Academic Journal of Science and Technology
ISSN: 2771-3032 | Vol. 10, No. 2, 2024

The Evolutionary Process of Acid Adaptation and Deep Transcriptional Analysis in *Bacillus subtilis 168*

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Abstract: This study is focused on investigating the variations in gene expression of *Bacillus subtilis 168* following acid adaptation evolution. Through simulating prolonged cultivation in a natural acidic setting, we have observed the occurrence of adaptive evolution within the strain and conducted an extensive analysis of gene expression in the evolved strain using high-throughput transcriptomics technology. The findings from this research shed light on the fundamental molecular mechanisms employed by *Bacillus subtilis* to adapt to acidic environments, thereby providing crucial scientific evidence for comprehending its survival strategies and potential applications in acidic settings.

1. 1 Introduction

Bacillus subtilis, a Gram-positive bacterium, is widely distributed in soil, plant surfaces, and air [1]. Its exceptional adaptability and versatility have led to significant applications across various fields. Since its initial description and classification in the 19th century, it has been a focal point of microbiological research [2]. From early investigations into biological characteristics to subsequent studies on gene expression, regulation, and genetic mechanisms, *Bacillus subtilis* has consistently played a pivotal role [3]. Notably, the completion of its genetic map in the mid-to-late 20th century further accelerated subsequent advancements in gene manipulation and functional studies [4].

The potential applications of *Bacillus subtilis* in biotechnology, agriculture, the food industry, and environmental science are continuously being revealed. With the advent of systems biology research methodologies, it has become an essential tool for investigating intricate biological systems and metabolic networks [5]. However, in practical applications, particularly in specific industrial processes requiring acidic fermentation conditions [6], the viability of *Bacillus subtilis* is often significantly compromised, thereby constraining production efficiency.

In order to tackle this issue, we propose a solution rooted in adaptive evolution [7]. Adaptive evolution refers to the process by which organisms bolster their survival and reproductive capabilities through genetic and physiological changes in response to specific environmental pressures [8]. In an acidic environment, genetic variations that promote adaptation to low pH conditions are favored by natural selection [9]. These variations may encompass various aspects, such as improved cell membrane stability, optimized adjustment of metabolic pathways, and the production of acid-resistant enzymes [10].

This study aims to investigate the gene expression changes of *Bacillus subtilis* in a low-pH environment using an experimental scheme designed for acid adaptation evolution combined with transcriptomics analysis [11-13]. We expect that this research will elucidate its adaptive mechanisms in acidic environments, offering new theoretical support and practical guidance for the application of microbiology in such conditions.

2. Materials and Methods

2.1. Strain and Medium

The strain utilized in this study was *Bacillus subtilis 168*, which had been maintained in the laboratory. It was cultivated and activated in the LB medium and underwent acid adaptation evolution in the M3G medium.

2.2. Preparation of Acid Adaptation Evolution Medium and Seed Solution

For the acid adaptation evolution experiment, we prepared M3G media with pH values of 4.5 and 7.0, respectively, and sterilized them at 115℃ to ensure aseptic conditions. Simultaneously, LB medium with a pH of 7.0 was also prepared and sterilized at 120℃ for subsequent strain activation.

To obtain the seed solution for acid adaptation evolution, a small amount of bacteria was extracted from the preserved *Bacillus subtilis 168* strain and streaked onto a solid LB plate for activation. The plate was then incubated in a constant temperature incubator at 37℃ for approximately 10 hours until distinct single colonies emerged. Subsequently, a single colony was selected from the plate and inoculated into a conical flask containing 50ml of fresh, sterilized LB liquid medium. The culture was then incubated at 37℃ with shaking at 200 rpm/min. After 10-12 hours of cultivation, the strain activation process was completed.

Subsequently, 1 ml of the activated bacterial solution was inoculated into a conical flask containing 50 ml of pH 7.0 M3G liquid medium and cultured under identical conditions (37℃, 200 rpm/min). Following an incubation period of 10-12 hours, the seed solution for acid adaptation evolution was obtained.

2.3. Acid Adaptation Evolution

In order to investigate the adaptive evolution in acidic environments, we developed and executed a comprehensive acid adaptation experiment. The experimental protocol was as follows:

During the initial adaptation phase, we evenly distributed a 1ml inoculum of the seed solution into nine sterilized conical flasks containing M3G medium at pH 7.0. These flasks were

then incubated in a constant temperature shaker at 37℃ and 200 rpm/min for 10 to 12 hours until reaching an optical density (OD_{600}) of approximately 4, indicating the attainment of a particular bacterial concentration. Subsequently, we transferred 80ml of culture from each flask into new conical flasks containing 20ml of M3G medium at pH 4.5, labeled A1 to A9, with the purpose of exposing the strain to an acidic environment for initial adaptation.

In the second round of evolution, we continued to culture the bacterial solutions in flasks A1 to A9 until reaching an OD_{600} of approximately 4. Subsequently, we selected the six flasks with the highest growth rates and transferred 60ml of culture from each into new conical flasks containing 40ml of fresh M3G medium at pH 4.5, designated as the B series. This step aimed to further enhance the adaptability of the strain to acidic environments by selectively cultivating more resilient strains.

The third round of evolution was conducted with even greater stringency. When the OD_{600} values of the B series cultures approached 4, we once again selected the bacterial solutions exhibiting the highest growth rates and transferred 40ml of each into new conical flasks containing 60ml of fresh M3G medium at pH 4.5, designated as the C series. This procedure facilitated the ongoing selection of strains with enhanced adaptability to acidic environments, progressively elevating their level of adaptation.

In the final phase of adaptation evolution, we selected the three most rapidly proliferating cultures from the C series and transferred 20ml of each into new conical flasks containing 80ml of M3G medium at pH 4.5, designated as the D series. These were cultivated under consistent temperature shaking conditions until reaching an OD_{600} of approximately 4, signifying the completion of the low pH adaptation evolution process for the strain.

To assess the efficacy of low pH adaptation evolution, we selected the fastest-growing cohort from the D series as the experimental group and inoculated it into a new pH 4.5 M3G medium. Concurrently, an equivalent amount of wild-type strain was inoculated as a control group. By monitoring growth curves over a 12-hour period, we confirmed the success of low acid adaptation evolution.

2.4. RNA Extraction and Transcriptome Sequencing

Samples of the adaptively evolved bacterial solution were collected and transferred to centrifuge tubes. The bacterial cells were then fully precipitated at 4℃ by centrifuging at a speed of 10,000 rpm, followed by removal of the supernatant.

Subsequently, the precipitated bacterial cells were rapidly frozen in liquid nitrogen for 15 minutes to stabilize the RNA and prevent degradation. Total RNA extraction was performed, and transcriptome sequencing was carried out using the Illumina sequencing platform. Rigorous quality control and preprocessing of the sequencing data ensured accurate results for subsequent gene expression analysis.

2.5. Transcriptome Data Analysis

A comprehensive bioinformatics analysis was conducted on the transcriptome data obtained from sequencing. Initially, differentially expressed genes (DEGs) were screened to identify genes exhibiting significant changes in expression levels during the acid adaptation evolution process. Subsequently, functional and pathway enrichment analyses were performed on the DEGs to elucidate their roles in biological processes and molecular functions, as well as their interactions and regulatory mechanisms within metabolic pathways.

3. Results and Discussion

3.1. Analysis of Acid Adaptation Evolution

Through meticulously planned iterative acid adaptation evolution experiments, we have effectively bolstered the adaptability of *Bacillus subtilis* to low-pH environments. The comprehensive results of our evolutionary study are outlined as follows:

During the initial adaptation stage, we assessed the growth of all nine bottles of bacterial solution by measuring their optical density at 600nm (OD_{600}). The OD_{600} values of all nine bottles were found to be significantly higher compared to the sterilized M3G medium control group, indicating robust growth. As a result, none of the bacterial solutions were eliminated in the first round of evolution.

In the second round of evolution, we implemented a more stringent screening process to monitor the growth of the nine bacterial solutions. The results revealed that bottles 1, 2, 4, 5, 6, and 9 exhibited OD_{600} values of 4.12, 4.19, 4.07, 4.23, 4.01, and 3.96 respectively - significantly higher than those of the remaining bottles. Based on these findings, we have selected these six bottles for further experimentation in our study.

In the third round of evolution, these six bottles continued to exhibit a consistently high OD_{600} growth trend, with no statistically significant differences observed among them. As a result, it was determined that all six bottles would be retained in the ongoing evolution experiment in anticipation of further enhancements in adaptability.

During the critical fourth round of evolution, bottles 1, 5, and 6 demonstrated more pronounced growth advantages. Notably, bottle 1 exhibited the highest OD_{600} value of 3.93, establishing itself as the frontrunner in this evolutionary stage. Consequently, bottle 1 was chosen as the representative for subsequent validation experiments.

To validate the success of acid adaptation evolution, we conducted a comparative experiment between the evolved strain from bottle 1 and the wild-type strain. Both strains were inoculated into a sterilized M3G liquid medium at pH 4.5 under identical conditions, and their growth rates were monitored. The results were remarkable: after a 12-hour incubation period, the evolved strain reached an OD_{600} value of 3.18, while the wild-type strain only achieved a growth of 1.32. This significant disparity unequivocally demonstrates the exceptional efficacy of our acid adaptation evolution strategy.

Through a series of acid adaptation evolution experiments and validation processes, we have successfully augmented the growth capacity of *Bacillus subtilis* in low-pH environments. This accomplishment provides robust evidence for a deeper comprehension of microbial adaptation mechanisms and unlocks new potential for biotechnology applications in related fields.

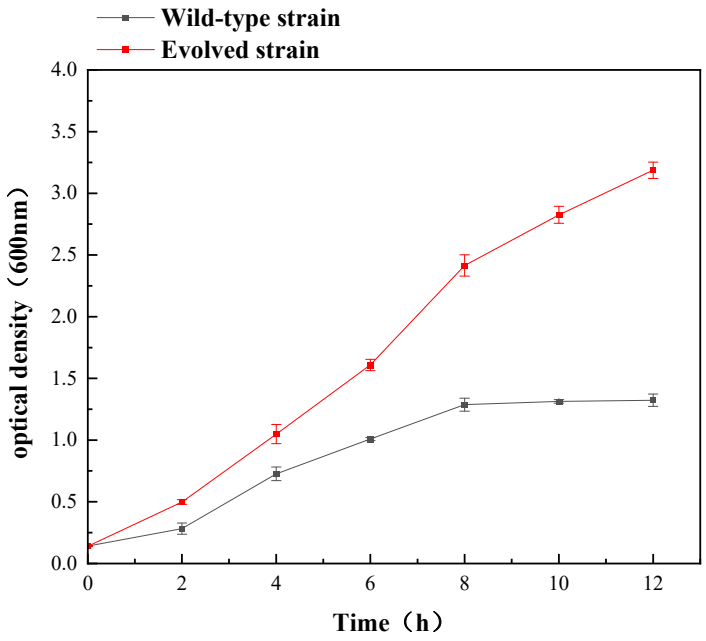

Figure 3-1. Growth verification experiment of evolved strains

3.2. Differential Expression of Genes in the Transcriptome and KEGG Analysis

Through comparison of the expressed genes in the experimental group, we identified 672 upregulated genes, including *gsiB, glpK*, and *ohrB*. Notably, the upregulation of the *glpK* gene, associated with glycerol kinase, may play a role in modulating cellular membrane lipid synthesis or energy metabolism to enhance cell survivability under acidic conditions. Among the 656 downregulated genes, the decrease in expression of *lctE* and *lctP*, related to lactic acid metabolism, likely signifies a reduction in lactic acid production by the cells under acidic conditions as a means to prevent further acidification.

Through a comprehensive KEGG enrichment analysis, we observed a significant enrichment of differentially expressed genes (DEGs) in four primary metabolic pathways: Cellular Processes, Environmental Information Processing, Human Disease, and Metabolism (refer to Figure 3-2 for detailed information). This distribution pattern provides insight into the gene expression regulation strategies and metabolic remodeling mechanisms of *Bacillus subtilis* in response to low pH environments.

Initially, it is noteworthy that while DEGs are enriched in Cellular Processes, Environmental Information Processing, and Human Disease, the number of enriched genes is relatively limited. This indicates that *Bacillus subtilis's* adaptive evolution under low pH conditions does not primarily depend on the regulation of these pathways. However, specific gene alterations within these pathways may still play a pivotal role in the survival and adaptability of cells under low pH conditions, necessitating further comprehensive investigations to elucidate their precise functions.

Contrastingly, there is a notable enrichment of genes in the Metabolism pathway, particularly in amino acid metabolism and biosynthesis-related pathways. Specifically, histidine metabolism, arginine biosynthesis, tryptophan metabolism, and cyanoamino acid metabolism represent a substantial proportion of the differentially expressed genes (DEGs). This discovery strongly suggests that amino acid metabolism plays a crucial role in *Bacillus subtilis's* ability to adapt to low pH conditions.

In the presence of low pH conditions, microbial cells are subjected to a multitude of stresses, including compromised membrane stability, altered proton motive force, and intracellular acid-base imbalances. Modulating specific metabolic pathways, particularly those related to amino acid metabolism, may represent a pivotal strategy for cells to adapt to these challenges. Amino acids play a crucial role not only in protein synthesis but also in various essential cellular processes such as energy metabolism, signal transduction, and cell wall synthesis. Augmenting the demand for and synthesis of amino acids could potentially aid cells in preserving membrane integrity and functionality, regulating intracellular pH levels, and ensuring the smooth operation of key biosynthetic pathways.

In conclusion, the results of the KEGG enrichment analysis reveal a significant adaptive strategy employed by *Bacillus subtilis* in response to low pH conditions: maintaining cell survival and normal physiological functions through metabolic network remodeling, particularly by enhancing amino acid metabolism and biosynthetic pathways. These findings not only enhance our comprehension of microbial adaptation mechanisms but also offer valuable insights for the development of industrial microbial strains with enhanced environmental adaptability.

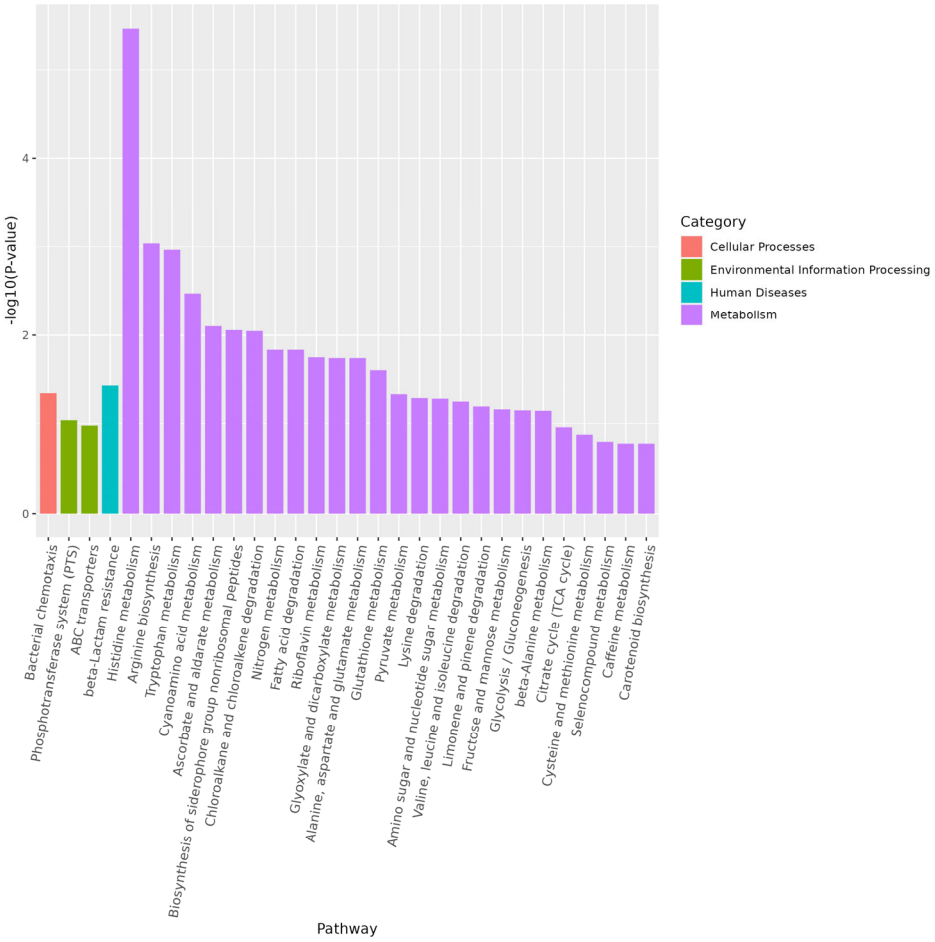

Figure 3-2. KEGG Pathway enrichment results histogram

3.3. GO Enrichment Analysis of the Transcriptome

In order to further elucidate the functional roles of differentially expressed genes during the adaptive evolution of *Bacillus subtilis 168* under low pH conditions, a Gene Ontology (GO) enrichment analysis of the transcriptome was conducted. This analytical approach reveals significantly regulated biological functions under specific conditions by quantifying the number of genes associated with each GO term (representing specific biological functions, processes, or cellular components) and calculating enrichment significance using a hypergeometric distribution.

After categorizing the results of GO enrichment analysis into Molecular Function (MF), Biological Process (BP), and Cellular Component (CC), we have identified and selected the top 10 most significantly enriched GO terms from each category for detailed presentation (Figure 3-3). These findings reveal the primary biological functions associated with the differentially expressed genes.

Within the Cellular Component (CC) category, significantly enriched GO terms are predominantly associated with the cell membrane and its constituents, such as "membrane" (GO:0016020) and "plasma membrane" (GO:0005886). This suggests that under acidic conditions, *Bacillus subtilis* enhances the stability and material transport capabilities of the cell membrane by significantly upregulating the expression of genes related to membrane structure and function, thereby adapting to the stress of the low pH environment.

In the Molecular Function (MF) category, enriched GO terms primarily focus on transporter activity, such as "transmembrane transporter activity" (GO:0022857) and "transporter activity" (GO:0005215). These findings suggest that under acidic conditions, *Bacillus subtilis* enhances the regulation of genes associated with transporter activity to facilitate the exchange and equilibrium of intracellular and extracellular substances, thereby maintaining normal physiological functions and metabolic activities within the cell.

In the Biological Process (BP) category, significantly enriched GO terms are associated with compound transport and metabolic processes, such as "organic substance transport" (GO:0071702) and "glutamine family amino acid metabolic process" (GO:0009064). These findings suggest that under acidic conditions, *Bacillus subtilis* upregulates the regulation of compound transport and metabolic processes, thereby enhancing nutrient uptake efficiency and utilization while expediting the excretion of toxic substances. This ultimately contributes to improved cell survival and adaptability.

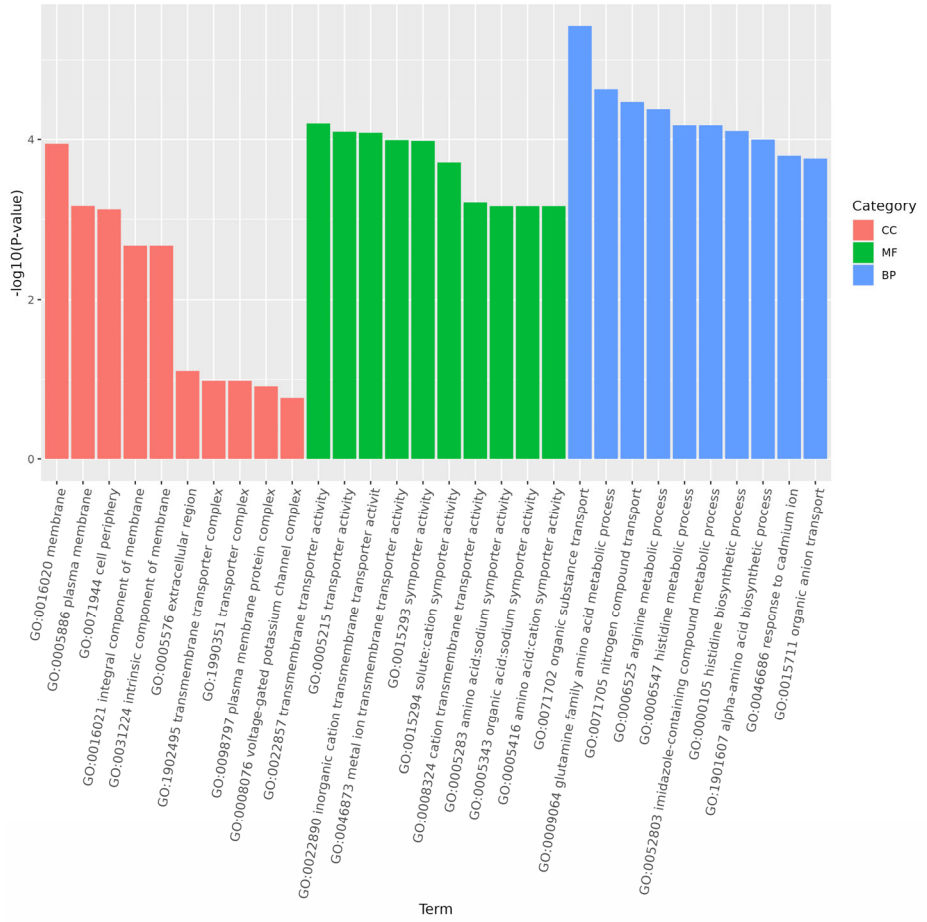

Figure 3-3. GO enrichment analysis histogram

In conclusion, our GO enrichment analysis of the transcriptome has revealed crucial mechanisms underlying the adaptive evolution of *Bacillus subtilis 168* in response to low pH conditions. These mechanisms primarily involve the enhancement of cell membrane structure and regulation of function to maintain cellular stability, upregulation of transporter activity-related gene expression to facilitate material exchange and homeostasis, and reinforcement of compound transport and metabolic process regulation to improve cell survival and adaptability. These coordinated adaptive changes enable *Bacillus subtilis* to better cope with acidic stress, sustaining normal growth and metabolic activities in a low pH environment. Future research can further explore the specific mechanistic roles and regulatory networks of these key genes and pathways in the process of adaptive evolution.

4. Conclusion

In this study, we successfully identified acid-tolerant strains of *Bacillus subtilis 168* through induced adaptive evolution under acidic conditions. These strains demonstrated significant growth advantages in low-pH media. To further elucidate their mechanisms of acid tolerance, we conducted transcriptome sequencing analysis.

The KEGG enrichment analysis results revealed that the primary adaptive strategy of *Bacillus subtilis* in low pH conditions involves restructuring its metabolic network. Particularly, there was a significant enhancement in amino acid metabolism and biosynthetic pathways, which play a crucial role in maintaining normal physiological functions and ensuring cell survival in an acidic environment. Through GO enrichment analysis of the transcriptome, we observed significant enrichment of differentially expressed genes in cellular components, molecular functions, and biological processes. Specifically, under low pH stress conditions, *Bacillus subtilis* upregulated the expression of genes associated with cell membrane structure and function, transporter activity, and compound transport and metabolic processes. The upregulation of these genes contributes to enhancing cellular tolerance and adaptability, enabling the bacterium to maintain cell stability, promote material exchange and homeostasis, and improve the uptake and utilization efficiency of nutrients in an acidic environment.

In conclusion, this study not only provides valuable insights into the adaptive mechanisms of microorganisms in extreme environmental conditions but also identifies potential targets for enhancing and optimizing industrial microbial strains. These findings will facilitate the development of more efficient and resilient industrial microbial strains capable of adapting to diverse extreme environmental conditions.

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