**Academic Journal of Science and Technology**
ISSN: 2771-3032 | Vol. 12, No. 2, 2024

# Research Progress of CRISPR -based Bacillus Subtilis System

**Siyu Liu, Wenke Zhou ***

School of Environmental Ecology and Biological Engineering, Wuhan Institute of Technology University, Wuhan 430200, China
*** Corresponding author:** Wenke Zhou

**Abstract:** As a typical industrial model of food safety, the Bacillus Subtilis has the characteristics of non -pathogenicity, strong extracellular secretion protein ability, and no obvious password preferences, which have been widely used in the field of metabolic engineering. In recent years, with the rapid development of molecular biology and genetic engineering technology, various technologies have been applied to Bacillus Subtilis, thereby synthesizing biological products. This article detailed the application of CRISPR and CRISPR on Bacillus Subtilis, and summarized the biological products of Bacillus Subtilis in related fields and the outlook on its future research direction.

**Keywords:** Bacillus Subtilis; CRISPR; Metabolic Engineering.

## 1. Bacillus Subtilis

### 1.1. Introduction to Bacillus Subtilis

*Bacillus subtilis*. It is a Gram-positive bacterium, which has flagella on its surface and endospore formation in its body that can survive against harsh external environments. In addition, *Bacillus subtilis* is attractive because of its broad substrate range and its ability to survive harsh industrial fermentation conditions. It digests lignocellulosic materials, produces a wide range of industrial enzymes, and has excellent secretion capabilities, thus reducing the cost of biomass pretreatment and minimizing the need for downstream processing [1, 2]. Until recently, *Bacillus subtilis* has been shown to be a potentially excellent chassis host for the production of high-value chemicals and pharmaceuticals (e.g., shark inositol, etc.) [3, 4]. *Bacillus subtilis* is a Gram-positive bacterium, which is capable of forming a bacteriophage and has only one bacteriophage in the ascospores. Resistance *Bacillus subtilis* has been widely used and recognized as a safe host for the production of recombinant proteins, high-value chemicals, and pharmaceuticals, which contributes to the easier purification of heterologous proteins or metabolites [5, 6].

### 1.2. Application Products of Bacillus Subtilis as an Engineered Bacterium

During the development of natural compound machine derivatives, they mainly rely on plant or animal extraction, or chemical synthesis, etc., which face problems such as high cost, low quantity, high difficulty of purification, and ecological damage, etc. Therefore, by using microorganisms as engineered cells for gene editing, so as to re-construct or optimize the synthetic pathway of the target products, the large-scale preparation of the products can be achieved. *Bacillus subtilis*, as an excellent host for engineering bacteria, is currently favored and has successfully synthesized numerous high-value products, such as squalene [7], echinocandin [8], n-acetylglucosamine [9-12], hyaluronic acid [13], chondroitin [14], menaquinone-7 [15, 16], riboflavin [16, 17], and paclitaxel [18], among others.

## 2. CRISPR

### 2.1. Introduction to CRISPR

CRISPR, an acquired immune system for bacteria and archaea to defend themselves against invasion by foreign viruses, has now evolved into a powerful gene editing tool in genetic engineering. When a bacterium is infected by an exogenous phage, a fragment of the phage genome DNA is recognized and integrated into CRISPR, and the corresponding crRNA precursor (pre-crRNA) is transcribed. pre-crRNA is modified to generate guide RNA (gRNA, guide-RNA). gRNA contains DNA from the phage genome, and can recognize the phage genome through the principle of base-complementary pairing. The gRNA contains DNA from the phage genome and can recognize the phage genome through the base complementary pairing principle. At this time, gRNA and Cas protein specifically bind to recognize the phage genome to cut DNA. CRISPR gene editing system is to guide Cas protein (e.g. Cas9, Cpf1, etc.) through sgRNA to locate to the target gene site, and then Cas protein cuts the DNA at the specific site in the genome, resulting in DNA double-stranded breaks, and in the process of homology-directed repair introduction of specific modified fragments into the genome through homology arms, resulting in gene knockout, insertion or mutation [19].

### 2.2. History of CRISPR Technology in Bacillus Subtilis

*Bacillus subtilis* is a typical Gram-positive bacterium and has been recognized as a safe strain (GRAS) with superior protein secretion capacity compared to other microorganisms. B. subtilis has a clear genetic background and a well-established platform for molecular modification [20-23]. The creation and expansion of databases such as BsubCyc, SubtiWiki, DBTBS, etc. have allowed us to conveniently query genetic and metabolism-related information of B. subtilis [24, 25]. In summary, *Bacillus subtilis* becomes a powerful host capable of producing platform chemicals, biopolymers, and proteins. Currently, *Bacillus* subtilis has been used to produce a variety of products such as riboflavin, chondroitin, hyaluronic acid, and poly-y-glutamic acid [26,

27]. As a cell factory, *Bacillus subtilis* needs convenient and efficient gene editing tools to modify its genome-related genes as well as to solve problems such as genetic instability of free plasmids. Before the emergence of CRISPR technology, the commonly used gene editing tools are anti-selective marker system and site-specific recombination system. They are inefficient and complicated in the process of editing the genome [28-30]. With the gradual development of CRISPR-Cas technology, several efficient CRISPR systems have been developed in *Bacillus subtilis*, and their development will be systematically described below.

Altenbuchner constructed a single-plasmid CRISPR-Cas9 system, which is the first application of CRISPR system in *Bacillus subtilis*. gRNAs can be rapidly constructed into editing plasmids by restriction endonuclease BsaI combined with blue-white-spotting screening, and the function of CRISPR-Cas9 vectors can be performed by introduction of two large deletions and repair of the trpC2 mutation in *Bacillus subtilis* 168.Westbrook et al. constructed a CRISPR-Cas9-based genome editing toolkit for *Bacillus subtilis*, which integrated the Cas9 protein and gRNA expression frames into the genome in an integrative manner to achieve single-site and two-site mutations on the genome, with efficiencies of up to 100% and 85%, respectively. And the hyaluronan synthase gene, which is 2.9 kb long, was integrated into the genome with an efficiency of 69% [31].Zhang et al. constructed a dual-plasmid CRISPR-Cas9 system and utilized its highly efficient genome editing ability to knock out five genes on the genome of *Bacillus subtilis* ATCC6051a: srfC, spoIIAC, nprE, aprE and amyE, which solved the problems of foam production and bacillus production in the industrial enzyme in the production process [32].So et al. achieved the knockdown of pps manipulators up to 38 kb long with 80% efficiency using CRISPR-Cas9 system combined with post-culture strategy [33].Liu et al. developed a CRISPR/Cas9n-based *Bacillus subtilis* multiple genome editing system for iterative editing of the *Bacillus subtilis* genome. This system has an efficiency of at least 80% for 1-8kb gene deletions, at least 90% for 1-2kb gene insertions, close to 100% for sentinel mutations, 23.6% for large segmental DNA deletions, and close to 50% for three simultaneous point mutations. The efficiency of multiple gene editing was further improved by regulating NHEJ mediated by the ligD gene, resulting in an efficiency of about 65% for the introduction of 3-point mutations on chromosomes. The system was also applied to simultaneously fine-tune three genes in the riboflavin manipulator, and riboflavin production was significantly improved in one cycle [34].

# 3. Summary and Outlook

*Bacillus subtilis*, as a GRAS strain, has a huge application market and prospects in industries such as food, medicine, and breeding. Utilizing metabolic engineering technology to purposefully modify cells and construct highly efficient chassis cells is one of the effective strategies to enhance its industrial application potential and scope. Based on the CRISPR gene editing system, strategies such as promoter engineering, enzyme engineering, cofactor engineering, and dynamic regulation can achieve a relative unity of high yield, high productivity, and high production intensity of chassis cells. However, the current design and construction process of chassis cells is still rather cumbersome, and it is difficult to obtain universal metabolic rules after complex testing steps. Therefore, how to improve the efficiency of the design-build-test-learn cycle is one of the problems awaiting a solution. The intersection and combination of synthetic biology, systems biology, and multiple disciplines will become a breakthrough to solve this problem. On the one hand, the intersection and combination of artificial intelligence, machine learning, and multi-omics disciplines provides guarantees for annotating gene functions, analyzing regulatory mechanisms, and revealing the laws of life. On the other hand, directed evolution, computational tools, or de novo synthesis of non-natural enzymes or regulatory proteins also bring new opportunities for constructing high-version chassis cells.

# References


[1] M. Maki, K. T. Leung and W. S. Qin: The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass, International Journal of Biological Sciences, Vol. 5 (2009) No.5, p.500-516.

[2] M. S. Ou, N. Mohammed, L. O. Ingram and K. T. Shanmugam: Thermophilic Bacillus coagulans Requires Less Cellulases for Simultaneous Saccharification and Fermentation of Cellulose to Products than Mesophilic Microbial Biocatalysts, Applied Biochemistry and Biotechnology, Vol. 155 (2009) No.1-3, p.379-385.

[3] C. Michon, C. M. Kang, S. Karpenko, K. Tanaka, S. Ishikawa and K. Yoshida: A bacterial cell factory converting glucose into scyllo-inositol, a therapeutic agent for Alzheimer's disease, Communications Biology, Vol. 3 (2020) No.1, p.93-99.

[4] K. Tanaka, A. Natsume, S. Ishikawa, S. Takenaka and K. Yoshida: A new-generation of Bacillus subtilis cell factory for further elevated scyllo-inositol production, Microbial Cell Factories, Vol. 16 (2017), p.67-74.

[5] L. Liu, Y. F. Liu, H. D. Shin, R. R. Chen, N. S. Wang, J. H. Li, G. C. Du and J. Chen: Developing Bacillus spp. as a cell factory for production of microbial enzymes and industrially important biochemicals in the context of systems and synthetic biology, Applied Microbiology and Biotechnology, Vol. 97 (2013) No.14, p.6113-6127.

[6] Z. Guan, D. Xue, Abdallah, II, L. Dijkshoorn, R. Setroikromo, G. Y. Lv and W. J. Quax: Metabolic engineering of Bacillus subtilis for terpenoid production, Applied Microbiology and Biotechnology, Vol. 99 (2015) No.22, p.9395-9406.

[7] Y. F. Song, Z. Guan, R. van Merkerk, H. Pramastya, Abdallah, II, R. Setroikromo and W. J. Quax: Production of Squalene in Bacillus subtilis by Squalene Synthase Screening and Metabolic Engineering, Journal of Agricultural and Food Chemistry, Vol. 68 (2015) No.15, p.4447-4455.

[8] K. Zhou, R. Y. Zou, C. Q. Zhang, G. Stephanopoulos and H. P. Too: Optimization of Amorphadiene Synthesis in Bacillus Subtilis Via Transcriptional, Translational, and Media Modulation, Biotechnology and Bioengineering, Vol. 110 (2015) No.9, p.2556-2561.

[9] Y. F. Liu, H. Link, L. Liu, G. C. Du, J. Chen and U. Sauer: A dynamic pathway analysis approach reveals a limiting futile cycle in N-acetylglucosamine overproducing Bacillus subtilis, Nature Communications, Vol. 7 (2016), p.11933-11940.

[10] T. F. Niu, Y. F. Liu, J. H. Li, M. Koffas, G. C. Du, H. S. Alper and L. Liu: Engineering a Glucosamine-6-phosphate Responsive glmS Ribozyme Switch Enables Dynamic Control of Metabolic Flux in Bacillus subtilis for Overproduction of Acetylglucosamine, Acs Synthetic Biology, Vol. 7 (2018) No.10, p.2423-2435.

[11] Y. F. Liu, L. Liu, H. D. Shin, R. R. Chen, J. H. Li, G. C. Du and J. Chen: Pathway engineering of Bacillus subtilis for

microbial production of acetylglucosamine, Metabolic Engineering, Vol. 19 (2013), p.107-115.

[12] Y. Gu, X. Q. Lv, Y. F. Liu, J. H. Li, G. C. Du, J. Chen, L. A. Rodrigo and L. Liu: Synthetic redesign of central carbon and redox metabolism for high yield production of acetylglucosamine in Bacillus subtilis, Metabolic Engineering, Vol. 51 (2019), p.59-69.

[13] S. Yang, Y. Wang, C. B. Wei, Q. T. Liu, X. R. Jin, G. C. Du, J. Chen and Z. Kang: A new sRNA-mediated posttranscriptional regulation system for Bacillus subtilis, Biotechnology and Bioengineering, Vol. 115 (2018) No.12, p.2986-2995.

[14] P. Jin, L. P. Zhang, P. H. Yuan, Z. Kang, G. C. Du and J. Chen: Efficient biosynthesis of polysaccharides chondroitin and heparosan by metabolically engineered Bacillus subtilis, Carbohydrate Polymers, Vol. 140 (2018), p.424-432.

[15] S. X. Cui, X. Q. Lv, Y. K. Wu, J. H. Li, G. C. Du, R. Ledesma-Amaro and L. Liu: Engineering a Bifunctional Phr60-Rap60-Spo0A Quorum-Sensing Molecular Switch for Dynamic Fine-Tuning of Menaquinone-7 Synthesis in Bacillus subtilis, Acs Synthetic Biology, Vol. 8 (2019) No.8, p.1826-1837.

[16] S. B. Shi, T. Chen, Z. G. Zhang, X. Chen and X. M. Zhao: Transcriptome analysis guided metabolic engineering of Bacillus subtilis for riboflavin production, Metabolic Engineering, Vol. 11 (2009) No.4-5, p.243-252.

[17] T. Shi, Y. C. Wang, Z. W. Wang, G. L. Wang, D. Y. Liu, J. Fu, T. Chen and X. M. Zhao: Deregulation of purine pathway in Bacillus subtilis and its use in riboflavin biosynthesis, Microbial Cell Factories, Vol. 13 (2014), p.101-116.

[18] Abdallah, II, H. Pramastya, R. Van Merkerk, Sukrasno and W. J. Quax: Metabolic Engineering of Bacillus subtilis Toward Taxadiene Biosynthesis as the First Committed Step for Taxol Production, Frontiers in Microbiology, Vol. 10 (2019), p.218-228.

[19] Y. Gu, X. H. Xu, Y. K. Wu, T. F. Niu, Y. F. Liu, J. H. Li, G. C. Du and L. Liu: Advances and prospects of Bacillus subtilis cellular factories: From rational design to industrial applications, Metabolic Engineering, Vol. 50 (2018) No.4, p.109-121.

[20] Y. F. Liu, L. Liu, J. H. Li, G. C. Du and J. Che: Synthetic Biology Toolbox and Chassis Development in Bacillus subtilis, Trends in Biotechnology, Vol. 37 (2019) No.5, p.548-562.

[21] M. J. Xiang, Q. Kang and D. W. Zhang: Advances on systems metabolic engineering of Bacillus subtilis as a chassis cell, Synthetic and Systems Biotechnology, Vol. 5 (2020) No.4, p.245-251.

[22] S. Yang, Z. Kang, W. L. Cao, G. C. Du and J. Chen: Construction of a novel, stable, food-grade expression system by engineering the endogenous toxin-antitoxin system in Bacillus subtilis, Journal of Biotechnology, Vol. 219 (2022), p.40-47.

[23] L. A. Flórez, S. F. Roppel, A. G. Schmeisky, C. R. Lammers and J. Stülke: A community-curated consensual annotation that is continuously updated: the Bacillus subtilis centred wiki Subti Wiki, Database-the Journal of Biological Databases and Curation, Vol. 2009 (2009), p.9.

[24] T. Ishii, K. Yoshida, G. Terai, Y. Fujita and K. Nakai: DBTBS: a database of Bacillus subtilis promoters and transcription factors, Nucleic acids research, Vol. 29 (2001) No.1, p.278-80.

[25] P. D. Karp, C. A. Ouzounis, C. Moore-Kochlacs, L. Goldovsky, P. Kaipa, D. Ahren, S. Tsoka, N. Darzentas, V. Kunin and N. Lopez-Bigas: Expansion of the BioCyc collection of pathway/genome databases to 160 genomes, Nucleic acids research, Vol. 33 (2005) No.19, p.6083-9.

[26] M. X. Zhang, X. C. Zhao, X. Chen, M. Y. Li and X. D. Wang: Enhancement of riboflavin production in Bacillus subtilis via in vitro and in vivo metabolic engineering of pentose phosphate pathway, Biotechnology Letters, Vol. 43 (2021) No.12, p.2209-2216.

[27] P. Jin, Z. Kang, P. H. Yuan, G. C. Du and J. Chen: Production of specific-molecular-weight hyaluronan by metabolically engineered Bacillus subtilis 168, Metabolic Engineering, Vol. 35 (2016), p.21-30

[28] Y. Wang, J. Weng, R. Waseem, X. H. Yin, R. F. Zhang and Q. R. Shen: Bacillus subtilis genome editing using ssDNA with short homology regions, Nucleic Acids Research 2012, 40: 9

[29] X. Yan, H. J. Yu, Q. Hong and S. P. Li: Crelox system and PCR-based genome engineering in Bacillus subtilis, Applied and Environmental Microbiology, Vol. 74 (2008) No.17, p.5556-62.

[30] P. F. Popp, M. Dotzler, J. Radeck, J. Bartels and T. Mascher: The Bacillus BioBrick Box 2.0: expanding the genetic toolbox for the standardized work with Bacillus subtilis, Scientific Reports, Vol. 7 (2017) No.15058, p.16

[31] A. W. Westbrook, M. Moo-Young and C. P. Chou: Development of a CRISPR-Cas9 Tool Kit for Comprehensive Engineering of Bacillus subtilis, Applied and Environmental Microbiology, Vol. 82 (2016) No.16, p.4876-4895.

[32] K. Zhang, X. G. Duan and J. Wu: Multigene disruption in undomesticated Bacillus subtilis ATCC 6051a using the CRISPR/Cas9 system, Scientific Reports, Vol. 6 (2016), p.27943-27953.

[33] Y. So, S. Y. Park, E. H. Park, S. H. Park, E. J. Kim, J. G. Pan and S. K. Choi: A Highly Efficient CRISPR-Cas9-Mediated Large Genomic Deletion in Bacillus subtilis, Frontiers in Microbiology, Vol. 8 (2017), p.1167-1178.

[34] D. Y. Liu, C. Huang, J. X. Guo, P. J. Zhang, T. Chen, Z. W. Wang and X. M. Zhao: Development and characterization of a CRISPR/Cas9n-based multiplex genome editing system for Bacillus subtilis, Biotechnology for Biofuels, Vol. 12 (2019) No.1, p.197-213.