolated from a hospital patient in the UK and is one of the internationally prevalent *E. coli* O25:H4-ST131 clones prevalent in the UK [2]. All bacteria were grown separately in Luria–Bertani broth at 37°C with shaking at 200 rpm with or without antimicrobial (ampicillin 64 mg/L, cefotaxime 256 mg/L, imipenem 0.06 mg/L or ciprofloxacin 0.06 mg/L). Bacteria were pelleted by centrifugation at 3000 rpm for 15 min, re-suspended in ammonium bicarbonate (1 mL, 50 mM, pH 7.8) and sonicated on ice in 10-s bursts five times. The lysate was centrifuged at 13 000 rpm and the supernatant was quantified using a Qubit™ quantification system (Invitrogen). The protein sample was reduced by adding 5 μL of 0.2 M dithiothreitol (DTT) and was incubated at 95°C for 10 min, followed by alkylation with 0.55 M iodoacetamide (4 μL) at room temperature in the dark for 45 min. Alkylation was stopped by adding DTT (20 μL, 0.2 M) and incubation for 45 min at 25°C. Sequencing Grade Trypsin (Promega) (0.5 μg/μL) was added to the proteins and incubated at 37°C for 18 h. The digested protein sample was dried via SpeedVac concentrator (Thermo Scientific Savant™ DNA120). The samples were prepared for mass spectrometry (MS) as described previously [7]. Three independent biological replicates for each group were analysed.

2.2. Mass spectrometry: LC/MS Xcalibur™ instrument parameters for proteomic data acquisition

Digested proteins (1 μg) isolated from the replicates for each *E. coli* sample were loaded onto a Q Exactive™ (Thermo Fisher Scientific) high-resolution accurate-mass spectrometer connected to a Dionex UltiMate™ 3000 RSLCnano chromatography system (Thermo Fisher Scientific). Peptides were separated using an increasing acetonitrile gradient on a 50 cm EASY-Spray™ PepMap C18 column with 75 μm diameter (2 μm particle size), using a 180-min reverse phase gradient at a flow rate of 300 nL/min. All data were acquired over 141 min with the mass spectrometer operating in an automatic dependent switching mode. A full MS scan at 140 000 resolution and a range of 300–1700 *m/z* was followed by an MS/MS scan at 17 500 resolution with a range of 200–2000 *m/z* to select the 15 most intense ions prior to MS/MS.

Quantitative analysis [protein quantification and label-free quantification (LFQ) normalisation of the MS/MS data] of the *E. coli* proteome arising from exposure to the different antimicrobials was performed using MaxQuant v.1.6.3.3 (http://www.maxquant.org) following the general procedures and settings outlined in Hubner et al. [8].

2.3. Data analysis of the proteome

The Perseus v.1.5.5.3 software platform (www.maxquant.org/) was used for data analysis, processing and visualisation. Normalised intensity values were used for principal component analysis (PCA). Data analysis was performed as previously described [7]. The MS proteomics data and MaxQuant search output files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD027164 [9].

3. Results and discussion

In this study, quantitative and qualitative proteomics was employed to provide novel insights into the response of MDR plasmid-mediated *E. coli* to different antimicrobials. While antimicrobials have specific targets on which they exert their mechanism of action, the response of bacteria to these drugs is not limited to the target sites alone [10,11]. Proteomics analysis revealed similarities in the proteome of bacteria in response to antimicrobial-induced stress despite differences in the types of antimicrobials to which they were exposed. Moreover, the data presented here highlight the significance of the chromosome-mediated response to antibiotic-induced stress coupled with the resistance mechanism response of the bacteria.

The PCA performed on all filtered proteins resolved only the ciprofloxacin-treated *E. coli* and separated those samples from all other samples along component 1 (Fig. 1). Volcano plots were produced by pairwise Student's *t*-test ($P < 0.05$) on the post-imputated data set to determine differences in protein abundance between two groups (Fig. 2). The proteomic data arising from pairwise *t*-tests revealed an increase in the relative abundance of several proteins common to groups exposed to ampicillin, cefotaxime and imipenem compared with the control (Supplementary datasets 3–6). All increases and decreases in the relative abundances described are relative to the control. Among these were stress-related proteins including ecotin (Eco) and methionine-R-sulfoxide reductase (MsrC). Compared with the controls, a statistical difference in the relative abundance of two β-lactamases (CTX-M-15 and TEM-1) was detected in the cefotaxime- and imipenem-exposed groups, respectively, but not in ampicillin-exposed bacteria. In addition, the relative abundances of proteins involved in detoxification were increased in bacteria treated with these cell wall inhibitors, including superoxide dismutase SodA (ampicillin- and cefotaxime-treated), peroxiredoxin OsmC, glutaredoxin 3 (GrxC) and 4 (GrxD) (cefotaxime-treated), hydrogen peroxide-inducible genes activator (OxyR) (imipenem-treated) and thiosulfate sulfurtransferase PspE (cefotaxime- and imipenem-treated) [12–15]. Cold shock proteins (CspE and CspA in cefotaxime-treated and CspE in imipenem-treated groups) were detected at higher levels in these groups and in ampicillin-treated bacteria. Our results do not concur with most of the findings of Møller et al. except the increase in CTX-M under cefotaxime exposure [5]. However, our experiments contained 256 mg/L and $bla_{CTX\text{-}M\text{-}15}$ rather than 128 mg/L cefotaxime and $bla_{CTX\text{-}M\text{-}1}$, and a MDR plasmid rather than a single AMR-encoding plasmid, so this may have influenced the variation in proteomes. In addition Møller et al. suggest that upregulation of the *tra* genes in the presence of cefotaxime was dependent on the presence of $bla_{CTX\text{-}M\text{-}1}$ [5]. Our study also identified other proteins of potential interest in response to antimicrobial treatment that may

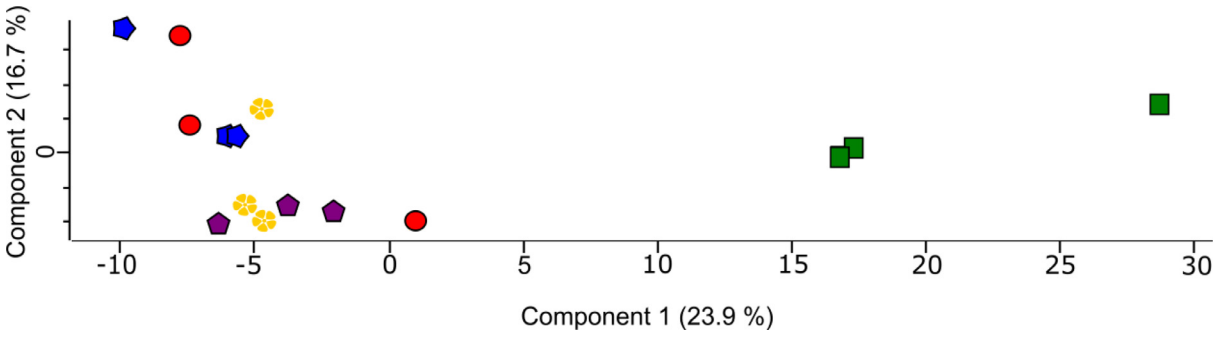

Fig. 1. Principal component analysis (PCA) of the proteomes of pEK499-containing *Escherichia coli* treated with ampicillin (red), cefotaxime (blue), imipenem (yellow) or ciprofloxacin (green) and the control untreated bacteria (purple).

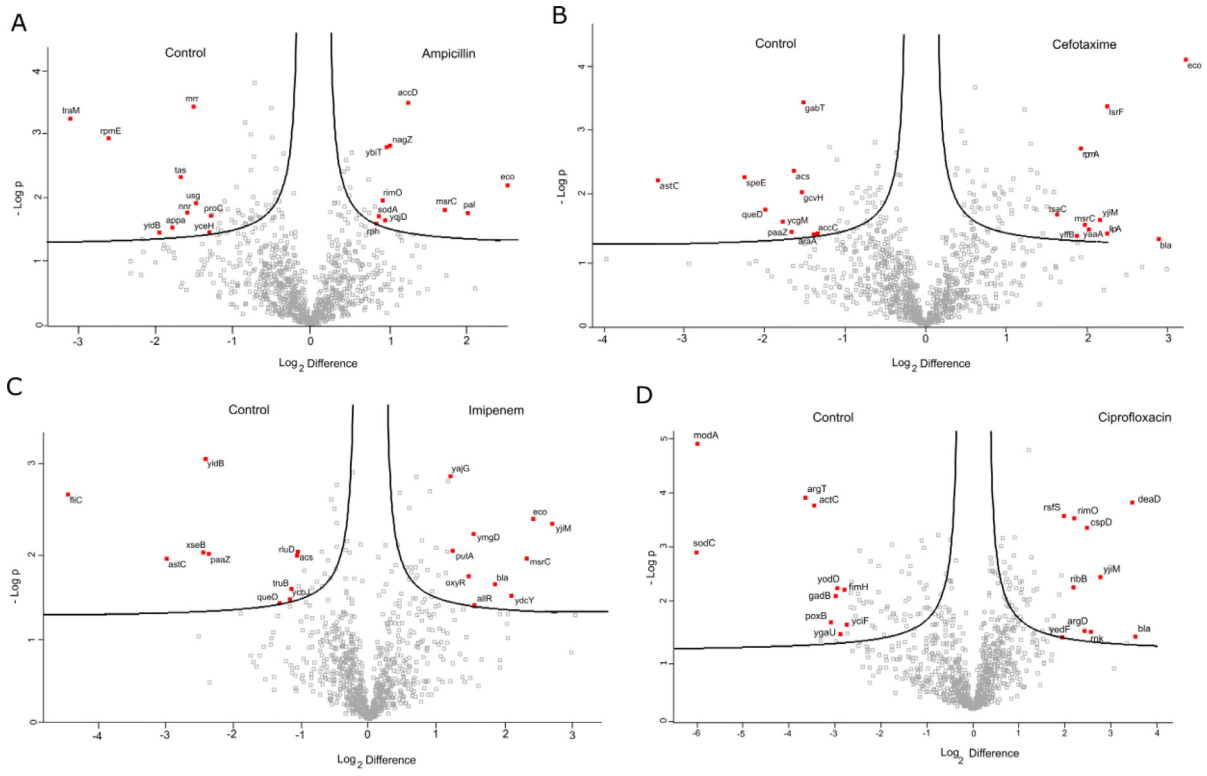

Fig. 2. Volcano plots derived from pairwise comparisons between pEK499-containing *Escherichia coli* treated with various antimicrobials and the control: (A) ampicillin-treated; (B) cefotaxime-treated; (C) imipenem-treated; and (D) ciprofloxacin-treated. The distribution of quantified proteins according to *P*-value ($-\log_{10}$ *P*-value) and fold change ($\log_2$ mean LFQ intensity difference) are shown. Proteins above the line are considered statistically significant ($P < 0.05$). The top 20 most differentially abundant proteins are shown for each group. LFQ, label-free quantification.

aid in understanding the control or production of AMR proteins from the plasmid. A study of carbapenemase-producing *E. coli* under carbapenem stress identified an increased abundance in GroES in *E. coli* containing bla_{IMP} or bla_{KPC} or bla_{NDM} (in increasing order of abundance) [11]. Our study also identified increased GroES in the presence of imipenem, but not the other β-lactams. However, pEK499 does not contain any carbapenemase gene and is imipenem susceptible. Thus, we suggest that this is a carbapenem-induced response rather than a resistance response, which may be increased by carbapenemase-degraded carbapenem as well as whole carbapenem.

In the ampicillin-treated samples, the proteins peptidoglycan-associated lipoprotein (Pal) and β-hexosaminidase (NagZ) were increased, indicating an apparent attempt to maintain cell wall integrity during antimicrobial challenge. It has been reported that ampicillin enhances the release of outer membrane vesicles (OMVs) in which Pal is contained, thereby increasing Pal levels [16]. This may be of clinical importance because OMVs containing Pal also contain lipopolysaccharides and other inflammatory molecules, and the ampicillin-mediated release of OMVs from the bacterial cell may contribute to inflammation in the host [16]. In this data set, Pal was not detected at statistically significant levels in any other samples.

In the ampicillin-treated group, proteins with the greatest decrease in relative abundance were associated with the uptake of foreign DNA and DNA processing [relaxosome protein TraM, 8.61-fold decrease due to absence of the protein (Table 1), YidB, 3.73-fold decrease; Mrr restriction system protein, 2.88-fold decrease] and in addition macrolide resistance protein MphA (2.38-fold decrease). The TraM and Mrr data for the ampicillin-treated *E. coli* suggest fewer plasmid transfer events compared with the control, which is in contrast to the finding by Liu et al. who demonstrated that sub-MIC levels of cefotaxime, ampicillin and ciprofloxacin in fact increase the level of plasmid transfer [17]. Differences in

Table 1
pEK499 plasmid-derived proteins detected by mass spectrometry. Proteins encoded by genes present on the pEK499 plasmid were detected in all groups or were exclusive to specific groups of *Escherichia coli*

Protein ID	Protein name	Sample presence	Sample absence
ACQ41977.1	Orf1176 protein (SopA)	All bacterial samples	None
ACQ42024.1	β-Lactamase (TEM)		
ACQ42045.1	AAC(6′)-Ib-cr		
ACQ42046.1	β-Lactamase (OXA)		
ACQ42051.1	β-Lactamase (CTX-M-15)		
ACQ42056.1	Dihydrofolate reductase		
ACQ42065.1	Macrolide 2 phosphotransferase [Mph(A)]		
ACQ42094.1	Putative HTH-type transcriptional regulator		
ACQ42102.1	(YfaX)		
ACQ42108.1	Hypothetical protein XCV		
	Hypothetical protein		
ACQ41973.1	Antitoxin CcdA	Cefotaxime-, imipenem- and ciprofloxacin-treated bacteria	Control, ampicillin-treated bacteria
ACQ41974.1	Toxin CcdB	Control, ampicillin-, cefotaxime- and ciprofloxacin-treated bacteria	Imipenem-treated bacteria
ACQ42006.1	Relaxosome protein TraM	Control, cefotaxime- and imipenem-treated bacteria	Ampicillin- and ciprofloxacin-treated bacteria
ACQ42109.1	Uncharacterised protein	Control and imipenem-treated bacteria	Ampicillin-, cefotaxime- and ciprofloxacin-treated bacteria
ACQ42036.1	mRNA interferase (PemK)	Control, ampicillin-, cefotaxime- and imipenem-treated bacteria	Ciprofloxacin-treated bacteria
ACQ42069.1	34-kDa membrane antigen (Tpd)		

NOTE: The data presented here are from the pre-imputated data set (Suppl 1) and identify the proteins present in each sample.

plasmids and plasmid-mediated resistance to ampicillin may account for the different findings here. However, the pEK499 plasmid does not confer resistance to ciprofloxacin but reduced susceptibility, and in this study the relative abundance of TraM was also decreased in this group indicating lower levels of DNA transfer. Although the levels of TraM are decreased during stationary phase [18], this does not explain the lower levels of TraM in the ciprofloxacin- and ampicillin-treated groups observed in this data set. Overproduction of reactive oxygen species (ROS) is known to trigger conjugative transfer [19]. In this study the levels of proteins associated with a response to ROS in groups treated with ampicillin or ciprofloxacin were relatively low, thus reduced levels of oxidative stress in these groups may be responsible for a decrease in the relative abundance of proteins associated with gene transfer. Of the β-lactam-exposed groups, the levels of proteins associated with an increase in oxidative stress were greater in groups treated with cefotaxime. In contrast, enrichment analysis performed in STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) on statistically significant differentially abundant (SSDA) proteins revealed a general decrease in the pathways associated with glycolysis and glyoxylate metabolism. This indicates that the energy used to combat oxidative stress is at the expense of carbohydrate metabolism [20]. STRING analysis also revealed an increase in protein levels associated with the ribosome. Because oxidative stress presents bacterial cells with unfavourable environmental conditions, the ability to alter RNA turnover is essential for survival via adaptation to harsh environments. Oxidative stress alters ribosomal activity in bacteria allowing cells to adapt to unfavourable environmental conditions [21]. DEAD-box helicases are a group of proteins associated with ensuring continuation of optimal ribosomal activity [22]. In addition to the range of proteins associated with translation, the proteomic data set here identified the protein product of DeaD, which increased by 3.02-fold in cefotaxime-treated cells. Taken together, comparative proteomics analysis of cefotaxime-exposed cells and untreated cells indicated that cefotaxime induces an oxidative stress response in *E. coli* that is met by an increase in ribosomal activity and a decrease in carbohydrate metabolism.

Analysis of the ampicillin-treated group using STRING revealed a decrease in the relative abundance of several proteins associated with the ribosome and translation (Supplementary Fig. S1B). In contrast, there was an increase in the relative abundance of proteins associated with these pathways in the cefotaxime-treated group (Supplementary Fig. S2A) and, interestingly, a decrease in the relative abundance of proteins involved in amino acid metabolism (Supplementary Fig. S2B). The relative abundance of proteins involved in carbohydrate metabolism was decreased in the cefotaxime- and imipenem-treated groups (Supplementary Fig. S2B and S3B). Specifically, the levels of proteins involved in the glycolytic pathway were reduced in the imipenem-treated group (Supplementary Fig. S3B).

Imipenem also induced stress in bacterial cells as demonstrated by the number of proteins associated with oxidative stress and the levels of the flagellin protein FliC were reduced greatly. This suggests that imipenem induces morphological changes in the bacterial cell that may ultimately affect the motility and adherence properties of the cells. Subinhibitory concentrations of antimicrobials are known to induce morphological changes in bacterial cells [23,24]. Understanding these changes and how they may affect bacterial interactions with the host cell are important for developing therapeutic strategies [24]. The relative abundance of several proteins involved in carbohydrate metabolism, specifically glucose metabolism, was decreased in this group, but the levels of proteins associated with monosaccharide transport into the cell increased. High-affinity transport systems are known to increase under conditions of nutrient limitation [25]. It is possible that a decrease in glucose availability induced an increase in the uptake of alternative carbon sources such as xylose and arabinose, causing an increase in the levels of transporters. One of the transporters identified in the data set arising from imipenem-treated bacteria was D-xylose-binding periplasmic protein, encoded by the *xylF* gene. This gene is upregulated in response to cold shock [26]. Cold-shock-inducible genes, while providing protection against temperature decline, also play a role in the bacterial response to antimicrobial stress [26–28]. In total, there are nine cold shock proteins (Csp) in *E. coli* (CspA–CspI). In this study, the relative abundance of one of these Csp (CspE) was increased in imipenem-, cefotaxime- and ciprofloxacin-treated groups. CspE is constitutively expressed and is responsible for the stability of RNA transcripts arising from genes associated with a general stress response, specifically the

Table 2
Variations in protein abundance in comparison with control in proteins produced from plasmid genes (post-imputation)

Protein; gene position on the plasmid	Fold change			
	Ampicillin	Cefotaxime	Imipenem	Ciprofloxacin
TraM; nt 26 494–26 877	−3.11	–	–	−2.73
MphA; nt 74 827–75 732	−1.25	–	–	
CTX-M-15; nt 62 953–63 828	–	+7.43	–	+3.54
TEM-1; nt 39 864–40 676	–	–	+3.64	+1.68
DhfrVII; nt 66 537–67 211	–	–	–	−0.5
Hypothetical protein; nt 111 726–112 226	–	–	–	−0.91

nt, nucleotides.

master regulator RpoS [29]. The relative abundance of four Csp was increased in bacteria exposed to ciprofloxacin (CspA, CspC, CspD and CspE), suggesting a major role for these proteins in response to ciprofloxacin-induced stress. There was a 5.58-fold increase in the level of CspD. CspD is generally associated with a carbon-starvation-induced stress response during stationary-phase growth [30]. This protein binds to single-stranded DNA and inhibits its replication [31]. The significant increase in its abundance in this study indicates that CspD may have a role to play in protection against ciprofloxacin, perhaps by inhibiting DNA replication thereby reducing the effect of ciprofloxacin on this process. Furthermore, there was a 1.41-fold decrease in dihydrofolate reductase, a crucial enzyme for the biosynthesis of DNA precursors. This indicates a reduction in the biosynthesis of DNA in bacterial cells exposed to ciprofloxacin. CspD is involved in the MqsR/MqsA-mediated toxin/antitoxin (TA) system that regulates the formation of persister cells by inducing biofilm formation [32,33]. Other proteins associated with TA system-dependent persister cell formation are Lon, ClpX and Fis, all of which were increased in relative abundance in ciprofloxacin-treated bacteria [32,33]. In addition to CspE, CspA and CspC are proteins involved in the stabilisation of DNA and RNA transcripts under cellular stress and in response to low temperatures [34,35]. These proteins increase the half-life of RNA transcripts arising from the expression of stress-induced genes and interfere with the formation of secondary structures in RNA that can result in transcriptional termination [34,36]. It was therefore interesting to observe an increase in the relative abundance of a substantial number of proteins associated with translation in ciprofloxacin-exposed bacteria. The levels of these proteins indicate increased translational activity in this group. In contrast, there was a decrease in the relative abundance of several proteins associated with oxidative stress, including superoxide dismutase (SodC), which was reduced by almost 65-fold. Taken together, the data in this study suggest that exposure to subinhibitory levels of ciprofloxacin induces a Csp response that may, in part, be responsible for the increased levels of ribosomal proteins and decrease in proteins associated with oxidative stress. The increases in the levels of β-lactamases suggests a secondary effect of ciprofloxacin, one that impacts the bacterial cell wall. This observation supports the theory that antimicrobials may serve as an environmental signal for bacteria which induces physiological alterations that provide cells with a competitive advantage [37].

Post-imputation analysis revealed several changes in the relative abundance of proteins originating from the plasmid (Table 2). Only the addition of cefotaxime was associated with an increase in the protein of the corresponding resistance mechanism (CTX-M-15). Ampicillin and ciprofloxacin did not results in increased protein abundances of any β-lactamases or the Aac(6′)-Ib-cr protein associated with reduced susceptibility to fluoroquinolones. However, these proteins were present in the control and thus it appears that this demonstrates that there is no additional regulation of their protein production in the presence of these antimicrobials. There were no carbapenemase genes present on the plasmid.

In the presence of ampicillin or ciprofloxacin there was an absence of the protein TraM. TraM is a mating signal that is used in combination with the integration host factor to bind *oriT* and prepare the plasmid for transfer. Some of the repression systems of TraM include the H-NS repression or the Hfq binding of mRNA transcripts of *traM* or by GroEL chaperone proteins that directly activate proteolysis. The relative abundances of these proteins were not increased in the ampicillin- or ciprofloxacin-treated *E. coli*. Thus, the lack of TraM was not a direct result of known repression proteins. The proteins with increased abundance under ampicillin or ciprofloxacin stress but absent or with reduced abundance under cefotaxime and imipenem stress comprised 13 proteins (RimO, RfbB, MetK, GalM, RplD, NagZ, RplC, GreA, Apt, SeqA, FumA, SucB and TufB).

The relative increases in the amount of CTX-M-15 in the presence of cefotaxime and ciprofloxacin suggest that production of this protein is regulated, but not only by the direct presence of the cephalosporin. However, no common protein or pathways were identifiable as potential control systems. The common proteins with increased abundances were YjiM (uncharacterised protein), CspA and CspE (cold shock proteins), DeaD (ATP-dependent RNA helicase) and LsrF [terminal protein in the quorum-sensing signal autoinducer-2 (AI-2) processing pathway]. YjiM and CspE also had increased abundances in the imipenem-treated samples. How the other proteins interact with the plasmid and specifically CTX-M-15 protein production remains to be determined and requires further investigation. The CspA and DeaD proteins are both stress response proteins, but the link to LsrF is unknown. LsrF is produced in response to the quorum-sensing AI-2 signal and is thought to promote AI-2 degradation or feedback control to the Lsr operon but has not been associated with AMR [10]. There was no commonality identified between these proteins with reduced abundances.

SeqA has been identified as a negative modulator of initiation of replication and of plasmid replication [38]. We propose that under ampicillin and ciprofloxacin stress, SeqA performs this function thus reducing the relative abundance of TraM. As the TEM-1 protein and the acid stress response were increased in the presence of imipenem or ciprofloxacin, but not ampicillin or cefotaxime, we question whether TEM-1 protein production was increased in response to these stress protein increases or the antimicrobials, or if the acid stress response is activated in response to increased TEM production. The FruB and YciF proteins present in reduced abundance unique to imipenem and ciprofloxacin have been reported to be upregulated in response to acid stress. Thus, while components of the response to acid stress were increased, only some of the proteins required for response and resistance to acid stress were associated with these experiments.

The responses of HdeB and OsmY were increased in imipenem-treated but decreased in ciprofloxacin-treated samples only. In the presence of ciprofloxacin but not the other antimicrobials the level

of GadB was reduced 7.95-fold, and in the imipenem-treated samples the GadC protein was reduced in abundance 1.84-fold. The GadBC proteins are usually increased in response to acid stress. A GadB knockout mutant demonstrated increased persister formation under ampicillin stress [39]. In addition, HdeAB, OsmY and OsmE were repressed in persister-forming cells [39]. The relative protein abundances of HdeB, GadB and OsmY were reduced in the ciprofloxacin-treated bacteria, suggesting that these bacteria were persisters. The opposite occurred in the imipenem-treated bacteria, as both HdeB and OsmY were increased. Hong et al. described both bacterial resistance and persistence in response to stress, such as acid or antimicrobials [39]. This proteomics study suggests the specific antimicrobial responses of *E. coli* to these stresses as resistance in relation to ampicillin, cefotaxime and imipenem and persistence in relation to ciprofloxacin. Persistence was demonstrated to occur due to downregulation of the acid (*gadB, gadX*), osmotic (*osmY*) and multidrug (*mdtF*) resistance systems owing to the degradation of MqsA by proteases (ClpXP and Lon) [39]. In the presence of ciprofloxacin, in our study the Lon and ClpX proteins were increased in abundance and the GadB, HdeB and OsmY proteins were decreased in abundance. This pattern is described in the persister formation- rather than the resistance formation-induced pathways. In addition, CspD is involved in the MqsR/MqsA-mediated TA system that regulates the formation of persister cells by inducing biofilm formation [32,33]. While we detected increased CspD only in ciprofloxacin-treated bacteria, we did not detect MqsR or MsqA proteins in any sample.

4. Conclusions

This study provides novel insights into the changes that occur in the proteome of multidrug-resistant *E. coli* under antimicrobial pressure and highlights a significant role for chromosomally-encoded genes. The data arising from experiments with three different β-lactam antibiotics identified distinct differences in the cellular response to each drug. These data also identified novel proteins (CspA, CspE, DeaD, LsrF and YjiM) not previously associated with AMR or antimicrobial responses in pathogens.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2021.12.006.

References

[1] World Health Organization (WHO) Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics, Geneva, Switzerland: WHO; 2017. https://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf, [accessed 14 July 2021].

[2] Woodford N, Carattoli A, Karisik E, Underwood A, Ellington MJ, Livermore DM. Complete nucleotide sequences of plasmids pEK204, pEK499, and pEK516, encoding CTX-M enzymes in three major *Escherichia coli* lineages from the United Kingdom, all belonging to the international O25:H4-ST131 clone. Antimicrob Agents Chemother 2009;53:4472–82.

[3] Matsumura Y, Pitout JD, Gomi R, Matsuda T, Noguchi T, Yamamoto M, et al. Global *Escherichia coli* sequence type 131 clade with $bla_{CTX-M-27}$ gene. Emerg Infect Dis 2016;22:1900–7.

[4] Blumenscheit C, Pfeifer Y, Werner G, John C, Schneider A, Lasch P, et al. Unbiased antimicrobial resistance detection from clinical bacterial isolates using proteomics. Anal Chem 2021;93:14599–608.

[5] Møller TSB, Liu G, Boysen A, Thomsen LE, Lüthje FL, Mortensen S, et al. Treatment with cefotaxime affects expression of conjugation associated proteins and conjugation transfer frequency of an IncI1 plasmid in *Escherichia coli*. Front Microbiol 2017;8:2365.

[6] Møller TSB, Liu G, Hartman HB, Rau MH, Mortensen S, Thamsborg K, et al. Global responses to oxytetracycline treatment in tetracycline-resistant. *Escherichia coli*. Sci Rep 2020;10:8438.

[7] Margalit A, Carolan JC, Sheehan D, Kavanagh K. The *Aspergillus fumigatus* secretome alters the proteome of *Pseudomonas aeruginosa* to stimulate bacterial growth: implications for co-infection. Mol Cell Proteomics 2020;19:1346–59.

[8] Hubner NC, Bird AW, Cox J, Splettstoesser B, Bandilla P, Poser I, et al. Quantitative proteomics combined with BAC TransgeneOmics reveals in vivo protein interactions. J Cell Biol 2010;189:739–54.

[9] Côté RG, Griss J, Dianes JA, Wang Wright JC, van den Toorn HWP, et al. The PRoteomics IDEntification (PRIDE) Converter 2 framework: an improved suite of tools to facilitate data submission to the PRIDE database and the ProteomeXchange Consortium. Mol Cell Proteomics 2012;11:1682–9.

[10] Kapoor G, Saigal S, Elongavan A. Action and resistance mechanisms of antibiotics: a guide for clinicians. J Anaesthesiol Clin Pharmacol 2017;33:300–5.

[11] Sidjabat HE, Gien J, Kvaskoff D, Ashman Vaswani K, Reed S, et al. The use of SWATH to analyse the dynamic changes of bacterial proteome of carbapenemase-producing *Escherichia coli* under antibiotic pressure. Sci Rep 2018;8:3871.

[12] Lesniak J, Barton WA, Nikolov DB. Structural and functional features of the *Escherichia coli* hydroperoxide resistance protein OsmC. Protein Sci 2003;12:2838–43.

[13] Molina-Heredia FP, Houée-Levin C, Berthomieu C, Touati D, Tremey E, Favaudon V, et al. Detoxification of superoxide without production of H_2O_2: antioxidant activity of superoxide reductase complexed with ferrocyanide. Proc Natl Acad Sci U S A 2006;103:14750–5.

[14] Prieto-Alamo MJ, Jurado J, Gallardo-Madueno R, Monje-Casas F, Holmgren A, Pueyo C. Transcriptional regulation of glutaredoxin and thioredoxin pathways and related enzymes in response to oxidative stress. J Biol Chem 2000;275:13398–405.

[15] Smirnova G, Muzyka N, Lepekhina E, Oktyabrsky O. Roles of the glutathione- and thioredoxin-dependent systems in the *Escherichia coli* responses to ciprofloxacin and ampicillin. Arch Microbiol 2016;198:913–21.

[16] Michel LV, Gallardo L, Konovalova A, Bauer Jackson N, Zavorin M, et al. Ampicillin triggers the release of Pal in toxic vesicles from *Escherichia coli*. Int J Antimicrob Agents 2020;56:106163.

[17] Liu G, Bogaj K, Bortolaia V, Olsen JE, Thomsen LE. Antibiotic-induced, increased conjugative transfer is common to diverse naturally occurring ESBL plasmids in *Escherichia coli*. Front Microbiol 2019;10:2119.

[18] Zatyka M, Thomas CM. Control of genes for conjugative transfer of plasmids and other mobile elements. FEMS Microbiol Rev 1998;21:291–319.

[19] Zhang S, Wang Y, Song H, Lu Yuan Z, Guo J. Copper nanoparticles and copper ions promote horizontal transfer of plasmid-mediated multi-antibiotic resistance genes across bacterial genera. Environ Int 2019;129:478–87.

[20] Mullarky E, Cantley LC. Diverting glycolysis to combat oxidative stress. In: Nakao K, Minato N, Uemoto S, editors. Innovative medicine: basic research and development. Tokyo, Japan: Springer; 2015. p. 3–23.

[21] Leiva LE, Pincheira A, Elgamal S, Kienast SD, Bravo V, Leufken J, et al. Modulation of *Escherichia coli* translation by the specific inactivation of $tRNA^{Gly}$ under oxidative stress. Front Genet 2020;11:856.

[22] Redder P, Hausmann S, Khemici V, Yasrebi H, Linder P. Bacterial versatility requires DEAD-box RNA helicases. FEMS Microbiol Rev 2015;39:392–412.

[23] Lorian V. Medical relevance of low concentrations of antibiotics. J Antimicrob Chemother 1993;31(Suppl D):137–48.

[24] Fonseca AP, Sousa JC. Effect of antibiotic-induced morphological changes on surface properties, motility and adhesion of nosocomial *Pseudomonas aeruginosa* strains under different physiological states. J Appl Microbiol 2007;103:1828–37.

[25] Ferenci T. Adaptation to life at micromolar nutrient levels: the regulation of *Escherichia coli* glucose transport by endoinduction and cAMP. FEMS Microbiol Rev 1996;18:301–17.

[26] Phadtare S, Inouye M. Genome-wide transcriptional analysis of the cold shock response in wild-type and cold-sensitive, quadruple-*csp*-deletion strains of *Escherichia coli*. J Bacteriol 2004;186:7007–14.

[27] Xia B, Ke H, Inouye M. Acquirement of cold sensitivity by quadruple deletion of the *cspA* family and its suppression by PNPase S1 domain in *Escherichia coli*. Mol Microbiol 2001;40:179–88.

[28] Cruz-Loya M, Kang TM, Lozano NA, Watanabe R, Tekin E, Damoiseaux R, et al. Stressor interaction networks suggest antibiotic resistance co-opted from stress responses to temperature. ISME J 2019;13:12–23.

[29] Shenhar Y, Biran D, Ron EZ. Resistance to environmental stress requires the RNA chaperones CspC and CspE. Environ Microbiol Rep 2012;4:532–9.

[30] Yamanaka K, Inouye M. Growth-phase-dependent expression of *cspD*, encoding a member of the CspA family in *Escherichia coli*. J Bacteriol 1997;179:5126–30.
[31] Yamanaka K, Zheng W, Crooke E, Wang YH, Inouye M. CspD, a novel DNA replication inhibitor induced during the stationary phase in *Escherichia coli*. Mol Microbiol 2001;39:1572–84.
[32] Kim Y, Wood TK. Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in *Escherichia coli*. Biochem Biophys Res Commun 2010;391:209–13.
[33] Kim Y, Wang X, Zhang XS, Grigoriu Page R, Peti W, et al. *Escherichia coli* toxin/antitoxin pair MqsR/MqsA regulate toxin CspD. Environ Microbiol 2010;12:1105–21.
[34] Bae W, Xia B, Inouye M, Severinov K. *Escherichia coli* CspA-family RNA chaperones are transcription antiterminators. Proc Natl Acad Sci U S A 2000;97:7784–9.
[35] Phadtare S, Tadigotla V, Shin WH, Sengupta A, Severinov K. Analysis of *Escherichia coli* global gene expression profiles in response to overexpression and deletion of CspC and CspE. J Bacteriol 2006;188:2521–7.
[36] Phadtare S, Inouye M. Role of CspC and CspE in regulation of expression of RpoS and UspA, the stress response proteins in *Escherichia coli*. J Bacteriol 2001;183:1205–14.
[37] Linares JF, Gustafsson I, Baquero F, Martinez JL. Antibiotics as intermicrobial signaling agents instead of weapons. Proc Natl Acad Sci U S A 2006;103:19484–9.
[38] Douraid D, Ahmed L. SeqA, the *Escherichia coli* origin sequestration protein, can regulate the replication of the pBR322 plasmid. Plasmid 2011;65:15–19.
[39] Hong SH, Wang X, O'Connor HF, Benedik MJ, Wood TK. Bacterial persistence increases as environmental fitness decreases. Microb Biotechnol 2012;5:509–22.