Research Article

TMT-Based Quantitative Proteomics Analysis of the Fish-Borne Spoiler Shewanella putrefaciens Subjected to Cold Stress Using LC-MS/MS

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Shewanella putrefaciens is a specific spoilage bacterium for fish during cold storage. To better understand the molecular mechanisms of cold stress adaptation of S. putrefaciens, tandem mass tag- (TMT-) based quantitative proteomic analysis was performed to detect the effects of cold stress on protein expression profiles in S. putrefaciens which had been cultivated at 4°C and 30°C, respectively. A total of 266670 peptide spectrum matching numbers were quantified proteins after data analysis. Of the 2292 proteins quantitatively analyzed, a total of 274 were found to be differentially expressed (DE) under cold stress compared with the nonstress control. By integrating the results of Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, 9 common KEGG terms were found notable for the cold-responsive proteins. Generally, the DE proteins involved in carbohydrate, amino acid, and fatty acid biosynthesis and metabolism were significantly upregulated, leading to a specific energy conservation survival mode. The DE proteins related to DNA repair, transcription, and translation were upregulated, implicating change of gene expression and more protein biosynthesis needed in response to cold stress.

1. Introduction

Fresh fish is very perishable due to endogenous enzymes and microbial activities, which can result in large economic losses [1]. Indeed, the intrinsic properties of fresh fish are favourable for growth and enzymatic activity of spoilage bacteria, with the consequent off-flavours, off-odours, discoloration, textural changes, and slime formation [2]. Cold storage is widely used to maintain the quality of fish and prolong the shelf life [3, 4]. Shewanella putrefaciens is considered as the common specific spoilage organisms (SSOs) in fish during cold storage [5, 6]. S. putrefaciens has been reported to be able to use electron acceptors, such as TMAO, instead of oxygen to survive under oxygen or hypoxia conditions. It can produce proteolytic and lipolytic enzymes broken down proteins and produce various flavour defects to lower the fish quality [7]. In addition, S. putrefaciens is a cold-adapted bacterium in refrigerated fish and exhibits many special characteristics and molecular mechanisms that allow them to adapt to the cold stress environment [8, 9].

Some bacteria show a variety of physiological adaptation mechanisms, in order to cope with cold stress, survive, and grow in the cold stress environment. The mechanism is as follows:

(i) Increased fluidity of cell membranes
(ii) The freezing point of the aqueous phase in the cytoplasm decreased
ample, proteomics methods were used to investigate the research studies have been performed to determine the of proteins in complex biological samples [16, 17]. Some (TMT)issuitableforanalyzingtheabundanceofthousands
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tification, proteome analysis based on the tandem mass tag (TMT) is suitable for analyzing the abundance of thousands of proteins in complex biological samples [16, 17]. Some research studies have been performed to determine the proteomics changes of bacteria under cold stress. For example, proteomics methods were used to investigate the quantitative proteomics of Edwardsiella tarda in the mid-exponential growth phase at the optimal temperature of 37°C for 24 h and then through the hatch at 4°C for two weeks without vibration. Several key proteins related to DNA synthesis and transcription were significantly upregulated [18]. Similar comprehensive studies for S. putrefaciens have yet to be carried out. To provide insight into potential mechanisms underlying the ability of S. putrefaciens to grow at a temperature of 4°C, we investigated the whole proteome response of S. putrefaciens exposed to cold stress using mass spectrometry.

2. Materials and Methods

2.1. Bacterial Strain and Growth Conditions. Broth cultures of S putrefaciens (ATCC 8071) were prepared as follows: 1 mL aliquots of logarithmic phase grown broth cultures were transferred to 250 mL Erlenmeyer flasks containing 100 mL medium. The flasks were incubated aerobically at 30°C and 150 rpm, respectively, until an absorbance (OD600) of 0.4 was attained. Six independent replicates were collected for each sample. The cell pellets prepared by centrifugation of the bacterial culture were resuspended and washed three times with phosphate-buffered saline (PBS).

2.2. Protein Extraction and Quantification. The spoilage cells were resuspended in a 600 L lysis buffer and subjected to high-intensity probe ultrasound in a 200 w ice bath (UP-250S sonicator, Scientz, Ningbo, China). The mixtures were centrifuged at 16000 g at 4°C for 5 min. The supernatant was collected, and the protein concentration was quantified using the bicinchoninic acid method. 10 μg protein samples were added to 5X loading buffer at a rate of 5:1 (V/V), and then, the mixture was put in the boiling water for 5 min. The purity of proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on the basis of Hou et al. [19].

2.3. Protein Enzymatic Hydrolysis and Peptide Desalting. 300 μg of each sample and 0.1 M DTT were mixed together. The mixture was heated in the boiling water for 5 min and then cooled to room temperature. Then, 200 μL UA buffer (8 M urea, 150 mM Tris-HCl, pH 8.0) was added to the mixture, and the protein was collected by centrifugation with 10 kDa ultrafiltration centrifuge tube at 12,000 × g for 15 min. This process was repeated twice. Subsequently, the protein was added with 100 μL IAA (50 mM IAA in UA) and oscillated at 600 rpm for 1 min and centrifuged at 12,000 × g for 10 min at room temperature in dark. The protein was collected by centrifugation with 10 kDa ultrafiltration centrifuge tube at 12,000 × g for 15 min, and this procedure was repeated twice.

2.4. TMT Labelling. Desalted peptides were reconstituted in 0.1% FA, and the concentration of peptide was determined with the total protein assay kit (BCA method, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Peptides were reconstituted in 50 mM 2-hydroxyethyl (pH 8.5) and TMT zero reagent (Thermo Fisher, Waltham, USA) was added from stocks dissolved in 100% anhydrous ACN. The peptide-TMT mixture was incubated for 1 h at 25°C and 400 rpm, and the labelling reaction was stopped by the addition of either 5% hydroxylamine to a final concentration of 0.4% or 8 μL of 1 M Tris, pH 8.0, and incubation for 15 min at 25°C and 400 rpm. Peptide solutions were acidified with 45% (v/v) of 10% FA in 10% ACN. The peptide-TMT mixture was incubated for 1 h at 25°C and 400 rpm, and the labelling reaction was stopped by the addition of either 5% hydroxylamine to a final concentration of 0.4% or 8 μL of 1 M Tris, pH 8.0, and incubation for 15 min at 25°C and 400 rpm. Peptide solutions were acidified with 45% (v/v) of 10% FA in 10% ACN prior to drying or directly frozen at 80°C and dried by vacuum centrifugation. For in-depth proteome analyses, peptides derived from Lys-C/trypsin digests of luminal and basal PDX tumors were processed as described in Fang et al. [4] but following the optimized TMT labelling protocol.

Briefly, 300 μg peptides were dissolved in 60 μL of 50 mM HEPES (pH 8.5), and the labelling reaction was started by the addition of 300 μg TMT reagents (15 μL of 56.7 mM (20 μg/μL) TMT stocks). Samples were incubated for 1 h at 25°C and 1,000 rpm, and the labelling reaction was quenched using 5 μL of 5% hydroxylamine (15 min; 25°C, 1,000 rpm). Peptide solutions were pooled, frozen at 80°C, and dried by vacuum centrifugation. Subsequently, TMT-labelled samples were desalted using Tc18, RP solid-phase extraction cartridges (Waters Corp.; wash solvent: 0.1% TFA; elution solvent: 0.1% FA in 50% ACN), frozen at −80°C, and dried by vacuum centrifugation. TMT-labelled peptides were fractionated using a high-pH reversed-phase column (PierceTM High-pH Reversed-Phase Peptide Fractionation Kit, Thermo Fisher). Peptides were pooled into 15 fractions. Enrichment was performed using Ni-nitrilotriacetic acid superf low agarose beads (Qiagen) loaded with iron (III) ions. Subsequently, phosphopeptides were desalted using self-packed StageTips (wash solvent: 0.1% FA; elution solvent: 0.1% FA in 50% ACN), frozen at 80°C, and dried by vacuum centrifugation.
2.5. LC-MS/MS Measurements. Tryptic peptides for one-shot analyses were analyzed on an EASY-nLC 12000 (Thermo Scientific) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). After reconstitution in 0.1% FA, an amount corresponding to 500 ng peptides was injected. Peptides were separated on an analytical column (EASY column, 75 μm × 45 cm, Thermo Fisher Scientific) applying a flow rate of 300 nL/min and following elution program: 0–2 min: from 5 to 8% solvent B (0.1% formic acid + 98% acetonitrile); 2–42 min: from 8 to 23% solvent B; 42–50 min: from 23 to 40% solvent B; 50–52 min: from 40 to 100% solvent B; and 52–60 min: 100% solvent B, respectively. Mass spectrometers were operated in data-dependent and positive ionization mode. On the Q Exactive Plus, MS1 spectra were recorded at a resolution of 70 k using an automatic gain control (AGC) target value of 1e6 charges and maximum injection time (maxIT) of 50 ms. After peptide fragmentation via higher energy collisional dissociation, MS2 spectra of up to 10 precursors were acquired at 17.5 k resolution using an AGC target value of 1e5 and a maxIT of 50.

2.6. Database Searching. MaxQuant: for peptide and TMT titration experiments, peptide identification and quantification were performed using MaxQuant (version 1.6.0.16) with its built-in search engine, Andromeda (15, 16). Tandem cation were performed using MaxQuant (version 1.6.0.16) with its built-in search engine, Andromeda (15, 16). Tandem cation were performed using MaxQuant: for peptide and TMT quantification, and differential protein classification analysis.

3. Results and Discussion

3.1. Identification of Proteins by Quantitative Proteomics Analysis. The results of spectrometry in the present research included protein identification, peptide identification, protein quantification, and differential protein classification analysis. A total of 266670 peptide spectrum matching (PSM) numbers, 19483 unique peptides, and 2292 quantified proteins were obtained after data analysis. The intensity histogram for each sample is shown in Figure 1(a). Figure 1(b) was the box plot of normalized density and represented the box plots of log 2 protein intensity average for each sample.

3.2. Identification of Proteins and Their Total and Differential Abundances. TMT-based quantitative proteomic analysis was developed to identify, quantify, and statistically verify quantitative differences in protein abundance from S. putrefaciens grown at 4°C versus 30°C. The use of this approach ensured that the quantitative differences recorded were reliable, irrespective of the magnitude of the quantitative differences, and provided a robust means of interpreting the biological relevance of the data. A total of six experiments were analyzed by LC-MS/MS. Of these proteins, 274 were significant DE proteins with abundances that changed >1.5-fold (cultivated at 30°C/cultivated at 4°C) and P values of <0.05. A total of 189 proteins were upregulated and 85 proteins were downregulated (red and green background colors, respectively, in Table 1).

3.3. Bioinformatics Analysis of DE Proteins Identified by TMT. The upregulated and downregulated DE proteins were annotated by Gene Ontology (GO) with Fisher’s exact test to better understand the roles that these proteins may play in cold adaptation. The significantly upregulated and downregulated DE proteins were classified into three categories using GO terms: biological process (BP), cell component (CC), and molecular function (MF). The downregulated DE proteins were clustered into 50 BP terms (the most representative term was “organic substance metabolic process”), 20 CC terms (the most representative term was “cell”), and 18 MF terms (the most representative term was “binding”). Each of the first ten terms in BP, CC, and MF determined based on P values is listed in Figure 2.

3.4. Categorization of Differentially Downregulated Ribosomal Proteins (RPs). Ribosomes are thought to act as sensors for the heat and cold shock response networks in bacteria and are involved as signals linking environmental stimulus (temperature) with the increased heat shock gene expression [20]. Numerous studies have shown that RPs have a strong functional role, especially in regulating protein synthesis and maintaining the stability of ribosomal complexes [21]. Among the quantified proteins, 44 RPs were identified including 23 30S RPs and 21 50S RPs. Of the 183 significant DE proteins, 31 RPs were upregulated (14 30S and 17 50S, Figure 3) and 21 RPs were downregulated (6 30S and 15 50S). This result is consistent with the fact that RPs may be important for the correct assembly of rRNA under cold stress. All of these RPs had a significant score based on FC, and the very large number indicates that more RPs were likely downregulated in the cold stress environment.

3.5. Main Energy Source Metabolism Network Analysis. The metabolic network of the main energy sources was established, including the citrate cycle, glycolysis/glucoseogenesis, fatty acid degradation, and main amino acid (valine, leucine, and isoleucine) degradation, to reveal the energy change profiles of S. putrefaciens in cold temperature (Figure 4). Some of these energy change profiles contained several proteins, and the preferred upregulated proteins are shown. Overall, 13 proteins were identified and quantified in the constructed energy metabolism network. In total, 6 proteins (46.15%) showed a downregulated trend (FC < 1), including 2 tricarboxylic acid cycle, 3 fatty acid degradation, and 3 main amino acid. Fumarate hydratase class I (EC 4.2.1.2) and isovaleryl-CoA dehydrogenase (EC 1.3.8.4) were slightly upregulated (1.2 < FC < 1.5, P < 0.05), and phosphoglycerate kinase (EC 2.7.2.3), enolase (EC 4.2.1.11), malate dehydrogenase (EC 1.1.1.37), aconitate hydratase B (EC 4.2.1.3), and acetyl-CoA acetyltransferase (EC 2.3.1.9) were significantly upregulated (FC ≥ 1.5, P < 0.05). In addition, these two enzymes are involved in other metabolic processes, such as fatty acid degradation and degradation of valine, leucine, and isoleucine. In addition, retinal dehydrogenase 1 and 3-ketoacyl coenzyme A thiolase are also involved in the degradation of fatty acids and metabolic processes such as valine, leucine, and isoleucine [22]. In
3.6. Interaction Network of Upregulated DE Proteins. Studies have shown that proteins in living cells do not exist as a single entity, but rather as functional associations within the cell [23]. It was of great significance to reveal the qualitative characteristics of proteins through the interaction between the formations of the network [24]. By using Cytoscape software against the *S. oneidensis* database, we explored protein interaction networks altered in the cold stress of *S. putrefaciens* by extracting a putative PPI network. According to the filter criteria of score > 400, 23 proteins in PPIs showed significantly differential abundance between *S. putrefaciens* cultivated at 4°C and 30°C (Figure 5). The proteins were mainly associated with the cellular metabolic process, organonitrogen compound biosynthetic process, plasma membrane ATP synthesis coupled proton transport, protein metabolic process, ATP synthesis coupled proton transport, and ATP biosynthetic process. Among these proteins, 30S and 50S RPs were upregulated, including those encoded by rpl and rps. Proteins linked to ATP synthase, such as atpA and atpG, were downregulated significantly. We speculated that these proteins had a pivotal role in the network.

Bacteria may encounter a variety of physiological threats under low temperature stress, such as less and less membrane fluidity, less and less enzyme activity, irregular protein folding, lower and lower ice formation and transcription rate in cells, nutrient transport, translation, cell changes, and

**Figure 1:** (a) Histogram of log2 protein intensity for each sample. (b) Box plots of log2 protein (or reporter ion) intensity average for each sample.
Table 1: Detailed information regarding upregulated and downregulated differentially expressed proteins in *S. putrefaciens* cultivated at 4°C and 30°C.

| Accession1 | Sequence length | FC2 | Gene names | Locus | Protein names |
|------------|-----------------|-----|------------|-------|---------------|
| **Amino acid transport and metabolism** |
| A4Y849     | 505             | 0.52| Sputcn32_2411 | L-Lysine 6-monoxygenase, EC 1.14.13.59 |
| A4Y315     | 797             | 0.61| Sputcn32_0617 | Bifunctional aspartokinase/homoserine dehydrogenase (includes aspartokinase, EC 2.7.2.4; homoserine dehydrogenase, EC 1.1.1.3) |
| A4Y550     | 456             | 0.70| Sputcn32_1355 | Zinc metalloprotease, EC 3.4.24.- |
| A4YD8      | 451             | 0.72| Sputcn32_1093 | L-Glutamine synthetase, EC 6.3.1.2 |
| A4YC19     | 582             | 0.80| Sputcn32_3795 | Urocanate reductase, EC 1.3.99.33 |
| A4Y713     | 1614            | 0.82| Sputcn32_2195 | Glutamate dehydrogenase, EC 1.4.1.2 |
| A4Y7Q7     | 939             | 1.20| Sputcn32_2269 | 2-Oxoglutarate dehydrogenase E1 component, EC 1.2.4.2 |
| A4Y4E5     | 425             | 1.22| Sputcn32_1100 | 4-Aminobutyrate aminotransferase, EC 2.6.1.19 |
| A4Y656     | 234             | 1.28| Sputcn32_1714 | 3-Oxoadid CoA-transferase, A subunit, EC 2.8.3.5 |
| A4Y1J0     | 679             | 1.31| Sputcn32_0087 | Oligopeptidase A, EC 3.4.24.70 |
| A4Y3F6     | 358             | 1.32| alr         | Alanine racemase, EC 5.1.1.1 |
| A4Y661     | 389             | 1.35| Sputcn32_1719 | Isovaleryl-CoA dehydrogenase, EC 1.3.9.4 |
| A4Y4D3     | 285             | 1.39| Sputcn32_1088 | 3-Mercapto analogue sulfurtransferase, EC 2.8.3.12 |
| A4Y657     | 315             | 1.42| Sputcn32_1715 | Hydroxymethylglutaryl-CoA lyase, EC 4.1.3.4 |
| A4Y3U2     | 189             | 1.43| Sputcn32_0896 | Alkyl hydroperoxide reductase C, EC 1.1.1.15 |
| A4Y219     | 524             | 1.44| Sputcn32_0440 | Histidine ammonia-lyase, EC 4.3.1.13 |
| A4Y540     | 274             | 1.45| dapD        | 2,3,4,5-Tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase, EC 2.3.1.117 |
| A4Y542     | 278             | 1.45| map         | Methionine aminopeptidase, EC 3.4.11.18 |
| A4YBI5     | 377             | 1.46| Sputcn32_3610 | Alanine-glyoxylate aminotransferase, EC 2.6.1.44 |
| A4Y342     | 405             | 1.46| argD        | Acetylornithine amonotransferase, EC 2.6.1.11 |
| A4YCC5     | 341             | 1.47| Sputcn32_3902 | L-Threonine 3-dehydrogenase, EC 1.1.1.103 |
| A4Y3C6     | 386             | 1.54| Sputcn32_0729 | Methionine gamma-lyase, EC 4.4.1.11 |
| A4Y6M4     | 344             | 1.55| astE        | Succinylglutamate desuccinylase, EC 3.5.1.96 |
| A4YCC4     | 397             | 1.56| kbl         | 2-Amino-3-ketobutyrate coenzyme A ligase, EC 2.3.1.29 |
| A4Y7N9     | 444             | 1.61| astB        | N-succinylarginine dihydrolase, EC 3.5.3.23 |
| A4Y1I2     | 555             | 1.82| hutU        | Urocanate hydratase, EC 4.2.1.49 |
| A4Y110     | 408             | 1.85| hutl        | Imidazolonepropionase, EC 3.5.2.7 |
| A4Y1I7     | 451             | 1.89| Sputcn32_0084 | NADPH-glutathione reductase, EC 1.8.1.7 |
| A4Y596     | 300             | 1.89| Sputcn32_1402 | 3-Hydroxyisobutyrate dehydrogenase, EC 1.1.1.31 |
| A4YAE0     | 364             | 1.93| gcvT        | Aminomethyltransferase, EC 2.1.2.10 |
| A4Y612     | 346             | 2.05| Sputcn32_1670 | 4-Hydroxyphenylpyruvate dioxygenase, EC 1.13.11.27 |
| A4Y659     | 288             | 2.09| Sputcn32_1717 | Methylglutacoyl-CoA hydratase, EC 4.2.1.18 |
| A4Y8W2     | 197             | 2.18| Sputcn32_2676 | Thiol peroxidase, EC 1.11.1.15 |
| A4Y946     | 370             | 2.20| aguA        | Putative agmatine deiminase, EC 3.5.3.12 |
| A4Y3Y4     | 169             | 2.21| luxS        | S-Ribosylhomocysteine lyase, EC 4.4.1.21 |
| A4Y733     | 396             | 2.26| Sputcn32_2045 | Aminotransferase, EC 2.6.1.- |
| A4YAD9     | 129             | 2.31| gcvH        | Glycine cleavage system H protein |
| **Carbohydrate transport and metabolism** |
| A4Y4N4     | 707             | 0.66| Sputcn32_1189 | Aldehyde ferredoxin oxidoreductase, EC 1.2.7.5 |
| A4Y7R1     | 131             | 0.67| Sputcn32_2273 | Succinate dehydrogenase subunit C, EC 1.3.5.1 |
| A4Y9V1     | 594             | 0.80| opgD        | Glucan biosynthesis protein D |
| A4Y93      | 545             | 1.32| Sputcn32_1048 | Glucose-6-phosphate isomerase, EC 5.3.1.9 |
| A4Y6B4     | 164             | 1.41| Sputcn32_1772 | 4-Hydroxy-4-methyl-2-oxoglutarate aldolase, EC 4.1.1.112 |
| A4YA6      | 404             | 1.43| Sputcn32_2820 | Phosphopentomutase, EC 5.4.2.7 |
| A4Y566     | 305             | 1.49| Sputcn32_1371 | N-Acetylglucosamine kinase, EC 2.7.1.59 |
| A4Y6K9     | 227             | 1.53| Sputcn32_1869 | 2-Keto-3-deoxy-phosphogluconate aldolase, EC 4.1.2.14 |
| A4Y579     | 302             | 1.60| Sputcn32_1385 | UTP--glucose-1-phosphate uridylyltransferase, EC 2.7.7.9 |
| A4Y769     | 544             | 1.76| Sputcn32_2081 | Glucan biosynthesis protein G |
| **Cell cycle control, cell division, chromosome partitioning** |
| A4Y3E0     | 370             | 0.73| zapE        | Cell division protein ZapE |
| Accession | Sequence length | FC | Gene names | Locus | Protein names |
|-----------|-----------------|----|------------|-------|---------------|
| A4Y6J0    | 192             | 0.59 | rnfA       | Sputcn32_1850 | Ion-translocating oxidoreductase complex subunit A, EC 7.1.1.23 |
| A4YBG8    | 460             | 0.59 | pal        | Sputcn32_3593 | Membrane fusion protein (MFP) family protein |
| A4Y5V0    | 178             | 0.61 | ftsW       | Sputcn32_1608 | Peptidoglycan-associated protein |
| A4Y2N5    | 403             | 0.69 | ftsW       | Sputcn32_0486 | Probable peptidoglycan glycosyltransferase FtsW, EC 2.4.1.129 |
| A4YB04    | 244             | 0.70 | zapD       | Sputcn32_3427 | Cell division protein ZapD |
| A4Y378    | 330             | 0.71 | Sputcn32_0681 | Mg2+ transporter protein, CorA family protein |
| A4Y3B5    | 186             | 0.75 | lptC       | Sputcn32_0718 | Lipopolysaccharide export system protein LptC |
| A4Y8T7    | 395             | 0.77 | Sputcn32_2649 | Outer-membrane protein assembly factor BamB |
| A4Y3B6    | 183             | 1.72 | Sputcn32_0719 | 3-Deoxy-D-manno-octulosonate 8-phosphate phosphatase |
| A4YBR9    | 197             | 1.78 | gmhA       | Sputcn32_3694 | Phosphohexose isomerase, EC 5.3.1.28 |

**Cell wall/membrane/envelope biogenesis**

**Energy production and conversion**

| Accession | Sequence length | FC | Gene names | Locus | Protein names |
|-----------|-----------------|----|------------|-------|---------------|
| A4Y920    | 320             | 0.58 | thrB       | Sputcn32_2734 | Homoserine kinase, EC 2.7.1.39 |
| A4Y6S9    | 334             | 0.62 | ruvB       | Sputcn32_1939 | Holliday junction ATP-dependent DNA helicase RuvB, EC 3.6.4.12 |
| A4Y398    | 545             | 0.68 | groL       | Sputcn32_0701 | ATP synthase epsilon chain (ATP synthase F1 sector epsilon subunit) (F-ATPase epsilon subunit) |
| A4Y7T7    | 637             | 0.68 | htpG       | Sputcn32_2299 | Chaperone protein HtpG |
| A4YCH7    | 142             | 0.68 | atpC       | Sputcn32_3955 | ATP synthase epsilon chain (ATP synthase F1 sector epsilon subunit) (F-ATPase epsilon subunit) |
| A4YCH9    | 286             | 0.69 | atpG       | Sputcn32_3957 | ATP synthase gamma chain |
| A4Y6J2    | 668             | 0.69 | uvrB       | Sputcn32_1852 | UvrABC system protein B, protein UvrB |
| A4Y3P6    | 410             | 0.69 | nqrB       | Sputcn32_0850 | Na (+)-translocating NADH:quinone reductase subunit B, Na (+)-NQR subunit B, Na (+)-translocating NQR subunit B, EC 7.2.1.1 |
| A4Y2C0    | 470             | 0.70 | ntrC       | Sputcn32_0371 | DNA-binding transcriptional regulator NtrC |
| A4Y4K2    | 596             | 0.70 | lepA       | Sputcn32_1157 | Elongation factor 4, EF-4, EC 3.6.5.11 |
| A4YBH4    | 670             | 0.73 | rep        | Sputcn32_3599 | ATP-dependent DNA helicase Rep, EC 3.6.4.12 |
| A4YAH6    | 604             | 0.73 | dsbD       | Sputcn32_3247 | Thiol/disulfide interchange protein DsbD, EC 1.8.1.8 |
| A4Y3E5    | 468             | 0.74 | Sputcn32_0758 | Replicative DNA helicase, EC 3.6.4.12 |
| A4YCH8    | 463             | 0.75 | atpD       | Sputcn32_3956 | ATP synthase epsilon chain (ATP synthase F1 sector epsilon subunit) (F-ATPase epsilon subunit) |
| A4YAV2    | 722             | 0.75 | Sputcn32_3373 | DNA helicase, EC 3.6.4.12 |
| A4Y1A4    | 461             | 0.76 | dnaA       | Sputcn32_0001 | Chromosomal replication initiator protein DnaA |
| A4Y934    | 856             | 0.76 | mutS       | Sputcn32_2748 | DNA mismatch repair protein MutS |
| A4YCI0    | 513             | 0.76 | atpA       | Sputcn32_3958 | ATP synthase subunit alpha, EC 7.1.2.2 |
| A4Y4B7    | 429             | 0.76 | Sputcn32_1072 | NADH dehydrogenase, EC 1.6.99.3 |
| A4Y675    | 720             | 0.77 | Sputcn32_1733 | Polysphosphate kinase, EC 2.7.1.41 |
| A4Y965    | 149             | 0.77 | nrdR       | Sputcn32_2779 | Transcriptional repressor NrdR |
| A4YBZ9    | 319             | 0.77 | birA       | Sputcn32_3775 | Bipolarized and repressor BirA |
| A4Y406    | 274             | 0.78 | hmuV       | Sputcn32_0960 | Hemin import ATP-binding protein HmuV, EC 7.6.2.- |
| A4Y9Z6    | 494             | 0.78 | Sputcn32_3066 | UDP-N-acetylmuramate-L-alanyl-gamma-D-glutamyl-meso-2,6-diaminoheptanodioic acid, EC 6.3.2.45 |
| A4Y3M4    | 464             | 0.79 | mpl        | Sputcn32_0827 | DNA repair protein RadA |
| A4Y9A0    | 454             | 0.79 | radA       | Sputcn32_2814 | DNA repair protein RadA |
| A4Y2J9    | 696             | 0.80 | recG       | Sputcn32_0450 | ATP-dependent DNA helicase RecG, EC 3.6.4.12 |
| A4Y470    | 857             | 0.80 | clpB       | Sputcn32_1024 | Chaperone protein ClpB |
| A4Y8E4    | 192             | 0.80 | tdk        | Sputcn32_2506 | Thymidine kinase, EC 2.7.1.21 |
| A4YN9     | 207             | 0.81 | Sputcn32_2957 | Corrinoid adenosyltransferase, EC 2.5.1.17 |
| A4Y5P9    | 602             | 0.81 | msaA       | Sputcn32_1557 | Lipid A export ATP-binding/permease protein MsaA, EC 7.5.2.6 |
| A4Y427    | 388             | 0.82 | obg         | Sputcn32_0981 | GTPase Obg, EC 3.6.5.- |
| A4Y4E0    | 378             | 0.83 | potA       | Sputcn32_1095 | Spermidine/putrescine import ATP-binding protein PotA, EC 7.6.2.11 |
| A4Y8T3    | 525             | 1.20 | guaA       | Sputcn32_2645 | GMP synthase (glutamine-hydrolyzing), EC 6.3.5.2 |
| A4YBZ8    | 316             | 1.21 | coaA       | Sputcn32_3774 | Pantothenate kinase, EC 2.7.1.33 |
| A4Y8F6    | 571             | 1.22 | proS       | Sputcn32_2518 | Proline-tRNA ligase, EC 6.1.1.15 |
| A4Y9M1    | 1074            | 1.22 | carB       | Sputcn32_2939 | Carbamoyl-phosphate synthase large chain, EC 6.3.5.5 |
| Accession | Sequence length | FC | Gene names | Locus | Protein names |
|-----------|----------------|----|------------|-------|---------------|
| A4Y5H4    | 556            | 1.24| glnS       | Sputcn32_1482 | Glutamine--tRNA ligase, EC 6.1.1.18 |
| A4Y334    | 640            | 1.25| deaD       | Sputcn32_0637 | ATP-dependent RNA helicase DeaD, EC 3.6.4.13 |
| A4YBS3    | 332            | 1.25| trpS       | Sputcn32_3698 | Tryptophan--tRNA ligase, EC 6.1.1.2 |
| A4YN71    | 789            | 1.25|           | Sputcn32_2243 | Phosphoenolpyruvate synthase, EC 2.7.9.2 |
| A4YNM2    | 386            | 1.29| carbA      | Sputcn32_2940 | Carbamoyl-phosphate synthase small chain, EC 6.3.5.5 |
| A4YN78    | 345            | 1.32| purM       | Sputcn32_1596 | Phosphoribosylformylglycinamidine cyclo-ligase, EC 6.3.3.1 |
| A4YNM8    | 360            | 1.33| nadA       | Sputcn32_1888 | Quinolinate synthase A, EC 2.5.1.72 |
| A4Y545    | 245            | 1.34| pyrH       | Sputcn32_1350 | Uridylate kinase, EC 2.7.4.22 |
| A4YN24    | 439            | 1.37| murD       | Sputcn32_0485 | UDP-N-acetylmuramoylalanine--D-glutamate ligase, EC 6.3.2.9 |
| A4YN4H3   | 397            | 1.37| tyrS       | Sputcn32_1128 | Tyrosine--tRNA ligase, EC 6.1.1.11 |
| A4YN4A    | 550            | 1.37| rhoE       | Sputcn32_3108 | ATP-dependent RNA helicase RhoE, EC 3.6.4.13 |
| A4YN928   | 958            | 1.38| valS       | Sputcn32_2742 | Valine--tRNA ligase, EC 6.1.1.9 |
| A4YN9Z3   | 565            | 1.40| cysI       | Sputcn32_3063 | Sulfite reductase [NADPH] hemoprotein beta-component, EC 1.8.1.2 |
| A4YN59    | 323            | 1.41| thlL       | Sputcn32_2773 | Thiamine-monophosphate kinase, EC 2.7.4.16 |
| A4YN6G8   | 327            | 1.42| pheS       | Sputcn32_1828 | Phenylalanine--tRNA ligase alpha subunit, EC 6.1.1.20 |
| A4YN7E3   | 231            | 1.43| loI0D      | Sputcn32_2155 | Lipoprotein-releasing system ATP-binding protein LoID, EC 7.6.2.- |
| A4YNBL9   | 292            | 1.44| prpB       | Sputcn32_3644 | 2-Methylisocitrate lyase, 2-MIC, EC 4.1.3.30 |
| A4YN4A4   | 940            | 1.46| ileS       | Sputcn32_1059 | Isoleucine--tRNA ligase, EC 6.1.1.15 |
| A4YN57    | 401            | 1.49| fabV       | Sputcn32_1495 | Enoyl-[acyl-carrier-protein] reductase [NADH], EC 1.3.1.9 |
| A4YN4A    | 317            | 1.51| gshA       | Sputcn32_1362 | Glutathione synthetase, EC 6.3.2.3 |
| A4YN7C9   | 143            | 1.53| ndk        | Sputcn32_2141 | Nucleoside diphosphate kinase, EC 2.7.4.6 |
| A4YN6Z5   | 428            | 1.55| serS       | Sputcn32_2007 | Serine--tRNA ligase, EC 6.1.1.11 |
| A4YN9Q3   | 639            | 1.60| dnaK       | Sputcn32_2971 | Chaperone protein DnaK |
| A4YN2H2   | 198            | 1.71| azoR       | Sputcn32_0423 | FMN-dependent NADH-azoreductase, EC 1.7.1.17 |
| A4YNAYO   | 432            | 2.11| purD       | Sputcn32_3402 | Phosphoribosylamine--glycine ligase, EC 6.3.4.13 |
| A4YN8O    | 391            | 2.25| pgk         | Sputcn32_0874 | Phosphoglycerate kinase, EC 2.7.2.3 |
| A4YN397   | 96             | 3.88| groS       | Sputcn32_2940 | 10 kDa chaperonin |

**Lipid transport and metabolism**

| Accession | Sequence length | FC | Gene names | Locus | Protein names |
|-----------|----------------|----|------------|-------|---------------|
| A4YF4     | 203            | 0.54| plsY       | Sputcn32_1109 | G3P acyltransferase, GPAT, EC 2.3.1.25 |
| A4YN9    | 219            | 0.70| lipB       | Sputcn32_2875 | Octanoyltransferase, EC 2.3.1.181 |
| A4YN625   | 238            | 0.72| fabR       | Sputcn32_1683 | Fatty acid metabolism regulator protein |
| A4YN8     | 436            | 0.72| fabD       | Sputcn32_2460 | 3-Ketoacyl-CoA thiolase, EC 2.3.1.16 |
| A4YN553   | 341            | 0.75| lpxD       | Sputcn32_1358 | UDP-3-O-acylglucosamine N-acetyltransferase, EC 2.3.1.7 |
| A4YN54    | 248            | 1.22|           | Sputcn32_1582 | 3-Oxoacyl-[acyl-carrier-protein] reductase, EC 1.1.1.100 |
| A4YN4D0   | 498            | 1.34|           | Sputcn32_1085 | Aldehyde dehydrogenase (acceptor), EC 1.2.5.2 |
| A4YN7Z3   | 354            | 1.38| fabH       | Sputcn32_2355 | 3-Oxoacyl-[acyl-carrier-protein] synthase 3, EC 2.3.1.180 |
| A4YN3G1   | 245            | 1.49|           | Sputcn32_0764 | Enol-CoA hydratase, EC 4.2.1.17 |
| A4YN5S3   | 308            | 1.74|           | Sputcn32_1581 | Malonyl CoA-acyl carrier protein transacylase, EC 2.3.1.39 |
| A4YN883   | 411            | 1.83|           | Sputcn32_2445 | 3-Oxoacyl-[acyl-carrier-protein] synthase I, EC 2.3.1.41 |
| A4YNX9    | 171            | 2.05| fabA       | Sputcn32_1637 | 3-Hydroxydecanoyl-[acyl-carrier-protein] dehydratase, EC 4.2.1.59 |
| A4YN5S5   | 77             | 2.54| acpP       | Sputcn32_1583 | Acyl carrier protein, ACP |

**Nucleotide transport and metabolism**

| Accession | Sequence length | FC | Gene names | Locus | Protein names |
|-----------|----------------|----|------------|-------|---------------|
| A4YN51    | 735            | 0.64|           | Sputcn32_2765 | (P)ppGpp synthetase I, SpoT/RelA, EC 2.7.6.5 |
| A4YN58    | 343            | 1.32| pyrC       | Sputcn32_2994 | Dihydroorotase, EC 3.5.2.3 |
| A4YN79    | 208            | 1.33| upp         | Sputcn32_1597 | Uracil phosphoribosyltransferase, EC 2.4.2.9 |
| A4YN72    | 339            | 1.37| pyrD        | Sputcn32_2194 | Dihydroorotate dehydrogenase, EC 1.3.5.2 |
| A4YNX9    | 543            | 1.46| purH        | Sputcn32_3401 | Bifunctional purine biosynthesis protein PurH (includes phosphoribosylaminomimidazolecarboxamide formyltransferase, EC 2.1.2.3 (AICAR transformylase); IMP cyclohydrolase, EC 3.5.4.10 (ATIC)) |
| A4YN8X    | 205            | 1.49|           | Sputcn32_2692 | dTTP/XTP pyrophosphatase, EC 3.6.1.66 |
| A4YN97    | 443            | 1.54| deoA        | Sputcn32_2821 | Thymidine phosphorlyase, EC 2.4.2.4 |
| A4YN2U    | 252            | 1.55|           | Sputcn32_0543 | Uridine phosphorlyase, EC 2.4.2.3 |
Table 1: Continued.

| Accession | Sequence length | FC² | Gene names | Locus | Protein names |
|-----------|----------------|-----|------------|-------|---------------|
| A4YCD7    | 331            | 1.62 | add        | Sputcn32_3914 | Adenosine deaminase, EC 3.5.4.4 |
| A4Y2C8    | 103            | 1.64 | pppP       | Sputcn32_0379 | Pyrimidine/purine nucleoside phosphorylase, EC 2.4.2.2 |
| A4YSR0    | 296            | 1.71 | cdd        | Sputcn32_1568 | Cytidine deaminase, EC 3.5.4.5 |
| A4Y7B5    | 245            | 1.80 | kdsB       | Sputcn32_2127 | 8-Amino-3,8-dideoxy-manno-octulosonate cytidylyltransferase, EC 2.7.7.90 |
| A4Y7K5    | 193            | 1.99 | dcd        | Sputcn32_2217 | dCTP deaminase, EC 3.5.4.13 |
| A4Y726    | 231            | 2.08 | pyrF       | Sputcn32_2038 | Orotidine 5′-phosphate decarboxylase, EC 4.1.1.23 |
| A4Y4P9    | 145            | 2.97 |           | Sputcn32_1204 | Cytosine deaminase, EC 3.5.4.1 |

Replication, recombination, and repair

| Accession | Sequence length | FC² | Gene names | Locus | Protein names |
|-----------|----------------|-----|------------|-------|---------------|
| A4Y6I4    | 213            | 0.74 | nth        | Sputcn32_1844 | Endonuclease III, EC 4.2.99.18 |
| A4Y5F1    | 609            | 0.82 | uvrC       | Sputcn32_1640 | UvrABC system protein C, protein UvrC |
| A4Y6M1    | 185            | 1.24 | seqA       | Sputcn32_1881 | Negative modulator of initiation of replication |
| A4Y8J7    | 237            | 1.24 | fliA       | Sputcn32_2559 | RNA polymerase sigma factor FliA |
| A4Y729    | 95             | 1.43 | ihfB       | Sputcn32_2041 | Integration host factor subunit beta, IHF-beta |
| A4YBK6    | 92             | 1.63 | rpoZ       | Sputcn32_3631 | DNA-directed RNA polymerase subunit omega, EC 2.7.7.6 |
| A4Y9D3    | 158            | 1.65 | greA       | Sputcn32_2847 | Transcription elongation factor GreA |
| A4Y805    | 69             | 2.50 | yacG       | Sputcn32_3428 | DNA gyrase inhibitor YacG |

Secondary metabolites

| Accession | Sequence length | FC² | Gene names | Locus | Protein names |
|-----------|----------------|-----|------------|-------|---------------|
| A4YAT1    | 493            | 0.62 | ubiD       | Sputcn32_3352 | 3-Octaprenyl-4-hydroxybenzoate carboxy-lyase, EC 4.1.1.98 |
| A4Y760    | 222            | 0.64 |            | Sputcn32_2072 | HAD-superfamily hydrolase, subfamily IA, variant 1, EC 3.1.3.18 |
| A4Y3M6    | 176            | 0.69 |            | Sputcn32_0829 | Hypoxanthine phosphoribosyltransferase, EC 2.4.2.8 |
| A4Y930    | 418            | 0.71 |            | Sputcn32_2744 | Aspartokinase, EC 2.7.2.4 |
| A4YAL8    | 289            | 0.73 | psd        | Sputcn32_3289 | Phosphatidylserine decarboxylase proenzyme, EC 4.1.1.65 |
| A4YQ6     | 400            | 0.74 |            | Sputcn32_2268 | Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, EC 2.3.1.61 |
| A4Y9A3    | 331            | 0.75 |            | Sputcn32_2817 | Phosphoserine phosphatase, EC 3.1.3.3 |
| A4Y7C7    | 164            | 0.76 |            | Sputcn32_2139 | Acetolactate synthase, small subunit, EC 2.2.1.6 |
| A4Y7J9    | 437            | 0.79 |            | Sputcn32_2031 | Phospholipase D/transphosphatidylase, EC 2.7.8.8 |
| A4Y9C6    | 277            | 0.82 |            | Sputcn32_2840 | Dihydrolipoamide synthetase, EC 2.5.1.15 |
| A4Y2M0    | 522            | 1.21 | leuA       | Sputcn32_0471 | 2-Isopropylmalate synthase, EC 2.3.1.13 |
| A4Y9M3    | 270            | 1.22 | dapB       | Sputcn32_2941 | 4-Hydroxy-tetrahydrodipicolinate reductase, EC 1.17.1.8 |
| A4Y9W8    | 173            | 1.27 |            | Sputcn32_3038 | Protoporphyrinogen oxidase, EC 1.3.3.4 |
| A4Y2M2    | 474            | 1.29 | leuC       | Sputcn32_0473 | 3-Isopropylmalate dehydratase large subunit, EC 4.2.1.33 |
| A4Y7E1    | 267            | 1.31 |            | Sputcn32_2153 | Inositol-1-monophosphatase, EC 3.1.3.25 |
| A4Y4A7    | 318            | 1.32 | ispH       | Sputcn32_1062 | 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase, EC 1.17.7.4 |
| A4Y7T4    | 440            | 1.34 |            | Sputcn32_1239 | Isocitrate lyase, EC 4.1.3.1 |
| A4Y3R8    | 664            | 1.35 |            | Sputcn32_0872 | Transketolase, EC 2.2.1.1 |
| A4YAZ4    | 888            | 1.39 |            | Sputcn32_3417 | Pyruvate dehydrogenase E1 component, EC 1.2.4.1 |
| A4YBI7    | 619            | 1.40 | ilvD       | Sputcn32_3612 | Dihydroxy-acid dehydrogenase, EC 4.2.1.9 |
| A4YM1     | 364            | 1.42 | leuB       | Sputcn32_0472 | 3-Isopropylmalate dehydrogenase, EC 1.1.1.85 |
| A4Y486    | 163            | 1.44 | purE       | Sputcn32_1041 | N5-carboxyaminomimidazole ribonucleotide mutase, N5-CAIR mutase, EC 5.4.99.18 |
| A4Y6E8    | 514            | 1.48 |            | Sputcn32_1807 | Fumarate hydratase class I, EC 4.2.1.2 |
| A4Y4X1    | 293            | 1.49 |            | Sputcn32_1276 | Farnesyl-diphosphate synthase, EC 2.5.1.10 |
| A4Y5G2    | 394            | 1.49 |            | Sputcn32_1470 | Glycerate kinase, EC 2.7.1.31 |
| A4Y424    | 331            | 1.51 |            | Sputcn32_0978 | Trans-hexaprenyltransferase, EC 2.5.1.30 |
| A4Y7N3    | 357            | 1.54 |            | Sputcn32_2245 | Phospho-2-dehydro-3-deoxyheptonate aldolase, EC 2.5.1.54 |
| A4Y591    | 396            | 1.55 |            | Sputcn32_1397 | Acetyl-CoA acetyltransferase, EC 2.3.1.9 |
| A4YAY7    | 865            | 1.58 |            | Sputcn32_3409 | Acotinate hydratase B, EC 4.2.1.3 |
| A4Y9W4    | 217            | 1.60 |            | Sputcn32_3034 | Dihydropteridine reductase, EC 1.5.1.34 |
Table 1: Continued.

| Accession1 | Sequence length | FC2  | Gene names | Locus | Protein names                          |
|------------|----------------|------|------------|-------|----------------------------------------|
| A4Y670     | 288            | 1.63 | Sputcn32_1728 |    | Acyl-CoA thioesterase II, EC 3.1.2.2   |
| A4YAE8     | 311            | 1.66 | mdh        | Sputcn32_3219 | Malate dehydrogenase, EC 1.1.1.37     |
| A4Y494     | 318            | 1.66 | tal        | Sputcn32_1049 | Transaldolase, EC 2.2.1.2             |
| A4YAY4     | 354            | 1.68 | hemE       | Sputcn32_3406 | Uroporphyrinogen decarboxylase, EC 4.1.1.37 |
| A4YS5N5    | 203            | 1.74 | ribA       | Sputcn32_1543 | GTP cyclohydrolase-2, EC 3.5.4.25     |
| A4YID3     | 302            | 1.75 | hemF       | Sputcn32_0030 | Oxygen-dependent coproporphyrinogen-III oxidase, EC 1.3.3.3 |
| A4YBS4     | 226            | 1.75 | gph        | Sputcn32_3699 | Phosphoglycolate phosphatase, EC 3.1.1.18 |
| A4Y4G4     | 430            | 1.77 | hemL       | Sputcn32_1119 | Glutamate-1-semialdehyde 2,1-aminomutase, EC 2.5.1.47 |
| A4Y4H4     | 294            | 1.96 | dapA       | Sputcn32_1702 | 4-Hydroxy-tetrahydrodipicolinate synthase, EC 4.3.3.7 |
| A4Y1D3     | 278            | 2.00 | trpA       | Sputcn32_2408 | Tryptophan synthase alpha chain, EC 4.2.1.20 |
| A4YBG2     | 310            | 2.04 | hemC       | Sputcn32_3587 | Porphobilinogen deaminase, EC 2.5.1.61 |
| A4Y7T5     | 214            | 2.14 | adk        | Sputcn32_2297 | Adenylate kinase, EC 2.7.4.3          |
| A4Y1E3     | 514            | 2.14 | gpmI       | Sputcn32_0040 | 2,3-Bisphosphoglycerate-independent phosphoglycerate mutase, EC 5.4.2.12 |
| A4Y881     | 338            | 2.14 | truB       | Sputcn32_2832 | tRNA pseudouridine synthase B, EC 5.4.99.25 |
| A4Y9L3     | 220            | 2.15 | rpiA       | Sputcn32_2931 | Ribose-5-phosphate isomerase A, EC 5.3.1.6 |
| A4Y966     | 417            | 2.22 | glyA       | Sputcn32_2780 | Serine hydroxymethyltransferase, SHMT, EC 2.1.2.1 |
| A4Y3V1     | 597            | 2.22 | nusA       | Sputcn32_2835 | Transcription termination/antitermination protein NusA |
| A4Y4S5     | 292            | 2.27 | rlmJ       | Sputcn32_0098 | Ribosomal RNA large subunit methyltransferase J, EC 2.1.1.91 |
| A4Y4G4     | 237            | 2.31 | eno        | Sputcn32_2757 | Fumarate reductase flavoprotein subunit, EC 1.3.1.18 |
| A4Y7M6     | 344            | 2.41 | rpsC       | Sputcn32_3753 | 30S ribosomal protein S3 |
| A4Y9B8     | 318            | 0.63 | truB       | Sputcn32_2832 | tRNA pseudouridine synthase B, EC 5.4.99.25 |
| A4Y9L4     | 396            | 0.79 | rlmJ       | Sputcn32_2932 | Ribosomal RNA large subunit methyltransferase I, EC 2.1.1.91 |
| A4Y9C1     | 499            | 1.27 | nusA       | Sputcn32_2835 | Transcription termination/antitermination protein NusA |
| A4YB21     | 101            | 1.31 | fis        | Sputcn32_3444 | DNA-binding protein Fis |
| A4Y2K5     | 237            | 1.31 | rph        | Sputcn32_0456 | Ribonuclease PH, EC 2.7.7.56 |
| A4Y960     | 134            | 1.66 | nusB       | Sputcn32_2774 | Transcription antitermination protein NusB |
| A4Y9B8     | 228            | 0.67 | tolQ       | Sputcn32_1604 | Tol-Pal system protein TolQ |
| A4Y7L6     | 102            | 0.70 | clpS       | Sputcn32_2228 | ATP-dependent Clp protease adapter protein ClpS |
| A4YBZ6     | 123            | 0.71 | secE       | Sputcn32_3772 | Protein translocase subunit SecE |
| A4Y83      | 291            | 0.71 | rlmJ       | Sputcn32_2398 | Pseudouridine synthase, EC 5.4.99.25 |
| A4Y4C1     | 281            | 0.73 | rlmJ       | Sputcn32_0098 | Ribosomal RNA large subunit methyltransferase J, EC 2.1.1.266 |
| A4Y4B2     | 201            | 0.73 | rplD       | Sputcn32_3758 | 50S ribosomal protein L4 |
| A4Y5M6     | 291            | 0.75 | rpsD       | Sputcn32_3758 | 50S ribosomal protein S10 |
| A4Y3Y6     | 156            | 0.76 | atpF       | Sputcn32_3960 | ATP synthase subunit b |
| A4Y4S7     | 230            | 0.78 | rpsC       | Sputcn32_3753 | 30S ribosomal protein S3 |
| A4Y565     | 273            | 0.78 | atpB       | Sputcn32_3962 | ATP synthase subunit a |
| A4Y4B9     | 315            | 0.79 | secF       | Sputcn32_2481 | Protein-export membrane protein SecF |
| A4Y4C5     | 110            | 0.79 | yajC       | Sputcn32_2483 | Sec translacor accessory complex subunit YajC |
| A4Y5K5     | 338            | 0.80 | era         | Sputcn32_1160 | GTase Era |
| A4Y4B4     | 103            | 1.26 | rpsL       | Sputcn32_3760 | 30S ribosomal protein S10 |
| A4Y4S7     | 174            | 1.31 | bamE       | Sputcn32_1232 | Outer-membrane protein assembly factor BamE |
| A4Y4V9     | 206            | 1.32 | rpsD       | Sputcn32_3735 | 30S ribosomal protein S4 |
| A4Y3E7     | 246            | 1.33 | rlmB       | Sputcn32_0750 | 23S rRNA (guanosine-2’-O-)-methyltransferase RlmB, EC 2.1.1.185 |
| A4Y4W1     | 118            | 1.41 | rpsM       | Sputcn32_3737 | 30S ribosomal protein S13 |
| A4Y3E1     | 142            | 1.42 | rplM       | Sputcn32_0744 | 50S ribosomal protein L13 |
| A4Y9C2     | 151            | 1.42 | rimP       | Sputcn32_2836 | Ribosome maturation factor RimP |
| A4Y4W4     | 144            | 1.44 | rpoO       | Sputcn32_3740 | 50S ribosomal protein L15 |
| A4Y544     | 283            | 1.45 | tsf         | Sputcn32_1349 | Elongation factor Ts, EF-Ts |
| A4Y4W0     | 130            | 1.47 | rpsK       | Sputcn32_3736 | 30S ribosomal protein S11 |
| A4Y3E2     | 130            | 1.49 | rpsL       | Sputcn32_0745 | 30S ribosomal protein S9 |
| A4Y7O5     | 143            | 1.56 | infC        | Sputcn32_2017 | Translation initiation factor IF-3 |
| A4Y6A5     | 85             | 1.57 | minE        | Sputcn32_1763 | Cell division topological specificity factor |
| A4Y3F3     | 150            | 1.61 | rplL       | Sputcn32_0756 | 50S ribosomal protein L9 |
| A4Y4X9     | 92             | 1.67 | rpsS       | Sputcn32_3755 | 30S ribosomal protein S19 |
3.7. Energy Metabolism. We detected 6 downregulated proteins and 7 upregulated proteins related to energy metabolism. A significantly higher level of enzymes involved in glycolysis, which may suggest that \textit{S. putrefaciens} under implicates the production of high energy intermediates, such as pyruvate (Figure 3). Upregulation of the key enzymes phosphoglycerate kinase and enolase showed similar results in glycolysis. Phosphoglycerate kinase is a housekeeping gene referred to carbon metabolism and energy production [26]. There are eight DE proteins participating in phosphoglycerate kinase (PGK, EC 2.7.2.3), glycolysis, and enolase, which were more abundant in \textit{S. putrefaciens} cultivated at 4°C than that at 30°C (\textit{S. putrefaciens} cultivated at 4°C/S. putrefaciens cultivated at 30°C > 1.5) (Figure 3).

Glycolysis was a more active energy-producing pathway in \textit{S. putrefaciens} cultivated at 4°C under nonfavorable temperature, which was also demonstrated by Sun et al. [30]. PGK is found in every domain and in almost all living organisms [31]. It is coded by pgk and PGK activity relates to the conversion of ADP + 3-phospho-d-glyceraldehyde to ATP + 3-phospho-d-glycerate. PGK is one of the oldest “housekeeping” enzymes, which is found in the most ubiquitous three-carbon portion of the best studied and probably the most ancient metabolic pathway—glycolysis, the Embden–Meyerhof–Parnas cycle (fermentation). PGK activity is also present in several other biochemical processes (e.g., Calvin–Benson–Bassham CO2 fixation cycle = “CBB”) and is often characterized as metabolically essential [32]. Enolase is a rich expression of cytoplasmic protein in many

### Table 1: Continued.

| Accession | Sequence length | FC | Genes | Locus | Protein names |
|-----------|----------------|----|-------|-------|---------------|
| A4YBY8    | 124            | 1.71| rpsL  | Sputcn32_3764 | 30S ribosomal protein S12 |
| A4YBV7    | 131            | 1.72| rplQ  | Sputcn32_3733 | 50S ribosomal protein L17 |
| A4YBW5    | 60             | 1.72| rpmD  | Sputcn32_3741 | 50S ribosomal protein L30 |
| A4YS80    | 56             | 1.75| rpmF  | Sputcn32_1578 | 50S ribosomal protein L32 |
| A4Y9F3    | 109            | 1.77| rpsS  | Sputcn32_2867 | Ribosomal silencing factor RsfS |
| A4Y9B7    | 89             | 1.84| rplO  | Sputcn32_2831 | 30S ribosomal protein S15 |
| A4YBY7    | 156            | 1.86| rpsG  | Sputcn32_3763 | 30S ribosomal protein S7 |
| A4Y704    | 64             | 1.87| rplM  | Sputcn32_2016 | 50S ribosomal protein L35 |
| A4Y7A0    | 72             | 1.90| infA  | Sputcn32_2226 | Translation initiation factor IF-1 |
| A4Y426    | 84             | 1.90| rpmA  | Sputcn32_0980 | 50S ribosomal protein L27 |
| A4Y4L3    | 83             | 1.90| rpsL  | Sputcn32_1168 | 30S ribosomal protein S16 |
| A4YBM9    | 73             | 1.92| zapB  | Sputcn32_3654 | Cell division protein ZapB |
| A4YBX6    | 136            | 1.93| rplP  | Sputcn32_3752 | 50S ribosomal protein L16 |
| A4Y6L5    | 186            | 1.96| efp   | Sputcn32_1875 | Elongation factor P, EF-P |
| A4Y3F2    | 75             | 2.05| rpsR  | Sputcn32_0755 | 30S ribosomal protein S18 |
| A4Y4A1    | 88             | 2.09| rpsT  | Sputcn32_1056 | 30S ribosomal protein S20 |
| A4Y5V1    | 249            | 2.09| cpoB  | Sputcn32_1609 | Outer-membrane lipoprotein carrier protein |
| A4Y6Z8    | 208            | 2.14| lolA  | Sputcn32_2010 | tRNA (cytidine (34)-2′-O)-methyltransferase, EC 2.1.1.207 |
| A4Y7B4    | 154            | 2.17| trmL  | Sputcn32_3498 | 50S ribosomal protein L22 |
| A4YBX8    | 110            | 2.21| rplV  | Sputcn32_3754 | 50S ribosomal protein L24 |
| A4YBX2    | 104            | 2.22| rplX  | Sputcn32_3748 | 50S ribosomal protein L31 |
| A4Y2V2    | 79             | 2.28| rpmE  | Sputcn32_0553 | Ribosome-recycling factor, RRF |
| A4Y546    | 185            | 2.28| frf   | Sputcn32_1351 | 50S ribosomal protein L18 |
| A4YBW7    | 116            | 2.44| rplR  | Sputcn32_3743 | 50S ribosomal protein L14 |
| A4YBX0    | 101            | 2.61| rpsN  | Sputcn32_3746 | 50S ribosomal protein L20 |
| A4Y703    | 118            | 2.62| rplT  | Sputcn32_2015 | 50S ribosomal protein L33 |
| A4Y2L3    | 57             | 3.30| rpmG  | Sputcn32_0464 | Ribosome modulation factor, RMF |
| A4Y5X8    | 58             | 3.61| rnf   | Sputcn32_1636 | 50S ribosomal protein L34 |
| A4YCM5    | 45             | 3.83| rpmH  | Sputcn32_4005 | 50S ribosomal protein L36 |
| A4YBW2    | 37             | 4.44| rplJ  | Sputcn32_3738 | 50S ribosomal protein L36 |

1UniProt and NCBI database accession numbers. 2Average ratio from three replicates by TMT experiment. A protein species was considered differentially accumulated if it exhibited a fold change > 1.5-fold (cultivated at 4°C/cultivated at 30°C) with a P value of < 0.05.

Not assigned

A4Y635 | 218 | 1.41 | Sputcn32_1713 | Butyryl-CoA:acetate CoA transferase, EC 2.8.3.8
organisms. It plays a very important role, especially at the end of the catabolic glycolytic pathway, it is a key glycolytic enzyme, and its main role is to catalyze the dehydration of 2-phosphate-d-glycerol (2-PGA) to produce phosphoenolpyruvate (PEP). In the presence of magnesium ions, it provides energy for organisms, so it is an important energy obtaining way for cells [33]. The upregulation of PGK and enolase was accompanied by the increase of glycolysis rate, which indicates the accumulation of pyruvate.

3.8. RPs. As mentioned above, RPs are one of the most abundant proteins. The effect of cold stress significantly increased their expression (Table 1), indicating their important role in the response to cold stress. Cold stress made the structure of ribosome subunits [34] incomplete, which stalled the translation process and reduced the number of polymers, but this reduction was temporary, accompanied by an increase in the number of a single 70 ribosome and 50 and 30 subunits [15]. Consistent with this, quantities of RPs increased when S. putrefaciens was cultivated at 4°C in the current research. For L. monocytogenes strains, the strongest and most active genomes are associated with ribosomal genes under cold stress [35]. We observed that nearly all of 30S and 50S RPs were upregulated, including those encoded by rpl, rps, and rpm. The RPs themself adapt to the new stress conditions [36]. In vitro experiments showed that the E. coli translation apparatus in the cold environment can preferentially act on mRNAs from cold-induced genes [37]. The drop in temperature reduces cell membrane fluidity at the cellular level, and the active transport and secretion of proteins are also affected. The role of RNA helicase in different desiccants was studied. Helicase helps to unlock the secondary structure of RNA so that efficient transcription and translation processes can be achieved under cold stress [38]. Some studies on cold-loving and mesothermal prokaryotes have shown that external ribonuclease and helicase (dead box protein) have this effect; chaperonin proteins have important roles in RNA degradation, nucleotide excision repair pathways, and cold adaptation [39]. There was evidence that RNA degradation under cold stress helps the cells to adapt its RNA metabolism for subsequent growth under cold stress [40]. With the stability of the secondary structure of DNA and RNA, in the case of reduced transcription and translation efficiency and low protein folding efficiency, RPs need to adapt to cold stress in order to function normally [41]. RPs are responsible for ribosome biogenesis and protein translation and play important roles in controlling cell growth, division, and development [42]. In the current research, 47 PRs were identified as being upregulated. The kinetic properties of RPs are different at 4°C than at 30°C, and a large increase in ribosome proteins may lead to the improvement of translation efficiency. Upregulated RPs
under cold stress could enhance the appropriate translation or function of ribosome assembly in response to growth requirements. Under this circumstance, ribosomes that are more active under cold stress might be very important for *S. putrefaciens* under cold stress.

3.9. Translation. In the current study, several factors are upregulated under low temperature stress, such as proteins involved in translation (such as initiation factors IF-2 and extension factors), chaperones involved in protein folding, and proteins involved in transcription (such as DNA-directed RNA polymerase) found in *Streptococcus putrefaciens*. This shows that *S. putrefaciens* entered a stabilized phase, which is distinct from the state upon cold shock [15]. In the case of elongation factors, GreA, EF-P, and EF-Ts were upregulated. All were produced at a high level in *S. putrefaciens* under cold stress, indicating that protein synthesis was maintained to live under cold stress. The transcript cleavage factor, GreA, interacts with the RNAP secondary channel and stimulates the intrinsic transcript cleavage activity of RNAP for the removal of the aberrant RNA 3' ends. Therefore, polymerization activity can be restarted from the end of a cleaved RNA allowing transcription to resume [43]. GreA is essential for the survival of bacteria under stress [44] and it can facilitate RecBCD-mediated resection and inhibits RecA, which plays an important role in impeding DNA break repair in *E. coli* [45], indicating the role of GreA in adapting to the stressful environments. The eukaryotic and archaea extension factor 5A (E/A EF-5A) and its prokaryotic bacterial translation extension factor P (EF-P) would slow the ribosome stagneation. The L-type EF-P is also composed of three bucket domains. EF-p binds to the polyproline-stalled ribosomes between the peptide-TRNA binding site (P site) and the exiting tRNA (E site) and stimulates the formation of peptide bonds by stabilizing the CCA end of prolyl-TRNA at P site. During translation elongation, the ribosome, with the help of translation elongation factors EF-G, EF-TU, and EF-TS, binds the corresponding amino acid of each codon to the growing polypeptide chain and drives along the coding sequence of the mRNA. Ef-ts are involved in protein synthesis and translation extension, and their expression is increased [46–49].

3.10. Lipid Transport and Metabolism. The effect of temperature on bacterial membrane lipids has been extensively studied [50, 51]. Bacteria adapt their membranes to lower the phase-transition temperature below which their membrane
changes from a “fluid” (liquid-crystalline) to a “rigid” phase to maintain sufficient membrane fluidity under cold stress [52, 53]. In the current research, 13 proteins (5 downregulated and 8 upregulated) were associated with lipid transport and metabolism in \textit{S. putrefaciens} under cold stress. PlsY, lipB, fadR, fadI, and lpxD related to the lipid transport and metabolism were downregulated in \textit{S. putrefaciens} under cold stress. FadR regulatory protein acts as a regulator controlling bacterial lipid metabolism by inhibiting the fatty acid degradation (fad) system and activating the synthesis of unsaturated fatty acids [54]. In \textit{E. coli}, FadR is a key gene for the synthesis of unsaturated fatty acids and positively regulates fabA and fabB. However, the FadR in \textit{S. putrefaciens} under cold stress only regulates

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{The metabolism pathway of the main energy sources. Different colors indicate the ranges of the fold change (cultivated at 4°C/30°C) values.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{PPI network: significantly upregulated differentially expressed proteins of \textit{S. putrefaciens} cultivated at 4°C and 30°C, respectively. The proteins upregulated in \textit{S. putrefaciens} are colored in a gradient color from white to red, 1.52 ≤ FC ≤ 6.36.}
\end{figure}
fabA fatty acid synthesis gene and the fabB protein did not change significantly, which was similar to the result of Yang et al. [54]. Fatty acid degradation occurs through the well-characterized β-oxidation cycle and produces acetyl-CoA, which is further metabolized for energy and precursors of cellular biosynthesis [55]. Acyl-CoA dehydrogenases could catalyze the initial steps in fatty acid β-oxidation. The long-chain fatty acyl-CoA ligase could activate free fatty acids to acyl-CoA thioesters and DE proteins related to lipid synthesis including ketoacyl-ACP synthase III (FabH), which was upregulated under cold stress, which shows the protein expression in S. putrefaciens involved in fatty acid elongation and plays a critical role in maintaining the fluidity of membrane [56]. In some research studies, the FabH enzyme of L. monocytogenes prefers 2-methylbutyryl-CoA as the precursor of odd-numbered anteiso fatty acids under cold stress and increases the synthesis of anteiso fatty acids [57].

4. Conclusions

The ability to canvass a high proportion of the expressed proteome and define quantitatively large or small changes in protein abundance with strict statistical rigour has provided a strong view of S. putrefaciens under cold stress. The proteomics provided functional evidence supporting the importance of specific functional classes of genes that were identified as genomic markers of cold adaptation (e.g., energy metabolism). The quantitative analyses particularly showed the factor in S. putrefaciens under cold stress and the study identified specific pathways that were linked to the cold stress environment. The study provides a platform for comparative analyses of cold adaptation of other bacteria.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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