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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Resistance mechanism of *Escherichia coli* strains with different ampicillin resistance levels

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Abstract

Antibiotic resistance is an important problem that threatens medical treatment. Differences in the resistance levels of microorganisms cause great difficulties in understanding the mechanisms of antibiotic resistance. Therefore, the molecular reasons underlying the differences in the level of antibiotic resistance need to be clarified. For this purpose, genomic and transcriptomic analyses were performed on three *Escherichia coli* strains with varying degrees of adaptive resistance to ampicillin. Whole-genome sequencing of strains with different levels of resistance detected five mutations in strains with 10-fold resistance and two additional mutations in strains with 95-fold resistance. Overall, three of the seven mutations occurred as a single base change, while the other four occurred as insertions or deletions. While it was thought that 10-fold resistance was achieved by the effect of mutations in the *ftsI*, *marAR*, and *rpoC* genes, it was found that 95-fold resistance was achieved by the synergistic effect of five mutations and the *ampC* mutation. In addition, when the general transcriptomic profiles were examined, it was found that similar transcriptomic responses were elicited in strains with different levels of resistance. This study will improve our view of resistance mechanisms in bacteria with different levels of resistance and provide the basis for our understanding of the molecular mechanism of antibiotic resistance in ampicillin-resistant *E. coli* strains.

Key points

- *The mutation of the ampC promoter may act synergistically with other mutations and lead to higher resistance.*
- *Similar transcriptomic responses to ampicillin are induced in strains with different levels of resistance.*
- *Low antibiotic concentrations are the steps that allow rapid achievement of high antibiotic resistance.*

Keywords Antibiotic resistance · Resistance evolution · Adaptive laboratory experiments · Ampicillin · Genomic and transcriptomic analyses

Introduction

Antibiotics are very important drugs used to treat infections caused by bacteria. Since their discovery, antibiotics have saved countless lives and extended human life by an average of 20 years (Hutchings et al. 2019). However, these weapons used against bacteria are losing their effectiveness today. In the course of antibiotic resistance, bacteria develop defense mechanisms against antibiotics that reduce or eliminate the effect of antibiotics. Antibiotic-resistant pathogens are one of the most common causes of death worldwide. Antibiotic resistance complicates the treatment of infectious diseases, prolongs treatment time, increases economic costs, and increases mortality rates. Therefore, human, animal, and environmental health are at great risk from resistant pathogens.

The use of antibiotics today has reached alarming proportions. According to one study, it is estimated that in 2018, there was an average of 14.3 doses of antibiotics per day for every 1000 people. This corresponds to 40.2 billion doses of antibiotics per day worldwide (Browne et al. 2021). The most commonly used antibiotics in the treatment of infectious diseases belong to the beta-lactam class. More than 60% of the antibiotics used are beta-lactam antibiotics (Eiamphungporn et al. 2018). There is a significant relationship between antibiotic resistance and antibiotic use. In a

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study conducted across Europe by the World Health Organization in 2020, 54.6% of clinical *Escherichia coli* isolates were found to be resistant to aminopenicillin, 14.9% to third-generation cephalosporins, and 0.2% to carpabenem (WHO 2022). Ampicillin, a semi-synthetic antibiotic, belongs to the aminopenicillin group in which *E. coli* resistance is most frequently observed (Monaghan et al. 2021). This antibiotic is used to treat or prevent many different types of infections, such as urinary tract infections (Heintz et al. 2010), gastrointestinal infections (Kim et al. 2019), pneumonia (Kato et al. 2022), neonatal streptococcal infections (Bratzler et al. 2013), chorioamnionitis (Conde-Agudelo et al. 2020), and carbapenem-resistant *Acinetobacter baumannii* infections (Tamma et al. 2022). High resistance to ampicillin, which has a wide range of uses, hinders the sustainability of treatment with a large number of beta-lactams.

It is very important to understand the molecular mechanisms involved in the emergence of antibiotic resistance and to identify the causes of different levels of resistance in order to prolong the life of antibiotics. In this study, it was aimed to analyze the genomic and transcriptomic differences of three strains (non-resistant, 10-fold resistant, and 95-fold resistant) that acquired adaptive resistance to ampicillin in vitro.

Materials and methods

Bacterial strains, media, and growth conditions

E. coli W3110 strain was selected for this study because it is sensitive to ampicillin and its whole-genome sequence was determined (Hayashi et al. 2006). Strains with 10-fold and 95-fold resistance obtained during the study were designated *E. coli* W3110-10 and *E. coli* W3110-95, respectively. Bacteria were stored at –80 °C in LB broth containing 40% glycerol before being used in the study. Bacteria removed from the freezer for use were transferred to LB agar medium and incubated at 37 °C for 18 h and then stored at 4 °C to be used as needed. A single selected colony was transferred to 5 ml LB broth and grown at 37 °C with shaking at 160 rpm overnight.

Ampicillin adaptation of *E. coli* W3110

E. coli W3110 was made resistant in our laboratory by adaptation experiments that lasted 50 passages. In these experiments, the organism was transferred to LB plates prepared with the minimum inhibitory concentration (MIC) of the antibiotic and then incubated at 37 °C for one night. One of the growing bacterial colonies was selected and transferred to the plates with a 10% higher antibiotic concentration than in the previous passage. The cycle was continued in this manner 50 times, resulting in more antibiotic-resistant strains after each passage. To test the persistence of resistance after the adaptation experiment, selected resistant strains were passaged 10 times on antibiotic-free LB plates. All resistant bacteria were stored at –80 °C in LB broth containing 40% glycerol.

Sensitivity determination

Antibiotic resistance of organisms was determined using the microbroth dilution method (CLSI 2018; Kaygusuz et al. 2020). The organism whose minimum inhibitory concentration (MIC) is to be determined was inoculated into 5 ml LB broth and incubated overnight at 37 °C and 160 rpm. After incubation, bacterial density was adjusted to 0.1 absorbance at OD_{600}, and 400 µl was taken from here and transferred to 60 ml sterile LB broth. In total, 170 or 180 µl of this culture was added to the first wells of the 96-well microplate and 100 µl to the next wells. Then, 30 or 20 µl of antibiotic (10 mg/ml or 50 mg/ml, respectively, depending on the resistance level) was added to the first wells containing 170 or 180 µl of bacterial culture, respectively, and the total volume was made up to 200 µl. Then, ½ serial dilution was made from the 1st to the 12th well and incubated at 37 °C for one night. The next day, the MIC values were determined. Then, to determine the minimum cidal concentration (MCC) of the compounds, 10 µl was taken from the wells, starting from the first well with no growth to the well with growth, and dropped onto plates containing LB agar. Plates were incubated at 37 °C for one night, and MCC values were determined the next day. Experiments were performed with at least three independent replicates. As a negative control, a ½ serial dilution was performed by adding antibiotic-containing LB broth to one of the wells and only the LB broth to the other.

Growth experiments

The growth of bacterial cultures was studied by growth assays. Each strain was inoculated in 5 ml of LB broth and incubated at 37 °C for 18 h with shaking (160 rpm). Bacterial density was then adjusted to 0.1 absorbance at OD_{600} in 15 ml of LB broth to ensure similar initial cell counts. The prepared samples were incubated at 37 °C and 160 rpm in a shaking incubator. Bacteria were sampled every 2 h for 8 h, OD_{600} absorbance was measured, and a growth curve was drawn. Experiments were performed with three independent replicates.

Whole-genome sequencing

E. coli W3110, *E. coli* W3110-10, and *E. coli* W3110-95 were grown in 5 ml LB broth at 160 rpm for 18 h. Then, the

cells obtained from these precultures were transferred to 10 ml of fresh LB broth by performing a 1/100 dilution and incubating at 160 rpm for 18 h. Cells were then harvested and genomic DNA isolation was performed according to the manufacturer's protocol using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). Sequencing library was prepared using Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA), and sequencing was done by Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) as paired-end (PE) 2×150 base reads.

Total RNA isolation and RNA-sequencing

E. coli W3110, *E. coli* W3110-10, and *E. coli* W3110-95 were grown in 5 ml LB broth with shaking at 160 rpm for 18 h. Growing cells were transferred to 15 ml of LB broth medium with an OD_{600} absorbance value of approximately 0.005 and grew until the OD_{600} absorbance value was 0.1. Then ampicillin was added to this culture at a concentration of ¾ of the MIC value, and it was grown until the OD_{600} absorbance value was 0.5–0.6. The culture was shaken at 160 rpm at each stage at which the cells were grown. Cells with an OD_{600} absorbance value of 0.5–0.6 were collected by centrifugation and washed with PBS, and RNA isolation was performed using TRI Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions.

To ensure the quality of the sample being sequenced, RNA purity was assessed using the NanoPhotometer spectrophotometer (Implen, Munich, Germany) and RNA integrity was assessed using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and the RNA Nano 6000 test kit (Agilent Technologies, Santa Clara, CA, USA). Sequencing libraries were generated using NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) following manufacturer's recommendations, and index codes were added to attribute sequences to each sample. For strand-specific libraries, NEBNext Ultra Directional RNA Library Preparation Kit for Illumina (NEB, Ipswich, MA, USA) is used. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform (Illumina, San Diego, CA, USA) and 150-bp paired-end reads were generated.

Bioinformatics analysis

An analysis line was established for the evaluation of the data obtained by genome sequencing. The raw sequence data (FASTQ) obtained in the first step of the analysis line were filtered according to the Q30 score, one of the standard error rates used to remove erroneous reads. Filtered data were mapped to the *E. coli* W3110 genome from Ensembl (GCA_000010245) using the Burrows-Wheeler Aligner (BWA) algorithm (Li and Durbin 2009). After mapping, variations were determined using Samtools and annotation was performed using SnpEff software (Li et al. 2009; Cingolani et al. 2012).

Omicsbox software was used for the analysis of RNA sequence data (OmicsBox 2019). After the RNA data were filtered, they were mapped to the above reference genome via Burrows-Wheeler Aligner (BWA). HTSeq was used to extract transcript expression from mapped data (Anders et al. 2015). EdgeR, a Bioconductor package, was used to identify differentially expressed genes (DEG) from the raw transcript data obtained (Robinson et al. 2010). Genes that changed significantly between control and experimental samples were calculated considering log2 (over twofold expression change) ≥ 1 and false discovery rate (FDR) < 0.001. Blast2GO methodology was used for functional annotation of differentially expressed genes (Conesa et al. 2005). Genes with altered expression were searched in online databases (NCBI, EMBL-EBI) with the help of various alignment algorithms (Blast, InterProScan). As a result of the search, it was determined which Gene Ontology (GO) class DEGs belong to and the groups that showed the most variation in three different GO classes (Biological Processes, Molecular Functions, and Cellular Components) were analyzed (Ashburner et al. 2000).

Validation of RNA-sequencing data by qRT-PCR

qRT-PCR was performed for 10 selected genes to confirm the DEGs detected by RNA-seq. Sequence information of selected genes was obtained from PEC, an *E. coli* genome database (Yamazaki et al. 2008). Primer candidates with a length of 18–22 nucleotides and a GC ratio of 45–55% were determined using a written Python script. Subsequently, the hairpin, self, and heterodimer possibilities of these primer candidates were checked using the oligoanalyzer tool (Owczarzy et al. 2008). After primer design, all primer pairs were simulated and verified using in silico PCR software (Bikandi et al. 2004). Primer sequences of 10 selected genes and one housekeeping gene are shown in Supplemental Table S1.

cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions. RealQ Plus 2x Master Mix (Ampliqon, Odense, Denmark) and AriaMx Real-time PCR System (Agilent, Santa Clara, CA, USA) were used for the qRT-PCR reaction. In total, 1 µl of cDNA (50 ng/µl), 0.5 µl of each primer (10 µM), 12.5 µl of RealQ Plus 2X Master Mix, and 10.5 µl of nuclease-free water were added to the reaction. Genes were amplified with an initial denaturation at 95 °C for 5

min, followed by 40 cycles at 95 °C for 30 s and 60 °C for 60 s. The $2^{-\Delta\Delta Ct}$ method was used to determine the changes in gene expression based on the results obtained (Pfaffl 2001). To minimize possible errors, three independent replicates were performed and the average of three values was taken.

Plasmid construction and bacterial transformation

The *ampC* gene containing the mutant promoter sequence, coding sequence, and terminator sequence was synthesized with reference to *E. coli* W3110-95. The determined sequence was synthesized and transferred to plasmid pEX-K248 by Eurofins Genomics (Ebersberg, Germany), and plasmid pEX-K248-*ampC* was generated (Supplemental Fig. S1). The accuracy of the synthesized gene region was verified using the Sanger sequencing method. After validation of the synthesized gene, pEX-K248-*ampC* was transformed into *E. coli* W3110 and *E. coli* W3110-10.

Results

Adaptive laboratory evolution of ampicillin resistance in *E. coli* cells

During the process of adaptive laboratory evolution, numerous *E. coli* strains with different levels of resistance were obtained. Among these strains, three strains representing different levels of resistance were selected. The first of these strains is the ancestral strain *E. coli* W3110 used at the beginning of the adaptation process, the second is *E. coli* W3110-10 obtained in the middle of the adaptation process, and the third is *E. coli* W3110-95 obtained at the end of the adaptation process. When the MIC and MCC results of these strains were examined, it was found that the cidal effect of the antibiotic continued after long-term adaptation experiments. While the ampicillin MIC of *E. coli* W3110, which had never been treated with antibiotics, was determined to be 2.93 µg/ml, the MIC of *E. coli* W3110-10 increased approximately 10-fold to 25 µg/ml. In addition, *E. coli* W3110-95 with an MIC of 275 µg/ml became about 95-fold resistant compared to the ancestral strain. According to the 2020 CLSI report, *Enterobacteria* with MICs less than 8 µg/ml are sensitive, while those with MICs greater than 32 µg/ml are resistant. The range between these two values is considered the intermediate range (CLSI 2020). When tested according to these standards, *E. coli* W3110 was placed in the sensitive category, *E. coli* W3110-95 was placed in the resistant category, and *E. coli* W3110-10 was placed in the intermediate category. After 10 passages of strains *E. coli* W3110-10 and *E. coli* W3110-95 in an antibiotic-free medium, no change in the resistance level was observed. When the growth status of the selected bacteria was examined, no significant difference was observed compared to ancestral strain *E. coli* W3110 (Supplemental Fig. S2).

Genome re-sequencing

Whole-genome sequencing of *E. coli* W3110, *E. coli* W3110-10, and *E. coli* W3110-95 was performed to determine the genetic basis of the different levels of antibiotic resistance. Mutations occurring during ampicillin adaptation were determined by comparing the genome sequences of *E. coli* W3110-10 and *E. coli* W3110-95 with the genome sequence of *E. coli* W3110. Whole-genome sequencing revealed that *E. coli* W3110-10 contained five mutations, whereas two additional mutations were detected in *E. coli* W3110-95. While three of these seven mutations were single nucleotide polymorphisms (SNP), four of them occurred as insertion or deletions (InDel). All mutations detected are listed in Table 1.

Mutations common to *E. coli* W3110-10 and *E. coli* W3110-95 occurred at positions 93022, 1621204, 2829040, 3450580, and 4371270 in the genome. The SNP occurring at position 93022 is located in the promoter region of the *murE* gene, while it is located in the coding region of the *ftsI* gene.

Table 1 Mutations detected in *E. coli* W3110-10 and *E. coli* W3110-95

Position	*E. coli* W3110	*E. coli* W3110-10	*E. coli* W3110-95	Gene	Gene position	Variation type	Effect
93022	-	X	X	*ftsI*	1610/1767	SNP (C>T)	Missense
				murE	−144		Upstream
1621204	-	X	X	*marR*	377/435	Del (A)	Frameshift
				marA	−83		Upstream
2829040	-	X	X	*gutQ*	575/966	Del (G)	Frameshift
3450580	-	X	X	*rpoC*	752/4224	SNP (C>T)	Missense
4371270	-	X	X	*dcuA*	−7_−6	Del (TT)	Upstream
4383697	-	-	X	*frdD*	349/360	Ins (G)	Frameshift
				ampC	−74		Upstream
4401616	-	-	X	*amiB*	872/1338	SNP (C>G)	Missense

This variation causes amino acid miscoding within the *ftsI* gene and a change from one base in the promoter region of the *murE* gene. The deletion at position 1621204 is located in the promoter of the *marA* gene and in the coding region of the *marR* gene. In the resulting deletion, a frameshift occurred in the *marR* gene, while a base was deleted in the promoter region of the *marA* gene. The deletion at position 2829040 corresponds to base 575 of the *gutQ* gene. The guanine base at this position was deleted and caused the formation of a stop codon, resulting in defective protein production. The SNP at position 3450580 corresponds to position 752 of the *rpoC* gene. In this mutation, an incorrect amino acid is encoded. The mutation at position 4371270 occurred by deletion of bases -6 and -7 in the upstream region of the *dcuA* gene. This mutation in the non-coding region does not coincide with any other gene.

E. coli W3110-95 has two additional mutations at positions 4383697 and 4401616. The insertion at position 4383697 corresponds to the coding region of the *frdD* gene, while it is located within the promoter region of the *ampC* gene. The insertion we determined at position −74 relative to the translation start point corresponds to position −15 according to the transcription start point. This position is located in the spacer region between boxes −35 and −10 within the *ampC* promoter. With the resulting insertion, the length of the spacer region was 17 bp. The mutation at position 4401616 is the cytosine-guanine change at position 827 of the *amiB* gene. This change resulted in the miscoding of an amino acid.

Transcriptome analysis of AMP-resistant strains

The transcriptomic response of *E. coli* W3110, *E. coli* W3110-10, and *E. coli* W3110-95 with different ampicillin resistance levels to ampicillin was determined by RNA-seq method and comparatively evaluated. As expected, significant differences in the number of DEGs were found. Although there was no significant difference in the number of up- and downregulated genes, it was observed that the number of DEGs increased with the level of resistance (Fig. 1A). Gene expression in *E. coli* W3110-10 and *E. coli* W3110-95 was determined by comparison with *E. coli* W3110. The expression of more than 900 genes changed more than twofold, indicating markedly different responses in ampicillin-resistant strains. *E. coli* W3110-10 and *E. coli* W3110-95 treated with sub-MIC antibiotics showed at least a 10-fold change in expression in 79 and 109 DEGs, respectively, at least a 50-fold change in expression in seven and 14 DEGs, respectively, and at least a 100-fold change in expression in two and five DEGs, respectively (Fig. 1B). When *E. coli* W3110 and *E. coli* W3110-10 were compared, the expression of 938 genes was found to have changed significantly. The expression of 470 of these genes is upregulated, while 468 of them are downregulated. As a result of the comparison of *E. coli* W3110 and *E. coli* W3110-95, it was found that the number of DEGs in the sample numbered *E. coli* W3110-95 increased to 1107. The expressions of 597 of them were upregulated, while the expressions of 510 of them were downregulated. The results show that the number of DEGs increases with the increase of the resistance level.

Venn plots were generated to identify unique and common DEGs in resistant strains (Fig. 1C). Of the DEGs whose gene expression changes at least twofold, 606 are common to *E. coli* W3110-10 and *E. coli* W3110-95, 332 are specific to *E. coli* W3110-10, and 501 are specific to *E. coli* W3110-95. The higher the threshold for the change in gene expression, the lower the proportion of common genes to differentially expressed genes. These results show that in addition to a common response to ampicillin, there are significant changes in the expression of various genes depending on the resistance level.

Gene Ontology (GO) is an internationally standardized system for classifying gene function that consists of three ontologies: molecular function, cellular component, and biological process (Harris et al. 2004). To group the detected DEGs, all these genes were annotated with terms from the GO database and assigned to at least one of these three main categories GO. The 15 most frequently enriched categories are shown in Fig. 2. DEGs detected in *E. coli* W3110-10 were annotated 1336 times in biological process, 715 times in cellular component, and 1089 times in molecular function. The most enriched groups in the biological process were carbohydrate metabolic process, transmembrane transport, and regulation of DNA-templated transcription, while plasma membrane and cytosol fell under the category of cellular components and oxidoreductase activity, hydrolase activity, transferase activity, DNA binding, and transporter activity fell under the category of molecular functions. For *E. coli* W3110-95, the annotation rate increased at a similar rate as DEGs. DEGs detected for this strain were annotated 1570 times in biological process, 788 times in cellular component, and 1256 times in molecular function. The most enriched groups in the biological process were carbohydrate metabolic process, regulation of DNA-templated transcription, and transmembrane transport while plasma membrane and cytosol fell under the category of cellular components and hydrolase activity, transferase activity, DNA binding, oxidoreductase activity, and transporter activity fell under the category of molecular function. It was found that similar GO groups were enriched at both resistance levels. Thus, there is no difference depending on the resistance level.

Relationship between gene expression changes and genome mutations

We compared whole-genome sequencing and transcriptome results to gain a better understanding of genomic and

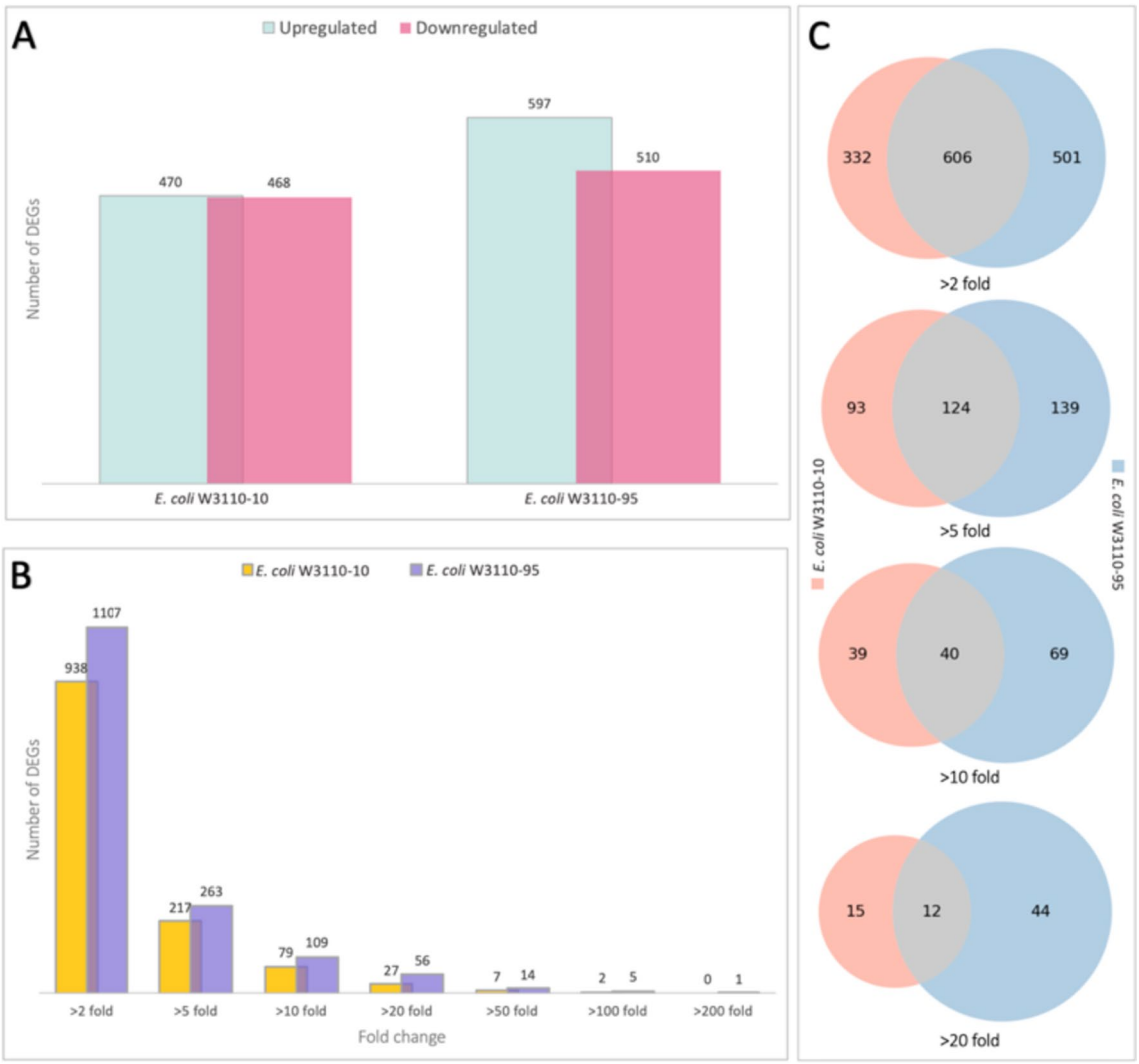

Fig. 1 Global expression profiles of *E. coli* W3110-10 and *E. coli* W3110-95. **A** Number of downregulated and upregulated genes compared to *E. coli* W3110 in the same condition. **B** Distribution of detected DEGs according to the change in gene expression. **C** Venn diagrams comparing DEG results from two conditions

transcriptomic alterations in ampicillin-resistant strains. None of the genes with mutations in the coding region is a regulatory gene. Therefore, the mutations contained in these genes affect only their own functions and do not cause a change in the expression of any gene other than itself. For this reason, in analyzing the relationship between mutation and change in gene expression, we examined only genes with mutations in the upstream region and those associated with regulatory genes. There are four genes with mutations in the upstream region. Of these four mutations, those in the *murE*, *dcuA*, and *marA* genes are found in *E. coli* W3110-10 and *E. coli* W3110-95, whereas the mutation in the *ampC* gene is present only in *E. coli* W3110-95. The expression changes of these four genes as a result of RNA-seq analysis are shown in Fig. 3. Expression of the *murE* gene did not differ in *E. coli* W3110-10 and *E. coli* W3110-95. This result shows that the mutation in the upstream region does not change the expression level of this gene. The *dcuA* gene contains a deletion in its upstream region. Expression of this gene decreased 2.29-fold in *E. coli* W3110-10 but remained unchanged in *E. coli* W3110-95. Despite the presence of this mutation in both strains, the differences in gene expression suggest that there is no relationship between the mutation and gene expression.

Expression of the *marA* gene increased 20.59-fold in *E. coli* W3110-10 and 16.90-fold in *E. coli* W3110-95. The increase in expression in both strains compared with *E. coli* W3110, which does not contain the mutation, indicates that the upstream mutation affects *marA* expression. The *marA* gene is a transcriptional activator that regulates the expression of many genes related to antibiotic resistance and stress response (Martin et al. 1996). Expression of the genes

Fig. 2 GO enrichment analysis of DEGs detected in *E. coli* W3110-10 and *E. coli* W3110-95. BP (biological process), CC (cellular component), MF (molecular function), 10 (*E. coli* W3110-10), 95 (*E. coli* W3110-95)

ymgA, *poxB*, *acnA*, *marR*, *marB*, *ybjC*, *inaA*, *nfsA*, and *ycgZ*, which are regulated by *marA*, was increased in both *E. coli* W3110-10 and *E. coli* W3110-95 (Supplemental Table S2, Supplemental Table S3). Therefore, the *marA* mutation has an important role in the 10-fold increase in resistance.

According to the RNA-seq data, a 52.98-fold increase in *ampC* gene expression was observed in *E. coli* W3110-95 containing the mutation, whereas no change was detected in *E. coli* W3110-10 without the mutation. Similarly, according to the qRT-PCR results, the expression of *ampC* gene increased 89.88-fold in *E. coli* W3110-95, while *E. coli* W3110-10 did not change (Supplemental Fig. S3). These results show that gene expression increases significantly with insertion in the promoter region of the *ampC* gene. It was found that *E. coli* W3110-95 with the mutation of the *ampC* promoter was about 95 times more resistant than the ancestral strain and about 10 times more resistant than *E. coli* W3110-10. This shows that mutation of the *ampC* promoter has a significant effect on increasing antibiotic resistance from 10-fold to 95-fold.

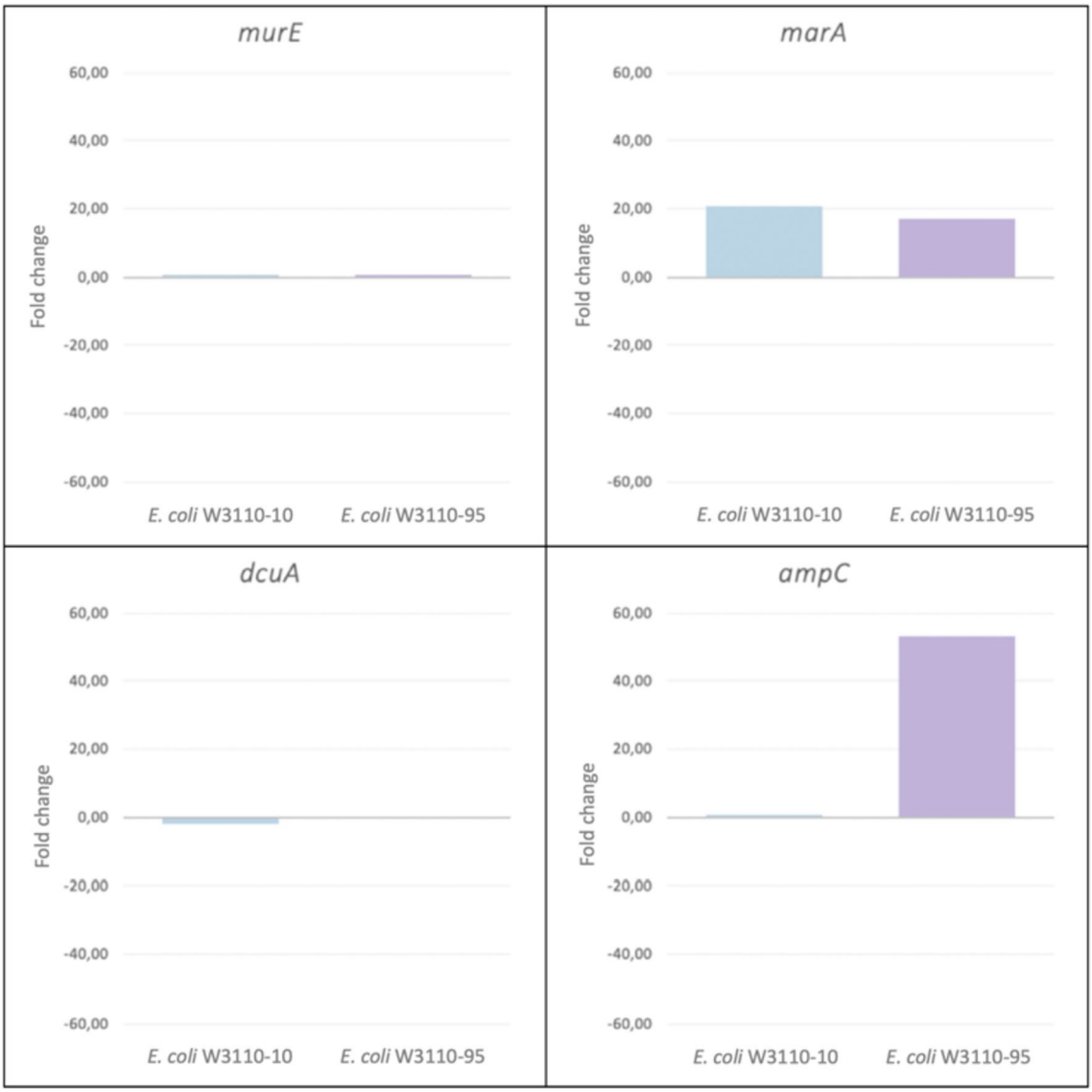

Fig. 3 Gene expression levels of genes with promoter mutation

Confirmation of the effect of *ampC* promoter mutation

To determine the individual and cumulative effect of the mutation of the *ampC* promoter, the *ampC* gene with this mutation was synthesized and transferred to plasmid pEX-K248. The obtained plasmid was transformed into *E. coli* W3110 and *E. coli* W3110-10 to generate *E. coli* W3110-*ampC* and *E. coli* W3110-10-*ampC*, respectively. Significant increases in both resistance and *ampC* expression were observed in these strains (Fig. 4). As a result of transformation of the plasmid containing the *ampC* gene, the MIC of *E. coli* W3110 increased from 2.94 to 375 µg/µl, whereas the MIC of *E. coli* W3110-10 increased from 25 to 750 µg/µl. (Fig. 4A). The resistance level of *E. coli* W3110-*ampC* and *E. coli* W3110-10-*ampC* was higher than that of *E. coli* W3110-95, which contains the mutation in its own chromosome (Fig. 4B). When the expression levels were examined, it was found that *ampC* expression of the *E. coli* W3110-*ampC* increased 221-fold and *ampC* expression of the *E. coli* W3110-10-*ampC* increased 205-fold. These results indicate that *ampC* expression was higher in both strains to which the plasmid was transferred than in *E. coli* W3110-95.

In *E. coli* W3110-*ampC*, the effect of the mutation on resistance and gene expression was clearly demonstrated because only the plasmid containing the mutation was present. In *E. coli* W3110-10-*ampC*, the combined effect of the mutation of the *ampC* promoter with five mutations was observed. While the previous five mutations resulted in a 10-fold resistance to the bacteria, the addition of the

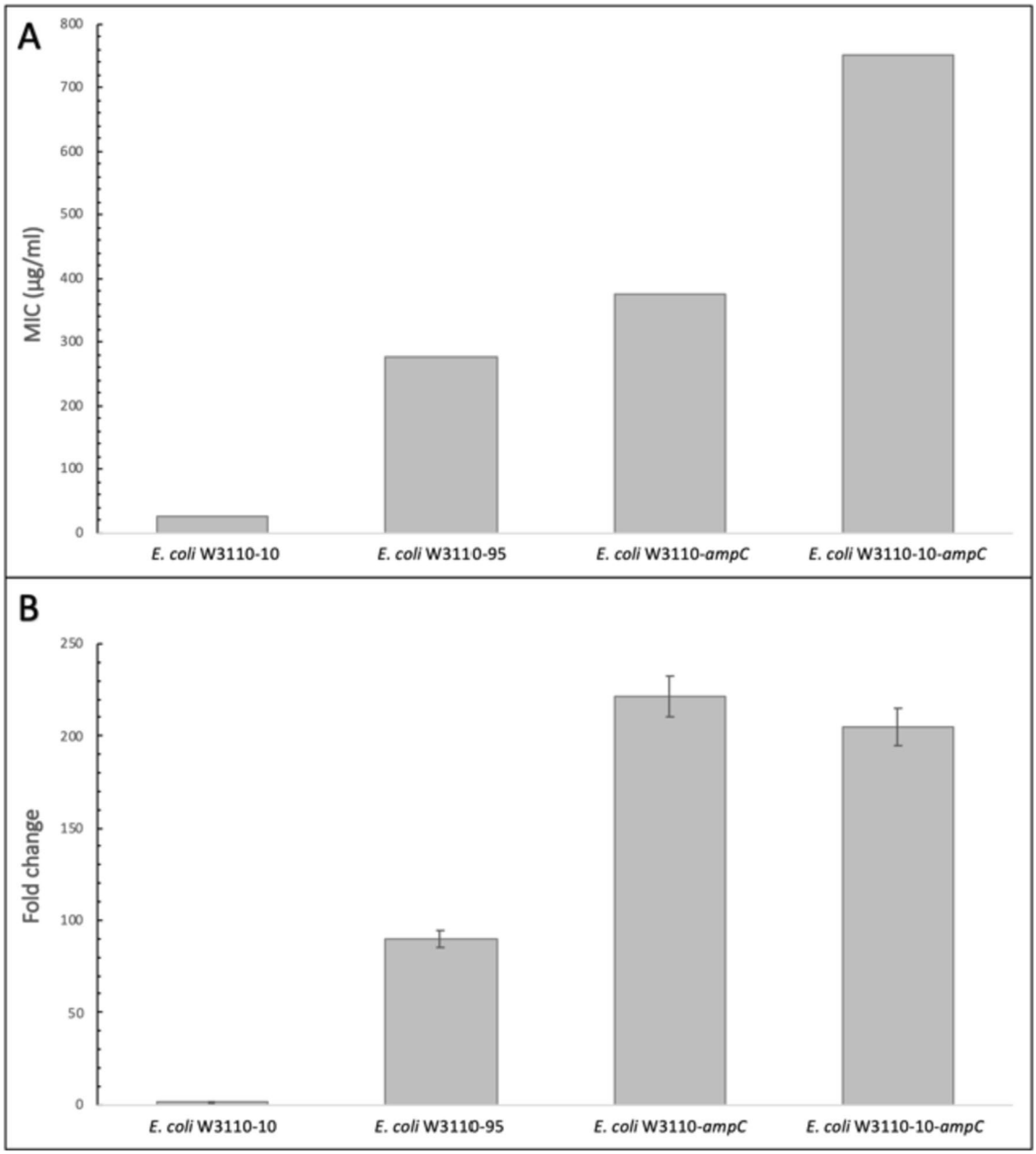

Fig. 4 Ampicillin MIC levels and *ampC* gene expression levels of *E. coli* W3110-10, *E. coli* W3110-95, *E. coli* W3110-*ampC*, and *E. coli* W3110-10-*ampC*. **A** Comparison of ampicillin MIC values of strains. **B** Comparison of *ampC* expression levels of strains in the presence of ampicillin

mutation of the *ampC* promoter increased the resistance 250-fold. Despite this increased resistance, the expression of the *ampC* gene was similar to that of *E. coli* W3110-*ampC*, which exhibited approximately 125-fold resistance. These results show that five mutations causing 10-fold resistance, together with the mutation of the *ampC* promoter, have a cumulative effect causing a much higher level of resistance than expected.

Validation of RNA-sequencing data by qRT-PCR

To test the accuracy of the RNA-seq results, 10 genes were selected and their gene expression levels were examined using the qRT-PCR method. Overall, qRT-PCR data showed similar profiles to gene expression results from RNA-seq (Supplemental Fig. S3).

Discussion

Antibiotic resistance is one of the most important health problems today. There are numerous studies on the emergence and mechanisms of antibiotic resistance. However, information on the mechanisms leading to the different levels of antibiotic resistance is very limited. Therefore, we performed genomic and transcriptomic analyses on different ampicillin-resistant strains. Adaptation experiments were performed in the presence of ampicillin to obtain strains with different levels of resistance. In the adaptation experiments, it was observed that the organism quickly adapts to the 10% increased concentration in each passage. Lindsey et al. (2013) showed that sudden exposure to high concentrations of antibiotics impedes adaptation. Similarly, another study showed that antibiotic treatment at intermediate concentrations shortened adaptation time (Baym et al. 2016). These results demonstrate the driving force of intermediate antibiotic concentrations to achieve high levels of antibiotic resistance. In light of this information, the importance of antibiotic dose should be considered, especially when formulating strategies for antibiotic use.

Whole-genome analysis of *E. coli* W3110, *E. coli* W3110-10, and *E. coli* W3110-95 was performed to understand the reasons for the differences in resistance. As a result of whole-genome sequencing, *E. coli* W3110-10 was found to contain five mutations compared to the *E. coli* W3110, while two additional mutations were detected in *E. coli* W3110-95. Mutations common to *E. coli* W3110-10 and *E. coli* W3110-95 occurred at positions 93022, 1621204, 2829040, 3450580, and 4371270 in the genome. The cumulative effect of these mutations resulted in a 10-fold increase in bacterial resistance.

The SNP at position 93022 is located in the promoter region of the *murE* gene, while it is located in the coding region of the *ftsI* gene. MurE is involved in processes such as cell wall synthesis, cell division, and regulation of cell shape (Michaud et al. 1990). The mutation was located in the promoter region of this gene but did not result in a significant change in expression, so the *murE* mutation was not associated with resistance. The *ftsI* gene is an essential molecule involved in cell division and is the target of beta-lactam antibiotics. Mutations in the *ftsI* gene can cause the cell to become resistant to beta-lactam antibiotics (Misawa et al. 2018). In one study, the 536th amino acid was found to be altered in three ampicillin-resistant strains (Xing et al. 2020). The 537th amino acid was found to be altered in *E. coli* W3110-10 and *E. coli* W3110-95. Based on this information, amino acids 536 and 537 in the *ftsI* region are thought to be important for interaction with ampicillin. Presumably, the mutation alters the structure of the protein and decreases its ability to bind to ampicillin. The deletion at position 1621204 corresponds to the promoter region of the *marA* gene and the coding region of the *marR* gene. It is known that *marABR* genes exhibit low resistance to various classes of antibiotics, disinfectants, and organic solvents (Randall and Woodward 2002). In addition, the *marA* gene is a transcriptional activator that regulates the expression of many genes related to antibiotic resistance and stress response (Martin et al. 1996). The resulting transcriptome data show significant changes in the expression of a number of genes regulated by *marA* and associated with stress response and antibiotic resistance (Martin and Rosner 2002; Kettles et al. 2019). Therefore, the mutation corresponding to the *marA* and *marR* genes is thought to contribute significantly to the intermediate resistance. In the SNP at position 3450580, the cytosine base at position 752 of the *rpoC* gene has been replaced by thymine. In this change, the amino acid proline at position 251 was replaced by leucine. RpoC forms the β'-subunit of RNA polymerase (Simpson 1979; Nedea et al. 1999). The β' subunit plays an important role in the initiation of transcription by providing target site recognition and promoter melting (Chen et al. 2010). Therefore, alterations in the *rpoC* gene can lead to cell death by disrupting the transcription mechanisms of cells. Young et al. (2004) investigated which regions are actively involved in an RNA polymerase that can initiate transcription in a study they performed in *E. coli*. According to the results, a small part of the σ factor and the first 314 amino acids of the β'-subunit were sufficient for transcription initiation and opening of the promoter region. Although the mutation we detected coincided with this region, it was observed that the organism did not have a significant problem initiating gene expression. The presence of stress factors leads to frequent mutations of the *rpoC* gene. Mutations in the *rpoC* gene were observed during the adaptation process to various factors such as osmotic stress (Guo et al. 2017), fatty acid (Chen et al. 2020), acidity (Harden et al. 2015), temperature (Sandberg et al. 2014), ionizing radiation (Bruckbauer et al. 2019), hydrogen peroxide (Pereira et al. 2021), and chlorhexidine (Pereira et al. 2021). As a result of hydrogen peroxide and chlorhexidine adaptation, a two-fold increase in ampicillin resistance of cells with *rpoC* mutation was observed (Pereira et al. 2021).

E. coli W3110-95 has 95-fold resistance compared to the ancestral strain and 10-fold resistance compared to *E. coli* W3110-10. The reason for this difference in resistance is due to two additional mutations found in *E. coli* W3110-95. The insertion at position 4383697 is located in the coding region of the *frdD* gene, while it is located in the promoter region of the *ampC* gene. The *frdD* gene encodes the D subunit of the enzyme fumarate reductase, which converts fumarate to succinate. This gene is 360 nucleotides long, and the region

between bases 303 and 360 overlaps with the promoter region of the *ampC* gene (Yamazaki et al. 2008; Tierrafría et al. 2022). There are some studies in the literature showing that mutations in the *frdD* gene cause beta-lactam resistance. In a study examining the effects of triclosan on antibiotic resistance, it was found that at the end of the 30-day adaptation period, the *fabI*, *frdD*, *marR*, *acrR*, and *soxR* genes had mutations and increased beta-lactam resistance (Lu et al. 2018). Similarly, in another study in which *E. coli* cells were adapted to ampicillin, the *frdD*, *ftsI*, *acrB*, *ompD*, *marR*, *vgrG*, and *envZ* genes were found to be mutated and ampicillin resistance increased 64-fold (Li et al. 2019). In a study using antibiotics with different mechanisms of action, the ampicillin-adapted strains were found to contain an insertion in the *frdD* gene (Karve and Wagner 2022). While two of the above studies found a base insertion in the *frdD* gene, a point mutation was found in the study by Li et al. (2019). These studies suggest that the *frdD* gene is involved in metabolic pathways related to ampicillin resistance in ways that are still unknown, but not all of these studies assumed that the promoter of the *ampC* gene is located in the *frdD* gene. In the study with triclosan, the insertion in the *frdD* gene was located at position 345. This insertion also coincides with the promoter region of the *ampC* gene. The other two studies did not show in which region of the *frdD* gene the mutations were located. Thus, because the *ampC* gene was not mentioned in these studies, an important cause of resistance was overlooked. In these studies, the mutations in the *frdD* gene need to be analyzed in more detail.

It is known that mutations of the *ampC* promoter in *E. coli* cells increase the expression of the *ampC* gene, a beta-lactamase gene, and resistance to beta-lactams is acquired (Tracz et al. 2005; Singh et al. 2020; McCann et al. 2022). The reported mutations are located in a broad region of the *ampC* gene, including the promoter and attenuator region. The most frequent are found in the −35 box, followed by the mutations in the spacer region and then in the −10 box (Tracz et al. 2007). The mutation detected in our study was caused by the introduction of a guanine between the bases at positions −15 and −16 in the spacer region. The optimum spacer length in *E. coli* cells is 17 bp (Harley and Reynolds 1987; Klein et al. 2021). This insertion increased the length of the spacer region in the *ampC* promoter to 17 bp. Previous studies have shown that mutations that cause elongation of the spacer region in the *ampC* promoter increase gene expression. In cells with the −21_−20insT mutation, *ampC* gene expression increased four- to sixfold (Singh et al. 2019). In a study examining the effects of the mutation we detected on promoter activity and antibiotic resistance, it was found that antibiotic resistance and promoter activity increased approximately 15-fold (Jaurin et al. 1982). This promoter activity and resistance is quite low compared to the levels we found in *E. coli* W3110-95 with the same mutation. The reason for the differences in resistance levels and promoter activity is that the genome of *E. coli* W3110-95 contains several mutations in addition to the *ampC* mutation.

Transformation of the plasmid containing the gene with the *ampC* promoter mutation into *E. coli* W3110 increased ampicillin resistance 125-fold and expression 221-fold. When the same plasmid was transformed into *E. coli* W3110-10 with a mutation at five positions, resistance increased 250-fold and expression increased 205-fold. The increase in resistance in both strains suggests that mutation of the *ampC* promoter increases ampicillin resistance. However, the ampicillin resistance of both strains is higher than that of *E. coli* W3110-95, which contains this mutation in its own chromosome. When gene expression levels were examined, the fact that expression was higher in *E. coli* W3110-*ampC* and *E. coli* W3110-10-*ampC* than in *E. coli* W3110-95 explains the high resistance level. It is common for more than one plasmid to enter the cell simultaneously during transformation (Weston et al. 1979; Goldsmith et al. 2007; Tomoiaga et al. 2022). Therefore, *E. coli* W3110-*ampC* and *E. coli* W3110-10-*ampC* carry more than one copy of the *ampC* gene. Thus, under similar conditions, it expresses more *ampC* than *E. coli* W3110-95.

Although the level of *ampC* expression in *E. coli* W3110-*ampC* is higher than in *E. coli* W3110-10-*ampC*, the resistance level is much lower. The difference between the two strains is the presence of five additional mutations in *E. coli* W3110-10-*ampC*. While these mutations resulted in a resistance of 25 µg/ml in *E. coli* W3110-10, they caused an increase in resistance from 375 to 750 µg/µl in plasmid-containing strains. These results show that the combination of five mutations that individually cause 10-fold resistance and one mutation that individually causes 125-fold resistance can lead to a much higher level of resistance than expected. This can be called a synergistic effect of mutations. In the synergistic effect, several mutations show a much greater effect than their individual effects (Friedman et al. 1984). This study is the first to identify mutations that exhibit synergistic effects with mutations of the *ampC* promoter.

In summary, bacteria rapidly acquire antibiotic resistance at low antibiotic concentrations. Resistance is acquired through mutations. This is the greatest weapon of bacteria to survive. While mutations that provide resistance to lower concentrations occur at the beginning of the adaptation process, with the increase of antibiotic concentration, mutations accumulate and a higher level of antibiotic resistance is provided. While these mutations alone provide some level of resistance to antibiotics, multiple mutations can have a synergistic effect and lead to the emergence of much higher levels of resistance. This study has demonstrated the importance of accurate analysis of genomic data obtained in antibiotic resistance and has provided new perspectives for

antibiotic resistance studies by demonstrating the synergistic effect of mutations in ampicillin resistance.

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Data availability Raw data for whole-genome sequencing and RNA sequencing were uploaded to the NCBI Sequence Read Archive under BioProject ID PRJNA998010 and PRJNA997788, respectively.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

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