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Research article

The effect of epetraborole on the transcriptome and proteome profiles of an *Escherichia coli* strain overexpressing *leuS*, Leucyl-tRNA Synthetase

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Abstract

Epetraborole (EP) is a boron-containing antibiotic known for its effectiveness against gram-negative enteric bacteria and *Mycobacterium* species. It is designed to bind and inhibit the LeuS enzyme (Leucyl-tRNA Synthetase), which is encoded by the essential gene *leuS* in *Escherichia coli*. EP inhibits protein translation, impeding bacterial growth. However, when *leuS* is overexpressed in a recombinant plasmid, the amount of EP required for growth inhibition needs to be increased. This study explored the impact of EP on the transcriptome and proteome of *E. coli* overexpressing *leuS*, aiming to reveal additional gene and pathway insights beyond LeuS, shedding light on the biochemical players orchestrating the bacterium's molecular response. 2D-PAGE Proteomics analysis identified four differentially regulated proteins influenced by EP in the *leuS* overexpression strain. Notably, LeuA and DeoA emerged as identified proteins. EP may affect LeuA in the cells overexpressing LeuS, which could result in truncated LeuA protein variants. Transcriptomics analyses, based on microarray data, revealed 23 up-regulated and 9 down-regulated genes responding to EP in the overexpression strain ($p<0.05$, fold change; FC>2). Based on the statistical analyses, the first five up-and down-regulated genes showing the highest fold differences in their mRNA levels are *yiaW*, *mglB*, *narH*, *ybiO*, *flgB* and *yhdY*, *deoR*, *recX*, *yobB*, *potF*, respectively. Analyses using the Omics Dashboard pathway and String indicate that the EP effect on the *leuS* overexpressing strain mainly induces alterations in the expression of genes related to the cell exterior, regulation, and response to stimuli. It is suggested that EP and higher levels of LeuS may interfere with the translational and transcriptional regulation of the expression of the *leuA* gene, which encodes the first enzyme, 2-isopropylmalate synthase, in L-leucine biosynthesis. This study offers new insights into the effects of EP on the bacterium, specifically when the level of the aminoacyl-tRNA synthetase LeuS is increased.

Keywords: *Epetraborole; Escherichia coli; LeuS; Leucyl-tRNA Synthetase; proteomics; transcriptomics*

1. Introduction

The global antibiotic crisis is a growing concern due to the rapid development of resistance by pathogenic bacteria to existing antibiotics, which has surpassed the discovery and introduction of new antibiotics to the market. Therefore, it is

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crucial to prioritize scientific research that not only identifies novel antibiotics but also comprehensively understands their impact on microorganisms. Recent investigations have revealed boron-containing metabolites with antibiotic properties in specific microorganisms (Monteferrante et al., 2016; Dibek et al., 2020). Examples of such antibiotics include boromycin (Kohno et al., 1996; Arai et al., 2004), aplasmomycin (Nakamura et al., 1977), and tartrolone (Irschik et al., 1995). Additionally, epetraborole (AN3365), a boron-containing molecule, has been synthesized and tested as an antibiotic by the ANACOR company in the United States. Epetraborole (EP) inhibits the leucyl-tRNA synthetase enzyme in the cell (Monteferrante et al., 2016). However, its effects on other molecules and systems within the cell are not yet fully understood.

EP binds specifically to the editing active site of leucyl-tRNA synthetase. The boron atom in the antibiotic binds to the *cis*-diol group of the ribose in the terminal nucleotide of $tRNA^{Leu}$, preventing the addition of leucine, disrupting the synthesis of the polypeptide chain, and inhibiting the survival of microbial cells (Monteferrante et al., 2016). EP has been reported to have bactericidal and bacteriostatic effects on various bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus pneumoniae*, and *Bacteroides fragilis*. Studies have shown that EP is effective against *E. coli* and *P. aeruginosa* in rat hip infection models (Hernandez et al., 2013).

The studies evaluated the effectiveness of EP (AN3365, GSK2251052, GSK052), a boron-containing protein synthesis inhibitor, against clinical isolates of Enterobacteriaceae and some gram-negative bacilli. Minimum Inhibitory Concentration (MIC) values were determined for selected clinical isolates. The antibiotic was found to be effective against some antibiotic-resistant isolates. AN3365 (EP) inhibited both wild-type and carbapenem-resistant strains of *Pseudomonas aeruginosa* (MIC 50/90, 2/8 μg/ml), as well as *Klebsiella pneumoniae* (MIC50/90, 1/2 μg/ml), *Acinetobacter baumannii* (MIC50/90, 2/8 μg/ml), and *Stenotrophomonas maltophilia* (MIC50/90, 2/4 μg/ml). However, the efficacy of this antibiotic was relatively lower against multidrug-resistant *Acinetobacter baumannii* (MIC50/90, 8/16 μg/ml) and *Burkholderia cepacia* (MIC50/90, 8/32 μg/ml) (Mendes et al., 2013). Furthermore, it has been demonstrated that this antibiotic inhibits Chlamydial growth and induces transcriptional changes (Hatch and Ouellette, 2020).

EP was found to be effective against *Mycobacterium abscessus* in both *in vitro* conditions and a mouse infection model (Ganapathy et al., 2021; Nguyen et al., 2023). EP has also been reported as a novel and effective candidate for *M. abscessus* treatment through *in vitro* screening against rough (R) and smooth (S) variants of *M. abscessus* (Kim et al., 2021). The effectiveness of EP antibiotic against the clinically relevant cystic fibrosis pathogen *M. abscessus* has been demonstrated. Co-treatment with EP and norvaline has been demonstrated to be effective in treating other Mycobacterial infections, including *M. abscessus* and *M. tuberculosis* (Sullivan et al., 2021).

A study was conducted to test the effect of epetraborole on *M. avium* complex (MAC) (Shafiee and Chanda, 2024). The results indicate that the EP antibiotic can be co-administered with some of the current standard care (SoC) antibiotics at clinically relevant concentrations, with a reduced likelihood of side effects from drug-drug interactions (DDI). Phase 2/3 clinical trials are currently underway to evaluate the safety and efficacy of EP in patients with MAC lung disease. Clinical trials are planned to assess the safety of EP in patients with melioidosis (Shafiee and Chanda, 2024).

Cummings et al. (2023) conducted a study to evaluate the *in vitro* activity and efficacy of epetraborole against *Burkholderia pseudomallei* infections in a mouse model. The results suggest that EP has potential as a treatment for melioidosis. Furthermore, the study identified leucyl-tRNA synthetase as a clinically relevant drug target in *B. pseudomallei*. A recent study by Sivasankar et al. (2023) reported that the EP antibiotic is highly effective against pan-drug resistant *Klebsiella pneumoniae* with low MIC values of 10 μM. These findings emphasize the growing significance of the EP antibiotic in recent years.

Further research is necessary to investigate the impact of EP on bacterial cells. EP is an antibiotic that targets the LeuS enzyme, and one of the mechanisms of antibiotic resistance is target overexpression. In this study, the *E. coli* strain AG1(pCA24N::*leuS*), in which *leuS* is overexpressed by IPTG, was exposed to sub-lethal concentrations of EP. Transcriptome and proteome analyses were conducted to investigate changes in mRNA and protein levels in the bacterial cell. The article presents and discusses the effects of EP on a strain that overexpresses the target gene by providing information on genes that are differently regulated.

2. Materials and methods

2.1. Bacterial strain and culture conditions

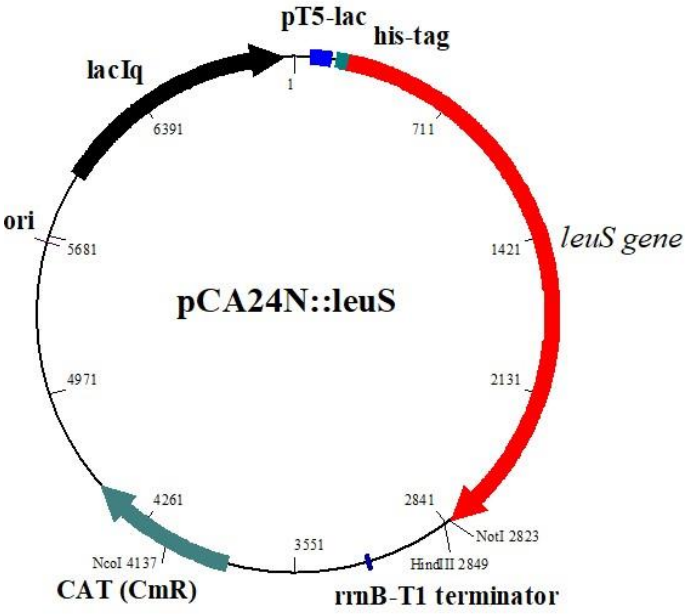

Fig. 1. Map of the plasmid pCA24N::*leuS*. The recombinant plasmid pCA24N::*leuS* is present in the strain that overexpresses the *leuS* gene. The gene was cloned into the MCS region and has a his-taq at the N terminus. Transcription is controlled by the T5 promoter, and the *lac* operator was cloned nearby the promoter region. The gene expression is negatively controlled by the Lac repressor, encoded by the *lacIq*, which is also present in the plasmid. IPTG removes the repressor from the operator region, allowing for the expression of the *leuS* gene. Transcription is terminated by the T1 terminator cloned downstream of the *leuS* gene. The plasmid contains a chloramphenicol resistance gene (CAT) and an ori region for replication in *E. coli* (Kitagawa et al., 2005).

The *Escherichia coli* AG1 strain containing the pCA24N::*leuS* plasmid was utilized. Fig. 1 displays the map of

the plasmid. The experiments were conducted in duplicate. For proteomic analysis, bacterial cells were cultured in 200 ml of Luria Bertani (LB) (Sigma, USA) medium supplemented with chloramphenicol (cm) (Sigma, USA). Growth curve experiments were conducted to determine the optimal concentration and exposure time for the antibiotic. The culture was initiated at an OD_{600} of 0.05 and grown until reaching the logarithmic phase (OD_{600} 0.5). Then, the cells were exposed to the culture medium both with and without the EP antibiotic, at a concentration of 0.25 µg/ml, for a duration of 1 hour. For the microarray experiments, bacterial cells were grown in 7 ml of LB-cm medium, starting from an OD_{600} of 0.05, and cultured until reaching the logarithmic phase (OD_{600} 0.5). The cells were incubated in the culture medium without EP and the culture medium containing 0.25 µg/ml of the EP antibiotic for 15 minutes. Total RNA isolation was then performed from the cells.

2.2. Protein extraction and TCA precipitation

The cells from the bacterial culture were centrifuged at 10,000 rpm for 10 minutes in a refrigerated centrifuge at +4°C. The supernatant was then discarded, and the resulting cell pellets were washed twice with cold Phosphate-Buffered Saline (PBS) (Sigma, USA). Afterward, the cell pellets were centrifuged again at +4°C to remove any remaining culture media, resulting in cell pellets. To disrupt the cells, 2D Rehydration buffer (8M Urea, 2M Thiourea, 2% CHAPS, 50 mM DTT, 0.2% Ampholytes, 0.002% Bromophenol blue) (Sigma, USA) was used. Additionally, the samples were sonicated for five repetitions with a 20-second cycle and then centrifuged at 10,000 rpm for 10 minutes at +4°C. The protein extracts were treated with a solution of 10% trichloroacetic acid (TCA) and 20 mM dithiothreitol (DTT), using at least three times the volume to eliminate salts and other unwanted components. After treatment, the samples were kept at -20°C overnight and then centrifuged at 15,200 rpm in a refrigerated centrifuge at +4°C for 20 minutes. The supernatants were carefully separated from the pellet, which was washed twice with 500 µl of cold acetone. After centrifugation at 15,200 rpm in a refrigerated centrifuge at +4°C for 10 minutes, 200 µl of 2D rehydration buffer was added to the pellet, and the mixture was vortexed at room temperature until fully dissolved. After centrifugation at 15,200 rpm in a refrigerated centrifuge at +4°C for 10 minutes, 200 µl of 2D rehydration buffer was added to the pellet, and the mixture was vortexed at room temperature until fully dissolved. The supernatants were then transferred to sterile Eppendorf tubes. Protein concentrations were measured using the Bradford method (Bradford, 1976).

2.2.1. SDS-PAGE

Protein separation was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A 12% SDS-PAGE separating gel and a 4% stacking (upper) gel were prepared. Each well was loaded with 20 µg of protein and underwent vertical electrophoresis in a tank containing 1X SDS running buffer (1X 25 mM Tris, 192 mM glycine, 0.1% SDS pH 8.3) at 180 volts and 400 milliamps for approximately 60 minutes. The gels were transferred to a fixation solution containing 50 ml of MeOH, 10 ml of acetic acid, and 40 ml of distilled water. They were then shaken on an orbital shaker for 30 minutes. After fixation, the gels were stained with Coomassie Brilliant Blue R-250 dye (BioRad, USA) for 30 minutes. Once the staining process was completed, the gels were immersed in distilled water and washed until free from dye.

2.2.2. 2D-PAGE and MALDI-TOF analysis

The protein extract (100 µg protein) was resuspended in 2D rehydration buffer and loaded onto an 11 cm IPG strip with a pH range of 3-10 (ReadyStrip, BioRad, USA). The IPG strips were left to rehydrate overnight without applying any voltage in the Passive Rehydration Program, which took approximately 13 hours at 20°C (BioRad, USA Protean IEF Cell). Increasing voltage values were applied during the focusing process at 20°C. The program for focusing comprised three steps, taking approximately 10-12 hours. The first step involved applying 250 Volts for 20 minutes using a linear ramp. The second step involved applying 4000 Volts for 2 hours using a linear ramp. Finally, a rapid ramp of 40,000 Volts V/H was applied using an IEF cell Protean from BioRad. The strips were focused and then transferred to an equilibration container. They were successively washed for 15 minutes each with Equilibration Buffer I (6 M Urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% Glycerol, 2% (w/v) DTT), Buffer II (6 M Urea, 0.375 M Tris-HCl pH 8.8, 2% SDS, 20% Glycerol, 2.5% (w/v) Iodoacetamide), and 1X SDS buffer.

The second-dimensional vertical SDS-PAGE was conducted using a 12% polyacrylamide gel. IPG strips and 3 µl of unstained protein marker (Fermentas, SM0431) were placed onto the separating gel. Electrophoresis was conducted using 1X SDS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) as a running buffer, and gels were run at 400 mA (maximum), approximately 180 volts for about 55 minutes (BioRad, Mini Protean Tetra Cell, USA). The gels were initially placed in a fixation solution consisting of 50% methanol and 10% acetic acid. They were then shaken on an orbital shaker for a period ranging from 6 to 24 hours. For staining, a freshly prepared Colloidal Coomassie dye (KeraFAST, Bloomoose, USA) in a 4:1 ratio (Reagent I: Reagent II) was used for each gel. The gels were gently shaken in an orbital shaker at room temperature for 6-24 hours until protein spots became visible. To fix and enhance spots on gels after removing excess dye, an "intensifying" solution was prepared. To obtain the solution, mix Reagent II and Milli-Q water in a 1:4 ratio and gently shake the gels in an orbital shaker at room temperature. Obtain gel images of protein spots using the Quantity One program (BioRad, USA). Before excising the spots in 2D gels, compare the protein profiles using the PD Quest Advanced program (BioRad, USA). Identify protein spots that show differences in regulation by checking spot densities between matched protein spots. The gel spots that were removed underwent several destaining steps. The proteins within were then digested using 10 ng/µl of trypsin enzyme from Promega. Afterward, MALDI TOF-TOF analysis was performed, and the mass spectra were identified using the Mascot protein identification search engine (Kocaeli University, Proteomics Laboratory).

2.3. Microarray analysis

Following cell growth and antibiotic treatment of the *Escherichia coli* AG1(pCA24N::*leuS*) strain, total RNA was isolated from the cell pellets using a commercial kit (PureLink RNA Mini Kit, 12183018A). DNaseI treatment was applied to the isolated RNA to remove any DNA contamination (Thermo, Scientific, EN0521). For microarray analysis, RNA at a

concentration of 100 ng/µl was used. Microarray analysis was conducted following the protocol of Agilent's "Single-Color Microarray-Based Gene Expression Analysis". According to the protocol, the process involved cRNA synthesis and amplification, followed by cRNA purification. RNA samples were labeled with Cy3 dye. After the labeling process, the amounts of RNA labeled with Cy3 dye were measured using a Nanodrop spectrophotometer (Implen, Germany). Subsequently, the samples underwent the hybridization process, using 600 ng of RNA. Prepared hybridization samples were applied to the 8-array Agilent chip without creating bubbles. The samples were left to hybridize with array slides at 65°C for 17 hours. Following the hybridization process, the samples were washed with wash buffers and prepared for imaging. The arrays were scanned using the Agilent Microarray scanner (Agilent, USA). The image intensity data were recorded and transferred into text files using the Agilent Feature Extraction Software, version A.4.0.45 (Agilent, USA).

2.4. Statistical and bioinformatic analyses

The data was collected using Agilent Feature Extraction Software 8.0 and analyzed with GeneSpring 14.9 Software (Agilent, USA) using the t-test and ANOVA. Datasets with p-values <0.05 were filtered to analyze the extent of change (increase or decrease) compared to the control condition. The microarray results, which contain gene information and fold differences, were analyzed using the EcoCyc (Keseler et al., 2005) and Omics Dashboard (Pathway Tools) programs (Paley et al., 2017) to identify metabolic pathways or cellular processes. Additionally, EcoCyc was used to obtain more information about the genes of interest, and relevant publications were searched for in the literature.

3. Results

3.1. Proteomics analysis of the leuS overexpressing strain exposed to EP

The protein profiles of the AG1(pCA24N::*leuS*) strain were examined under two conditions: with and without EP exposure. To assess the quality and integrity of the proteins, a 1D-SDS-PAGE page was performed to visualize the protein bands. After ensuring the quality of the protein extracts, 2D-PAGE was performed.

After the second dimension, proteins were stained, and the protein spots were observed and photographs were taken. Fig. 2A illustrates the images of 2D-PAGE gels showing the protein spots obtained from the cells as a result of exposure to EP antibiotic (0.25 µg/ml) and the control (no exposure). The images were captured using the Quantity One program and then compared using the PDQuest Advanced program (BioRad). Four protein spots with differential regulation were identified by examining the spot densities of the matched protein spots, as shown in Fig. 2B.

The EP effect on the *E. coli* AG1(pCA24N::*leuS*) strain resulted in the upregulation of two protein spots (spots 4103 and 9105) and the downregulation of two protein spots (spots 7405 and 8303) (Fig. 2B). MALDI TOF-TOF analysis of the selected protein spots identified 2-isopropylmalate synthase (LeuA) and thymidine phosphorylase (DeoA) (Table 1). Three spots were found to be associated with LeuA, indicating the possibility of truncated forms of this protein.

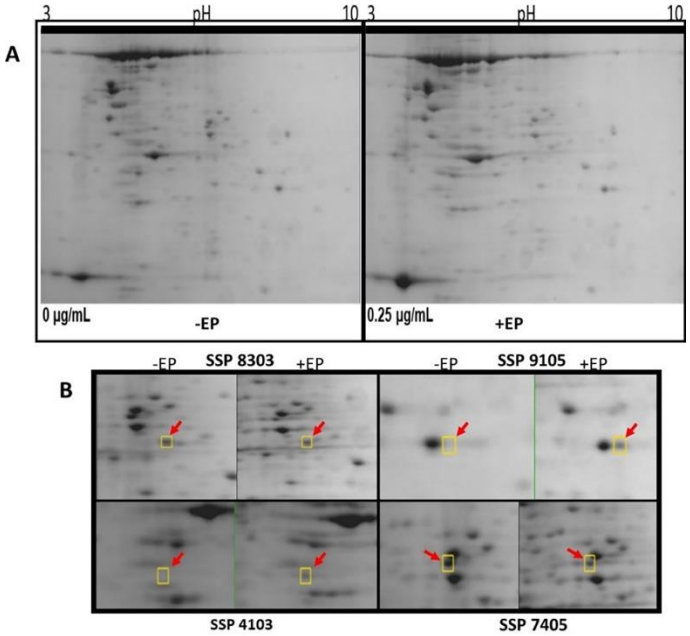

Fig. 2. 2D-PAGE profiles of the AG1(pCA24N::*leuS*) strain under EP treatment, highlighting spots exhibiting regulation. 2A. Protein spots obtained from the strain exposed to EP (right gel) and no treatment control (left gel). pI strip (3-10) was used and EP concentration of 0.25 µg/ml was applied, 2B. Comparative images of protein spots from the AG1(pCA24N::*leuS*) strain showing regulation differences without EP antibiotic (-EP) and with 0.25 µg/ml EP (+EP). Spots numbered 4103, 7405, 8303, and 9105 are shown with red arrows.

Table 1
Identification of proteins differentially regulated upon EP effect on the AG1(pCA24N::*leuS*) strain.

Spot No	Protein ID	Score	Regulation	Gene
7405	2-isopropylmalate synthase	51	down	*leuA*
4103	2-isopropylmalate synthase	37	up	*leuA*
8303	Thymidine phosphorylase	35	down	*deoA*
9105	2-isopropylmalate synthase	30	up	*leuA*

3.2. Microarray-based transcriptomic analysis results

Microarray analysis was performed to assess the levels of regulated mRNAs in the bacterial strain under both EP antibiotic (0.25 µg/ml) and antibiotic-free conditions. The analysis revealed that the mRNA levels of 23 genes increased ($p<0.05$, fold change; FC>2), while those of 9 genes decreased in response to the EP effect (Tables 2 and 3). Up-regulated genes were *yiaW*, *mglB*, *narH*, *ybiO*, *flgB*, *lacZ*, *yibH*, *yedN*, *yhbW*, *glpB*, *murB*, *hydN*, *ymfC*, *fadE*, *yidF*, *gltI*, *dkgB*, *frdA*, *yafU*, *yidJ*, *creB*, *yhfZ* and *yfhG* (Table 2). In summary, these genes in *E. coli* encode various proteins with diverse functions, including transport, metabolism, regulation, and biosynthesis. Many of them are involved in essential cellular processes, but specific details about some genes are not available in the literature. These genes were classified based on the cellular processes or metabolic pathways and were seen to be associated with chemotaxis, transport, biosynthesis, and metabolism of galactose, lactose, glycerol, peptidoglycan, fatty acids, hydrogen, and sulfate. Some genes have unknown functions (Tables 4 and 5). Down-regulated genes, on the other hand, were *yhdY*, *deoR*, *recX*, *yobB*, *potF*, *yccE*, *yraJ*, *ycaD*, and *mutH* genes, which are involved in various cellular functions, including DNA repair, regulation of gene expression, cell division, and transport processes.

Table 2
List of up-regulated genes as a result of EP effect on *E. coli* AG1(pCA24N::*leuS*) strain.

Gene	Definition	Fold change	Regulation
yiaW	orf, hypothetical protein [b3587]	5.1	up
mglB	galactose-binding transport protein; receptor for galactose taxis [b2150]	4.9	up
narH	nitrate reductase 1, beta subunit [b1225]	4.2	up
ybiO	putative transport protein [b0808]	4.2	up
flgB	flagellar biosynthesis, cell-proximal portion of basal-body rod [b1073]	4.1	up
lacZ	beta-D-galactosidase [b0344]	4	up
yibH	putative membrane protein [b3597]	3.9	up
yedN	orf, hypothetical protein [b1934]	3.8	up
yhbW	putative enzyme [b3160]	3.3	up
glpB	sn-glycerol-3-phosphate dehydrogenase [b2242]	3.2	up
murB	UDP-N-acetylenolpyruvoylglucosamine reductase [c_4931]	3.1	up
hydN	Electron transport protein hydN [c_3269]	2.9	up
ymfC	orf, hypothetical protein [Z1864]	2.5	up
fadE	putative acyl-CoA dehydrogenase [b0221]	2.4	up
	Putative cell cycle protein mesJ [c_0226]	2.4	up
yidF	putative transcriptional regulator [Z5169]	24	up
	Hypothetical protein [c_3755]	2.3	up
gltI	putative periplasmic binding transport protein [b0655]	2.3	up
dkgB	putative aldose reductase [b0207]	2.2	up
frdA	fumarate reductase, anaerobic, flavoprotein subunit [b4154]	2.2	up
yafU	orf, hypothetical protein [b0218]	2.2	up
yidJ	putative sulfatase [b3678]	2.1	up
creB	catabolic regulation response regulator [Z6001]	2	up
yhfZ	orf, hypothetical protein [b3383]	2	up
yfhG	putative alpha helix protein [b2555]	2	up

Table 3
List of down-regulated genes as a result of EP effect on *E. coli* AG1(pCA24N::*leuS*) strain.

Gene	Definition	Fold change	Regulation
yhdY	putative transport system permease protein	2.8	down
deoR	transcriptional repressor for deo operon, tsx, nupG [b0840]	2.6	down
recX	regulator, OraA protein [Z4001]	2.5	down
yobB	orf, hypothetical protein [b1843]	2.5	down
potF	periplasmic putrescine-binding protein; permease protein [b0854]	2.5	down
	Hypothetical protein ydcX [c_1870]	2.4	down
yccE	orf, hypothetical protein [b1001]	2.4	down
yraJ	putative outer membrane protein [b3144]	2.4	down
ycaD	Hypothetical protein ycaD [c_1037]	2.2	down
mutH	methyl-directed mismatch repair [b2831]	2	down

4. Discussion

The objective of this study was to examine the effect of epetraborole (EP) on the transcriptome and proteome of the *Escherichia coli* strain that overexpresses the *leuS* gene, which encodes Leucyl-tRNA synthetase. The study provides valuable insights into the molecular response of the bacterium to this boron-containing antibiotic, exploring changes at both the genetic and protein levels. The findings shed light on the broader effects beyond the primary target, LeuS.

Proteomics analysis based on 2D-PAGE and MALDI TOF-TOF revealed four differentially regulated protein spots influenced by EP in the *leuS* overexpression strain. The study identified two regulated proteins, LeuA and DeoA. LeuA catalyzes the initial step in leucine synthesis (Stieglitz and Calvo, 1974). EP and higher levels of LeuS may affect LeuA, potentially leading to truncated LeuA protein variants. LeuA was identified in three locations on the 2D-PAGE gel, with two spots up-regulated and one down-regulated (Table 2). Despite the relatively low identification scores for LeuA (51, 37, and 30), the regulation levels of the spots were visible on the 2D gel (refer to Fig. 2). While considering the possibility of misidentification, it is also possible that the cell may produce varying lengths of LeuA protein due to increased levels of LeuS and EP effect.

LeuA is a crucial enzyme in the biosynthesis of leucine. The activity of LeuA is regulated to ensure that leucine biosynthesis occurs only when necessary. Regulation commonly involves feedback inhibition, where the end product of the pathway, leucine, acts as an allosteric inhibitor of LeuA. This feedback mechanism helps to maintain optimal levels of leucine within the cell. Overexpression of LeuS and EP may indirectly affect the translational and transcriptional regulation of LeuA, resulting in the production of shorter LeuA proteins and subsequent impacts on leucine biosynthesis.

Wessler and Calvo (1981) proposed that the expression of the *leuA* gene is controlled by Ribosome-Mediated Attenuation. This mechanism involves the promotion of transcription termination at the attenuator by charged Leucyl-tRNA (L-leucyl-tRNA^{Leu}). Also, Gemmill et al. (1979) demonstrated that the operon of leucine biosynthesis genes, including *leuA*, is controlled by attenuation in *Salmonella typhimurium*. Therefore, it may be possible that, if there is a sufficient amount of L-leucyl-tRNA^{Leu} in the cell, premature translation of LeuA may occur through the involvement of ribosomes, facilitated by the attenuation of transcription. Due to the absence of a nuclear membrane in bacteria, transcription and translation are coupled. This may result in the synthesis of truncated versions of the LeuA protein, which can be detected by 2D-PAGE.

In the current study, the overexpression of *leuS* by a recombinant plasmid may have led to an increase in the L-leucyl-tRNA^{Leu} level. This increase, combined with the presence of EP, may have disrupted the balance between LeuS enzyme activity and the product L-leucyl-tRNA^{Leu}, leading to the detection of truncated forms of the LeuA protein. Future studies could explore and clarify the relationship between the LeuA attenuation mechanism and EP effect in the presence of higher levels of LeuS. Attenuation may indirectly regulate and maintain amino acid levels in the cell for other aminoacyl-tRNA synthetases and amino acid synthesis operons.

Transcriptomics analysis based on microarray data revealed 23 genes up-regulated and 9 genes down-regulated in response to EP in the *leuS* overexpression strain. The identified genes cover a range of functions, indicating a multifaceted

Table 4
Omics Dashboard Pathway analysis of the up-regulated genes.

System	Subsystem	Gene ID	Gene	Fold change	Regulation	Function
Biosynthesis		EG11205	*murB*	3.1	up	UDP-N-acetylenolpyruvoylglucosamine reductase
Degradation	Carbohydrates and Carboxylates Degradation	EG10392	*glpB*	3.2	up	anaerobic glycerol-3-phosphate dehydrogenase subunit B
		EG10527	*lacZ*	4.0	up	β-galactosidase
	Alcohol Degradation	EG10392	*glpB*	3.2	up	anaerobic glycerol-3-phosphate dehydrogenase subunit B
	Fatty Acid and Lipid Degradation	G6105	*fadE*	2.4	up	acyl-CoA dehydrogenase
	Other Degradation	EG11205	*murB*	3.1	up	UDP-N-acetylenolpyruvoylglucosamine reductase
Energy	Anaerobic Respiration	EG10392	*glpB*	3.2	up	anaerobic glycerol-3-phosphate dehydrogenase subunit B
		EG10639	*narH*	4.2	up	nitrate reductase A subunit β
	Other Energy	EG10392	*glpB*	3.2	up	anaerobic glycerol-3-phosphate dehydrogenase subunit B
Central Dogma		G6581	*ymfC*	2.5	up	23S rRNA pseudouridine2457 synthase
Regulation	Sigma and Transcription Factor Regulons	G358	*flgB*	4.1	up	flagellar basal-body rod protein
		EG10392	*glpB*	3.2	up	anaerobic glycerol-3-phosphate dehydrogenase subunit B
		EG11552	*hydN*	2.9	up	putative electron transport protein
		EG10593	*mglB*	4.9	up	D-galactose/methyl-galactoside ABC transporter periplasmic binding protein
	Transcription Factor Regulons	EG10639	*narH*	4.2	up	nitrate reductase A subunit β
		EG10527	*lacZ*	4.0	up	β-galactosidase
		G6581	*ymfC*	2.5	up	23S rRNA pseudouridine2457 synthase
		G7654	*yhbW*	3.3	up	putative luciferase-like monooxygenase
		G6417	*ybiO*	4.2	up	moderate conductance mechanosensitive channel
		EG12291	*yiaW*	5.1	up	DUF3302 domain-containing protein
		G6105	*fadE*	2.4	up	acyl-CoA dehydrogenase
Response to Stimulus	Proteins Involved in Response to DNA Damage	EG11764	*yibH*	3.9	up	inner membrane protein
	Proteins Involved in Response to Osmotic Stress	G6417	*ybiO*	4.2	up	moderate conductance mechanosensitive channel
	Other Proteins involved in Stimulus Response	G358	*flgB*	4.1	up	flagellar basal-body rod protein
		EG10593	*mglB*	4.9	up	D-galactose/methyl-galactoside ABC transporter periplasmic binding protein
Cellular Processes		EG11205	*murB*	3.1	up	UDP-N-acetylenolpyruvoylglucosamine reductase
Virulence-Related	Proteins Involved in Locomotion	G358	*flgB*	4.1	up	flagellar basal-body rod protein
		EG10593	*mglB*	4.9	up	D-galactose/methyl-galactoside ABC transporter periplasmic binding protein
Cell Exterior	Flagellar Proteins	G358	*flgB*	4.1	up	flagellar basal-body rod protein
	Plasma Membrane Proteins	EG10392	*glpB*	3.2	up	anaerobic glycerol-3-phosphate dehydrogenase subunit B
		G6417	*ybiO*	4.2	up	moderate conductance mechanosensitive channel
		EG11764	*yibH*	3.9	up	inner membrane protein
		EG10593	*mglB*	4.9	up	D-galactose/methyl-galactoside ABC transporter periplasmic binding protein
		G6105	*fadE*	2.4	up	acyl-CoA dehydrogenase
		EG12291	*yiaW*	5.1	up	DUF3302 domain-containing protein
		EG10639	*narH*	4.2	up	nitrate reductase A subunit β
	Periplasmic Proteins	G358	*flgB*	4.1	up	flagellar basal-body rod protein
		EG10593	*mglB*	4.9	up	D-galactose/methyl-galactoside ABC transporter periplasmic binding protein
	Transport Proteins	EG10593	*mglB*	4.9	up	D-galactose/methyl-galactoside ABC transporter periplasmic binding protein
	Cell Wall Biogenesis/Organization Proteins	EG11205	*murB*	3.1	up	UDP-N-acetylenolpyruvoylglucosamine reductase

*The evaluation of cellular systems and subsystems, based on the microarray analysis results of the up-regulated genes in response to Epetraborole in the *E. coli* AG1(pCA24N::*leuS*) strain, was performed using the Omics Dashboard Pathway (Tools) program.

Table 5
Omics Dashboard Pathway analysis of the down-regulated genes.

System	Subsystem	Gene ID	Gene	Fold change	Regulation	Function
Central Dogma	RNA Metabolism	EG10223	*deoR*	2.6	down	DNA-binding transcriptional
	DNA Metabolism	EG10624	*mutH*	2.0	down	DNA mismatch repair protein
		EG12080	*recX*	2.5	down	RecA inhibitor
Regulation	Sigma and Transcription Factor Regulons	EG12080	*recX*	2.5	down	RecA inhibitor
		EG11629	*potF*	2.5	down	putrescine ABC transporter periplasmic binding protein
		EG12836	*yhdY*	2.8	down	DNA-binding transcription regulator
	Signal transmission pathways	EG12196	*yccE*	2.4	down	uncharacterized protein
	Transcription Factor Regulons	EG10223	*deoR*	2.6	down	DNA-binding transcriptional repressor
		EG11242	*ycaD*	2.2	down	putative transporter
		G7015	*yobB*	2.5	down	putative carbon-nitrogen hydrolase family protein
Response to Stimulus	Proteins Involved in Response to DNA Damage	EG12080	*recX*	2.5	down	RecA inhibitor
		EG10624	*mutH*	2.0	down	DNA mismatch repair protein
		G7639	*yraJ*	2.4	down	putative fimbrial usher protein
Cell Exterior	Plasma Membrane Proteins	EG11629	*potF*	2.5	down	putrescine ABC transporter periplasmic binding protein
		EG12836	*yhdY*	2.8	down	putative ABC transporter membrane subunit
		G7015	*yobB*	2.5	down	putative carbon-nitrogen hydrolase family protein
		EG11242	*ycaD*	2.2	down	putative transporter
	Periplasmic Proteins	EG11629	*potF*	2.5	down	putrescine ABC transporter periplasmic binding protein
	Transport Proteins	EG11629	*potF*	2.5	down	putrescine ABC transporter periplasmic binding protein
	Outer Membrane Proteins	G7639	*yraJ*	2.4	down	putative fimbrial usher protein

cellular response. Upon evaluating differentially regulated genes, it is evident that up-regulated genes are associated with biosynthesis, cellular processes, virulence-related degradation, and energy metabolism within the cell, in contrast to down-regulated genes. The study found that genes that were both up-regulated and down-regulated played a role in regulating responses to stimuli, cell exterior, and the central dogma.

Analyses were conducted using Omics Dashboard pathway (EcoCyc) (Paley et al., 2017) and String DB (Snel et al., 2000) to interpret the microarray results. The results suggest that EP has a significant impact on the *leuS* overexpressing strain, particularly in terms of altering the expression of genes associated with the cell exterior, regulation, and response to stimuli. This broader impact on cellular processes suggests a systemic response to EP beyond the direct inhibition of LeuS.

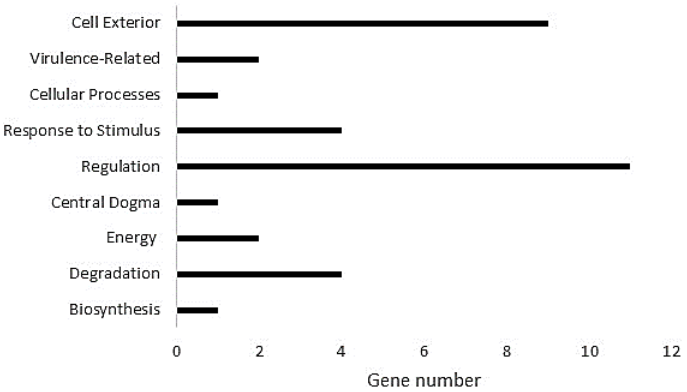

Fig. 3. Enrichment analysis of up-regulated genes. The results related to the cellular functions of the genes showing upregulation in the microarray analysis of the AG1(pCA24N::*leuS*) strain are provided. Several genes match with more than one subsystem. The numbers are derived based on the Omics Dashboard analysis results in the EcoCyc database.

The Omics Dashboard analysis (BioCyc, 2024) is a useful tool for analyzing large datasets, such as transcriptomics results,

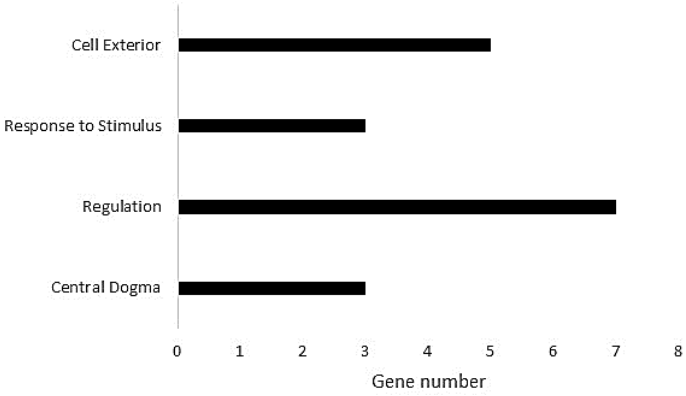

Fig. 4. Enrichment analysis of down-regulated genes. The results related to the cellular functions of the genes showing downregulation in the microarray analysis of the AG1(pCA24N::*leuS*) strain are provided. Several genes match with more than one subsystem. The numbers are derived based on the Omics Dashboard analysis results in the EcoCyc database.

to identify pathways and cell processes related to the topic of interest (Paley et al., 2017). Separate analyses were conducted for up-regulated and down-regulated genes, and the affected pathways and processes are presented in Tables 4 and 5, respectively. Additionally, the up-regulated genes were analyzed using the "Enrichment" tool (NIH, 2024), which revealed that the most affected pathways or cellular processes were regulation (n=11 genes), cell exterior (n=9), response to stimulus (n=4), and degradation (n=4) (Fig. 3). According to the enrichment analysis module of the Omics Dashboard, the down-regulated genes showed a greater impact on regulation, cell exterior, response to stimulus, and central dogma genes compared to others (Fig. 4). The gene information related to these processes and pathways can be utilized for further studies to investigate specific gene involvement and understand the impact of EP on bacteria.

Furthermore, String analysis was also performed (STRING, 2024) on both up-regulated and down-regulated

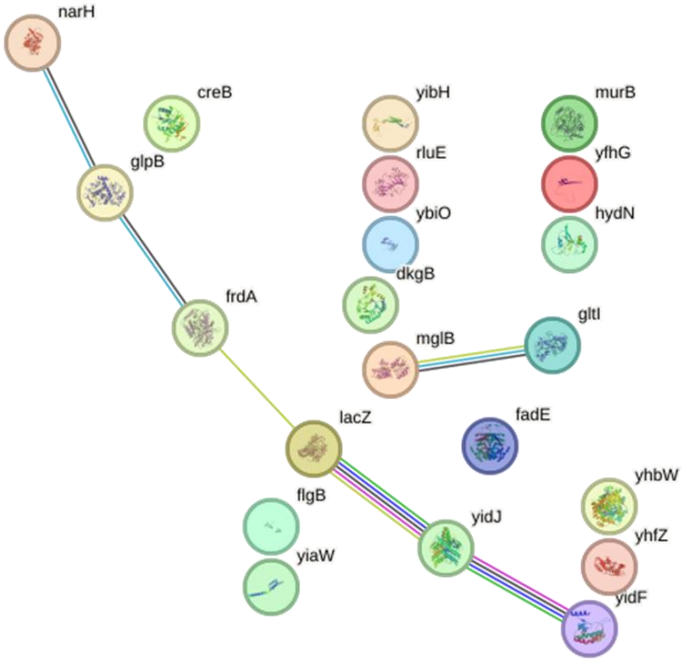

Fig. 5. STRING network analysis of the up-regulated genes. A connection map illustrating the predicted functional relationships among the genes up-regulated in response to epetraborole on the *E. coli* AG1(pCA24N::*leuS*) strain. The thickness of the lines indicates the degree of interaction based on fusion, co-occurrence, experimental, and co-expression data. The figure was generated using STRING (version 12.0) with a medium confidence score of 0.4 (approximate probability).

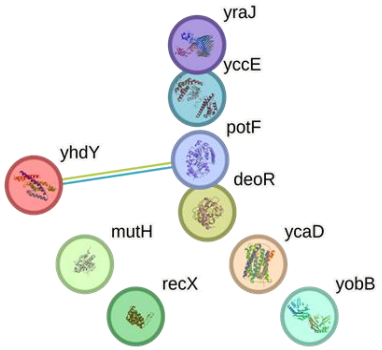

Fig. 6. STRING network analysis of the down-regulated genes. A connection map illustrating the predicted functional relationships among the genes down-regulated in response to epetraborole on the *E. coli* AG1(pCA24N::*leuS*) strain. The thickness of the lines indicates the degree of interaction based on fusion, co-occurrence, experimental, and co-expression data. The figure was generated using STRING (version 12.0) with a medium confidence score of 0.4 (approximate probability).

genes (Mering et al., 2003). Among the up-regulated genes, *yidF*, *yidJ*, *lacZ*, *frdA*, *glpB*, and *narF* formed a network, while *mlgB* and *gltL* formed another network (Fig. 5) (interaction score values: *narH* vs *glpB*- 0. 505, *glpB* vs *frdA*- 0.515, *frdA* vs *lacZ*- 0.524, *lacZ* vs *yidJ*- 0.523, *yidJ* vs *yidF*- 0.669, *mglB* vs *gltL*- 0.783). A link was observed between *potF* and *yhdY* for down-regulated genes (Fig. 6) with an interaction score value of 0.654. The network nodes represent proteins, and the edges represent protein-protein associations. These associations should be specific and meaningful, indicating that the proteins contribute jointly to a shared function. However, this does not necessarily imply that they are physically bound to each other (Fig. 5 and Fig. 6). These protein interactions are displayed in three ways. The program displays protein interactions using blue to indicate information from selected databases and purple to indicate experimentally determined information. The proximity of gene regions is represented by the color green, gene fusion interactions by red, and gene co-occurrence by dark blue. During the interaction, the color yellow indicates text mining, black indicates co-expression and light navy blue indicates protein homology.

The genes *mglB* and *flgB* are involved in chemotaxis and flagellar biosynthesis, respectively, while genes *glpB*, *murB*, and *fadE* are associated with metabolic pathways, including glycerol metabolism, peptidoglycan biosynthesis, and fatty acid metabolism (Scholle et al., 1987; Spoering et al., 2006; Egan et al., 2020; Pavoncello et al., 2022; Sun et al., 2022).

The literature indicates that *yidF* is involved in activating sulfatases in *E. coli* (Benjdia et al., 2007; Alméciga-Díaz et al., 2017). Overexpression of *yidF* enhances resistance to D-serine (Soo et al., 2011). The function of the *yidJ* gene is unknown, but it is a putative Cys-type sulfatase (Schirmer and Kolter, 1998). The *frdA* gene is one of four genes involved in acetyl-CoA consumption (Seong et al., 2020). The *glpB* gene encodes a subunit of anaerobic glycerol-3-phosphate dehydrogenase B, which converts glycerol-3-phosphate to dihydroxyacetone phosphate using fumarate under anaerobic conditions (Cole et al., 1988). Metcalf et al. (1990) found that this gene was induced during phosphate starvation. The literature information suggests that the *gltL* gene is the putative ATP-binding subunit of a glutamate/aspartate ABC transporter complex (Linton and Higgins, 1998). The *potF* gene is a putrescine-specific binding protein, as reported by Pistocchi et al. (1993). The *yhdY* gene, on the other hand, is predicted to be an inner membrane protein with eight transmembrane domains (Daley et al., 2005) and is predicted to be one of the two inner membrane subunits of a putative ABC transporter (Saier et al., 2016). A transcriptomic analysis of an *E. coli* strain in the presence of poly-gamma-glutamic acid (gamma-PGA) revealed an upregulation in the mRNA level of the *yhdY* gene (Jiang et al., 2006).

Harayama et al. (1983) conducted a study on MglB, a periplasmic binding protein of a D-galactose/methyl-galactose ABC transporter. The study found that *mglB*$^{+}$ plasmids synthesized both the precursor and mature forms of the galactose binding protein in the cells. According to Ito et al. (2008), FlgB, one of the four proteins that make up the rod of the flagellar basal body in bacteria, exhibited increased expression in *ΔrpoS* biofilms. However, DNA microarray analysis revealed no difference in expression in wild-type biofilms.

Genes *yiaW*, *yibH*, *yedN*, *yhbW*, *ymfC*, *yafU*, *yhfZ*, and *yfhG* have unknown functions, indicating areas for further investigation. This study has shown that these genes are differentially regulated by the EP antibiotic.

Furthermore, the literature contains additional studies on antibiotics. For example, Xu et al. (2006) conducted a proteomics study to screen the outer membrane proteome of *E. coli* K-12. They identified 11 protein spots representing nine proteins associated with tetracycline resistance and nine protein spots representing eight proteins associated with ampicillin resistance. Another proteomic study was conducted on an *E. coli* strain resistant to piperacillin/tazobactam antibiotics. The study found that 12 proteins were up-regulated and 14 proteins were down-regulated ($p<0.05$) (dos Santos et al., 2010). Bie et al. (2023) conducted a transcriptomic analysis to investigate the effect of nine different antibiotics on the transcriptome of *E. coli*. The study revealed that mRNA levels of eight genes were up-regulated, while 30 genes were down-regulated. Transcriptomic analysis was performed to examine the impact of ciprofloxacin

antibiotic on the transcriptome of *E. coli* MG1655 strain. The analysis showed differential regulation of mRNA levels of 41 genes (Goswami and Narayana Rao, 2018). In another study, Zhao et al. (2023) conducted a transcriptomic analysis to investigate the effects of gentamicin and ampicillin antibiotics. The study found that gentamicin caused differential expression of 51 genes, while ampicillin caused differential expression of 23 genes.

Inhibition of essential aminoacyl tRNA synthetases in various bacteria can be a potential target for antibiotics. Blocking these enzymes interrupts protein synthesis and prevents cell growth (Hurdle et al., 2005). Further research is necessary to understand the relation between these enzymes and antibiotic susceptibility or tolerance, and to combat antibiotic resistance. After reviewing the literature, it is evident that mutations in certain tRNA synthetase genes can reduce susceptibility to ciprofloxacin and other antibiotics. A study demonstrated that mutations in three different aminoacyl tRNA synthetase genes (*leuS*, *aspS*, and *thrS*) decreased susceptibility to ciprofloxacin. Transcriptome analysis showed that two mutations, independently selected in *leuS* (Asp162Asn and Ser496Pro), specifically up-regulated three loci (*mdtK*, *acrZ*, and *ydhIJK*). Genetic analysis showed that the bacterium's reduced susceptibility was linked to the activity of these loci. Additional antimicrobial sensitivity tests revealed that *leuS* mutations also decreased sensitivity to other antibiotic classes, including chloramphenicol, rifampicin, mecillinam, ampicillin, and trimethoprim (Garoff et al., 2018). Mutations in the *alaS*, *argS*, *ileS*, and *leuS* tRNA synthetase genes have also been linked to *E. coli*'s resistance to the antibiotic novobiocin (Milija et al., 1999). Vinella et al. (1993) reported a mutant of *E. coli* aminoacyl-tRNA synthetase that was resistant to amdinocillin (mecillinam), a beta-lactam antibiotic that binds to penicillin-binding protein 2 (PBP2), preventing cell wall elongation and leading to cell death.

Novikova et al. (2007) conducted a study analyzing a random transposon library using the antibiotic Microcin C (McC), which targets aspartyl-tRNA synthetase, and identified McC-resistant *E. coli* mutants. The study found that *yej* gene mutations interfere with McC uptake. YejABEF, the inner membrane transporter, was identified as responsible for McC uptake in *E. coli*.

Targeting aminoacyl tRNA synthetases with specific antibiotics, including boron-containing compounds, may lead to effective treatment strategies, especially against *Mycobacterium* species. Engineering structural variations has been shown to be effective in fine-tuning the antibacterial properties of these compounds, opening up new possibilities for combating bacterial infections (Cardenas, 2023). The effects of EP and other boron-containing antibiotics on bacterial cells should be studied across various microorganism species. The results of this study will likely prove useful in this field.

In summary, the results of the current study contribute novel insights into the impact of EP, a boron-containing antibiotic, on *E. coli*, unraveling molecular mechanisms governing cellular responses. This is particularly relevant when the level of an aminoacyl-tRNA synthetase, namely LeuS, is increased. In conclusion, the integrated proteomic and transcriptomic approach provides a comprehensive understanding of the molecular dynamics induced by EP in an *E. coli* strain overexpressing *leuS*. The genes and proteins discussed above offer a foundation for additional research into the broader impacts of EP and its potential in combating antibiotic-resistant bacteria.

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