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Transcriptome of Escherichia coli DH5α cultured under static liquid conditions in a gas-permeable film bag

Yuki Naka
Kindai University
Motomu Akita
935603@waka.kindai.ac.jp

Kindai University

Article

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Abstract

We previously proposed an air-permeable film bag culture as a novel method in microbiological studies. In this bag, oxygen molecules are passively supplied through the film. Here, we report the transcriptome differences between aerobic static liquid cultured cells and conventional shaking liquid cultured cells. The comparison was performed using *Escherichia coli* DH5α in early exponential phase, a period when cell growth is accelerating and the effects of substrate depletion and secreted products are considered to be negligible. We found a significant difference in the transcriptome between the shaking-cultured cells and the static film-cultured cells. Notably, a greater number of prophage sequences and small proteins, mainly predicted as gene expression regulators and cold shock proteins, were detected in the static-cultured cells. Furthermore, the enhanced expression of flagella-related genes, genes involved in nitrate/nitrite metabolism, and several chaperones (with different predicted targets) were detected in the static liquid culture compared with the shaken culture. Our results suggest that the aerobic static liquid culture method offers a novel insight into the physiology of microorganisms.

Introduction

We previously reported that the culture of microorganisms in gas-permeable film bags may provide novel insights into microbiology[1]. A gas-permeable film bag made with a polymethylpentene copolymer can be used to culture several microorganisms. The growth was either comparable to or greater in some cases than that observed in shaking liquid culture. This finding indicated that this static liquid culture technique may reveal the effect of shaking on liquid culture growth. Shaking liquid culture is the standard technique for the *in vitro* growth of aerobic microorganisms in liquid media. However, it is worth considering that during such culturing, cells are continuously agitated in the liquid, likely colliding with air bubbles and the wall of the culture vessel, potentially exposing the cells to continuous physical (shear) stress. Shaking or agitating the liquid medium has been thought to be indispensable for culture because of limitations on oxygen transfer and the amount of dissolved oxygen in water (1.38 mmol L^{-1} at 20°C, 1 atm). However, static liquid culture using the gas-permeable film bags provides physical-stress-free conditions in which the oxygen supply is not notably restricted. In static culture, cells mainly sink to the bottom of the medium, coming into contact with the surface of the film. Oxygen can pass through the film and dissolve into the medium and consequently the oxygen concentration will not be completely uniform throughout the medium, with a micro-gradient developing around the film. It is also worth considering that the cells touching the film surface may directly take up oxygen molecules from the film surface to a certain extent. However, the film bag offers the opportunity to culture cells in liquid containing sufficient oxygen, free of physical stress factors, and we were therefore interested to compare the characteristics and properties of cells grown in shaking and static culture. In this study, we compared the transcriptome of *Escherichia coli* between the different culture conditions of liquid cultured bacteria.

E. coli is an organism that primarily inhabits the intestine, a habitat characterized by high levels of mechanical stress, such as peristalsis and fluid flow. However, laboratory-used strains, such as DH5α engineered from strain K-12, have acquired mutations such as partial deletion of the *lacZ* gene. These

strains also appear to have a significantly reduced ability to form biofilms due to the loss of adaptive genes (e.g., Ghigo[2]). This limited ability to form biofilm makes laboratory strains of *E. coli* ideal candidates to study the effects of shaking on culture, without the need to consider the complex environment conferred by biofilm, including the limitation of oxygen transfer for organisms within the biofilm. The complete genome sequence of DH5α is also readily availible[3]. We previously observed that cell growth in the initial phase of culture is similar between shaking culture and the static film bag culture[1]. Thus, we compared the transcriptome of DH5α cells cultured in a shaking tube with cells cultured in a gas-permeable film bag during the early stage of culture when the influence of nutritional depletion and the effect of secreted products would be minimal. Note that the comparison simply reflects the relative difference observed between culture conditions and is not necessarily a reflection of the “natural state” of *E. coli* growth.

Results and Discussion

The transcriptome data generated in this study have been deposited in the DDBJ BioProject database under accession number PRJDB40068. After quality checking using FastQC software, filtering of the sequencing data was performed, and then a consolidated summary report covering all samples was generated by MultiQC software. The results confirmed that high-quality datasets were achieved for all of the samples. Specifically, 95.15% to 96.58% (average 95.79%) of the sequences were successfully mapped onto the reference genome sequence of DH5α (Genome assembly ASM284822v1). Principal component analysis (PCA) results are shown in Fig. 1. Samples from S (shaking) and T (static) conditions clearly separated into distinct clusters, suggesting that they possess significantly different expression profiles. This result was further confirmed by hierarchical clustering analysis (Fig. 2). Subsequently, expression quantification analysis and differential expression analysis between the two groups were performed. A total of 1,420 differentially expressed genes showing a false discovery rate (FDR) < 0.05 were identified (Supplementary Table S1). The distribution is shown in the volcano plot (Supplementary Fig. S1). Genes with |logFC|> 1 and a significantly low FDR (< 0.05) were selected and are shown in Supplementary Tables S2 (S<T) and S3 (S>T). The genes listed in these tables reflect the expression differences between shaking and static culture conditions. However, it should be noted that some genes do not necessarily reflect physical conditions directly (e.g., shear stress), such as genes related to cell-to-cell interactions and cell density. This is because cells cultured in the bag were not homogeneously distributed in the medium; instead, they formed relatively dense clusters scattered throughout the bag. For simplicity, the terms “enhancement” and “activation” are used hereafter; however, these terms describe relative differences between the two conditions and do not necessarily imply direct gene upregulation.

Expression levels corresponding to S01 to S03 and T01 to T03 were extracted and converted to numeric values. After exclusion of genes with no expression variability, PCA was performed. Group information corresponding to the S and T conditions was added, and the proportion of variance explained by each principal component (PC) is indicated in the axis labels. The PCA plot was generated using ggplot2,

visualizing PC1 on the x-axis and PC2 on the y-axis.

For clustering, the Euclidean distance was calculated and the complete linkage method was used. A heatmap was generated using the heatmap.2 function from the gplots package, reflecting the results of hierarchical clustering.

Table 1 Characteristic upregulated genes in static culture (T) compared with shaking culture (S)

The complete list of upregulated genes is shown in Supplementary Tables S2 and S3. Note that genes belonging to multiple groups (ex. prophage as small protein) are redundantly counted.

Interestingly, we found characteristic differences among the enhanced genes (Table 1). In total, 15 out of 74 genes (20.3%) were annotated as prophage[4,5] or prophage-related genes (based on the description on EcoCyc; https://ecocyc.org/) in the static cultured cells, whereas 3 out of 95 genes (3.2 9%) were identified in the shaking cultured cells. This result may be further supported by the fact that *cspE* was among the enhanced genes, suggesting that this antiterminator—which can also act as a negative regulator of *cspA*[6,7]—was activated. The number of small-sized proteins (set as < 100 amino acids for the purpose of this study) detected, including prophage, was 22 (29.7%) under static culture conditions and 11 (11.6%) under shaking culture conditions. Although it is premature to speculate further without more detailed data, this finding that nearly 30% of the listed genes were small proteins may suggest that static liquid cultured cells actively mobilize and modulate the expression of genes; at least half of these small proteins are annotated as being involved in the regulation of gene expression, such as heat/cold shock proteins (HSPs). By contrast, no HSP-like small proteins were detected in cell cultures under shaking conditions. In the static liquid culture, genes that were characteristically enhanced included those encoding proteins related to flagella, nitrate/nitrite metabolism, and chaperone functions. Specifically, higher expression was detected for a series of *flg* genes (*flgB, C, D, E, F, G*) and *fli* genes (*fliG, H, I, M, P*). The enhancement of flagellar genes may correlate with cells exhibiting high motility within the liquid medium. The cells also highly expressed genes encoding fimbriae (pili) proteins, *fimA* and *fimI*, which may indicate that the cell population is in a transitional state between planktonic spread and sessile colonization. This hypothesis is supported by our observation that cells formed relatively dense, scattered clusters throughout the medium. Additionally, interesting differences were noted regarding the type of chaperon proteins detected. The *fimC* (P31697) gene encodes a chaperone required for the biogenesis of type 1 fimbriae, which is consistent with the higher expression of *fimA* in static culture. By contrast, the chaperonins encoded by the *groL* and *groS* genes, which were enhanced under shaking conditions, are involved in cytoplasmic protein folding. It should be noted that DH5α has lost the ability to form biofilm; for example, genes previously reported to be important for fitness in competitive biofilms[8] were not detected. The above-mentioned appearance of the culture, with relatively dense and scattered clusters, may also be consistent with the higher expression of a series of genes for nitrate/nitrite metabolism (*narG, H, I, J, K*). Although cell growth was not significantly different between culture conditions, we cannot completely rule out the possibility that the net oxygen supply was more

restricted in the bag than in the shaken culture. The oxygen transfer rate may be locally reduced in high-cell-density areas, or a signal against the transition to high cell density may be generated, thereby anaerobic metabolism might be induced in static cultured cells. Under shaking conditions, the number of genes for carbohydrate utilization, transport, and associated regulation (e.g., *glpA*, *B*, *fadB*, and *uhpT*) was enhanced. This suggests that the cells were actively undergoing aerobic metabolism, sugar metabolism, nutrient uptake, and nutrient consumption. Gene ontology (GO) analysis also indicated that genes for energy metabolism were differently expressed between the culture methods. The enhancement of *groL* and *groS* chaperonins may reflect rapid cell proliferation, as upregulation of these genes is required during rapid protein synthesis. However, the growth rate of the cells was similar between the two types of culture as previously mentioned (see also Supplementary Table S4). In nutrient-rich medium, the final cell yield was slightly higher than in static culture[1]. Higher expression of the *csgB* and *yadK, L, N* genes might reflect the tendency of cells to form aggregates through cell-to-cell contact in response to continuous shaking.

Dot size represents the number of differentially expressed genes (DEGs) assigned to each GO term. Numbers within the dots indicate the significant number of DEGs. The color gradient represents the negative logarithm of the P-value (−log10 P-value) derived from Fisher's exact test. Detailed results including other categories are shown in Supplementary Table S4.

According to the GO results, the following terms were enriched: "organic acid metabolism" and "amino acid metabolism" within the Biological Process category (Fig. 3, Supplementary Table S5), and "oxidoreductase activity related to energy production" within the Molecular Function and Cellular Component categories. The gene ratio for the leucine metabolic pathway is 3/3 (1.0), and indeed, all genes of the leucine operon (*leuA, B, C, D*) were enhanced in the static cultured cells indicating that the requirement for this amino acid was increased in the static culture. Notably, the Lipoprotein Transport and Location categories also exhibited the highest gene ratios, potentially indicating that cellular resources are redirected toward outer membrane biogenesis. Overall, these findings indicate that genes involved in amino acid metabolism, membrane biogenesis, metabolic pathways associated with energy metabolism, and redox reactions are differentially expressed between the shaking and static conditions; however, this difference seems to be uncoupled from cell proliferation. We can speculate that the cells in shaking culture were subjected to a combination of three stress factors: shear stress, stress associated with rapid metabolic turnover, and oxidative stress. Enhanced expression of carbohydrate utilization, transport, and associated regulatory genes, such as the *lac* group of genes and *araB,* may suggest that cells were subjected to an acceleration of metabolic turnover. The enhancement of the *als* genes and *rbsA* may indicate that alternative carbon sources were used or were ready to be used, but the incremental change in biomass was comparable to that detected under static conditions.

Conclusions

Mechanical aeration, such as shaking and agitation, is a standard operation for the cultivation of aerobic organisms in liquid medium. It is particularly indispensable for large-scale culture to overcome oxygen

supply limitations. Today, we can commonly observe sophisticated and well-programmed liquid culture systems designed to optimize culture efficiency and productivity. Nevertheless, physical stress caused by this operation remains an important issue in liquid culture. In this study, by comparing differently expressed genes, we demonstrate a significant difference in the transcriptomes between shaking culture and static culture. These data may reflect complex factors such as shear stress, a planktonic habitat, cell-to-cell contact, the effect of aggregation factors, quorum sensing, and resource competition among cells. Notably, more putative transcriptional regulators were observed in the static culture than in the shaking culture, suggesting that transcription may be differently and/or more actively regulated under static conditions; conversely, a series of regulative genes may be less essential under shaking conditions. Our static culture method may contribute to providing novel approaches to elucidate the expression patterns and function of these small proteins[9]. In summary, static liquid culture using air-permeable film bags has great potential in understanding liquid-cultured microorganisms.

Methods

Preparation of RNA

Escherichia coli DH5α was cultured in LB medium. The culture conditions were the same as reported in our previous study[1]. To prepare samples, a single colony was inoculated into 5 mL of LB medium in a 50 mL conical tube and incubated with shaking. After 5 h, 2 mL of the resulting cell suspension was transferred to 300 mL of fresh LB medium and mixed thoroughly. Subsequently, 2 mL aliquots of this suspension were transferred into nine 50 mL conical tubes for shaking culture and nine film bags (50 mm × 100 mm, Okudake, Cell Film Lab. Co. Ltd., Kaizu, Gifu, Japan) for static culture. After 3 h of incubation, to ensure sufficient RNA yield, the contents of every three tubes or film bags were randomly selected and pooled into one single tube. This process was performed in triplicate, resulting in three biological replicates (n=3 per group). The consistency among the replicates was confirmed by their similar turbidity (Supplementary Table S4). Total RNA was then extracted using a commercial RNA isolation kit (PureLink™ RNA Mini, Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol.

Sequencing and alignment

Total RNA samples were further purified and subjected to next-generation sequencing and analysis at Amelieff Corporation, Tokyo, Japan. After rRNA depletion using the TIANSeq rRNA Depletion Kit (NR101_T5, Tiangen Biotech (Beijing) Co., Ltd, Beijing, China), libraries were prepared using the Fast RNA-seq Lib Prep Kit V2 (RK20306, ABclonal Technol., Woburn, MA, USA). Paired-end sequencing was performed on the NovaSeq X Plus platform (Illumina, San Diego, CA, USA), generating approximately 2 Gb of 150-bp raw reads per sample. Following quality control with FastQC (0.12.1) and MultiQC (1.22.3), adapter sequences were removed using Trimmomatic (0.39) with the following parameters: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 and MINLEN:30. Subsequently, the reads were processed using PRINSEQ-lite (0.20.4) to remove poly-A/T tails and low-quality sequences with the following settings: -

trim_tail_left 5, -trim_tail_right 5, -trim_qual_left 30, -trim_qual_right 30, -ns_max_p 20, and -min_len 30. Finally, mapping was performed using STAR (2.7.11b) against the *E. coli* DH5α reference genome (Assembly: ASM284822v1, NCBI Accession: GCF_002848225.1).

Bioinformatic and statistical analyses

The software used for bioinformatic and statistical analyses are listed in Supplementary Table S6. Principal component analysis (PCA) was conducted on all gene expression levels using the prcomp function in R, based on Euclidean distance. Genes with ≥1 counts per million (cpm) in at least two samples were clustered using the amap and gplots packages. The PCA results were visualized by plotting the first two principal components (PC1 and PC2) using ggfortify. Hierarchical clustering was performed using Euclidean distance with complete linkage, and heatmaps were generated using $\log_{10}$(cpm + 0.01) values. Gene expression levels were quantified using the featureCounts program (Subread package). Differential expression analysis between treatments was performed using the edgeR package. Prior to analysis, genes were filtered to retain only those with CPM ≥1 in two or more samples. Read counts were normalized using the Trimmed Mean of M-values (TMM) method. To account for biological variation, dispersion parameters were estimated according to the edgeR classic pipeline. Given the three biological replicates per group, both common and tagwise dispersions were calculated using the estimateCommonDisp and estimateTagwiseDisp functions, respectively. Statistical significance was determined via the exact test; P-values adjusted by the Benjamini-Hochberg method. Genes exhibiting a False Discovery Rate (FDR) < 0.05 identified as significantly differentially expressed. GO enrichment analysis (Biological Process, Cellular Component, Molecular Function) was performed using topGO in R with processed GAF files.

Declarations

Acknowledgments

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Author contributions

M.A. conceptualized the study. Y.N. conducted *in vitro* experiments including extraction and purification of RNA. M.A. was involved in the experimental design shown in Fig. 3 and the tables. Y.N was involved in the experimental design shown in the other figures. M.A. wrote the draft manuscript. Both authors provided feedback and contributed to manuscript preparation. Both authors reviewed the manuscript.

Data availability statement

The transcriptome data have been deposited in the DDBJ BioProject database under accession number PRJDB40068 and in the DDBJ Genomic Expression Archive under accession E-GEAD-1218. The data will be released publicly upon publication. Data generated during this study are included in Supplementary Information.

Competing interest statement

M.A. holds a position in Cell Film Lab. Co., Ltd. The authors have no competing interests to declare.

AI-assisted writing

The authors used ChatGPT (OpenAI) and Gemini (Google) for improving the clarity and readability of the manuscript. All content was reviewed and approved by the authors.

Funding Declaration

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Table

Table 1 is available in the Supplementary Files section.

Figures

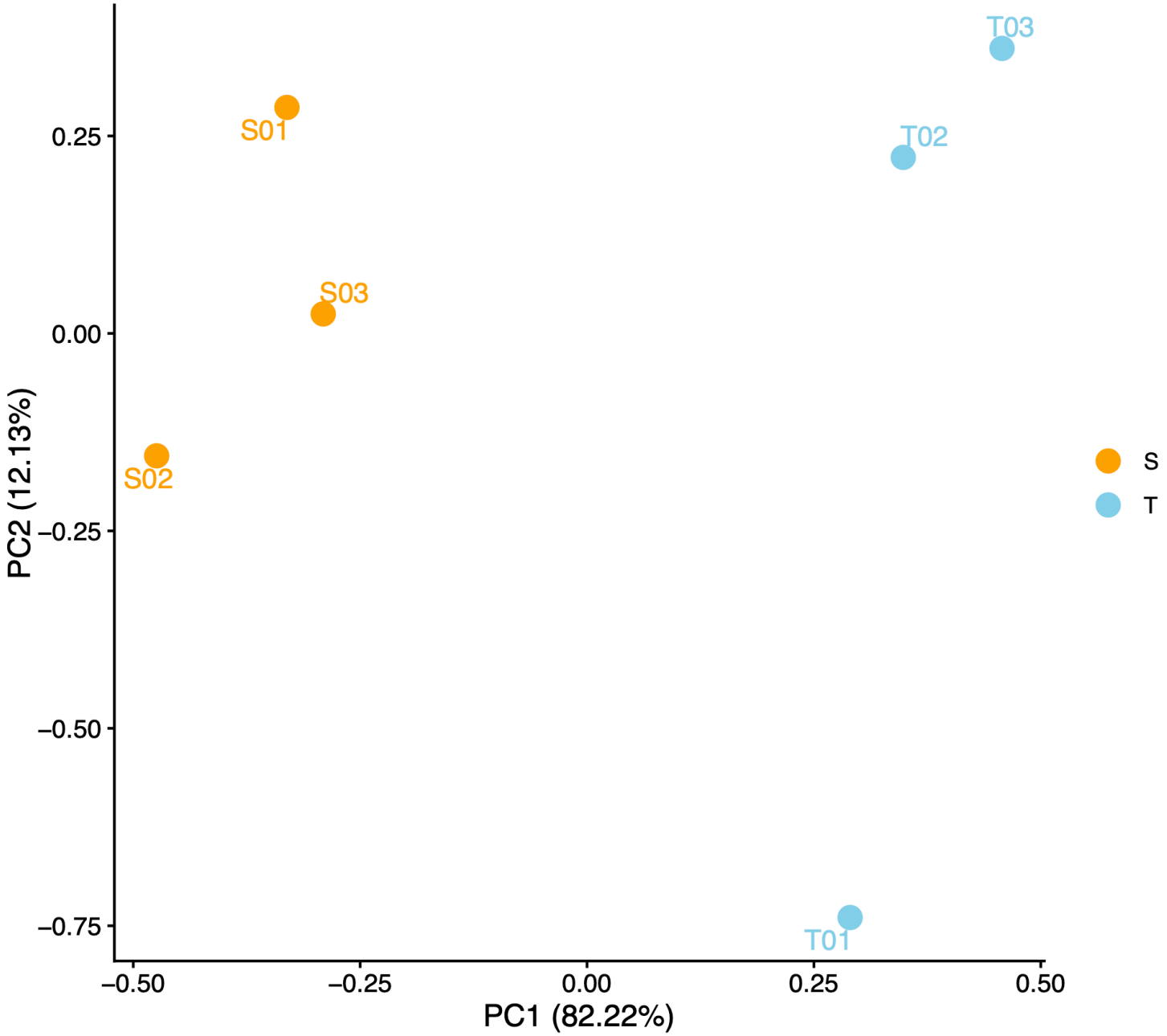


Figure 1

Principal component analysis (PCA) of shaking (S) and static (T) culture conditions

Expression levels corresponding to S01 to S03 and T01 to T03 were extracted and converted to numeric values. After exclusion of genes with no expression variability, PCA was performed. Group information corresponding to the S and T conditions was added, and the proportion of variance explained by each principal component (PC) is indicated in the axis labels. The PCA plot was generated using ggplot2, visualizing PC1 on the x-axis and PC2 on the y-axis.

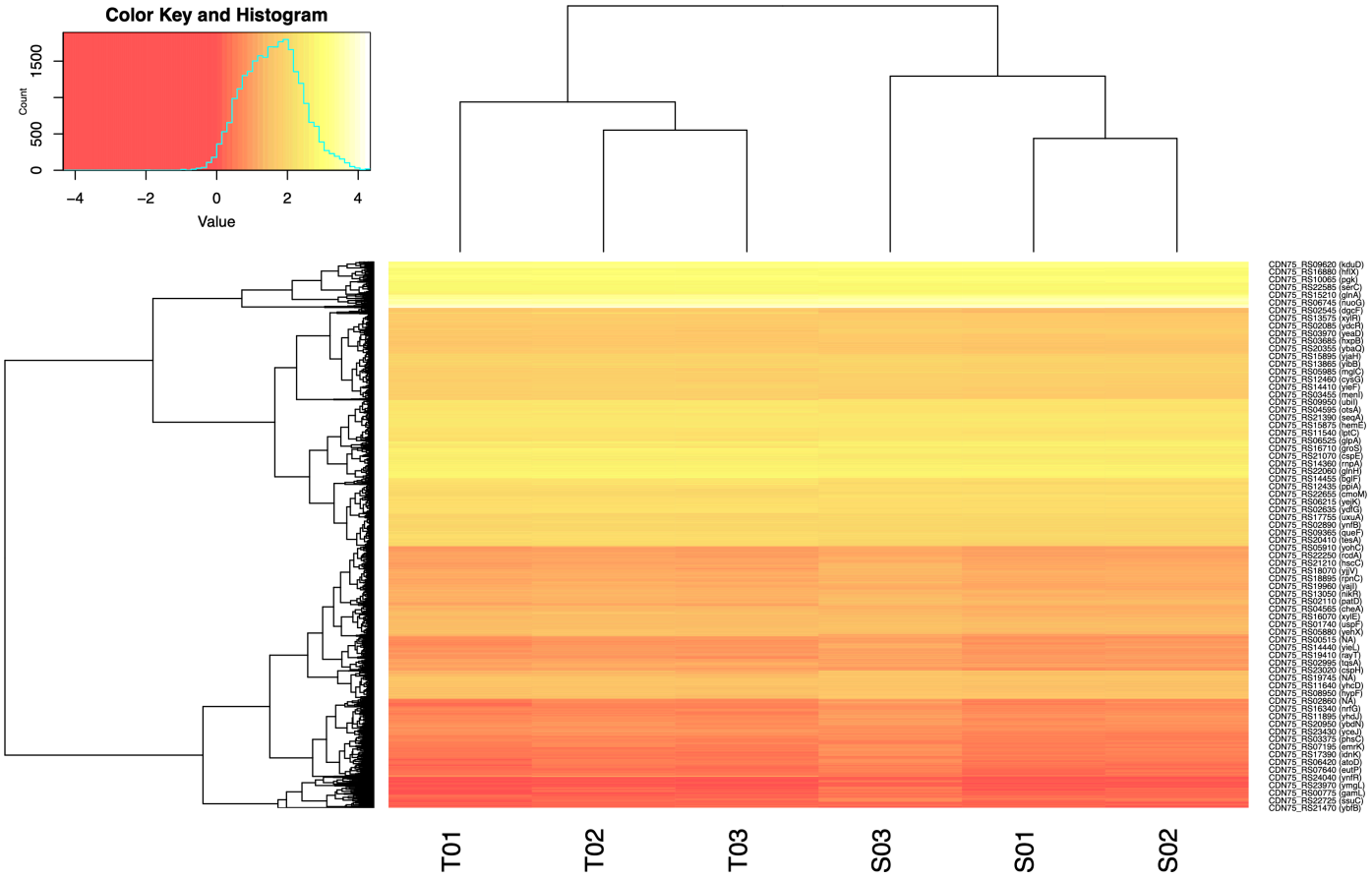


Figure 2

Heatmap and hierarchical clustering analysis of the shaking (S) and static (T) culture conditions

For clustering, the Euclidean distance was calculated and the complete linkage method was used. A heatmap was generated using the heatmap.2 function from the gplots package, reflecting the results of hierarchical clustering.

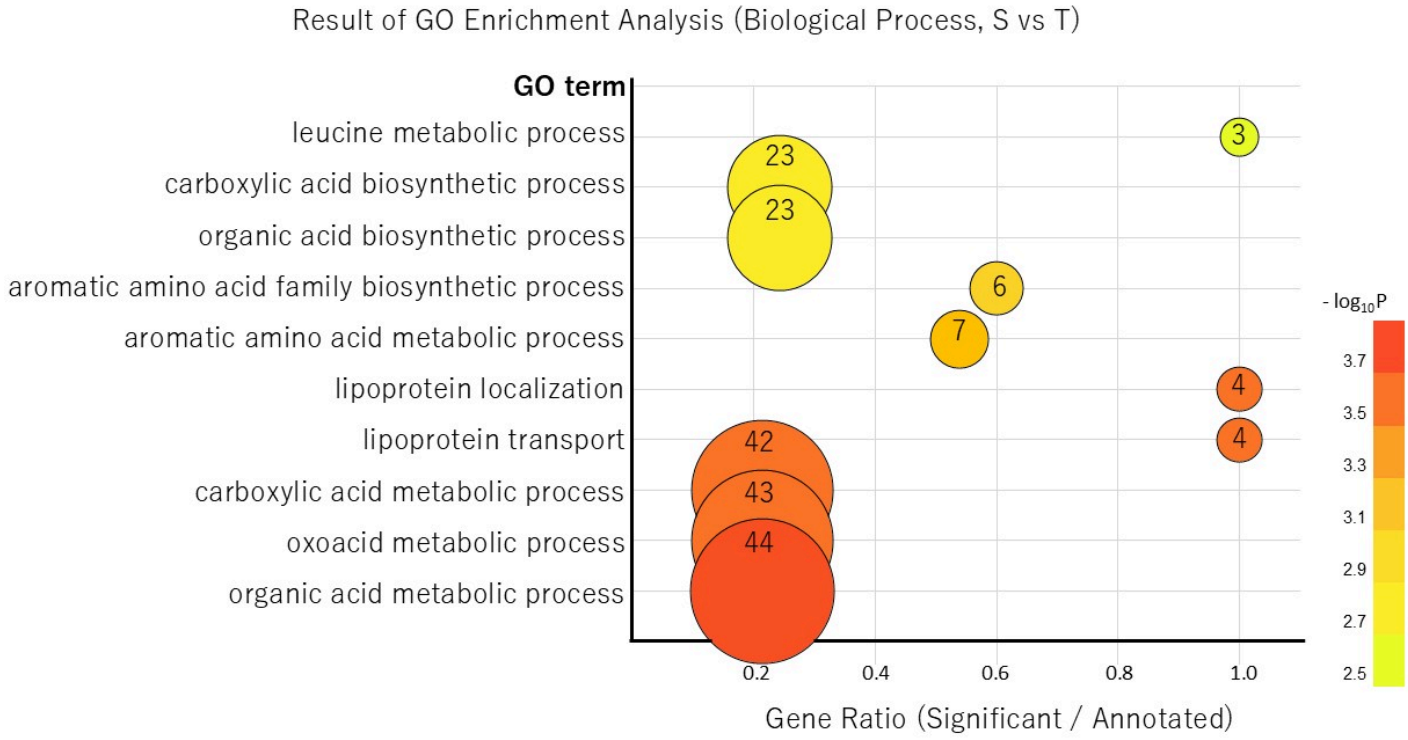


Figure 3

Results of gene ontology (GO) enrichment analysis [Biological Process, shaking (S) vs static (T)]

Dot size represents the number of differentially expressed genes (DEGs) assigned to each GO term. Numbers within the dots indicate the significant number of DEGs. The color gradient represents the negative logarithm of the P-value (log10 P-value) derived from Fisher's exact test. Detailed results including other categories are shown in Supplementary Table S4.

Supplementary Files

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- Table1numberofcharacteristicgenes.pdf
- NAKASupplementary.pdf