Comprehensive Analysis of Proteomic Differences between *Escherichia coli* K-12 and B Strains Using Multiplexed Isobaric Tandem Mass Tag (TMT) Labeling

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The *Escherichia coli* K-12 and B strains are among the most frequently used bacterial hosts for scientific research and biotechnological applications. However, omics analyses have revealed that *E. coli* K-12 and B exhibit notably different genotypic and phenotypic attributes, even though they were derived from the same ancestor. In a previous study, we identified a limited number of proteins from the two strains using two-dimensional gel electrophoresis and tandem mass spectrometry (MS/MS). In this study, an in-depth analysis of the physiological behavior of the *E. coli* K-12 and B strains at the proteomic level was performed using six-plex isobaric tandem mass tag-based quantitative MS. Additionally, the best lysis buffer for increasing the efficiency of protein extraction was selected from three tested buffers prior to the quantitative proteomic analysis. This study identifies the largest number of proteins in the two *E. coli* strains reported to date and is the first to show the dynamics of these proteins. Notable differences in proteins associated with key cellular properties, including some metabolic pathways, the biosynthesis and degradation of amino acids, membrane integrity, cellular tolerance, and motility, were found between the two representative strains. Compared with previous studies, these proteomic results provide a more holistic view of the overall state of *E. coli* cells based on a single proteomic study and reveal significant insights into why the two strains show distinct phenotypes. Additionally, the resulting data provide in-depth information that will help fine-tune processes in the future.

Keywords: *Escherichia coli*, proteome, tandem mass tag (TMT), isobaric tag, quantitative proteomics, mass spectrometry

Introduction

The *E. coli* K-12 and B strains are among the most widely used bacterial hosts in laboratories and industry for producing recombinant proteins and a variety of biomolecular chemicals, such as amino acids and metabolites. Interestingly, distinctive phenotypic differences between these two *E. coli* strains have been observed; for example, in their growth characteristics, acetate accumulation and recombinant protein production capacity [1, 2]. Thus, several comparative analyses of these two *E. coli* strains (rather than comparisons with other microorganisms) have been performed at various molecular levels to identify their cellular physiological and metabolic differences based on omics data, such as genomic, transcriptomic, proteomic, or phenomic data [3–8]. In particular, Yoon *et al.* [3] systematically analyzed the physiological differences of these two *E. coli* strains cultured in shake flasks containing Luria-Bertani (LB) medium. These omics analyses have shown that *E. coli* B has a greater capacity for acetate assimilation and amino acid biosynthesis and that it lacks proteases and flagella. Moreover, the B strain exhibits greater membrane permeability and an additional secretion system, making it more favorable for protein secretion purposes. In contrast, the K-12 strain displays a rigid membrane structure with flagellar motility and higher levels of stress-responsive proteins, showing

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resistance under stress conditions. However, among the available omics results, proteomic data acquired via two-dimensional gel electrophoresis (2-DE) or subcellular fractionation following 2-DE, to reduce cellular complexity, provide an incomplete story for the purpose of deciphering and understanding complex cellular behaviors or metabolism. Thus, a more comprehensive proteomic analysis of the two *E. coli* strains is required using a recent quantitative proteomics method.

Proteomic analysis is critical for understanding the cellular physiology and metabolism of an organism and is a more attractive method for discovering potential targets of engineering for strain improvement. The advancement of proteomic technologies and instruments has radically improved throughput and detection limits. Despite the advantages of typical gel-based methods, such as 2-DE and 2-D difference in-gel electrophoresis, these techniques present a number of problems, in that they are relatively labor intensive, less reproducible, unsuitable for high-throughput purposes, and less able to detect a trace amount of protein than other methods. Recently, mass spectrometry (MS)-based approaches, such as isotope-coded affinity tags, isobaric tags for relative and absolute quantitation (iTRAQ), stable isotope labeling with amino acids in cell culture (SILAC), and tandem mass tags (TMTs), have been developed and used widely in quantitative proteomics to solve these problems [9]. Several groups have employed iTRAQ labeling or SILAC techniques to identify the largest *E. coli* proteome [10, 11]. However, a comparative physiological study of the *E. coli* B and K-12 hosts has not yet been performed using quantitative proteomic methods. One of the most popular methods for quantification is TMT labeling of biological samples prior to MS analysis [9]. Interestingly, TMT quantification enables multiple biological samples (until 10 samples) to be analyzed under the same chromatography and MS conditions, leading to greater precision and accuracy.

Therefore, in this study, we employed six-plex TMTs to quantitate and compare the protein profiles of the *E. coli* K-12 and B strains cultured in LB medium. In particular, the largest proteomes of the *E. coli* K-12 and B strains were compared to determine the proteomic differences between the two strains using a quantitative MS-based method.

**Materials and Methods**

**Bacterial Strains and Culture Conditions**

The *E. coli* K-12 and B strains employed in this study were MG1655 and BL21(DE3), respectively. The *E. coli* strains were cultivated in a 250-ml flask containing 100 ml of LB medium (10 g/l bacto-tryptone, 5 g/l bacto-yeast extract, and 5 g/l NaCl) at 37°C and 250 rpm. Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using a spectrophotometer (DU650; BECKMAN, USA).

**Protein Extraction for Quantitative Proteomics**

The cell culture broth (OD_{600} = 5) was centrifuged at 3,500 × g for 5 min at 4°C. The cell pellets were then resuspended and homogenized in 0.5 ml of one of three lysis buffers: Epicentre buffer (PeriPreps Periplasting Kit (Cat. No. PS81100); Epicentre Technologies Corp., USA); urea buffer containing 8 M urea, 4% CHAPS, 5 mM magnesium acetate, and 30 mM Tris (pH 8.5), which is typically used for 2-DE; or RIPA buffer (Cat. No. 9806; Cell Signaling Technology Inc., USA). Homogenization was performed using a Precellys 24 homogenizer (Bertin Technologies, France) at a frequency of 6,500 cycles/min for 20 sec; this procedure was repeated three times with a 3-min pause to minimize heat generation. The supernatant was subsequently separated via centrifugation at 18,000 × g for 30 min at 4°C (for urea buffer, at 18°C). The protein concentration was finally determined using a Bradford protein assay (Bio-Rad, USA), and aliquots were stored at −80°C.

**TMT Labeling**

TMT labeling was performed according to the manufacturer’s instructions (Thermo Fisher Scientific, USA) with some modifications. The protein extracts (100 µg) from each bacterial sample were reduced with 500 mM Tris(2-carboxyethyl)phosphine at room temperature for 60 min and then alkylated with 500 mM iodoacetamide at room temperature in the dark for 60 min. Next, the samples were desalted using a membrane filter of 10kMW and dissolved in 200 mM triethylammonium bicarbonate (TEAB) buffer to a final concentration of 1 µg/µl. Sequencing-grade trypsin (Promega, USA) was added to the proteins at 1:20 (w/w) in TEAB buffer, followed by incubation overnight at 37°C. The six-plex labeling scheme allows up to six samples to be compared in a single MS run, thereby increasing the experimental throughput for protein quantitation. The quantitative proteomic experiment was performed independently of the two biological replicates. In the first TMT set, each peptide sample (30 µg) digested from individual proteins was individually labeled with six-plex tags. In the second TMT set, each peptide sample was reverse labeled with the different tags to exclude labeling bias and experimental variation between two independent *E. coli* cultures. Aqueous hydroxylamine solution (5% (w/v)) was added to quench the labeling reaction. The six samples were then combined, speed-vacuum dried, and dissolved in 50 µl of Milli-Q water containing 0.1% formic acid for 2D–liquid chromatography (LC)-MS/MS analysis.

**2D-LC-MS/MS Analysis**

The TMT-labeled samples were analyzed using a 2D-LC-MS/MS system consisting of a nanoACQUITY UltraPerformance LC System (Waters, USA) and an LTQ Orbitrap Elite mass spectrometer.
(Thermo Scientific) equipped with a nanoelectrospray source. 2D-LC-MS/MS analysis was performed as previously reported by Washburn et al. [12] with slight modifications. Briefly, a strong cation exchange (SCX, 5 µm, 3 cm) column was placed just before the C18 trap column (id 180 µm, length 20 mm, and particle size 5 µm; Waters). Peptide solutions were loaded in 5 µl aliquots for each run. The peptides were displaced from the SCX phase to the C18 phase via a salt gradient introduced through an autosampler loop and then desalted for 10 min at a flow rate of 4 µl/min. Next, the trapped peptides were separated in a 200-µm homemade microcapillary column consisting of C18 (Aqua; particle size 3 µm) packed into 100-µm silica tubing with an orifice id of 5 µm. A 10-step salt gradient was performed using 3 µl of 0, 25, 50, 100, 250, and 500 mM ammonium acetate (0.1% formic acid in 5% acetonitrile), followed by 4, 5, and 9 µl and an additional 9 µl of 500 mM ammonium acetate (0.1% formic acid in 30% acetonitrile). Mobile phases A and B were composed of 0 and 100% acetonitrile, respectively, and each contained 0.1% formic acid. The LC gradient began with 5% B for 1 min and was ramped up to 20% B over 5 min, then to 50% B over 80 min, 95% B over 4 min, 95% B over 10 min, and 5% B for another 5 min. The column was re-equilibrated with 5% B for 15 min before the next run. The voltage applied to produce the electrospray was 2.0 kV. During chromatographic separation, the LTQ Orbitrap Elite was operated in data-dependent mode. The MS data were acquired using the following parameters: five data-dependent collision-induced dissociation-high-energy collision dissociation (CID–HCD) dual MS/MS scans per full scan; CID scans acquired in linear trap quadrupole (LTQ) mode with two-microscan averaging; full scans and HCD scans acquired in Orbitrap mode at resolutions of 60,000 and 15,000 respectively, with two-microscan averaging; 35% normalized collision energy (NCE) in CID and 45% NCE in HCD; and a ±1 Da isolation window. Previously, fragmented ions were excluded for 60 sec. In CID–HCD dual scans, each selected parent ion was fragmented first by CID and then by HCD.

Protein Identification and Quantification

MS/MS spectra were analyzed using the following software analysis protocols, employing the E. coli MG1655 (NC_000913) and BL21(DE3) (NC_012971) genome databases. The reversed sequences of all proteins were appended into the database for calculation of the false discovery rate (FDR). ProLucid [13] was employed to identify the peptides, with a precursor mass error of 25 ppm and a fragment ion mass error of 600 ppm. Trypsin was selected as the enzyme, with three potential missed cleavages. TMT modification (+ 229.1629) at the N-terminus and lysine residues by the labeling reagent, and carbamidomethylation at cysteine, were chosen as static modifications. Oxidation at methionine was chosen as the variable modification. The CID and HCD tandem MS spectra from the same precursor ion are often combined by software to allow better peptide identification and quantification [9]. We used homemade software in which reporter ions from the HCD spectrum were inserted into the CID spectrum with the same precursor ion in the previous scan. Reporter ions were extracted from small windows (± 20 ppm) around their expected m/z in the HCD spectrum. The output data files were filtered and sorted to generate the protein list, using DTASelect [14] with two or more peptide assignments for protein identification and a false-positive rate of less than 0.01.

Quantitative analysis was conducted using Census in the IP2 pipeline (Integrated Proteomics). The intensity of a protein at a reporter ion channel was calculated as the average of the reporter ion intensities from all constituent peptides from the identified protein [15]. The measured intensity ratios of the proteins were transformed to the log, scale. The FDR was calculated by selecting a p-value (p < 0.05) from the two biological replicates and ensuring that the fold change was significant under the other two conditions. The functions of the differentially expressed proteins were assigned using the BRITE functional hierarchies at the KEGG Website (http://www.genome.jp/kegg/). Genes encoding unknown functions were not included in the analysis.

Results and Discussion

Optimization of E. coli K-12 and B Proteome Sample Preparation for Quantitative Proteomics

Prior to the proteomic analysis, three kinds of lysis buffers employed in homogenization were evaluated to increase the efficiency of protein extraction from E. coli cells: lysis buffer containing urea, which is typically used in 2-DE (lysis buffer I); the lysis buffer provided by Epicentre Biotechnologies (lysis buffer II); and the RIPA buffer provided by Cell Signaling Technology (lysis buffer III). The results showed that the final protein concentrations obtained with lysis buffers I and III were much higher than those obtained with lysis buffer II (Fig. 1A). The assessment of protein recovery as well as separation quality and reproducibility can be performed via SDS–PAGE analysis (Fig. 1B). Based on the obtained results, both lysis buffer I and III were considered good. However, it is necessary to carefully handle the proteins solubilized in the lysis buffer containing urea at low temperature, owing to its recrystallization, and to carefully control the sample boiling conditions for SDS–PAGE analysis owing to loss of protein.

Thus, lysis buffer III was selected as the best of the three tested buffers for isolating a greater number of E. coli proteins for quantitative proteomics.

Growth of E. coli K-12 and B Strains and Experimental Design of Quantitative Proteomics

Two representative strains, the E. coli K-12 strain MG1655 and B strain BL21(DE3), were cultured in LB medium. The two strains grew similarly to reports of their growth in a previous study [3] (Fig. 2A). The sampling points for
quantitative proteomics were in the exponential (E), early stationary (ES), and stationary (S) phases for both strains, as indicated in Fig. 2A. In addition, each protein sample extracted with lysis buffer III (i.e., RIPA buffer) was analyzed on a 10% SDS–PAGE gel (Fig. 2B).

For comparative analyses, the *E. coli* K-12 and B strains
were labeled with six TMTs. The workflow of the quantitative proteomic analysis using TMT labeling, following MS analysis, is schematically illustrated in Fig. 2C. In the first set of experiments, each peptide sample digested with trypsin was individually labeled with the six different tags. In the second set of experiments, each peptide sample was reverse labeled with the different tags to exclude labeling bias and experimental variations between the two independent *E. coli* cultures. The labeled peptides in each experiment were mixed in equal amounts and subjected to identification and quantification via tandem MS. Each experiment resulted in more than 8,000 high-confidence peptide identifications and quantifications, with an average FDR of 0.12%. For a representative example, the quantitative level of a maltose-binding periplasmic protein in the first set of experiments was very close to that in the second set of experiments (Fig. S1), indicating that there was no major labeling bias and that there were minimal technical variations. The results of the identification and quantitation of proteins and their MS data are summarized in Table S1.

**Growth-Dependent Proteins in *E. coli* K-12 or B Strains**

Based on our criteria, we analyzed the proteins showing significant differences that were commonly identified in the two sets of proteomic experiments in the *E. coli* K-12 or B strain. In the *E. coli* K-12 strain, the number of differentially expressed proteins was 131 among 208 proteins (63%) in the ES phase (K-ES) and 253 among 318 proteins (80%) in the S phase (K-S), as compared with the E phase (K-E) (Fig. 4A). In the comparison of K-E and K-ES, 92 proteins were found to present increased levels in K-ES in the K-12 strain, whereas 39 showed decreased levels (Table S3). The majority of proteins related to carbohydrate metabolic processes (TCA cycle and oxidative phosphorylation) and all seven proteins involved in cell motility (CheA, FlgM, FliC, MalE, MglB, RbsB, and Tar) were synthesized at higher levels in K-ES compared with those in K-E. In contrast, most of the proteins exhibiting decreases were involved in translation (especially ribosomal proteins) or were transcription factors.

In the comparison of K-E and K-S, a greater number of proteins were increased in K-S in the K-12 strain (173 proteins were increased, and 80 were decreased) (Table S3). Similar to the comparison of K-E and K-ES, carbohydrate metabolic processes and cell motility were further enhanced, and the biosynthesis of amino acids and energy metabolism were significantly upregulated in K-S. In contrast, many proteins related to transcription and translation were further repressed in K-S.

In the *E. coli* B strain, the proteins showing significant differences included 99 of 146 proteins (68%) in the ES phase (B-ES) and 222 of 306 proteins (80%) in the S phase (B-S), compared with those in the E phase (B-E) (Fig. 4B). In the comparison of B-E and B-ES, 64 proteins were present

**Fig. 3.** Functional classification of 834 proteins commonly identified in two sets of mass spectrometry (MS) experiments for the *E. coli* K-12 and B strains via tandem mass tag labeling and MS analyses. Functions were assigned using the BRITE functional hierarchies at the KEGG Website (http://www.genome.jp/kegg/). The numbers in parentheses refer to the protein numbers (see Table S2 for more detailed information).
at increased levels in B-ES in the B strain, whereas 35 showed decreased levels (Table S4). A large number of proteins with functions in carbohydrate and amino acid metabolism and a small number of proteins involved in nucleotide and lipid metabolism were synthesized at higher levels in B-ES compared with those in B-E. Interestingly, we observed strong induction of enzymes involved in the biosynthesis of amino acids in the B strain. In contrast, proteins involved in translation (especially ribosomal proteins) were decreased, which was expected.

In the comparison of B-E and B-S, 122 proteins were present at increased levels in B-S, whereas 100 showed decreased levels (Table S4). Similar to the comparison of the B-E and B-ES phases, carbohydrate metabolic processes were observed to be significantly enhanced, and protein translation was further repressed in B-S. Moreover, proteins involved in folding, sorting, and degradation (DeaD, GrxB, HdeB, HscA, IscS, SecY, and YidC) were markedly decreased in B-S.

**Strain-Specific Proteins between the *E. coli* K-12 and B Strains**

Although *E. coli* K-12 and B strains exhibit similar growth patterns in LB medium (Fig. 1A), they show quite different proteome profiles. Among the differentially expressed proteins between the two strains, the proteins that were commonly identified in the two sets of experiments included 116 of 178 proteins (65%) in the E phase, 118 of 158 proteins (75%) in the ES phase, and 149 of 205 proteins (72%) in the S phase (Fig. 4C). In general, the majority of proteins showing significant differences between the two strains from the E phase to the S phase were mainly classified into the carbohydrate metabolism, amino acid metabolism, transporter, and cell motility categories (Table S5).

The proteomic differences related to metabolic pathways between the two strains are a notable characteristic (Fig. 5). In particular, the glyoxylate shunt and anaplerotic reactions were significantly enhanced in the K-12 strain, but no significant change in the glycolytic pathway or the TCA cycle was observed in either strain. Enzymes (AceA, AceB, and GlcB) involved in the glyoxylate shunt were synthesized at substantially elevated levels in the K-12 strain compared with those in the B strain, which is similar to the results of previous transcriptomic and proteomic analyses [3]. Activation of the glyoxylate shunt may be enhanced by acetyl-CoA, which is produced at high levels from pyruvate in the presence of increased enzyme levels (AceE and AceF). In addition, the levels of enzymes involved in the production of various metabolites, such as formate, acetate, and ethanol, were higher in K-12 than in the B strain. Therefore, metabolic intermediates such as pyruvate and acetyl-CoA were consumed in considerable amounts, due to conversion of the by-products in the K-12 strain.

However, most enzymes involved in the TCA cycle (GltA, AcrB, Icd, SucABCD, SdhAB, FumABC, and Mdh) did not differ significantly between the two strains during all growth phases. Only FrdA and FrdB in the TCA cycle, which are responsible for the anaerobic catalysis of fumarate and succinate interconversion, were notably synthesized at a higher level in the K-12 strain. These proteins were shown to exhibit an unexpected function associated with the bacterial flagellar switch for both flagellar assembly and switching the direction of flagellar rotation [16]. Thus,

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**Fig. 4.** Venn diagrams indicating the number of proteins identified from two different mass spectrometry datasets for the *E. coli* K-12 or B strains. (A) Comparisons between the exponential and early stationary phases or the exponential and stationary phases in the K-12 strain (K-E versus K-ES or K-S); (B) comparisons between the exponential and early stationary phases or the exponential and stationary phases in the B strain (B-E versus B-ES or B-S); and (C) comparisons between the K-12 and B strains in three different growth phases (K-E versus B-E, K-ES versus B-ES, and K-S versus B-S).
enhanced FrdAB in this case may function as an important component of flagellar motility as a distinct characteristic of the K-12 strain, as mentioned below. Furthermore, it was observed that metabolic pathways
related to the utilization of carbon sources exhibited some differences between the two strains. For instance, enzymes required for glycerol utilization (GlpD, GlpF, and GlpK) were synthesized at significantly higher levels in the K-12 strain than in the B strain. Additionally, an enzyme involved in D-tagatose 6-phosphate degradation, GatY, was synthesized at high levels in the K-12 strain. Activation of these metabolic pathways was commonly enhanced in the pool of C3 compounds, such as D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, which are intermediates of the glycolytic pathway. Therefore, the metabolic differences in the two E. coli strains may reflect a global physiological response to cope with the changing proteomic demands of energy biogenesis and biomass synthesis under given growth conditions.

Another obvious proteomic difference related to metabolic pathways between the two strains occurred in the synthesis of amino acids (Fig. 5). The enzymes involved in the biosynthesis of certain amino acids, such as arginine (ArgBDEGH), serine (SerC), aspartate (AspC) and branched-chain amino acids (ilvC and ilvD), were synthesized at higher levels in the B strain than in the K-12 strain, which was in accord with proteomic results we previously obtained via a gel-based method [3]. These enhanced pools of amino acids can provide more favorable conditions for the production of heterologous proteins in the E. coli B strain. On the other hand, enzymes involved in the degradation pathways of some amino acids, including tryptophan and cysteine (TnaA), threonine (ThrC and ThrD), and glutamate (GadA and GadB), were synthesized at lower levels in the B strain. These results regarding enhanced biosynthetic pathways and repressed degradation pathways of amino acids support the notion that E. coli B strains are better hosts for producing recombinant proteins.

Additional distinct differences between the two strains are related to bacterial flagellar motility, chemotaxis, and a cellular adhesion component (Flu). FliC and FliY, which are involved in flagellar motility and machinery, were synthesized at high levels in the proteome of the K-12 strain (except for a mild reduction of FlgE), whereas FlgM, a negative regulator of flagellin synthesis (anti-σ factor), was significantly decreased, to enhance the flagellar assembly of proteins. This result is consistent with the genomic finding that the B strain lacks the flagellar biosynthesis gene cluster owing to the deletion of a 38-kb genomic region in K-12 from yecF to yedS, encompassing the fliYZACDSTEGHJIKLMNOPQR genes [17]. Furthermore, the B strain was confirmed to be non-motile in a swarming motility assay [18]. In addition, chemotaxis-related proteins (Tsr, Tar, RbsB, CheA, CheZ, and MalE) were synthesized at high levels in the K-12 strain. These findings also corroborate several previous omic studies [3, 4, 6] showing that proteins involved in motility and chemotaxis are highly expressed in the K-12 strain, whereas they mostly exhibited no detectable level of synthesis in the B strain. From these results, we can conclude that diverse chemotaxis and motility behaviors are K-12 strain-specific properties.

Moreover, we observed a clear difference in the expression of cellular membrane proteins between the two strains. E. coli B produced high levels of OmpF porins with a larger pore size due to deficiency of mfeF, which post-transcriptionally prevents the synthesis of OmpF [19]. In contrast, E. coli K-12 synthesized significantly higher levels of OmpW and OmpA, which are cellular adaptations involved in the response to various stresses and the structural integrity of the membrane, respectively. These findings are consistent with previous transcriptomic and proteomic results [3, 6]. Additionally, stress-responsive proteins (HdeB, UspF, and Dps) were strongly induced in the K-12 strain. The difference in these proteins can explain the greater susceptibility of the B strain to various antibiotics and stresses, whereas the K-12 strain shows greater tolerance. Furthermore, these strain-specific differences may influence the permeability and integrity of the E. coli membrane.

In the S phase, one interesting difference is that many ribosomal proteins (RplA, RplB, RplD, RplL, RpsD, RpsG, RpsR, and RpsS) and membrane-anchored ribosome-binding proteins (ElbA and YqjD) are required for protein synthesis. These proteins showed higher levels of synthesis in the S phase in the K-12 strain than in the B strain. It was noted that the proteins constituting the ribosome, a complex that is central to translation, play an important role in proteostasis [20]. Thus, these alterations might affect the regulation of proteostasis in E. coli in the S phase. Alternatively, they could be implicated in the higher production of enzymes that are specifically required for bacterial motility, various metabolites, and stress resistance in the K-12 strain.

In this study, the greatest number of proteins identified in comparative proteomic analysis of the E. coli K-12 and B strains was found via coupled TMT labeling and MS analysis, providing a more detailed picture of the physiological differences of these E. coli strains at the proteome level than the limited observations of proteins associated with metabolic pathways made via gel-based proteomic methods. Furthermore, most of the results obtained in this study are consistent with previous findings obtained using combined
omics tools (especially, transcriptomic and proteomic analyses). Therefore, the proteomic results obtained in this study contribute significant insights into the distinct phenotypes of the two strains studied. Furthermore, these results can be employed for the improvement of *Escherichia coli* strains in biotechnology.

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