

Article

Biosynthesis of Guanidinoacetate by *Bacillus subtilis* Whole-Cell Catalysis

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Abstract: Guanidinoacetate (GAA) is a naturally occurring amino acid derivative and the direct precursor of creatine, which is widely used in feed additives and the pharmaceutical industry. The current industrial synthesis of GAA is based on chemical methods, which limits the application of GAA. Here, a biological approach is developed for food safety GAA production via whole-cell biocatalysis by the generally regarded as safe (GRAS) bacterium *Bacillus subtilis*. First, we introduced a heterologous arginine: glycine amidinotransferase (AgaT) from *Amycolatopsis kentuckyensis* into *B. subtilis* and optimized its expression level using strategies including: promoter optimization, ribosome binding site (RBS) and N-terminal coding sequence (NCS) screening. In order to alleviate the waste of arginine and the inhibition of AgaT by ornithine, we optimized the natural ornithine cycle in *B. subtilis*. At the same time, the first gene in the glycine degradation pathway was knocked out. After optimization using these strategies, the titer of GAA was 4.26 g/L with a productivity of 0.21 g/L/h in 20 h, which provides a new method for the biosynthesis of GAA.

Keywords: *B. subtilis*; guanidinoacetate; ornithine cycle; whole-cell catalysis



Citation: Yan, K.; Tian, R.; Zhang, L.; Lv, X.; Liu, L.; Liu, Y. Biosynthesis of Guanidinoacetate by *Bacillus subtilis* Whole-Cell Catalysis. *Fermentation* **2022**, *8*, 116. <https://doi.org/10.3390/fermentation8030116>

Academic Editor: Nancy N. Nichols

Received: 30 December 2021

Accepted: 1 March 2022

Published: 7 March 2022

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1. Introduction

Guanidinoacetate (GAA) has the physiological functions of promoting insulin secretion, influencing neuromodulation, changing arginine metabolism, etc. It is widely used as an intermediate in pharmaceutical organic synthesis; GAA can promote energy metabolism and improve antioxidant capacity, so it is often used in feed additives and food additives [1,2]. In mammalian organisms, creatine has an important role in the intracellular energy transport process, assisting in providing energy to muscle and nerve cells [3]. Creatine can be synthesized from GAA and S-adenosine methionine by guanidinoacetate N-methyltransferase (GamT, EC: 2.1.1.2). The human body is able to synthesize 60–80% of its own creatine requirements, so additional supplementation from food is required [4]. However, the stability of supplemented creatine is poor, and the utilization rate is low, so nowadays GAA is used to replace creatine for the purpose of increasing tissue load.

Due to the wide application of GAA in food, feed and medicine, a green, environmentally friendly and safe method of GAA production is needed [5]. At present, the synthesis of GAA in industry is based on the chemical synthesis method. However, there are some disadvantages of synthesizing GAA by chemical methods. For example, the end product GAA is easily contaminated, the purification process of GAA is quite tedious, and the production process is not good for the environment.

The whole-cell catalysis method provides the possibility for the efficient and green production of GAA. In prokaryotes, GAA can be synthesized from glycine and arginine by arginine:glycine amidinotransferase (AgaT, EC: 2.1.4.1) [6,7]. AgaT transfers the amidino group of arginine to the amino group of glycine to obtain GAA, while generating the by-product ornithine. Previous studies have shown that the by-product ornithine inhibits AgaT, which is one of the main reasons that limits the synthesis of GAA via whole-cell catalysis [8–10]. In order to solve this problem, methods such as optimizing the source of *agaT* and strengthening the ornithine cycle have been tried in *Escherichia coli*, which proved the effectiveness of GAA production via whole-cell catalysis [11]. However, the production of food safety compounds by *E. coli* has many problems, such as the secretion of endotoxins that may cause disease. Here, we aimed to establish a whole-cell catalytic factory of GAA in *Bacillus subtilis*, which is generally regarded as food safe (GRAS).

As a typical industrial model microorganism of food safety grade, *B. subtilis* has a clear genetic background and mature gene manipulation technology [12]. Due to its non-pathogenicity and strong protein synthesis ability, it is very suitable for GAA biocatalysis [13]. In this study, we first introduced a heterologous *agaT* gene from *Amycolatopsis kentuckyensis* into *B. subtilis* and constructed a GAA biosynthetic pathway. Next, in order to improve the expression level of AgaT, we adopted strategies such as promoter screening, optimization of ribosome binding site (RBS) and N-terminal coding sequence (NCS). Then, we blocked the conversion of glycine and arginine into other products in *B. subtilis*, which reduced the waste of arginine and glycine. Since the by-product ornithine can inhibit AgaT, we optimized the natural ornithine cycle in *B. subtilis*. Finally, a GAA production process using *B. subtilis* as a whole-cell biocatalyst was demonstrated, which should be more favorable for food-grade GAA production. The strategies used here may be useful for engineering *B. subtilis* strains for the production of other guanidino compounds or GAA derivatives via whole-cell catalysis.

2. Materials and Methods

2.1. Plasmids and Strains

The plasmids and strains used in this study are listed in Table 1. *E. coli* JM109 was used as the cloning host, and *B. subtilis* (*B. subtilis* 168, *B. subtilis* WB600) was used as the expression host for this experiment [14]. All strains and plasmids are stored in our laboratory. Gibson assembly was used to construct all plasmids used in this study with pHT01 as backbones [15,16]. To construct the plasmid pHT01-AgaT, first, the *agaT* gene encoding AgaT from *Amycolatopsis kentuckyensis* was codon-optimized and synthesized by Suzhou GENEWIZ Biotech Company (Suzhou, China). Then, the codon-optimized *agaT* gene and different promoters, P_{lytR}, P₄₃, P₃₃₃, and P₅₆₆, were used as templates to obtain the gene fragment and promoter fragments, respectively. The different promoters were obtained from our laboratory storage, and the sequences of the promoters are listed in Table 2. Finally, the gene fragment and different promoters' fragments were cloned into the pHT01 plasmid by Gibson assembly, respectively. Ultimately, we obtained a series of plasmids containing different AgaT expression intensities. They were then extracted from *E. coli* JM109 that was used for cloning purposes using a plasmid extraction kit purchased from Shanghai Sangon (Shanghai, China) and transported into *B. subtilis* to construct the strains listed in Table 1. The DNA polymerase and DNA marker were obtained from TAKARA (Dalian, China).

Table 1. Strains and plasmids used in this study.

Plasmids	
pHT01	Amp ^r , Cm ^r , <i>E. coli</i> - <i>B. subtilis</i> shuttle vector
pHT01-P _{lytR} -AgaT	pHT01 derivative with <i>agaT</i> cloned under the control of P _{lytR} promoter
pHT01-P ₄₃ -AgaT	pHT01 derivative with <i>agaT</i> cloned under the control of P ₄₃ promoter
pHT01-P ₃₃₃ -AgaT	pHT01 derivative with <i>agaT</i> cloned under the control of P ₃₃₃ promoter
pHT01-P ₅₆₆ -AgaT	pHT01 derivative with <i>agaT</i> cloned under the control of P ₅₆₆ promoter
pHT01-Px-AgaT-GFP	pHT01-Px-AgaT derivative with GFP via a short linker, X stands for different promoters, including P _{lytR} , P ₄₃ , P ₃₃₃ , P ₅₆₆
pHT01-AgaT-X	pHT01-P ₅₆₆ -AgaT derivative with random mutation between the ribosome binding site and the first ten amino acids of <i>agaT</i> . X is from 1 to 8, which stands for eight different mutations. The sequences of the eight mutant plasmids are shown in Table 2.
Strains	
<i>E. coli</i> JM109	<i>recA1, endA1, thi, gyrA96, supE44, hsdR17Δ (lac-proAB)/F'[traD36, proAB⁺, lacI^q, lacZΔ M15]</i>
<i>B. subtilis</i> 168	<i>trpC2</i>
<i>B. subtilis</i> WB600	<i>B. subtilis</i> 168 derived strain, Δ <i>nprBΔ aprEΔ eprΔ bprΔ nprEΔ mpr</i>
B1-1,2,3,4	<i>B. subtilis</i> 168 harboring plasmid pHT01-Px-AgaT; X stands for different promoters; B1-1 through B1-4 contain plasmid pHT01-P _{lytR} -AgaT, pHT01-P ₄₃ -AgaT, pHT01-P ₃₃₃ -AgaT, and pHT01-P ₅₆₆ -AgaT, respectively.
B2-1,2,3,4	<i>B. subtilis</i> 168 harboring plasmid pHT01-Px-AgaT-GFP; X stands for different promoters; B2-1 through B2-4 contain plasmid pHT01-P _{lytR} -AgaT-GFP, pHT01-P ₄₃ -AgaT-GFP, pHT01-P ₃₃₃ -AgaT-GFP and pHT01-P ₅₆₆ -AgaT-GFP, respectively.
B3-1,2,3,4,5,6,7,8	<i>B. subtilis</i> 168 harboring plasmid pHT01-AgaT-X; X is from 1 to 8. B3-1 through B3-8 contain plasmid pHT01-AgaT-1,2,3,4,5,6,7,8, respectively.
B4-1,2	<i>B. subtilis</i> WB600 harboring plasmid pHT01-AgaT-1 and pHT01-AgaT-7
B5-1	B4-2Δ <i>gcvP</i> (accession no. CP053102.1)
B5-2	B4-2Δ <i>argI</i>
B6-1	B4-2Δ <i>argIΔgcvP</i>
B6-2	B4-2Δ <i>argIΔnosA</i> (accession no. CP053102.1)
B6-3	B4-2Δ <i>argIΔgcvPΔnosA</i>
B7	B6-2 overexpresses <i>argF</i> (accession no. CP053102.1), <i>carAB</i> (accession no. CP053102.1) <i>glnA</i> (accession no. CP053102.1)
B8	B6-2 overexpresses <i>argG</i> (accession no. CP053102.1), <i>argH</i> (accession no. CP052842.1)
B9	B6-2 overexpresses <i>aspA</i> (accession no. CP054177.1)
B10	B6-2 overexpresses <i>argF</i> , <i>carAB</i> , <i>glnA</i> , <i>argG</i> , <i>argH</i> , <i>aspA</i>

For the knockout and overexpression of genes, a homologous recombination box with 1000 bp homology arms was used to perform genome editing in *B. subtilis*, and the Cre-lox system was used to eliminate resistance selection markers on the genome [17]. For the knockout of the genes on the genome of *B. subtilis*, we used resistance genes to replace the genes to be knocked out. A PCR product containing lox72- and lox66-specific sites and a cassette encoding zeocin (30 µg/mL) resistance was amplified. Then, 1000 bp homologous arms of the target gene were constructed into a homologous recombination box with the resistance gene cassette. The recombination fragment was obtained by overlap-extension PCR and transformed into *B. subtilis* strains. For the overexpression of genes, the difference was that we amplified the 1000 bp homologous arms of the *argI* (accession no. CP053102.1) gene site. The homologous arms, the resistance gene cassette, the promoter P_{veg} and the gene to be overexpressed were constructed into a homologous recombination box. Other

operations were the same as the knockout of the genes, and the sequence of the promoter P_{veg} is listed in Table 2. In this way, we achieved overexpression of the gene while knocking out the *argI* gene on the genome of *B. subtilis*.

Table 2. Sequences of the promoters used in this study.

Promoters	Sequence
P_{lytR}	cgaacggtagaatcgctgacctgAAACAATGAAACTTTTTTTTATAAAAAACGACTATTTTAGGATTTCATTC TTGTATTAAATAGAGTTGTATTATTGGAAATTTAACTCATAATGAAAGTAATTaaaggaggtgataaaa
P_{43}	tgataggtggtatgttttcgctgaacttttaatacacccattgaacatacgggtgatttaataactgacaacatcacctcttgctaaagcgccaag gacgccgcccgggctgtttgcttcttccgtgatttcgtgtaccattgggttacttatttttccaaggctgtaaggctgaaaattctacattat ttacatttttagaaatggcggtgaaaaaagcgcgattatgtaaaataaaagtatacgggtaccattataggttaagaggaatgtacac
P_{333}	ttgaggaatcatagaattttgacttaaaatttcagttgcttaatctacaattcttgatataatattctcatagtttgaaaaaggaggtgataaaa
P_{566}	aaaaaacggcctctcgaaatagagggttgacactcttttgagaatatgttatattatcagaaaggaggtgataaaa
P_{veg}	ATTTTGTCAAAATAATTTTATTGACAACGCTTATTAAACGTTGATATAATTTA AATTTTATTGACAAAAATGGGCTCGTGTGTGTACAATAAATGTAGTG

The uppercase and lowercase of bases in the sequence have no special meaning.

2.2. Whole-Cell Catalysis of GAA

The engineered strains were cultured in Luria–Bertani (LB) broth and agar plates. The seed culture was LB broth containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. The tryptone and yeast extract used in this study were obtained from Thermo Fisher Scientific (Shanghai, China). For the cultivation of *B. subtilis* cells, the culture of cells was grown in a 50 mL centrifuge tube containing 3 mL LB medium with Chloramphenicol (5 mg/L) at 37 °C for 8 h while shaking at 220 rpm. Then, we inoculated 2.5 mL of the seed culture to the 50 mL fermentation medium (pH 7.0) containing the following: 12 g/L yeast extract, 6 g/L peptone, 6 g/L $(NH_4)_2SO_4$, 12.5 g/L $K_2HPO_4 \cdot 3H_2O$, 2.5 g/L KH_2PO_4 , 3 g/L $MgSO_4 \cdot 7H_2O$ and 40 g/L glucose within a 500 mL shake flask. The cells were cultivated at 37 °C for 17 h with Chloramphenicol (5 mg/L) while shaking at 220 rpm. After 17 h of culture, the *B. subtilis* fermentation broth was centrifuged at 4 °C, 7000 × g rpm/min for 10 min to collect the precipitate of the bacterium. The fermentation broth was then washed once with 20 mL of 0.01 M PBS buffer (136.89 mM NaCl, 2.67 mM KCl, 8.1 mM Na_2HPO_4 , 1.76 mM KH_2PO_4 , pH 7.4 adjusted with HCl). Then, all cells were resuspended in $OD_{600} = 60$ using PBS buffer containing initial concentrations of 20 g/L arginine and 20 g/L glycine. Five milliliters of the above reaction system was taken and the whole-cell catalysis reaction was performed at 30 °C in a constant temperature shaker at 220 rpm/min.

2.3. Analytical Methods

A liquid chromatograph-mass spectrometer (LC-MS, SHIMADZU) was used to detect the concentrations of GAA on a system equipped with a Waters XBridge NH_2 3.5 μm column (4.6 mm × 150 mm) in each sample. The analysis was performed at 30 °C with a mobile phase comprising 75% acetonitrile in water (pH adjusted to 10 with ammonia) at a flow rate of 1 mL/min, and GAA was detected at an OD of 210 nm. This method can also detect Arginine and Glycine simultaneously.

The biomass and fluorescence intensity were measured directly on the 96-well plates, which contained 200 μL of cell culture using a Cytation 3 Multi-Mode Reader (BIOTEK, Cytation 3). The fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 523 nm. OD_{600} is the absorbance at 600 nm measured on the Cytation 3 Multi-Mode Reader when the amount of liquid in each well of a 96-well plate is 200 μL . The cell concentration was measured following a 50-fold dilution during whole-cell catalysis.

2.4. Design and Flow Screening of the Degenerate Primers

According to the degenerate bases and genetic code tables, we constructed a random mutation library based on pHT01-P₅₆₆-AgaT-GFP by random A, T, G, and C insertions 13 bp between the −35 and −10 regions of promoter P₅₆₆ and 35 bp in NCS of *agaT* via degenerate primer design and PCR. Snapgene 4.3.6 was used for gene sequence analysis and primer design. The PCR reaction condition was 98 °C 3 min, 98 °C 10 s, 55 °C 10 s, 72 °C 3 min, 72 °C 5 min, 12 °C 10 min, and 35 cycles from step 2 to step 4. The random mutation library was transformed into *B. subtilis* 168.

The organisms were washed using PBS buffer and diluted to an OD₆₀₀ = 0.1, and single cells with high relative fluorescence intensity were screened by flow cytometry and coated onto plates. Further screening was performed in 96-well plates to detect the biomass and fluorescence intensity of each single colony. Data were analyzed and visualized using FlowJo_V10 (FlowJo, LLC, Ashland, OR, USA) software.

3. Results

3.1. Constructing GAA Biosynthetic Pathway in *B. subtilis*

To convert glycine and arginine to GAA, *agaT* was first introduced into *B. subtilis* (Figure 1). The sequence of *agaT* was optimized according to the codon preference of *B. subtilis*. We used plasmid pHT01 as the expression vector to express *agaT* under the control of a strong promoter P_{lytR}. Then, the plasmid was transformed into wild-type *B. subtilis* 168, and strain B1-1 was obtained. However, no GAA was detected after whole-cell catalysis. We hypothesized that the low expression level of AgaT was the main limitation [18]. Therefore, we first selected three other strong promoters, P₄₃, P₃₃₃, and P₅₆₆, to replace promoter P_{lytR}, resulting in strain B1-2, B1-3, and B1-4, respectively.

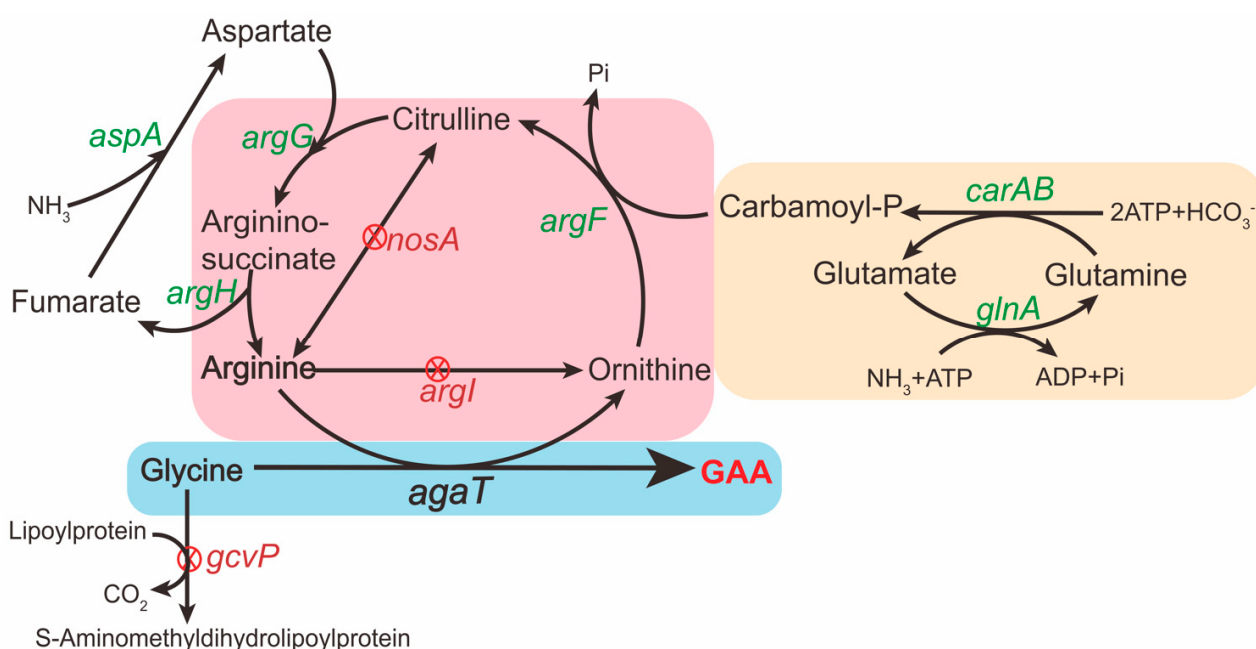


Figure 1. Schematic presentation of the GAA production in *B. subtilis*. The green font indicates overexpression of the corresponding genes on the genome of *B. subtilis*. The red “X” in a red circle indicates deletion of the corresponding genes. The ornithine cycle in *B. subtilis* is included in the pink box. The heterogeneous GAA synthesis pathway in *B. subtilis* is included in the blue box. The pathway in the yellow box is to promote the endogenous ornithine cycle in *B. subtilis*.

To screen for the best promoter that regulates AgaT expression with the highest intensity, we attached a green fluorescent protein (GFP, accession no. AAG29470.1) after AgaT by a short linker (GGAGGAGGATCTGGAGGCGG), and strains B2-1, B2-2, B2-3, and

B2-4 were obtained [19] (Figure 2a). We characterized the OD₆₀₀ and fluorescence intensity in *B. subtilis*. The expression intensity of AgaT under the control of different promoters was characterized according to the relative fluorescence intensity. We found that the relative fluorescence intensity of AgaT-GFP under the control of the promoter P₅₆₆ is the highest (Figure 2b). Finally, the *agaT* under the control of promoter P₅₆₆ was selected.

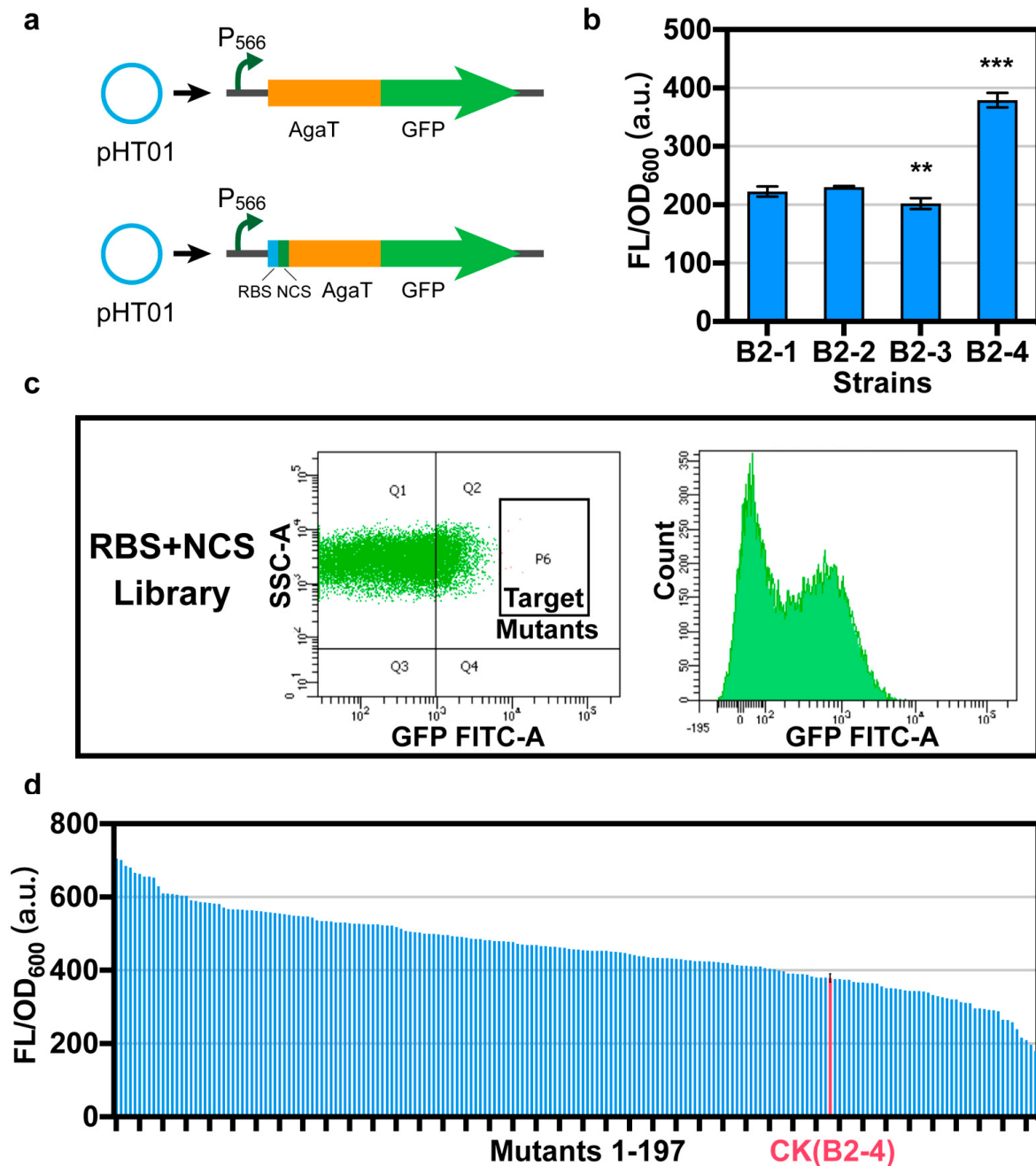


Figure 2. Optimizing RBS and NCS of *agaT*. (a) In order to optimize the RBS and NCS of *agaT*, we attached a *gfp* after *agaT* through a linker. Please refer to Table 3 for the starting sequence of RBS and NCS. (b) The relative fluorescence intensity of strains B2-1, B2-2, B2-3, and B2-4 under the control of different promoters. (c) Screening of the random mutant library for efficient mutant using flow cytometry. SSC-A: granularity, FITC-A: GFP fluorescence. (d) Characterization of 197 strains obtained from the library. We detected the relative fluorescence intensity of the 197 strains. Data are shown as mean $\pm$ S.D. from three ($n = 3$) biological replicate experiments, and significance determined from Student's *t*-test, where ** $p < 0.005$, *** $p < 0.0005$, and N.S. = not significant.

Table 3. The table shows the sequence of different mutants.

Strains	Sequence
B1-4	AGAAAGGAGGTGATAAAAATGAGAACAGATACAAGAATTGTTAATTCA
B3-1	TACGGGGAGGGGATAAAAATGAGAACGGATACAGAATCGTTAATTCT
B3-2	GATAGGGAGGGGATAAAAATGAGAACCGATACAGAATTGTTAATTCT
B3-3	TCGTGGGAGGGGATAAAAATGAGAACGGACACCAGAATTGTTAATTCT
B3-4	ATAGGGGAGGTCATAAAAAATGAGAACGGATACAGAATTGTTAATTCA
B3-5	TCGCGGGAGGGGATAAAAATGAGAACCGACACAAGAATCGTTAATTCC
B3-6	CCAAGGGAGGGGTAAAAATGAGAACCGATACGAGAATTGTAAATTCT
B3-7	GCCGAGGAGGGGTAAAAATGAGAACGGATACCAGAATTGTGAATTCT
B3-8	AACAGGGAGGGGATAAAAATGAGAACCGATACCAGAATCGTGAATTCT

After optimizing RBS and NCS of *agaT*, the sequences of different mutants are shown in this table. B1-4 was the initial strain. The red font indicates the mutation site.

3.2. Optimizing RBS and NCS of *agaT*

After optimizing the promoter, we still did not detect the production of GAA via whole-cell catalysis. However, the P₅₆₆ promoter is already the strongest promoter currently in use. Therefore, we assume that the main problem is the low level of translation, which led to the low expression of *agaT*. RBS and NCS of a gene could significantly impact the expression level in translation by influencing the efficiency of ribosome binding to mRNA and ribosome extension at the initial phase of translation [20]. Optimizing the RBSs and NCSs of genes is an important mechanism for fine-tuning gene expression in bacteria. To promote the expression of *agaT* in *B. subtilis*, we optimized the RBS and NCS in the pHT01-P₅₆₆-AgaT-GFP expression frame. The first step was to construct a random mutation library to screen RBS and NCS for efficiently driving *agaT* expression. We constructed a random mutation library based on 8 bp random A, T, G, and C substitution in the RBS region and 7 bp synonymous substitution of *agaT* NCS via degenerate primer design and PCR [21]. After screening a library containing 83,440 mutants using flow cytometry, 197 strains were further characterized using 96-well plates (Figure 2c). We detected the OD₆₀₀ and fluorescence intensity of all 197 strains (Figure 2d). The positive rate is 77.7%. Eight strains with the highest relative fluorescence intensity detected during rescreening were sequenced. The results showed that these eight single colonies had completely different mutations in both RBS and NCS of *agaT* (Table 3).

Then, we removed the *gfp* gene on the eight plasmids and obtained plasmids pHT01-AgaT-1 to pHT01-AgaT-8. We transferred these eight plasmids into *B. subtilis* 168, and obtained strains B3-1, B3-2, B3-3, B3-4, B3-5, B3-6, B3-7, and B3-8. After whole-cell catalysis, GAA was detected for the first time (Figure 3a). With an initial concentration of 0.871 g/L arginine and 0.504 g/L glycine, the strain with the highest titer was *B. subtilis* B3-7 containing the plasmid pHT01-AgaT-7 with a titer of 0.062 g/L, followed by *B. subtilis* B3-1 containing the plasmid pHT01-AgaT-1 with a titer of 0.039 g/L. The above results show that we successfully detected GAA production in *B. subtilis* by optimizing RBS and NCS.

Due to the differences in the ability of enzyme expression in different hosts, we compared the production of GAA from arginine and glycine catalyzed by AgaT in different hosts with an initial concentration of 20 g/L arginine and 20 g/L glycine. We transformed the plasmids pHT01-AgaT-1 and pHT01-AgaT-7 into *B. subtilis* WB600, respectively. We obtained the strains B4-1 and B4-2. It was found that regardless of which plasmid was introduced, GAA production was higher in *B. subtilis* WB600 than in *B. subtilis* 168 (Figure 3b). For pHT01-AgaT-7, the production of GAA in WB600 was 1.34-fold that in *B. subtilis* 168; for pHT01-AgaT-1, the production of GAA in WB600 was 2.13-fold that in *B. subtilis* 168. After optimizing the host of *B. subtilis*, the highest titer of GAA was 0.24 g/L.

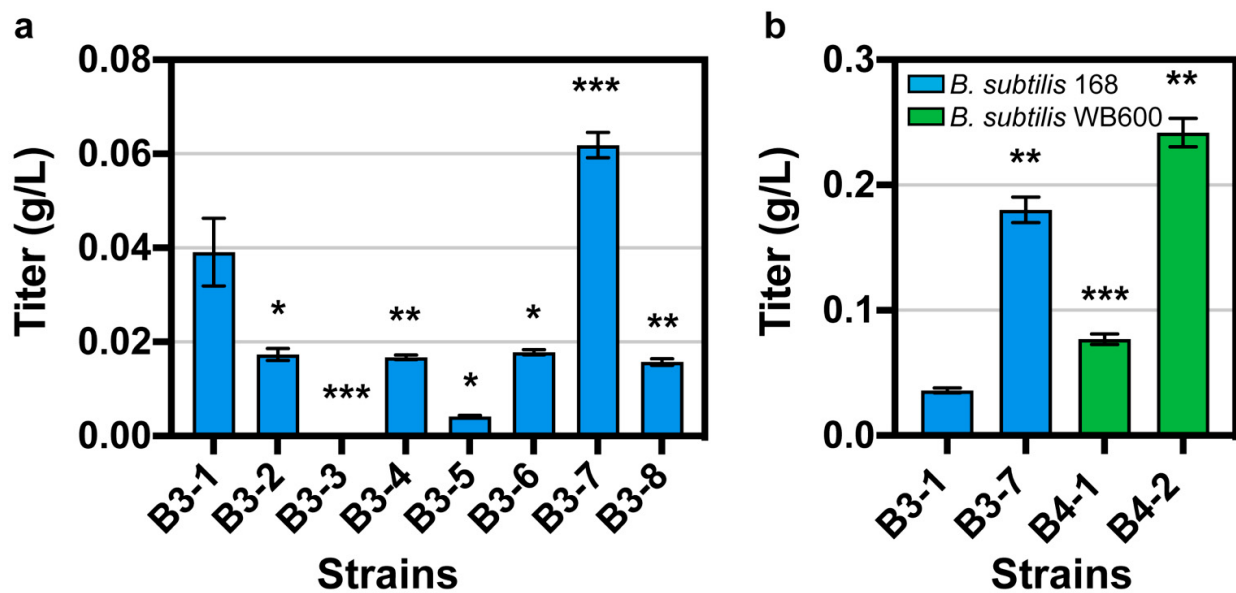


Figure 3. (a) The whole-cell catalysis results in the eight best mutants. (b) The whole-cell catalysis results in *B. subtilis* WB600 and *B. subtilis* 168. Data are shown as mean $\pm$ S.D. from three ($n = 3$) biological replicate experiments, and significance determined from Student's t-test, where * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, and N.S. = not significant.

3.3. Knocking-Out of the Substrate Catabolic Pathway

Blocking the substrate consumption pathway is an effective method to improve the yield of the target product. In the metabolic pathway of *B. subtilis*, glycine dehydrogenase, encoded by *gcvP*, is the first gene in the glycine degradation pathway. To make more glycine available for GAA biosynthesis, we knocked out *gcvP* on the genome of strain B4-2, yielding strain B5-1. Whole-cell catalysis with an initial concentration of 20 g/L arginine and 20 g/L glycine resulted in a GAA production of 0.18 g/L. Knock-out of *gcvP* on the *B. subtilis* genome was not as effective as knock-out of *argI*. We guessed that blocking the cellular utilization of glycine followed by affecting cell growth resulted in affecting GAA synthesis.

In *B. subtilis*, the ornithine cycle is complete. Arginase, encoded by *argI*, can catalyze the conversion of arginine to ornithine. Since ornithine has an inhibitory effect on AgaT, it would affect the synthesis of GAA from arginine and glycine catalyzed by AgaT. Therefore, it is essential to knock out *argI* in the metabolic pathway of *B. subtilis*. On the one hand, it would alleviate the inhibition of AgaT by ornithine, and on the other hand, it would reduce the consumption of arginine. Knocking out *argI* on the genome of strain B4-2 increased GAA titer to 0.46 g/L (strain B5-2).

After searching for metabolic pathways of *B. subtilis*, we found that arginine and citrulline interconvert in the ornithine cycle of *B*, which is catalyzed by the Nitric-Oxide synthase encoded by *nosA*. Next, we knocked out the above two or three genes simultaneously. *ArgI* and *gcvP* were simultaneously knocked out on the basis of strain B4-2 to obtain strain B6-1. On the basis of strain B4-2, *argI* and *nosA* were simultaneously knocked out to obtain strain B6-2. On the basis of strain B4-2, *argI*, *gcvP* and *nosA* were simultaneously knocked out to obtain strain B6-3. The best strain was B6-2, with a titer of 0.91 g/L; the titer of B6-1 was 0.57 g/L, and the titer of B6-3 was 0.84 g/L (Figure 4a). Strain B6-3 had the highest productivity of GAA at 0.56 g/L/h (Figure 4a). The knockout of *argI* and *nosA* significantly increased the production of GAA, even better than the simultaneous knockout of *argI*, *nosA* and *gcvP*, suggesting that arginine has a greater effect on GAA biosynthesis than glycine.

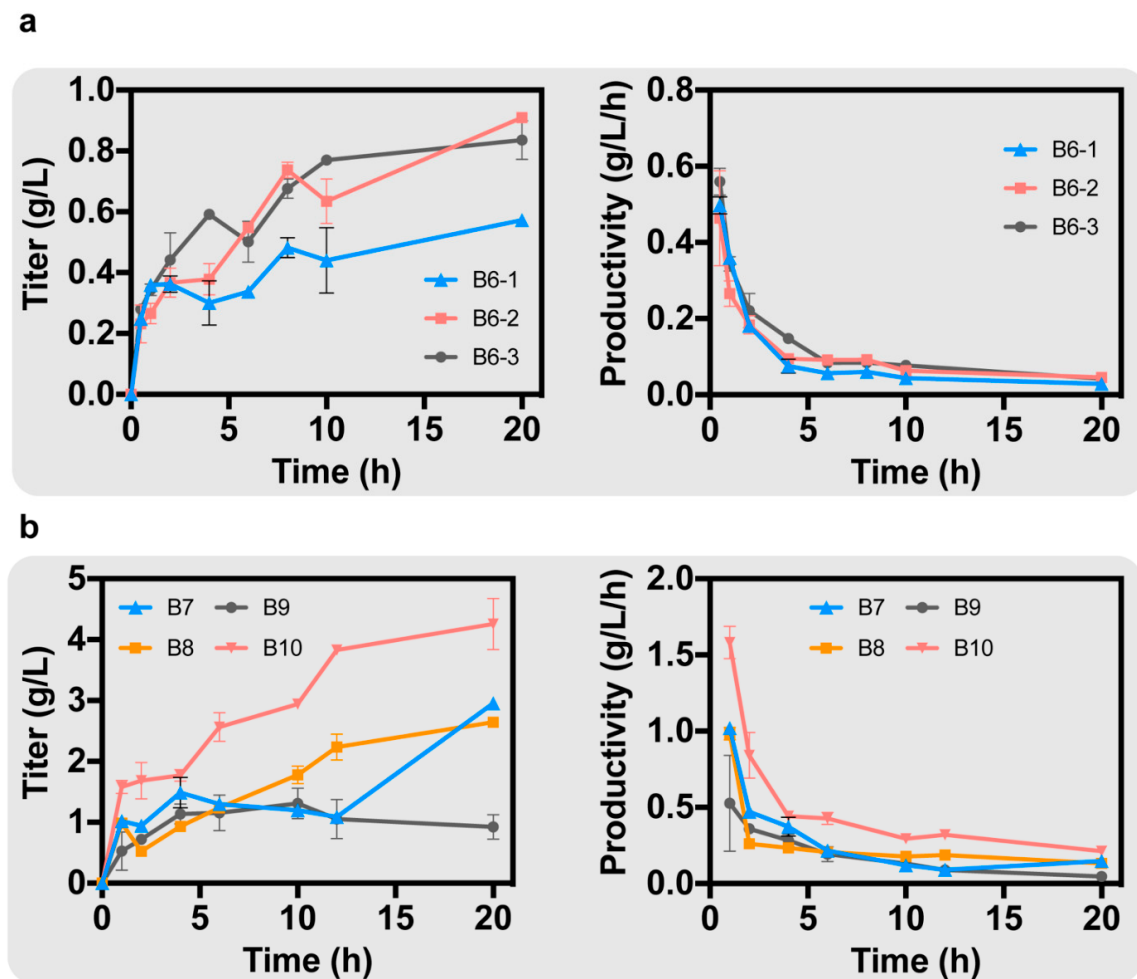


Figure 4. (a) After knocking out the degradation pathways of glycine and arginine in *B. subtilis*, the GAA production of different strains was obtained. (b) After enhancing the endogenous ornithine cycle in *B. subtilis*, the GAA production of different strains was obtained.

3.4. Enhancing Endogenous Ornithine Cycle of *B. subtilis*

Ornithine can revert to produce arginine in *B. subtilis* due to the endogenous ornithine cycle. If the metabolic flow of the ornithine cycle is increased to promote the production of arginine from ornithine, it can alleviate the inhibition of AgaT by ornithine on the one hand, and increase the arginine required for GAA biosynthesis on the other hand. Therefore, it was necessary to overexpress genes in the ornithine cycle on the *B. subtilis* genome. First, the ornithine carbamoyl transferase (EC: 2.1.3.3), encoded by *argF*, is able to catalyze the conversion of ornithine to citrulline. Overexpressing *argF* can alleviate the inhibition by ornithine. Meanwhile, carbamoyl-phosphate synthase (EC: 6.3.5.5), encoded by *carAB*, is able to increase carbamoyl phosphate (CP), which is involved in the synthesis of citrulline [22]. *glnA* was also overexpressed, which encodes glutamine synthetase (EC: 6.3.1.2) [23]. Glutamine synthetase catalyzes the conversion of glutamate to glutamine and is able to synergize with carbamoyl-phosphate synthase to provide the CP required for citrulline synthesis. On the genome of *B. subtilis*, *argF*, *carAB*, and *glnA* are on the same gene cluster. Whole-cell catalysis reactions were performed in PBS buffer containing 20 g/L arginine and 20 g/L glycine. Under the regulation of the Promoter P_{veg} , we simultaneously overexpressed *argF*, *carAB*, and *glnA* in strain B6-2, resulting in strain B7 with a GAA titer of 2.95 g/L (Figure 4b).

In the ornithine cycle, argininosuccinate synthase (EC: 6.3.4.5) and argininosuccinate lyase (EC: 4.3.2.1), encoded by *argG* and *argH*, respectively, are on a gene cluster that

promotes the production of arginine from citrulline. Overexpression of *argG* and *argH* would increase the metabolic flux of the ornithine cycle. Aspartate ammonia-lyase (EC: 4.3.1.1), encoded by *aspA*, catalyzes the conversion of fumarate to aspartate, providing aspartate required in the ornithine cycle [24]. We overexpressed *argG* and *argH* in strain B6-2 under the regulation of the Promoter P_{veg} , resulting in strain B8. After 20 h of whole-cell catalysis, the production of GAA was 2.45 g/L (Figure 4b). Meanwhile, we overexpressed *aspA* on strain B7 under the regulation of the Promoter P_{veg} , resulting in strain B9. The GAA production was only 0.92 g/L, suggesting that overexpression of *aspA* alone did not promote the synthesis of GAA (Figure 4b).

Finally, we simultaneously overexpressed *argF*, *carAB*, *glnA*, *argG*, *argH* and *aspA* under the regulation of the promoter P_{veg} in strain B6-2, resulting in strain B10. A total GAA production of 4.26 g/L was achieved in a 20 h whole-cell catalysis with a maximum productivity of 1.58 g/L/h in 1 h (Figure 4b).

4. Conclusions

This study demonstrated the feasibility of synthesizing GAA via whole-cell catalysis in *B. subtilis*. Due to the advantage of *B. subtilis* being a food-safe microorganism, we provided a safer and environmentally friendly production method. By optimizing RBS and NCS of *agaT* and other strategies, as well as optimizing the metabolic pathway in *B. subtilis* WB600, we obtained a recombinant *B. subtilis* with a highest GAA production of 4.26 g/L after a 20 h whole-cell catalysis. Compared to the currently reported GAA production of 8.61 g/L with a maximum productivity of 0.35 g/L/h after a 22 h whole-cell catalysis in *E. coli*, we obtained a higher maximum production intensity of 1.58 g/L/h [11]. This method has the advantages of easy operation and stable production, which provides a new strategy to realize the industrial production of GAA.

Author Contributions: Conceptualization, Y.L., L.L. and X.L.; methodology, K.Y., R.T. and L.Z.; validation, Y.L., R.T. and K.Y.; formal analysis, R.T. and K.Y.; investigation, Y.L.; resources, Y.L., K.Y. and R.T.; data curation, K.Y. and R.T.; writing—original draft preparation, K.Y. and R.T.; writing—review and editing, Y.L.; visualization, K.Y. and R.T.; supervision, Y.L. and L.L.; project administration, Y.L. and L.L.; funding acquisition, Y.L., K.Y. and R.T. contributed equally to this work. All authors have read and agreed to the published version of the manuscript.

Funding: This study was financially supported by the National Key Research and Development Program of China (2020YFA0908300), National Natural Science Foundation of China (32172349), Foundation for Innovative Research Groups of the National Natural Science Foundation of China (32021005), Natural Science Foundation of Jiangsu Province (BK20200085), and Key Research and Development Program of Jiangsu Province (BE2019628).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data information is available in this paper.

Conflicts of Interest: The authors declare no conflict of interest.

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