TROPICAL LIFE SCIENCES RESEARCH

A Meta-Analysis Approach to Understanding Stress Response and Cross-Protection in *Escherichia coli*

Authors:

Maria Genesis M. Acosta and Gil M. Penuliar*

***Correspondence:** gmpenuliar@up.edu.ph

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Highlights

- The meta-analysis of similar platform microarrays highlights key shared and stress-specific transcriptional responses of *E. coli* under various stressors, emphasizing its importance in understanding bacterial adaptation.

- The study identifies *ghoS* as a novel potential cross-protection gene, consistently responsive to antibiotic, oxidative, and osmotic stress.

- Cross-protection was linked to activation of SOS/DNA-repair and heat-shock pathways, along with repression of amino acid, nucleotide, and sulfur metabolism, illustrating a metabolic shift that enhances bacterial survival under stress.

EARLY VIEW

A Meta-Analysis Approach to Understanding Stress Response and Cross-Protection in *Escherichia coli*

Maria Genesis M. Acosta and Gil M. Penuliar*

Institute of Biology, National Science Complex, College of Science, University of the Philippines Diliman, Diliman, 1101 Quezon City, Philippines

***Corresponding author:** gmpenuliar@up.edu.ph

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Abstract: *Escherichia coli* is a Gram-negative bacterium capable of causing gastrointestinal diseases and responding to various environmental stressors. Previous studies, however, are often insufficient in evaluating how antimicrobials, alcohols, oxidants, and osmotics affect the overall fitness of *E. coli* when exposed to secondary stress conditions. The objective of this study was to investigate the molecular mechanisms involved in the cross-protective response of *E. coli* to multiple stress factors, through a comprehensive meta-analysis of microarray datasets. Gene expression data were acquired from the GEO database. Datasets were screened and processed based on published inclusion criteria and methodologies. Unsupervised clustering methods using k-means and hierarchical algorithms were used to visualize the expression patterns of genes, and to compare the output of the algorithms. The expression of genes were considered as significantly different based on the following criteria: (a) a p-value < 0.05, (b) an FDR value < 0.05, and (c) a fold change > 2. Identified DEGs were further examined through in silico functional and pathway analyses. The stress response of *E. coli* involves the upregulation of genes related to GhoT/GhoS antitoxin/toxin system, SOS response, DNA damage and repair, cold and heat shock proteins, and phage shock proteins that protect cells from stress and cause tolerance to other types of stress. The metabolism of *E. coli* was also altered in response to stress. Some genes responsible for synthesizing and utilizing trehalose, cysteine, nucleoside monophosphate, vitamin B12 iron-sulfur cluster, and

sulfur assimilation were significantly downregulated when exposed to unfavorable environments. Enriched pathways were identified in downregulated genes, which were related to metabolic pathways, biosynthesis of metabolites, and ABC transporters. These findings provide a comprehensive understanding of the molecular basis of cross-protection in *E. coli*, and suggest that metabolic shifts play a crucial role in the adaptation of *E. coli* to adverse environments.

Keywords: Meta-analysis, *Escherichia coli*, Transcription Profiles, Cross-protection

INTRODUCTION

Escherichia coli is a significant cause of morbidity and mortality across the world (Croxen et al. 2013). Infection with may lead to diarrhea, which is the second leading cause of morbidity in children under 5 years old (Farthing *et al*. 2013). Outbreaks occur in both developed and developing countries, and their impact on the healthcare system can be significant (Qadri *et al*. 2005). Russo and Johnson (2003) reported that billions of dollars are spent on health care and millions of working hours are lost annually due to infections and outbreaks caused by pathogenic *E. coli* (Adley & Ryan 2016).

Commensal and pathogenic *E. coli* experience fluctuations in environmental conditions when transmitted from their reservoirs to the gastrointestinal tract of their hosts (Lambrecht *et al*. 2019) Their adaptive response mechanisms are activated to survive these changes, which involve the differential expression of stress response genes (Mitchell *et al*. 2009).

Antimicrobials are generally used to treat infections caused by pathogenic microorganisms. These agents include antibacterials, antifungals, antivirals, and antiparasitics, with the purpose of either killing or slowing down the growth of microorganisms (WHO 2021). Antimicrobials are made from small molecular scaffolds that target critical cellular processes, activate alterations in bacterial metabolism and global gene expression, and promote the activation of stress response genes (Belenky *et al*., 2015). The activation of transcriptional regulators upregulates genes that are responsible for bacterial survival processes (Dawan & Ahn 2022). According to Mitosch *et al.* (2017), this cellular response may confer cross-protection, enabling *E. coli* to effectively withstand secondary stressors like acid, heat, oxidative, or osmotic stress. The investigators used *E. coli* to observe its genome-wide transcriptional response to four antibiotics, trimethoprim, tetracycline, nitrofurantoin, and chloramphenicol. The highlight of their study shows that the exposure of *E. coli* to trimethoprim led to the development of a rapid acid stress response, which protected the bacteria against subsequent exposure to acid. Reports, however, remain limited as to whether the changes in

gene expression induced by antibiotics can induce cross-protection against a second form of stress, such as heat, acid, oxidative, and osmotic stress (Mitosch *et al*. 2017).

Escherichia coli is the most studied microorganism and is often used as a model to study stress response in bacteria (Andersson 2016). It is used in studying gene expression and metabolic analysis, because of its simple nutritional requirements, small genome size, rapid growth, and ability to adapt in different environments (Cooper 2000). *E. coli* exhbits a high level of resistance to different classes of antimicrobial agents. (Vila *et al*. 2016), and serves as a donor and recipient of resistance genes, which often result in treatment failures (Poirel *et al*. 2018). AMR in *E. coli* is currently one of the most significant public health concerns and complicates the treatment of diseases caused by *E. coli* (Cepas & Soto 2020).

Different transcriptomic technologies are utilized to measure the expression levels of various genes. Microarrays and RNA sequencing (RNA-seq) are the predominant existing techniques that were developed during the 1990s and 2000s (Nelson 2001; Wang *et al.* 2009). Both approaches offer a comprehensive perspective on transcriptome activity; however, they vary in terms of procedure, data output, cost, and sensitivity (Lowe *et al.* 2017). RNA-seq offers an inclusive view of the transcriptome through sequencing RNA molecules that lead to the detection of novel transcripts and measurement of gene expression (Kukurba & Montgomery 2015). It has turned into a common tool in biological and medical research, offering benefits compared to microarrays (Haque *et al.* 2017; Liu *et al.* 2021). Microarray, on the other hand, studies gene expression by measuring the hybridization of complementary RNA sequences to identified probes. It allows for the identification of novel and highly reproducible gene expression patterns across a vast number of samples (Mantione *et al*. 2014). It is also known for its well-established method, standardized protocol, and data processing pipelines.

Despite the cost-effectiveness of RNA sequencing, microarrays continue to play a vital role in large-scale and cost-efficient studies for specific applications, especially when working with well-annotated genomes, like *E. coli* (Kim *et al.* 2024). Several studies have utilized microarray technology in cancer research to identify biomarkers and develop personalized drug therapies (Shi 2024; Tao *et al.* 2017). A review article has noted the evolving dynamics in this field, recognizing that while RNA sequencing is becoming more prevalent, microarrays remain reliable and economical for certain applications (Mantione *et al.* 2014). In cases where genomes are thoroughly annotated and specific targets are identified, microarrays offer dependable normalization across samples and continue to be valuable for retrospective and meta-analytic research, even as the use of RNA sequencing grows (Kim *et al.* 2024).

Studies have used microarray to evaluate the differential expression of genes of *E. coli* under various forms of stress (Allen *et al*. 2008). An integrative approach is lacking, however, in investigating the effects of antimicrobial agents in the overall fitness of *E. coli* when exposed

to other types of stress. The general objective of this study was to identify genes in *E. coli* potentially involved in cross-protection using in silico meta-analysis of published microarray datasets and to predict protein interactions that may underlie this mechanism. By understanding the molecular basis of cross-protection, we can gain insights into the complex mechanisms underlying bacterial survival and adaptation to multiple stresses, which may provide novel targets for the development of effective antimicrobial therapies.

METHODOLOGY

Data Selection of Microarray Datasets For Meta-Analysis

Microarray datasets were systematically retrieved from the NCBI-GEO repository, adhering to MIAME guidelines (Brazma *et al*. 2003). Based on MIAME guidelines, microarray studies should provide information about the experimental design, array design, source of the original sample, hybridization parameters, measurements of actual experimental results, and normalization controls. In addition, the following filter criteria were used based on the study of Fajarda *et al.* (2020): (a) Organism: *Escherichia coli*, (b) Study type: Expression profiling by array, (c) Affymetrix platform, and (d) Raw expression profiles (.cel files).

A single platform was applied to reduce technical variability across studies, which is one of the main concerns in transcriptomic meta-analysis (Campain & Yang, 2010). The Affymetrix platform offers standardized probe designs, well-established protocols, and extensive data availability in public repositories. Having datasets with a single platform allows consistent normalization and efficient probe annotation (Gao *et al.* 2025). Platform-specific method improves accuracy and reproducibility of the meta-analytic output through minimizing batch effects and enhancing annotation (Poli-Neto *et al.* 2020). It also allows comparable gene lists from different microarray studies. Data retrieval, preprocessing, statistical analyses, and visualization were performed using R studio (version 4.1.2) (Chambers 2008). The affy, clusterSim, limma, and FDRestimation packages were utilized for specific computational tasks (Murray & Blume 2021). Raw expression profiles in CEL format were retrieved using the GEOquery package and converted into a gene expression matrix for subsequent meta-analysis (Davis & Meltzer 2007).

Pre-processing of Datasets and Visualization of Data Quality

To ensure data comparability, probe set identifiers were converted to gene symbols, following the guidelines of Ramasamy *et al*. (2008). Gene expression profiles of shared genes across studies were normalized and transformed to eliminate technical variation and facilitate

meaningful comparisons (Kim *et al*. 2010). Raw CEL files were pre-processed using the Robust Multi-Array Average (RMA) algorithm in the affy package (Gautier *et al*. 2004), which corrected for background noise, normalized data, and summarized probe-level intensities. Probe IDs were mapped to gene symbols using the GEOquery package. For genes with multiple probes, the probe with the highest interquartile range was selected, a robust approach recommended by Hahne *et al*. (2010). To assess data quality, diagnostic plots were generated, as described by Ritchie *et al*. (2015). Box plots of normalized and transformed expression values were used to evaluate distribution and variability across datasets.

Identification of Co-regulated Genes

To identify co-regulated genes, gene-based and sample-based clustering analyses were performed. Gene-based clustering grouped genes with similar expression patterns across different stress conditions, while sample-based clustering compared expression patterns between conditions. Unsupervised machine learning techniques, including k-means and hierarchical clustering, were employed. K-means clustering partitioned genes into a specified number of clusters, determined using the elbow method. The algorithm iteratively assigns genes to the nearest cluster centroid, minimizing intra-cluster distances (Li & Wu 2012). Hierarchical clustering, on the other hand, creates a hierarchy of clusters, represented as a dendrogram. This method merges clusters based on inter-cluster distances (Murtagh & Contreras 2012). To assess the overall impact of stress, the average number of upregulated and downregulated differentially expressed genes (DEGs) per strain was calculated.

Identification of Differentially Expressed Genes

To identify genes differentially expressed in response to various stressors, a linear model with empirical Bayes moderation was employed (Li *et al*. 2015). This approach improves statistical power and reduces false positives, particularly for genes with low expression levels. P-values were adjusted using the Benjamini-Hochberg FDR correction to control for multiple testing. Design blocks were incorporated into the linear model to account for experimental design factors. Genes with an adjusted p-value < 0.05 and a fold change (FC) > 2 were considered significantly differentially expressed. Top hits were defined as genes with FC > 2 and adjusted p-value < 0.05, while high-fold genes had FC > 5 and adjusted p-value < 0.01. Heatmaps were generated to visualize the expression patterns of differentially expressed genes across different stress conditions, including antibiotic, oxidative, osmotic, and alcohol stress (Chung *et al*. 2006). Volcano plots were also created to compare upregulated and downregulated genes. All visualizations were generated using R.

Identification of Cross-Protective Genes

Upregulated and downregulated DEGs were analyzed to identify genes significantly affected by multiple stress conditions. Stress conditions were categorized as antibiotic, oxidative, osmotic, and alcohol (Chung *et al*. 2006). Functional annotations for DEGs were obtained from the EcoCyc *E. coli* Database (Karp *et al*. 2002). Genes differentially expressed in two or more stress conditions were considered potential cross-protective genes, potentially contributing to the stress response and enhancing survival under diverse stressors (Abdelwahed *et al*. 2022). A web-based Venn diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to visualize genes significantly affected by multiple stresses.

Functional Enrichment Analysis of Differentially Expressed Genes

Gene Ontology (GO) enrichment analysis was performed on upregulated and downregulated cross-protective differentially expressed genes (CP-DEGs) to identify enriched biological processes, cellular components, and molecular functions. The STRING database (https://string-db.org) was used for GO analysis, providing GO terms with Benjamini-Hochberg corrected p-values (FDR) for multiple testing correction. Significant enrichment was defined as FDR < 0.05. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was used to identify significantly enriched pathways associated with CP-DEGs. KEGG examines biochemical pathways and physiological processes affected by differential gene expression (te Pas *et al*. 2008). Pathway enrichment analysis of upregulated and downregulated CP-DEGs was performed using the STRING database. Pathways with FDR < 0.05 were considered significantly enriched (Mering *et al*. 2003).

Protein-Protein Interaction Network Analysis

Protein-protein interaction (PPI) networks were constructed using the STRING database to explore potential functional relationships between CP-DEGs. A confidence score cutoff of >0.4 was applied to filter interactions (Abdelwahed *et al*. 2022). The resulting PPI network was visualized using Cytoscape (v3.9.1) (Shannon *et al*. 2003). The Molecular Complex Detection (MCODE) algorithm was used to identify highly interconnected protein modules within the network, with parameters set to k-core = 2, degree cutoff = 2, maximum depth = 100, and node score cutoff = 0.2 (Bader & Hogue 2003).

RESULTS

Data Selection, Preprocessing and Quality Assessment

A total of 2,761 *E. coli* antimicrobial datasets were identified in NCBI-GEO (Table 1). After applying rigorous selection criteria, 138 datasets from 12 studies (Appendix A) were retained for analysis. These datasets, all utilizing the Affymetrix *E. coli* Genome 2.0 Array (GPL3154), included at least three biological replicates per stress condition and encompassed four primary stress types: antibiotic, oxidative, osmotic, and alcohol (Table 2). Antibiotic stress was the most prevalent, with eight different agents. Oxidative stress included hydrogen peroxide, chlorine, and CORM, while osmotic stress involved sodium chloride and urea. Alcohol stress was primarily induced by ethanol. The 138 datasets were derived from seven different *E. coli* strains, including both laboratory and pathogenic strains (Table 3).

Raw gene expression values were preprocessed to ensure data quality and consistency. The Affymetrix *E. coli* Genome 2.0 Array platform was used across all studies. After preprocessing, 8,349 probe IDs remained for subsequent analysis. Box plots of normalized expression values (Appendix B) indicate consistent distribution and variability across replicates within each microarray study, suggesting reliable data quality and suitability for meta-analysis.

Table 1. Summary of datasets available in microarray databases.

	Gene expression omnibus
Number of Datasets	2,761
Years included	2009-2020
Published articles	2536
Datasets with identical Affymetrix platform and raw data	1956
Relevant treatment conditions	309
Replicates	163
Included in the study	138

Table 2. Distribution of microarray datasets among different stress conditions.

	Stress conditions	Number of datasets
Antibiotic stress	Ampicillin	5
	Ciprofloxacin	4
	Gentamicin	9
	Kanamycin	3
	Norfloxacin	3

	Nalidixic acid	14
	Polymyxin B sulfate	6
	Glyphosate	3
Oxidative stress	Hydrogen peroxide	6
	Chlorine	2
	Carbon monoxide-releasing molecules (CORM)	4
Osmotic stress	Sodium chloride	3
	Urea	3
Alcohol stress	Ethanol	9
	Total:	74

Table 3. Distribution of the microarray datasets based on various E. coli strains.

Strains	Number of datasets
Escherichia coli D31	18
Escherichia coli DH5-alpha	14
Escherichia coli K-12 substr. BW25113	8
Escherichia coli K12 substr. JM109	6
Escherichia coli K-12 substr. MG1655	46
Escherichia coli MC4100relA+	20
Uropathogenic *Escherichia coli* O157:H7 (EHEC)	12
Enterohemorrhagic *Escherichia coli* CFT073 (UPEC)	8
Escherichia coli Trans10-nfiS	6
Total:	138

Identification of Gene Clusters Based on Expression Patterns

K-means clustering identified three distinct gene expression patterns: high, low, and moderate expression (Appendix C, Fig. 1). Sample-based clustering revealed that specific stress conditions within a group tended to cluster together, suggesting similar gene expression responses. Interestingly, oxidative stress conditions did not cluster together, in contrast to antibiotic stress. This difference may signify the distinct nature of oxidative stress, which involves chemically different agents that activate separate regulatory pathways in *E. coli*. For example, the OxyR regulon is primarily activated by hydrogen peroxide, while the SoxRS system is activated by superoxide-producing compounds (Imlay 2013). It means that various regulons are activated through different oxidants. Furthermore, the differences in experimental design across studies, such as dose of chemical agent, growth phase, and exposure time, affect the various transcriptional responses of *E. coli* (Imlay 2013). These combined factors might explain the different clustering of oxidative stress profiles in *E. coli*. To investigate strain-

specific responses, the average number of upregulated and downregulated differentially expressed genes (DEGs) per strain was calculated (Appendix D, E). Fig. 2 shows that *E. coli* Trans10 had the highest number of DEGs, while DH5-alpha had the lowest number of upregulated DEGs. MC4100 had a high number of downregulated DEGs, and D31 had the lowest number of downregulated DEGs. Pathogenic strains like EHEC and ETEC had a higher proportion of upregulated DEGs.

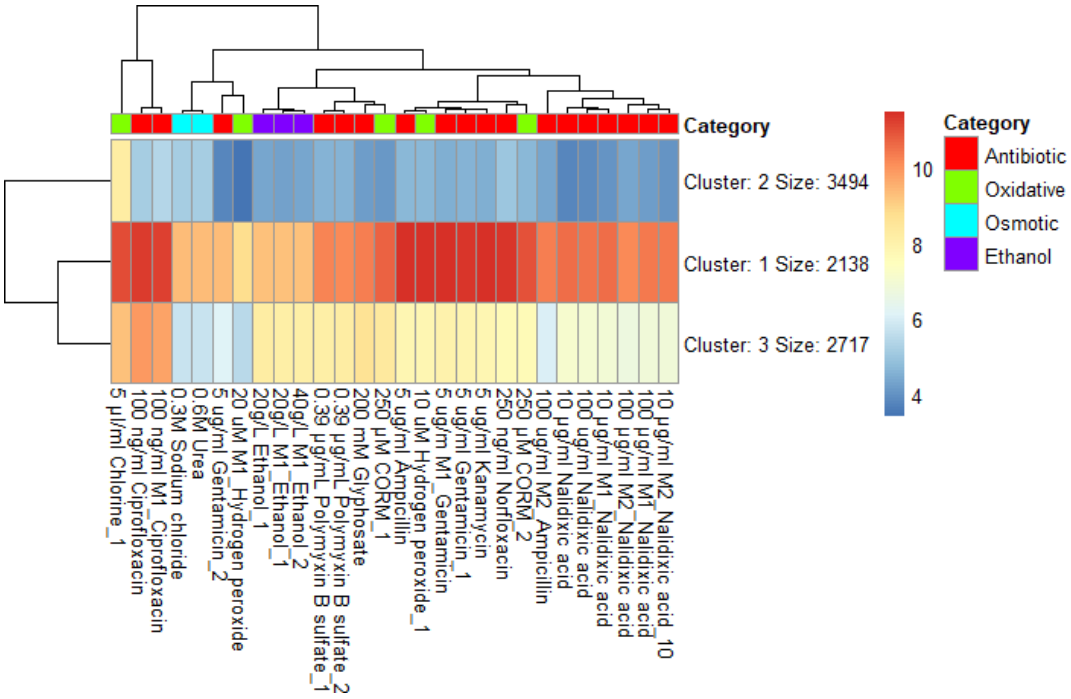


Figure 1. The clustering heatmap shows the gene expression estimates by microarray. Each column represents one treatment, and each row represents one cluster of genes based on similar expression levels. The colored dendrogram panel exhibits clustering of categorical treatments, where the treatments with related gene expression estimates were grouped.

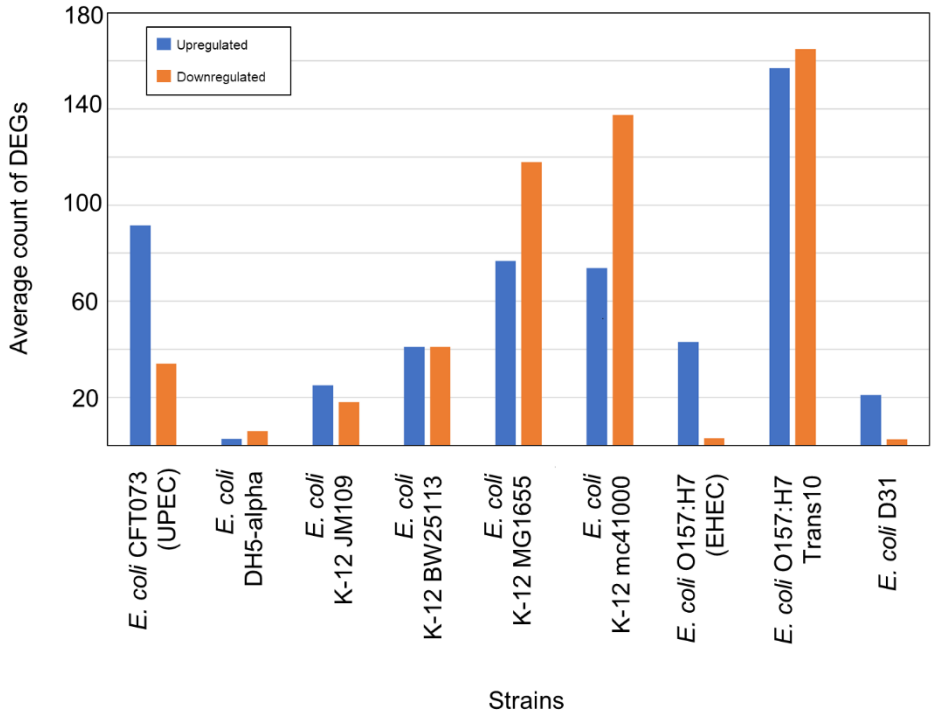


Figure 2. The average count of upregulated and downregulated DEGs per *E. coli* strain. Blue bar represents upregulated DEGs. Orange bar represents downregulated DEGs.

Differential Expression of Stress-Response Genes

Figure 3 illustrates the differential gene expression profiles under various stress conditions. Treatments with gentamicin (5 µg/ml gentamicin_2) and nalidixic acid (100 µg/ml M1_nalidixic acid and 100 µg/ml M2_nalidixic acid) in mutant *E. coli* strains, as well as hydrogen peroxide (20 µM M1_hydrogen peroxide), induced the most significant number of both upregulated and downregulated genes. Notably, the number of downregulated genes was higher in treatments involving mutant *E. coli* strains, such as 20 µM M1_hydrogen peroxide, 100 µg/ml M1_nalidixic acid, 100 µg/ml M2_nalidixic acid, and 100 µg/ml M1_ciprofloxacin. Interestingly, wild-type *E. coli* strains treated with gentamicin (5 µg/ml gentamicin_2) exhibited a higher number of differentially expressed genes compared to mutant strains under the same treatment. A similar trend was observed for ampicillin (5 µg/ml ampicillin) treatment. In contrast, both wild-type and mutant *E. coli* strains exposed to ethanol showed the fewest differentially expressed genes.

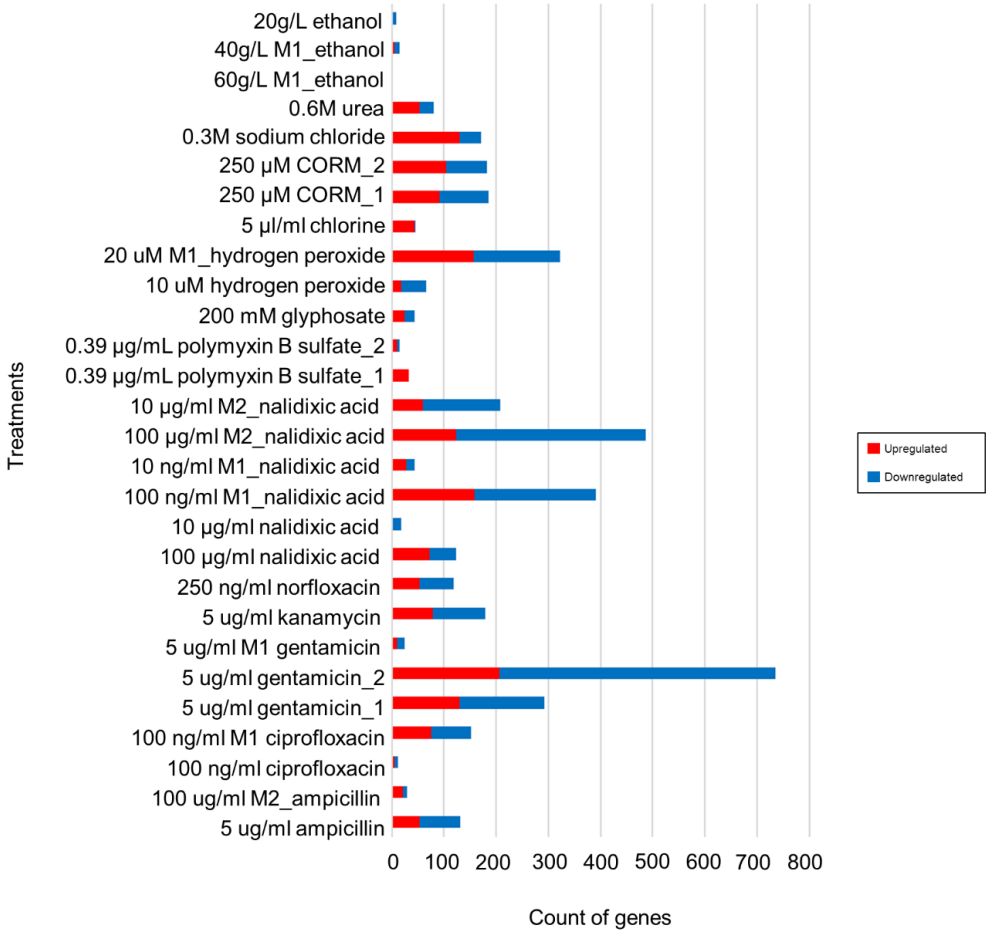


Figure 3. Overview of the differentially expressed genes under various stress conditions (cut-off fold change >2, adj. p-value < 0.05, FDR < 0.05). Treatments with the letter "M" (e.g., M1 and M2) stand for mutated *E. coli* strains. Treatments with the number in the end (e.g., treatment_1, treatment_2) signify the same treatment but a different sample and not a replicate.

Table 4 provides a detailed overview of the differentially expressed genes (DEGs) categorized into top hits (FC > 2, FDR < 0.05) and high-fold expression (FC > 5, FDR < 0.01) groups. Overall, a larger number of genes were downregulated than upregulated across most treatments. In the top hits group, 1,750 genes were upregulated and 2,363 genes were downregulated, while 35 genes were upregulated and 47 genes were downregulated in the high-fold expression group.

Table 4. The absolute number of differentially expressed genes (DEGs) after applying different stress conditions.

Category	Treatments	Top hits*		High fold**	
		Upregulated genes	Downregulated genes	Upregulated genes	Downregulated genes

Antibiotic	5 ug/ml ampicillin	54	78	0	0
	100 ug/ml M2_ampicillin	21	8	2	2
	100 ng/ml ciprofloxacin	6	6	0	0
	100 ng/ml M1_ciprofloxacin	76	76	0	0
	5 ug/ml gentamicin_1	130	162	2	1
	5 ug/ml gentamicin_2	206	529	7	27
	5 ug/ml M1_gentamicin	10	15	1	0
	5 ug/ml kanamycin	78	101	1	2
	250 ng/ml norfloxacin	53	66	7	0
	100 µg/ml nalidixic acid	73	50	0	0
	10 µg/ml nalidixic acid	1	17	0	0
	100 µg/ml M1_nalidixic acid	159	232	3	0
	10 µg/ml M1_nalidixic acid	28	15	0	0
	100 µg/ml M2_nalidixic acid	123	363	2	2
	10 µg/ml M2_nalidixic acid	59	148	0	0
	0.39 µg/mL polymyxin B sulfate_1	32	0	0	0
	0.39 µg/mL polymyxin B sulfate_2	10	5	0	0
	200 mM glyphosate	25	18	0	0

Oxidative	10 uM hydrogen peroxide	19	47	0	0
	20 uM M1_hydrogen peroxide	157	165	3	9
	5 µl/ml chlorine	43	3	0	0
	250 µM CORM_1	92	94	3	0
	250 µM CORM_2	104	79	1	2
Osmotic	0.3M sodium chloride	130	41	3	1
	0.6M urea	53	27	0	1
Alcohol	20g/L M1_ethanol	0	3	0	0
	40g/L M1_ethanol	6	9	0	0
	20g/L ethanol	2	6	0	0
	Total:	1,750	2,363	35	47

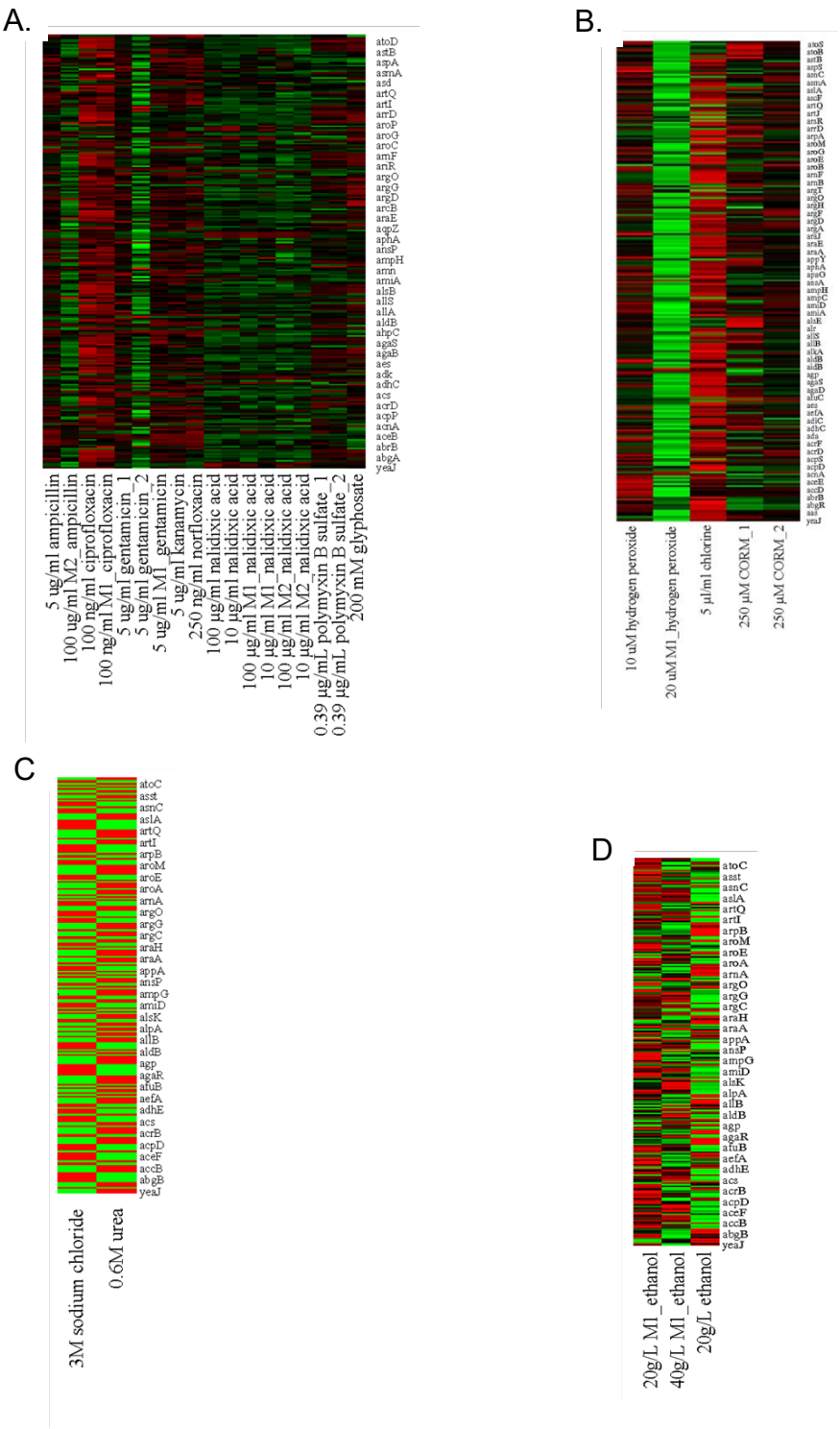


Figure 4. Heatmaps of the transcriptomic data per stress condition. A. Antibiotic; B. Oxidative; C. Osmotic; D. Alcohol. Shades of green and red represent high and low gene expression, respectively.

Under antibiotic stress, 5 µg/ml gentamicin_2 induced the highest number of both upregulated (206 genes) and downregulated (529 genes) genes in the top hits group. Furthermore, this treatment had the highest number of upregulated (7 genes) and downregulated (27 genes)

genes in the high-fold expression group. The substantial number of samples under antibiotic stress resulted in a high number of DEGs, many of which exhibited high-fold expression changes. These findings suggest that antibiotic stress, particularly 5 µg/ml gentamicin_2, has a significant impact on *E. coli* gene expression. In oxidative stress, 250 µM CORM_2 and 250 µM CORM_1 induced the highest number of upregulated (94 genes) and downregulated (94 genes) genes, respectively, in the top hits group. In the high-fold expression group, CORM_1 and CORM_2 had two downregulated genes and four upregulated genes, respectively. Chlorine treatment had the fewest DEGs in both groups. These results indicate that oxidative stress, particularly CORM_1 and CORM_2, induces substantial changes in *E. coli* gene expression.

Regarding osmotic stress, 0.3 M sodium chloride induced more DEGs than 0.6 M urea in the top hits group. However, 0.6 M urea had fewer high-fold expression changes. These findings suggest that different osmotic stressors, such as sodium chloride and urea, may elicit distinct gene expression patterns, with sodium chloride being more potent in inducing transcriptional changes (Withman *et al*. 2013). Alcohol stress had the fewest DEGs in both top hits and high-fold expression categories. However, within this stress condition, 40 g/L M1_ethanol induced the highest number of upregulated (6 genes) and downregulated (9 genes) genes in the top hits group. No high-fold expression changes were observed for any ethanol treatment. These results suggest that alcohol stress has a minimal impact on *E. coli* gene expression, except for 40 g/L M1_ethanol, which induced a moderate transcriptional response.

Heatmaps and volcano plots (Figure 4 and Appendix F) visually represent the transcriptional response of *E. coli* strains to different stressors. These analyses highlight the diverse transcriptional profiles induced by each stress condition, emphasizing the complexity of bacterial stress responses.

Identification of Potential Cross-Protective Genes

A total of 463 genes were differentially expressed under at least two stress conditions. To delve deeper into their functional roles, both upregulated and downregulated genes were categorized based on their biological functions and pathways (Tables 5 and 6).

Table 5 reveals the upregulation of 40 cellular response genes under antibiotic, oxidative, and osmotic stress. Notably, stress-response genes related to oxidative, osmotic, cold, and heat stress were significantly affected, even though the primary stressor was antibiotic. Two antibiotic resistance genes (*zraS* and *marB*) were also upregulated. Additionally, 11 genes involved in DNA damage response and three genes linked to bacterial adhesion and biofilm formation (*bdm*, *ydeT*, *ycfJ*) were induced. DNA repair genes, which play

a crucial role in stress adaptation, were also upregulated. Furthermore, stress-response genes involved in cell growth regulation and the toxin-antitoxin system were transcriptionally upregulated.

Table 6 shows a general downregulation of metabolic pathways at the transcriptional level. Specifically, genes involved in transport systems, sulfur metabolism, amino acid, and carbohydrate biosynthesis were downregulated. Antibiotic and oxidative stress induced significant downregulation of 11 genes involved in carbohydrate metabolism and seven genes associated with sulfur metabolism. Additionally, different types of antibiotics affected the transcription of genes responsible for vitamin B12 biosynthesis. These findings suggest that antibiotics and oxidative stress can have broad effects on metabolic pathways and the biosynthesis of essential compounds.

A total of 126 genes were upregulated under at least two stress conditions, regardless of the specific treatment (gentamicin, hydrogen peroxide, or different concentrations of nalidixic acid). Functional categorization of these upregulated cross-protective DEGs (CP-DEGs) (Figure 5A) revealed that 31% were involved in responses to DNA damage, multiple stress, antibiotic resistance, oxidative, osmotic, cold, and heat stress. Approximately 24% were involved in metabolic pathways, 18.6% in transcription regulation, 15.5% in transport, 4.7% in growth regulation, 3.9% in DNA repair, and 2.3% in motility. These observations suggest that these CP-DEGs may play crucial roles in microbial stress response, antimicrobial resistance, and survival under various stress conditions, likely through complex interactions within biological pathways.

A total of 334 genes were downregulated across different stress conditions. Of these, 38.6% were involved in metabolic processes, 19.5% in transport, 8.1% in cellular response, and 8.1% in transcription regulation. Additionally, 3.6% were associated with vitamin biosynthesis, 2.7% with peptidoglycan synthesis, 1.8% with other functions, 0.9% with cell adhesion, 0.6% with cell communication, 0.6% with DNA repair, and 0.3% with cell wall modification (Figure 5B). Interestingly, 40 putative genes (12%) were affected by both ampicillin and nalidixic acid treatments, suggesting a potential role in responding to multiple stress conditions. These putative genes were identified based on sequence similarity to known genes in the EcoCyc database, as described by Karp *et al*. (1999). A small number of genes were differentially expressed under alcohol stress, limiting the identification of potential cross-protective genes against this stressor.

Table 5. Upregulated gene functions significantly affected by at least 2 stress conditions.

Functional category	Functional type	Gene symbols	Count of genes	Antibiotic	Oxidative	Osmotic	Ethanol
Cellular response	DNA damage	*dinD, dinF, dinI, yebG, recX, mutM, fumB, sulA, symE, ybiJ, ybfE*	11	norfloxacin, gentamicin, nalidixic acid			
	Multiple stress	*cpxP, pspB, pspG, pspA, pspD, pspC, degP, sodA, bhsA, yjaA*	10	gentamicin, nalidixic acid	CORM		
	Antibiotic resistance	*zraS, marB*	2	gentamicin, kanamycin, nalidixic acid	CORM, hydrogen		
	Oxidative stress	*fumC, yqhD, yhcN, ytfK*	4	gentamicin, nalidixic acid			
	Osmotic stress	*osmB, osmY*	2	Ampicilin, Polymyxin, Glyphosate			
	Cold stress	*cspG, cspF, ydjR, ynaE*	4	gentamicin		urea	
	Heat stress	*ibpB, ibpA, hslJ, dnaJ, hslR, yccV, ybeD*	7	gentamicin, kanamycin	CORM		
Metabolic processes	Transferases	*upp, gpt, wcaE, eptB, lipB, pspE, yjhQ, ygfJ*	8	gentamicin	hydrogen peroxide		
	Hydroxylase	*trhO*	1	ampicilin	hydrogen peroxide		
	Transpeptidase	*ldtC*	1	gentamicin	CORM		
	Hydrogenase synthesis	*hyaF, hypE*	2	gentamicin, nalidixic acid			
	Cellular respiration	*fdoG, hycB, hycC*	3	nalidixic acid			

	Lipid metabolism	*fadE, yciA, fadD, citE, citD, citC*	6	kanamycin, nalidixic acid		
	Nucleic acid biosynthesis	*pyrL, add, nrdB, rihA, nrdA, mcrC, queG, deoA*	8	nalidixic acid		
	Nitrogen process	*yjjI, nirD*	2	nalidixic acid		sodium chloride
Transport	Carbohydrates	*gntP, uhpT, rbsA, rbsB, malE, yfdL*	6	nalidixic acid	hydrogen peroxide	
	Sulfur, sulfite	*ghoS, cysU, cysW, cysA, cysP, yeeE, sbp*	7		CORM, hydrogen peroxide	
	Nucleoside	*punC*	1	nalidixic acid		urea
	Autoinducer	*tqsA*	1	gentamicin	CORM	
	Efflux pump	*yojI, aaeA, aaeB, macB*	4	nalidixic acid		
Growth regulation		*ecnA, yjdK, ghoT, higB, ghoS*	5	nalidixic acid	sodium chloride	
Transcription regulation	small regulatory RNAs	*gadY, glmZ, micF, omrA, omrB, oxyS, rprA, rttR, rydB, sgrS*	10	gentamicin, nalidixic acid		
	repressors	*arsR, marR, puuR, uxuR, lacI, lexA, frmR, nfeR, stpA, frmR, mqsA, nemR, nfeR, mgrB*	14	gentamicin	CORM	
DNA repair		*polB, dinB, umuC, umuD, recN*	5	norfloxacin, nalidixic acid		
Motility		*bdm, ydeT, ycfJ*	3	ampicillin, polymyxin B sulfate	CORM	

Note: specific treatment denotes half or more of the genes within a functional category are differentially expressed. Genes were regarded as significantly differentially expressed if the p-value is < 0.05, FDR value is < 0.05, and the fold change cut off (FC) is > 2.

Table 6. Downregulated gene functions significantly affected by at least 2 stress conditions.

Functional category	Functional type	Gene symbols	Count of genes	Antibiotic	Oxidative	Osmotic	Ethanol
Transport	Iron	*efeB, fhuE, feoC, fhuF, feoA, feoB*	6	gentamicin, nalidixic acid			
	Sugar	*alsA, alsB, gudP, fruB, malK, malM, btsT, lamB, malE, malF, malG, srlA*	12	gentamicin, kanamycin			
	Amino acid	*aroP, artI, artJ, artM, artP, artQ, cydC, dppA, gsiA, gstB, ybjE, oppC, oppF, mppA, yddG, gadC, tyrP, tcyJ, shiA, napD, uraA, proV, dtpB, livG, nlpA, oppB, oppD, osmF, potC, potD, potF, tauA, tauB, dtpA, yeeO*	35	nalidixic acid, gentamicin			
	Fatty acids	*dcuC, focA*	2	ampicillin, gentamicin, kanamycin	hydrogen peroxide		
	Nitrogen, nitrite	*sapF, narK*	2	ampicillin, gentamicin, kanamycin	hydrogen peroxide		
	Sulfur, sulfate	*sbp, cysA, cysP, cysW, yeeE*	5		hydrogen peroxide	urea	
	Vitamin	*btuD*	1	gentamicin, nalidixic acid			
	Efflux pump	*mdtM*	1	nalidixic acid			
Metabolic pathways	Amino acid, protein	*argD, aroD, aroG, carA, carB, cysM, thrL, speD, glsA, speG, ppsR, ynjE, gdhA, alaC, hypB, hypC, ppnN, serA, npr, metA, tyrB, gltB, gltD, hisL, ilvH, ilvI, pheA, pheS, pheT, purI, pyrB, rimL, sppA, trpA*	34	gentamicin, nalidixic acid			
	Carbohydrate	*amyA, dhaL, dhaK, dhaM, galU, otsA, otsB, gatD, talA, malP, glgB, ghrB, rffH, ppc, melA, fruK, gldA, gnd, opgD, nadE, pgl, poxB, waaL, sdhC, sdhD, tktB, treF*	27	gentamicin, nalidixic acid			
	Nucleic acid	*pyrC, rluC, nrdA, nrdE, yjdK, pyrI, guaB, hsdR, hsdS, nrdF, purD, purU, yfcI*	13	gentamicin, nalidixic acid	CORM		
	Fatty acids	*aceA, aceB, aroH, atoS, serC, yciA, dmlA, garK, garL*	9	gentamicin			

	Lipids	*blc, cfa, eptC*	3	gentamicin, nalidixic acid	
	Sulfur	*cysC, cysD, cysH, cysI, cysJ, cysN, cysQ, sufE, sufS, sufD, sufB, sufC*	12	gentamicin, nalidixic acid	hydrogen peroxide
	Nitrogen	*sad, nirB, glnD, napA, narG, nrfA*	6	ampicillin, gentamicin, kanamycin	hydrogen peroxide
	Oxidoreductase	*msrC, yahK, curA*	3	salidixic acid	
	Inhibitor	*ycgK, pdeR*	2	salidixic acid	
Catabolic pathway	Amino acid	*ansB, astA, astD, dadA, dadX, dcp, ltaE, hinT, gadB, glsB, grcA, gcvP, tdcB, gadA, gcvT*	15	nalidixic	CORM
	Nucleic acid	*codA, ydfG, rne, rnlA*	4	nalidixic	
	Fatty acids	*garR*	1	kanamycin, gentamicin	
Cell adhesion		*csgA, dgcM, uspF*	3	nalidixic acid	
Cellular reponse		*asr, bcp, blr, btuE, cspG, dps, iraP, ycbX, mdtH, ychH, tehB, ydeI, nth, osmE, yeaG, yeaH, tar, yodD, zinT, napF, elaB, uspB, hdeB, katE, pphA, wrbA, zraP*	27	gentamicin, nalidixic acid	
Cell communication		*lsrG, rstB*	2	norfloxacin, gentamicin, nalidixic acid	
Cell division		*zapC, minD, minC, minE, mraY, murD, murE, mur*	8	gentamicin, nalidixic acid	
Cell wall modification		*ybhC*	1	gentamicin, nalidixic acid	
DNA repair		*yegP, RecD*	2	gentamicin, nalidixic acid	
Peptidoglycan		*dacC, mepK, mpaA, mepH, mipA, mepM, ldtA, elyC, ldtE*	9	nalidixic acid	
Transcription	Activator	*adiY, appY, atoC, cbl, cdaR, tdcA*	6	kanamycin, gentamicin	
	Repressor	*dgsA, lsrR, purR, hipB, putA, alsR*	6	nalidixic acid	
	Regulator	*csgD, cysB, yciT, mcbR, rstA, malY, yeaT, flhC, nac, btsR, gadW, gadX, ravA, melR, tyrR*	15	gentamicin, nalidixic acid	
Regulatory protein		*glnK, hnr*	2	gentamicin, nalidixic acid	
Vitamin biosynthesis		*bioA, bioB, bioD, bioF, cobS, cobT, cobU, folM, pdxH, ribC, menE, ribB*	12	gentamicin, nalidixic acid	

Others	*mocA, garD, slp, bluF, yhbU, yhhY*	6	ampicillin, gentamicin
Putative genes	*abrB, yncG, ydgD, ydhY, yiaG, yjgM, ytjA, chaB, yahN, ybaT, ybdK, ycaC, ycaD, yciW, ydcK, yddB, yneJ, ynfE, ydiJ, ydiK, yniA, yedE, yodB, tsuB, gatZ, yohC, elaA, yphA, yiaI, ptsA, psiE, lgoR, gcvR, ptsA, ycaN, yedL, yehD, ygjR, yjiM, yjiT*	40	nalidixic acid, ampicillin

Note: specific treatment denotes half or more of the genes within a functional category are differentially expressed. Genes were regarded as significantly differentially expressed if the p-value is < 0.05, FDR value is < 0.05, and the fold change cut off (FC) is > 2.

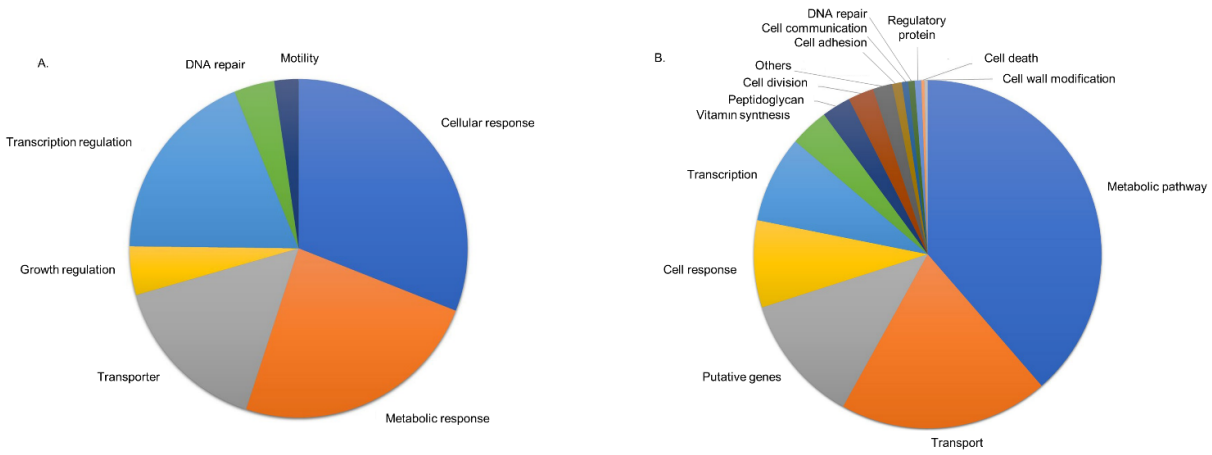


Figure 5. Distribution of functional categories among (A) upregulated genes and (B) downregulated genes common between at least two stress conditions.

Venn diagrams (Figure 6) illustrate the extent of shared gene expression between different treatments. Figure 6A shows that only one gene, *ghoS*, was upregulated under all three stress conditions. This gene encodes the antitoxin of the GhoT/GhoS toxin-antitoxin system. A total of 51 genes were commonly upregulated under both antibiotic and oxidative stress, while 11 were upregulated under both antibiotic and osmotic stress. Figure 6B shows that no genes were commonly downregulated under all three stress conditions. However, 36 genes were commonly downregulated under both antibiotic and oxidative stress, and five genes were commonly downregulated under both oxidative and osmotic stress. These findings provide insights into the unique and shared molecular responses to different stressors, informing future studies on stress adaptation and resilience in various biological systems.

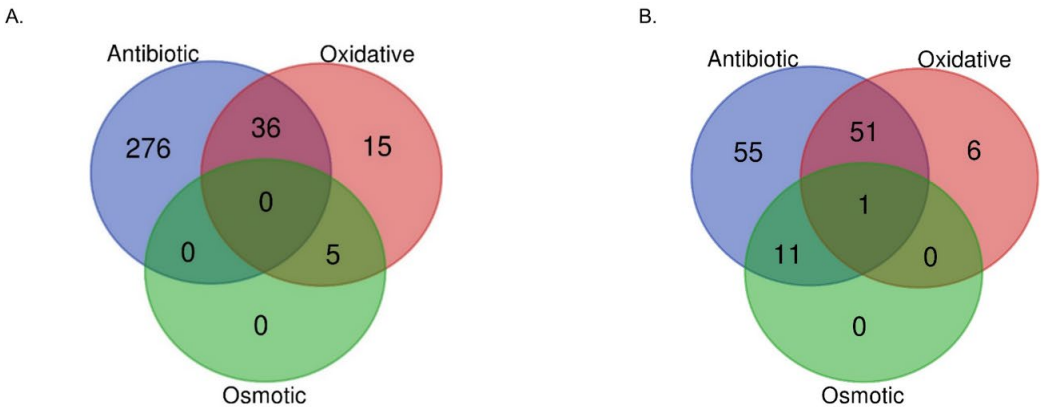
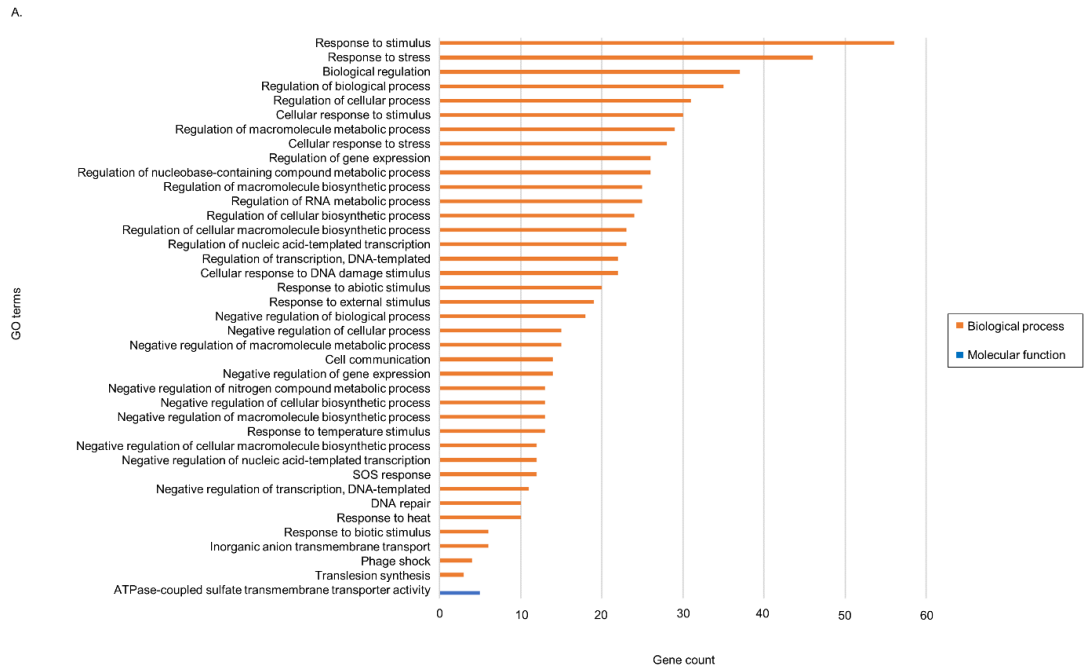


Figure 6. Venn diagrams displaying potential CP-DEGs between stress conditions. A. upregulated genes; B. downregulated genes.

Functional Analysis of Differentially Expressed Genes

To elucidate the potential functions of cross-protective differentially expressed genes (CP-DEGs), Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed using the STRING online tool (Appendices G-M). Identifying enriched biological processes, cellular components, molecular functions, and pathways is crucial for understanding the role of these genes in *E. coli* physiology, pathogenicity, and stress adaptation. GO and KEGG analyses revealed potential cellular mechanisms and pathways that provide insights into the molecular mechanisms underlying *E. coli*'s response to various environmental stressors. These findings may have implications for developing novel therapeutic strategies.

STRING analysis identified 38 significantly enriched biological process (BP) GO-terms and one molecular function (MF) GO-term in upregulated CP-DEGs (Figure 7a). The top three BP GO-terms, based on the number of associated genes, were response to stimulus (56 genes), response to stress (46 genes), and biological regulation (37 genes) (Appendix I). The only enriched MF GO-term was ATPase-coupled sulfate transmembrane transporter activity (Appendix J). Based on the strength of enrichment, the top three BP GO-terms were phage shock (1.56), translesion synthesis (1.56), and SOS response (1.16). These findings suggest that *E. coli* utilizes the phage shock protein response to protect itself against stress. The bacterial stress response involves DNA repair mechanisms, such as translesion synthesis, which may introduce mutations.



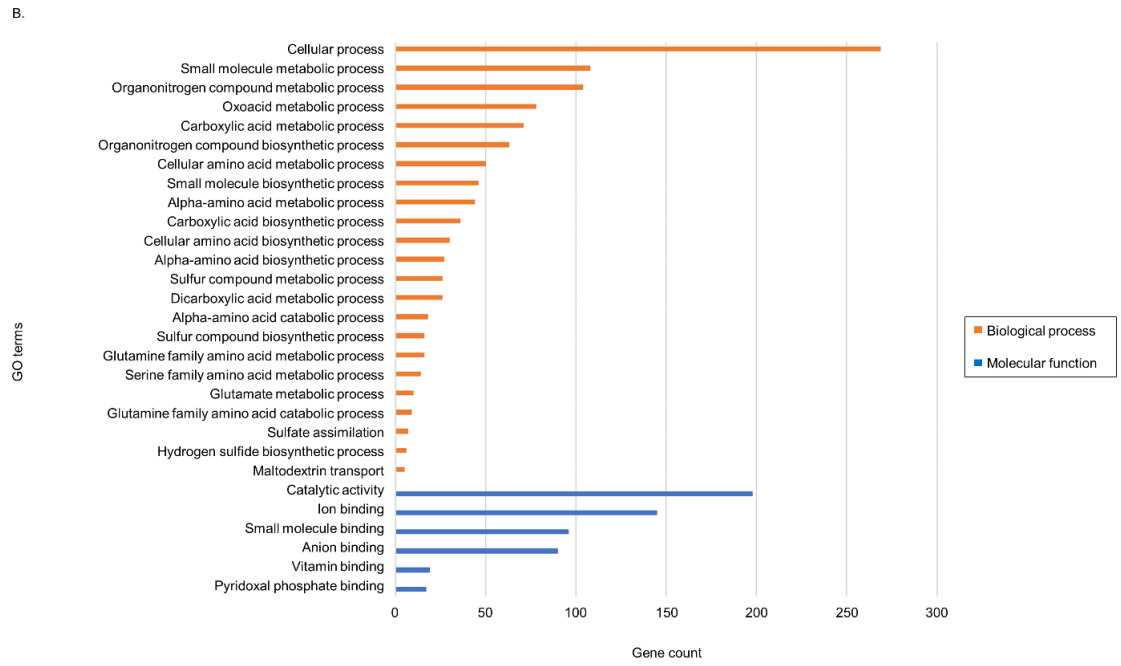


Figure 7. Gene ontology (GO) analysis of potential CP-DEGs. Orange shade represents biological processes. Blue shade pertains to molecular processes. The enriched terms were ranked based on number of genes. FDR values < 0.05 were considered significant.

Downregulated CP-DEGs were enriched in 23 BP and 6 MF GO-terms (Figure 7b). The transcriptional downregulation of genes in response to multiple stresses was primarily associated with cellular processes, organic molecule metabolism, and amino acid biosynthesis. The top three BP GO-terms, based on enrichment strength, were hydrogen sulfide biosynthesis, maltodextrin transport, and sulfate assimilation (Appendix K). The top three MF GO-terms, based on gene count, were catalytic activity (198 genes), ion binding (145 genes), and small molecule binding (96 genes) (Appendix L). In terms of GO-term enrichment, the top three MF GO-terms were pyridoxal phosphate binding (0.58), vitamin binding (0.47), and anion binding (0.19). These findings suggest that *E. coli* suppresses energy-consuming processes, such as metabolic and biosynthetic processes, under stress conditions.

KEGG analysis revealed 12 significantly enriched pathways in downregulated CP-DEGs (Figure 8). No enriched KEGG pathways were identified for upregulated CP-DEGs, likely due to the smaller number of genes and the specific pathway database used for enrichment analysis (Karp *et al*. 2021). The top three enriched pathways based on gene count were metabolic pathways (120), biosynthesis of secondary metabolites (52), and ABC transporters (35) (Appendix M). The top three enriched pathways based on strength value were glycerolipid (0.8), nitrogen (0.62), and glycine, serine, and threonine metabolism (0.53). These results indicate that the downregulated genes are commonly involved in metabolic pathways, including lipid biosynthesis, sulfur, and amino acid metabolism.

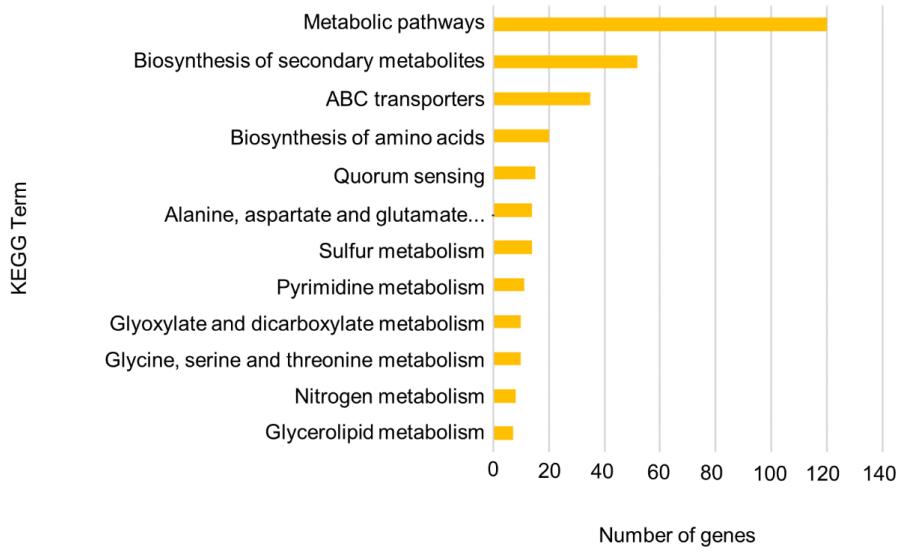


Figure 8. KEGG pathway enrichment analysis of downregulated potential CP-DEGs. The enriched terms were ranked based on the number of genes. FDR values < 0.05 were considered significant.

Protein-Protein Interaction Network Analysis of CP-DEGs

To further investigate the functional relationships among CP-DEGs, we constructed protein-protein interaction networks (PPINs) using the STRING database. DEGs identified in response to multiple stressors were used as input for STRING analysis. The resulting PPINs, visualized using Cytoscape software (v3.9.1), revealed extensive networks with numerous nodes and predicted interactions between gene products. Appendix N shows the PPIN for upregulated CP-DEGs, comprising 114 nodes and 234 edges, significantly exceeding the expected number of edges (79). Appendix O shows the PPIN for downregulated CP-DEGs, with 327 nodes and 1,197 predicted edges, surpassing the expected 609 edges. The larger number of nodes and edges in the downregulated CP-DEG network reflects the higher number of downregulated genes. Both upregulated and downregulated CP-DEGs formed networks with significantly more interactions than expected, indicating a high degree of connectivity among these genes. Both networks exhibited a PPI enrichment p-value of < 1.0e-16, suggesting that the observed interactions are not random. However, the downregulated CP-DEG network displayed a higher number of predicted connections overall. The PPIN analyses identified hub proteins that may play central roles in *E. coli*'s stress response. Figures 9A-9H depict the hub proteins for upregulated CP-DEGs, while Figures 10A-10I show the hub proteins for downregulated CP-DEGs.

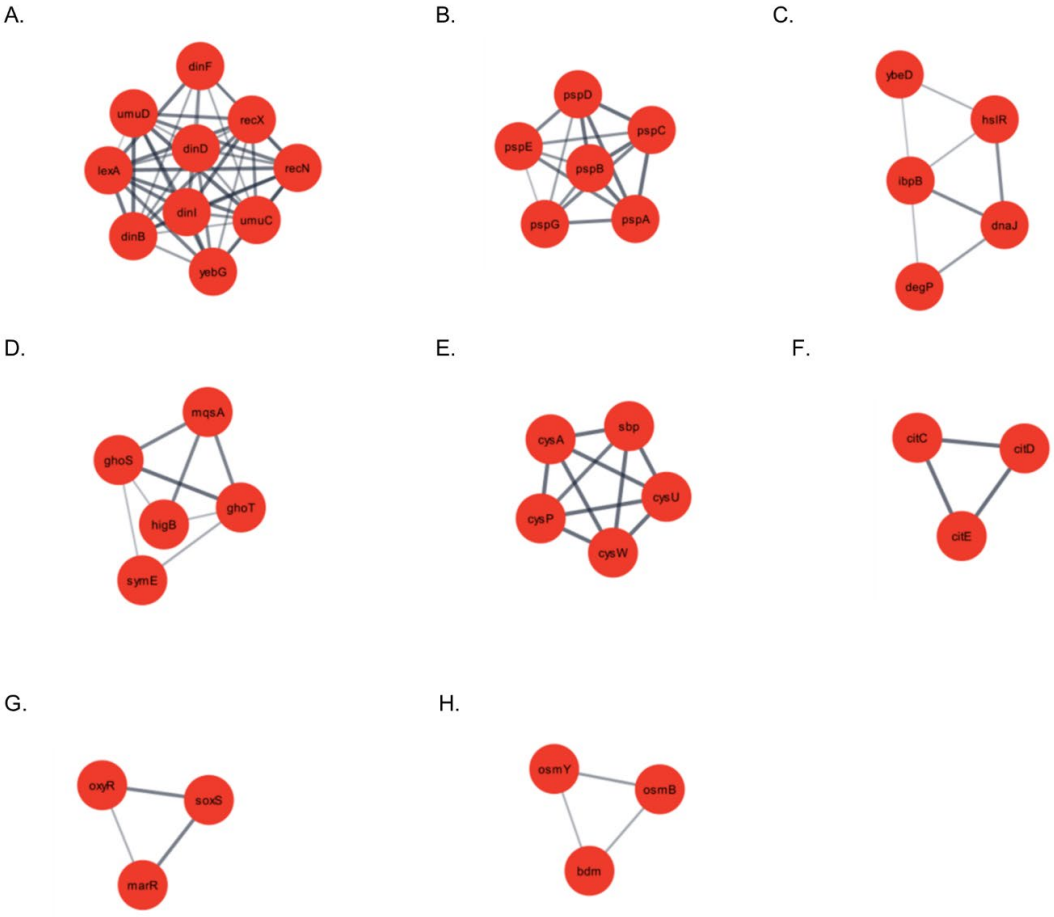


Figure 9. Eight clusters (A-I) were recognized by the MCODE clustering algorithm from upregulated CP-DEGs. Red circle represents upregulated proteins. Black line indicates predicted interaction.

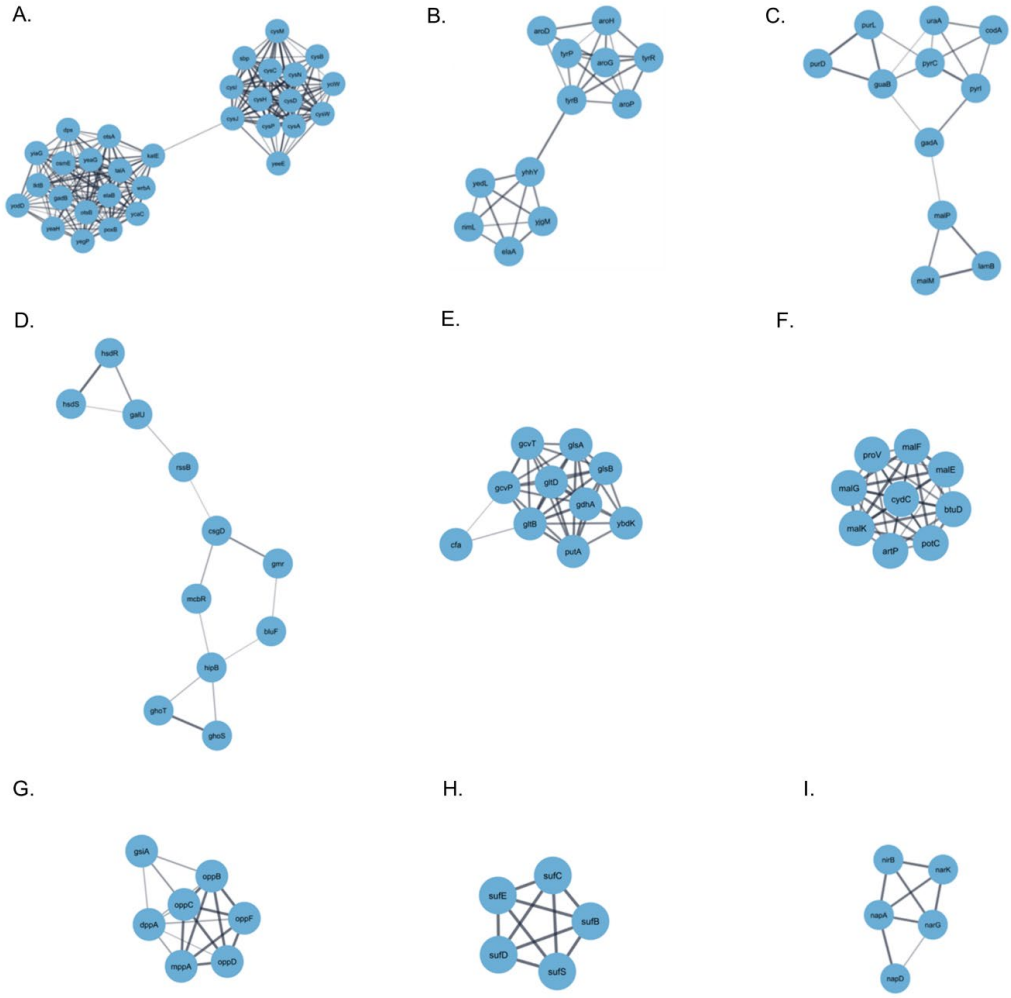


Figure 10. Nine clusters (A-I) were recognized by the MCODE clustering algorithm from downregulated CP-DEGs. Blue circle represents downregulated proteins. Black line indicates predicted interaction.

DISCUSSION

This study investigated the transcriptional stress response of *E. coli* by using a meta-analytic approach of microarray datasets under antibiotic, oxidative, and osmotic stresses. After data filtering and normalization, 8,349 probe sets were used for downstream analysis. K-means clustering of gene expression profiles identified three primary expression patterns, with samples under antibiotic stress clustering more closely compared to oxidative stress datasets, which showed greater variability in transcriptomic profiles. The results identified several DEGs under at least two stress conditions that might have cross-protection roles in *E. coli*. Interestingly, *ghoS* was identified as consistently regulated across several stress conditions.

Functional enrichment and network analysis of potential cross-protection genes revealed key stress response pathways involving DNA repair, oxidative resistance, osmoprotection, and persistence regulon (*ghoS*), giving insights into bacterial adaptation and survival mechanisms.

Differential gene expression (DEG) analysis revealed that genes within the same operon often exhibited similar expression patterns, leading to their clustering. However, it was also observed that specific genes within these operons were not consistently differentially expressed across different stress conditions. Heterogeneous expression of genes within the same operon under different stress conditions may occur due to mRNA stability and degradation, stress-specific transcriptional programming (Moll & Engelberg-Kulka 2012).

The type of media used in the assays and the specific stress conditions significantly influenced sample clustering. For instance, samples exposed to nalidixic acid and alcohol stress, with varying doses, clustered together due to the shared control conditions of media and temperature. Conversely, gentamicin-treated samples did not cluster, likely due to the use of alternative media containing additional components such as TRIS, glycerol, NaCl, and $ZnSO_4$ (Kasyap *et al*. 2017). Similarly, oxidative stress treatments did not cluster together, possibly due to the use of different media for each specific stress. Chlorine treatment, for example, was distinctly separated from others (Wang *et al*. 2009) due to the use of BHI media. In contrast, hydrogen peroxide treatments by Dwyer *et al*. (2014) and Hu *et al*. (2019) employed LB and M9 media, respectively. These findings highlight the substantial impact of media type and stress conditions on sample clustering.

Investigating the effects of various media types and stress conditions on *E. coli* provides valuable insights into its pathogenesis (Braz *et al*. 2020). By exposing *E. coli* to diverse stress factors, we can gain a better understanding of the conditions that promote antibiotic resistance and enhance pathogenicity. This study has explored the different pathotypes of *E. coli* that cause illnesses, such as gastrointestinal diseases and urinary tract infections (Pakbin *et al*. 2021). These pathotypes employ various virulence factors and effectors to disrupt host cell functions and induce disease. A notable example is *E. coli* O157:H7, a clinically significant pathogen that exhibits tolerance to acidic conditions, providing a significant fitness advantage (Van Elsas *et al*. 2011). By studying stress response genes under different stress conditions, we can develop strategies to target the cellular processes and vulnerabilities of pathogenic strains, including antibiotic-resistant bacteria (Monserrat-Martinez *et al*. 2019). One potential approach involves targeting quorum-sensing and biofilm formation, which can inhibit cell communication and prevent disease development. Peptide-based strategies that hijack bacterial autoinducers have shown promise in this regard (LaSarre & Federle 2013).

The clustering analysis revealed that norfloxacin and ciprofloxacin, two fluoroquinolones, did not cluster together. This discrepancy can be attributed to differences in

the media used (LB and M9 vs. LB only) and the *E. coli* strains employed (MG1655 vs. Trans10). Additionally, O'Rourke *et al*. (2020) emphasized that despite sharing similar mechanisms of action, antibiotics can induce distinct transcriptomic profiles in *E. coli*. Goh *et al*. (2002) proposed that the chemical structure and mode of action of an antibiotic can differentially affect gene transcription in *E. coli*. This can influence the antibiotic's activity and its ability to activate or inhibit gene transcription, particularly when using sub-inhibitory drug concentrations. These factors can impact the rate of mRNA production in response to stress.

Differential Gene Expression Between Treatments

Numerous studies have demonstrated significant alterations in gene expression profiles when *E. coli* is exposed to various stress conditions (Cao *et al*. 2017; Bhatia *et al*. 2022). In the present study, antibiotic and oxidative stress treatments induced a larger number of differentially expressed genes (DEGs) compared to other stress types. This is likely due to the higher number of samples exposed to these treatments. These findings align with the observations of Abdelwahed *et al*. (2022). In contrast, *E. coli* strains subjected to alcohol stress exhibited the fewest DEGs among all stress conditions. This result is consistent with previous research by Moen *et al*. (2009), who reported that ethanol treatment led to a relatively small number of DEGs in *E. coli*. Haft *et al*. (2014) suggested that ethanol toxicity, which can inhibit ribosome function and RNA polymerase activity, may contribute to this limited transcriptional response. Furthermore, Ferraro and Finkel (2019) demonstrated that high ethanol concentrations can induce cell death in *E. coli* K-12 strains.

A notable trend observed in this study was the predominance of downregulated genes compared to upregulated genes. This suggests that *E. coli* may downregulate genes involved in macromolecule biosynthesis and growth to conserve energy and focus on stress response mechanisms (Jozefczuk *et al*. 2010). Indeed, a significant proportion of the downregulated genes were associated with metabolic and biosynthetic processes. By allocating resources to activate stress response genes, *E. coli* can repair cellular damage and develop stress tolerance.

Strain-Specific Responses

The present study examined the stress responses of various *E. coli* strains, including pathogenic, non-pathogenic, and mutant strains. Previous research has shown that different *E. coli* strains can exhibit distinct stress response profiles (Peng *et al*. 2014). Stress exposure can lead to the development of stress resistance through mechanisms such as increased efflux pump activity (Langsrud *et al*. 2004). Moreover, the nature and intensity of the stress

response can vary significantly between pathogenic and non-pathogenic *E. coli* strains (Chung *et al*. 2006). Withman *et al*. (2013) investigated the stress response of uropathogenic *E. coli* CFT073 (UPEC) to sodium chloride and urea. UPEC, a causative agent of urinary tract infections, exhibited differential responses to these osmotic stressors. While genes associated with the osmotic stress response were upregulated in response to salt stress, a similar response was not observed in urea-treated cells. Additionally, genes involved in anaerobic metabolism were overexpressed under salt stress conditions. In contrast, urea stress induced the upregulation of molecular chaperone genes. Interestingly, urea exposure was also found to stimulate the expression of colonization factors, such as type 1 fimbriae, in UPEC.

Hu *et al*. (2019) studied the oxidative stress response of a mutant *E. coli* strain, Trans10, focusing on the role of the *nfiS* gene. The transcriptional profiling revealed significant alterations in the expression of 1184 genes, with 601 genes upregulated and 583 genes downregulated. These genes were associated with various cellular processes, including stress response, regulation, metabolism, transport, and membrane protein function. Notably, the mutant strain exhibited increased resistance to both oxidative and osmotic stress compared to the wild-type strain. Microarray analysis suggested that nfiS plays a crucial role in *E. coli*'s adaptation to hydrogen peroxide by protecting cells from oxidative damage. Alper and Stephanopoulos (2007) examined the ethanol tolerance of *E. coli* DH-alpha strains with mutant sigma factors. Microarray analysis revealed significant transcriptional changes in these mutant strains compared to the wild-type. The third-round mutant exhibited the most pronounced changes, with 72 differentially expressed genes. In contrast, the wild-type strain showed 354 DEGs, many of which were associated with the stress response. The mutant strains displayed pre-programmed expression of genes involved in ethanol stress tolerance, suggesting that specific mutations can enhance the expression of genes contributing to ethanol resistance.

Induction of the SOS Response

The overexpression of several stress-response genes, particularly those involved in DNA repair and the SOS response, was observed in *E. coli* strains exposed to antibiotic and oxidative stress, suggesting their important role in the stress adaptation of *E. coli*. In this study, lexA-regulated genes such as *dinD, yebG*, and *symE* were upregulated, reflecting the activation of DNA damage-inducible pathways in response to genotoxic agents like UV radiation, mitomycin C, and hydrogen peroxide (Bernstein et al., 1999; Wade et al., 2005; Kawano et al., 2007). These genes are important in bacterial adaptation through halting growth or degrading damaged transcripts under stress conditions. Regulators such as *dinI*, *recN*, and *recX* regulate SOS network to balance repair and survival (Yasuda et al., 2001; Stohl et al., 2003). The upregulation of the umuCD operon, encoding DNA polymerase V, functions in

error-prone translesion synthesis, allowing *E. coli* to repair DNA in response to environmental stress (Reuven et al., 1999; Sutton et al., 1999). Additional DEGs included *polB*, involved in interstrand cross-link and excision repair (Deatherage et al., 2018), and *mutM*, which removes 8-oxoguanine lesions to reduce mutation rates (Fowler & Schaaper, 1997). The cell division inhibitor *sulA*, which halts cell division by interfering with Z-ring formation, was also upregulated, enabling DNA repair before cell cycle progression resumes (Vedyaykin et al., 2017). Notably, *dinF*, implicated in oxidative stress defense, was upregulated, supporting its role in detoxifying reactive oxygen species (Rodriguez-Beltran et al., 2012).

The SOS response can lead to a high rate of mutagenesis, which is essential for adaptation, pathogenesis, and diversification in *E. coli* (Podlesek & Bortok 2020). The error-prone DNA replication mediated by the SOS response can introduce mutations, which may contribute to antibiotic resistance. Additionally, the SOS response can influence the expression of toxin-antitoxin systems, which play a role in biofilm formation, horizontal gene transfer, and bacterial persistence (Madsen *et al*. 2012). Exposure to sub-inhibitory concentrations of antibiotics can induce the SOS response, leading to the emergence of antibiotic-resistant mutants and biofilms (Ching *et al*. 2020). In this study, the overexpression of the *marB* and *marR* genes, which are part of the MarRAB multidrug resistance system, was observed in response to antibiotic and oxidative stress (Kunonga *et al*. 2000). Mutations in the *marR* gene can lead to increased expression of the MarRAB operon, resulting in enhanced antibiotic resistance.

Overlapping Stress Responses

The identification of *ghoS* as a potential cross-protection DEG (CP-DEG) responsive to antibiotic, oxidative, and osmotic stress implies a comprehensive role for this gene in *E. coli* stress adaptation. *ghoS* encodes an endoribonuclease antitoxin that is a part of the GhoT–GhoS toxin-antitoxin (TA) system. Under stress conditions, *E. coli* may initiate the activation of toxin-antitoxin systems to achieve bacterial persistence leading to enhanced tolerance to antibiotic stress (Yang *et al.* 2017). Notably, GhoS is more stable than other antitoxins, allowing it to persist under stress conditions (Wang et al. 2012). Changes in *E. coli* physiology under stress occurs when the toxin GhoT compromises membrane stability and lowers metabolic function, while GhoS counters this impact by specifically breaking down *ghoT* mRNA (Wang *et al*. 2012). The gene activation of *ghoS* may regulate bacterial entry into and recovery from a dormant state, improving survival in adverse environments. Bacterial dormancy is a recognized tactic that enables bacteria to enter a state of lowered metabolic activity and cellular growth to endure challenging environmental conditions. This strategy is recognized in bacterial persistence and cross-stress tolerance, where cellular growth arrest

lessens vulnerability to antibiotics, reactive oxygen species, and osmotic stress (Harms *et al.* 2016).

The result of the study revealed a downregulation of sulfur metabolism and other energy-intensive pathways under stress. The GhoT–GhoS system may support the energy spending shift through repressing any membrane activities and decreasing translation, thus saving energy and reducing cellular damage under stress (Wang *et al.* 2012). Likewise, ghoS regulation aligns with other stress-adaptive responses, including the upregulation of DNA repair and SOS genes, and the downregulation of sulfur metabolism examined in this study, indicating that *ghoS* participates in a synchronized resource reallocation and bacterial survival strategy.

Cold and Heat Stress Responses

The upregulation of cold shock proteins (Csps) in the current study suggests a potential role in stress tolerance. Csps are essential for rapid adaptation to cold temperatures (Phadtare 2004). Previous studies have shown that Csps contribute to tolerance to oxidative, osmotic, ethanol, and pH stress (Wang *et al*. 2014; Derman *et al*. 2015). Csps can also influence the expression of flagellar genes, which may enhance bacterial motility and virulence. The upregulation of flagellar genes in our study is consistent with this finding and may reflect a strategy to seek more favorable environments.

The overexpression of heat shock proteins (HSPs) under antibiotic and oxidative stress indicates a heat shock response. HSPs are essential for protein folding, refolding, and degradation under stress conditions (Arsene *et al*. 2000). The upregulated genes, including *hspQ*, *hslR*, *hslJ*, *ibpA*, *ibpB*, *dnaJ*, *degP*, *marR*, and *sodA*, play diverse roles in heat shock response and stress tolerance. HspQ is involved in protein degradation (Shimuta *et al*. 2004), Hsp15 is a ribosome-associated chaperone (Korber *et al*. 2000), and HspIJ is a lipoprotein associated with antibiotic resistance (Lilic *et al*. 2003). IbpA and IbpB are small heat shock proteins that prevent protein aggregation (Ratajczak *et al*. 2009). DnaJ is a molecular chaperone that assists in protein folding and refolding (Langer *et al*. 1992). DegP is a protease involved in protein degradation (Seol *et al*. 1991). MarR is a transcriptional repressor that regulates the MarRAB multidrug resistance operon (Reyes-Fernández & Schuldiner 2020). SodA is a superoxide dismutase that protects against oxidative stress (Britton & Fridovich, 1997).

Overexpression of Stress Response Genes

The upregulation of *osmB* and *osmY*, genes typically associated with osmotic stress, was observed in response to antibiotic stress. While Charoenwong *et al*. (2011) demonstrated the role of *osmB* in cell envelope integrity, Abdelwahed *et al*. (2022) reported its upregulation under various stress conditions, including antibiotic, oxidative, cold, heat, and nitrosative stress. Nitrosative stress, caused by reactive nitrogen species (RNS), can damage DNA, lipids, and proteins, compromising cell envelope integrity. Bacteria employ mechanisms to counteract the harmful effects of RNS, such as superoxide dismutases and nitric oxide reductases (Chautrand *et al*. 2022). In addition to osmotic stress, the expression of *osmB* can be induced by growth phase signals and is regulated by the Rcs phosphorelay system (McDade *et al*. 2017). While the Rcs system is involved in various cellular processes, including capsule synthesis and antibiotic resistance, its precise role in *osmB* regulation under different stress conditions warrants further investigation.

The overexpression of phage shock protein genes, such as *pspABCDE*, was observed in response to antibiotic and oxidative stress. These genes are typically induced by various stress conditions, including heat shock, ethanol stress, osmotic shock, and phage infection (Weiner & Model, 1994). The Psp proteins help maintain membrane integrity and proton motive force (Lloyd *et al*. 2004). The upregulation of efflux pumps, including MacB and YojI, was observed in response to stress. Efflux pumps play a crucial role in bacterial antibiotic resistance by exporting antimicrobial compounds out of the cell. MacB is a tripartite efflux pump that confers resistance to macrolide antibiotics and is involved in enterotoxin secretion (Lu & Zgurskaya 2012). YojI is an ABC transporter that confers resistance to microcin J25 and inhibits RNA polymerase activity (Hudson & Mitchell 2018).

The upregulation of aromatic carboxylic acid efflux pumps, such as AaeAB, was also observed. These pumps help maintain cellular homeostasis by removing toxic metabolites, including p-hydroxybenzoic acid (pHBA) (Kvist *et al*. 2008). The overexpression of various stress response genes, including those involved in DNA repair, osmotic stress, phage shock, and efflux, suggests that *E. coli* employs multiple strategies to survive under adverse conditions. These findings highlight the complex network of stress response mechanisms that contribute to bacterial resilience and the potential for the development of antibiotic resistance. As Sprouffske *et al*. (2018) demonstrated, stress exposure can lead to the emergence of more stress-tolerant progeny, further emphasizing the importance of understanding bacterial stress responses.

Adaptive Metabolic Shifts in Response to Environmental Stress

In response to environmental challenges, *E. coli* regulates significant metabolic pathways to improve survival and economize resources. In this study, the meta-analysis showed steady downregulation of sulfur assimilation genes (*cysDNC*, *cysC*, *cysHIJ*), demonstrating reduced biosynthesis of sulfur-containing amino acids during stress, which may redirect energy toward defense mechanisms (Leyh *et al.* 1988; Chartron *et al.* 2007; Kushkevych *et al.* 2020). Suppression of *cysH* has also been linked to increased biofilm formation, offering additional protection (Rossi *et al*. 2014). At the same time, oxidative stress upregulated the *suf* operon, assisting iron-sulfur cluster repair needed for redox homeostasis (Dai & Outten 2012; Blahut *et al*. 2020). Concurrently, the upregulation of trehalose metabolism suggests that this disaccharide acts as a universal protectant, stabilizing proteins and membranes while scavenging reactive oxygen species under osmotic and oxidative stress (Benaroudj & Goldberg 2001; Moruno-Algara *et al.* 2019; Purvis *et al.,* 2005). Additionally, stress-induced changes in *cob* operon expression point to altered cobalamin biosynthesis, possibly compensating for oxidative inactivation of vitamin B12-dependent enzymes like *metE*, which are critical for methionine and DNA metabolism (Lawrence & Roth 1995; Hondorp & Matthews 2004). Together, these regulatory shifts underscore *E. coli*'s capacity to reprioritize metabolic pathways under stress, promoting survival through resource reallocation and molecular protection.

To cope with stress, *E. coli* often adopts a survival strategy that prioritizes stress tolerance over growth. This involves downregulating energy-intensive processes, such as amino acid and nucleotide biosynthesis, and upregulating stress response genes. The downregulation of amino acid biosynthesis genes, including those involved in methionine, alanine, threonine, serine, and tryptophan metabolism, is consistent with the findings of Bie *et al.* (2023). Methionine limitation can further exacerbate stress responses, as methionine is essential for protein synthesis and other metabolic processes (Gold, 1988).

The downregulation of nucleotide biosynthesis genes and ATPase activity reflects a strategy to conserve energy. Nucleotide biosynthesis is an energy-intensive process, and reduced nucleotide synthesis can limit cell growth and division (Lopatkin & Yang 2021). ATPases are essential for energy generation and transport processes, and their downregulation can impact various cellular functions. The downregulation of ABC transporters, which are involved in nutrient uptake and efflux, may also contribute to stress tolerance. By reducing energy expenditure on transport processes, *E. coli* can conserve resources and focus on survival.

Network-Based Adaptation of *E. coli* under Stress

Network analysis revealed that *E. coli* activates an interconnected gene regulatory system to adapt to stress, with several hub genes identified in pathways related to DNA repair, the SOS response, and sulfur metabolism. Stress-induced downregulation of sulfate assimilation genes (*cysDNC*, *cysHIJ*), which are regulated by the CysB transcription factor, suggests a metabolic shift away from energy-intensive biosynthesis under unfavorable conditions (Loudon & Loughlin 1992). This suppression conserves sulfur resources and redirects cellular energy toward survival mechanisms. Concurrently, upregulation of the Suf system—a stress-tolerant iron-sulfur cluster assembly pathway—supports redox balance and essential metabolic functions during oxidative stress (Dai & Outten 2012). Unlike the Isc system, the Suf machinery is less sensitive to oxidative damage and more efficient in sulfur mobilization under stress (Schwartz *et al.* 2000). These regulatory shifts illustrate how *E. coli* coordinates gene expression through stress-responsive networks to optimize survival across varying environmental conditions (Smith *et al.* 2018).

CONCLUSIONS

In summary, this study used microarray datasets with the same platform to investigate genes associated with bacterial stress response and cross-protection. This study utilized a meta-analysis approach to investigate the transcriptional response of *E. coli* to various stress conditions, including antibiotic, oxidative, osmotic, and alcohol stress. The analysis revealed distinct stress-specific responses, with the upregulation of genes involved in the SOS response, DNA repair, and heat shock response. Additionally, the downregulation of genes involved in amino acid biosynthesis, nucleotide metabolism, and sulfur metabolism indicated a shift towards a survival-oriented metabolic state. Among all genes, the gene *ghoS* was notably identified for the first time as a potential cross-protection gene that is responsive to antibiotic, oxidative, and osmotic stress. These findings provide valuable insights into the complex mechanisms underlying *E. coli* stress response and cross-protection, and offer potential targets for developing strategies to combat antibiotic resistance.

While this study provides *in silico* insights regarding potential cross-protection genes in E. coli, experimental validation is essential to confirm these findings. In vitro assays involving multiple stress exposures—such as temperature shifts, nutrient deprivation, and nitrosative stress—are recommended to evaluate cross-protection responses. Gene expression patterns observed *in silico* should be validated through qPCR, protein abundance assays, and gene knockdown experiments to verify functional roles. The discrepancy observed within operon members suggests that some biologically relevant genes may have

been overlooked due to statistical filtering; thus, follow-up studies using relaxed thresholds or proteomic data may provide further insights. Structural characterization through sequence homology analysis may also help elucidate conserved motifs in uncharacterized genes. Ultimately, real-time stress response monitoring under sublethal antimicrobial exposures will be crucial to bridge computational predictions with functional biology.

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AUTHORS' CONTRIBUTIONS

Maria Genesis M. Acosta: contributed to writing the original draft, designing the methodology, creating data visualizations, validating data, and performing statistical analysis.
Gil M. Penuliar: contributed to the conceptualization, data interpretation, critical revision of the manuscript, and securing funding. Both authors approved the final version of the paper.

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