rocF affects the production of tetramethylpyrazine in fermented soybeans with Bacillus subtilis BJ3-2

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Abstract

Background: Tetramethylpyrazine (TTMP) is a flavoring additive that significantly contributes to the formation of flavor compounds in soybean-based fermented foods. Over recent years, the application of TTMP in the food industry and medicine has been widely investigated. In addition, several methods for the industrial-scale production of TTMP, including chemical and biological synthesis, have been proposed. However, there have been few reports on the synthesis of TTMP through amino acid metabolic flux. In this study, we investigated genetic alterations of arginine metabolic flux in solid-state fermentation (SSF) of soybeans with Bacillus subtilis (B. subtilis) BJ3-2 to enhance the TTMP yield.

Results: SSF of soybeans with BJ3-2 exhibited a strong Chi-flavour (a special flavour of ammonia-containing smelly distinct from natto) at 37 °C and a prominent soy sauce-like aroma at 45 °C. Transcriptome sequencing and RT-qPCR verification showed that the rocF gene was highly expressed at 45 °C but not at 37 °C. Moreover, the fermented soybeans with BJ3-2ΔrocF (a rocF knockout strain in B. subtilis BJ3-2 were obtained by homologous recombination) at 45 °C for 72 h displayed a lighter color and a slightly decreased pH, while exhibiting a higher arginine content (increased by 14%) than that of BJ3-2. However, the ammonia content of fermented soybeans with BJ3-2ΔrocF was 43% lower than that of BJ3-2. Inversely, the NH₄⁺ content in fermented soybeans with BJ3-2ΔrocF was increased by 28% (0.410 mg/kg). Notably, the TTMP content in fermented soybeans with BJ3-2ΔrocF and BJ3-2ΔrocF + Arg (treated with 0.05% arginine) were significantly increased by 8.6% (0.4617 mg/g) and 18.58% (0.504 mg/g) respectively than that of the BJ3-2.

Conclusion: The present study provides valuable information for understanding the underlying mechanism during the TTMP formation process through arginine metabolic flux.

Keywords: Bacillus subtilis, Transcriptome sequencing, rocF, Fermented soybeans, TTMP

Background

Tetramethylpyrazine (TTMP) is a nitrogen-containing heterocyclic compound that contributes to the formation of many aromas and flavors compounds [1, 2]. TTMP is a member of the class of pyrazines detected in fermented foods, such as Chinese Baijius (Chinese liquor), vinegar, and soybean-based fermented foods [3, 4]. It is commonly used in the food industry as an important aromatic compound additive to enhance flavor [5, 6]. Recent data suggested that TTMP possesses diet therapy functions.
and may be used as an effective therapeutic drug to treat cardiovascular health and enhance cognition [7–9]. Over the recent years, the application of TTMP in the food industry and medicine has been widely investigated. Most studies have focused on developing methods for the industrial-scale production of TTMP, including chemical and biological synthesis [10, 11]. The biological synthesis of TTMP provides several advantages over chemical syntheses, considering it is an environmentally friendly and cost-effective process [5]. Moreover, pyrazines have been widely reported as important microbial secondary metabolite, which implies that microbial metabolism can produce the TTMP [12].

Fermented soybeans (also named Douchi) are one of the most popular foods in China due to the strong Chi-flavour (namely soybean-flavour [13], a special flavor of ammonia-containing smelly distinct from natto). Bacillus subtilis (B. subtilis) is one of the most important microorganisms for the fermentation process of Douchi. More than half-century ago, Kosuge et al. [14] first suggested that B. subtilis can synthesize TTMP. Moreover, a recent study proved that TTMP in Chinese liquor is mainly generated from B. subtilis under Micro-Oxygen conditions [6]. Thus, it has been proposed that the metabolic engineering of B. subtilis can enhance the production of TTMP. A high yield (2.5 g/kg) of TTMP was obtained using B. subtilis IFO 3013 inoculated soybeans and fermentation for 14 days [15]. However, industrial production still has several difficulties, such as low production [16] and a low conversion rate of precursor substances [17].

Over the years, genetic engineering has emerged as a powerful tool in studying the behavior of the production of TTMP [7, 18]. Meng et al. [5] showed that knocking the 2,3-butanediol dehydrogenase gene (bdhA) and adding 2,3-butanediol exogenously improve the production of TTMP in B. subtilis. Similarly, a mutant bdhA and glucose uptake protein (GluU) were reported to affect the production of TTMP in B. subtilis 168 [6]. Moreover, two genes (BDH1 and another BDH2 coding 2,3-butanediol dehydrogenase) were deleted or overexpressed to improve the TTMP yield in Saccharomyces cerevisiae [19]. The TTMP can be generated from various precursors, including acetoin and ammonia [20]. Alterations of carbon flux into the acetoin biosynthesis pathway by blocking the degradation and competing pathways can enhance the TTMP yield [5]. Moreover, the TTMP has also been produced by condensing acetoin with NH₄⁺ [21, 22]. However, there have been few reports on the synthesis of TTMP through amino acid metabolic flux. Recently, a new theory has been proposed suggesting that amino acids have close relationship with the production of volatile compounds such as pyrazines [23].

Various metabolites were generated by using B. subtilis during the fermentation of natto, such as peptone, peptides, amino acids, sugars, and organic acids, enhancing organoleptic of the final products [24]. Yet, their contribution to the organoleptic properties of the final products has not yet been investigated in detail.

In this study, we investigated genetic alterations of arginine metabolic flux in order to enhance the TTMP yield. The results showed that the fermented soybeans with BJ3-2 ΔrocF had a significantly higher content of arginine and TTMP. Our findings provided detailed insights into the effects of rocF genes on the metabolism of arginine and TTMP in fermented soybeans with the B. subtilis BJ3-2.

**Table 1** Sensory evaluation of BJ3-2 at 37 °C and 45 °C

| Strain  | Soy sauce-like aroma (35) | Chi-flavor (35) | Ammonia (30) |
|---------|----------------------------|-----------------|--------------|
| BJ3-2 37 °C | 18                         | 32              | 22           |
| BJ3-2 45 °C | 33                         | 19              | 20           |

**Results**

**Summary of the sequencing data**

The sensory evaluation of the fermented broth was performed by well-trained panelists on the three components that included soy sauce-like aroma, Chi-flavour, and ammonia; individual scores were added together to provide a total score (Table 1). According to the sensory evaluation, we found that the Chi-flavour of broth fermented with BJ3-2 at 37 °C was prominent, whereas the soy sauce-like aroma of broth fermented with BJ3-2 at 45 °C was prominent; the ammonia was similar at 37 °C and 45 °C (Table 1). Therefore, we performed RNA sequencing (RNA-seq) analysis of BJ3-2 at 37 °C and 45 °C. For the sequencing data, we found that the base content was evenly distributed, and the nucleotide distributions were within a reasonable range (Additional file 1: Fig. S1A, D). The quality and error rate of the samples were within a normal range (Additional file 1: Fig. S1B, C, E and F). After filtering the original data, checking the sequencing error rate, and verifying the distribution of the GC content, clean reads for follow-up analysis were also obtained. The Q20 and Q30 values of BJ3-2 at 37 °C were 98.5% and 95.93%, respectively, while they were 98.42% and 95.7% at 45 °C (Table 2). Next, gene expression analysis indicated that the gene expression levels in different samples were uniformly distributed in density diagrams (Additional file 1: Fig. S2). After normalizing the sequencing data, we constructed a scatter and volcano diagram (Fig. 1A, B). As shown, 3648 genes were identified, including 67 up-regulated DEGs and 56
down-regulated DEGs ($P\text{-value} < 0.05$ and $|\log_2\text{FC}| \geq 1$) (Table 3).

**GO and KEGG enrichment analysis of the DEGs**

To determine the functions of the DEGs, GO analysis revealed that these genes were based on their roles in biological processes (BPs), cellular components (CCs), and molecular functions (MFs). The results indicated that these DEGs were mainly enriched in 28 GO terms (11 BPs, 10 CCs, and 7 MFs; Q-value $\leq 1$). For BPs, the exhaustive analysis showed that many DEGs were involved in cellular processes, metabolic processes, and single-organism processes. For CCs, most DEGs were enriched in the cell, cell part, and macromolecular complex. For MFs, many DEGs were involved in catalytic activity, binding, and transporter activity (Fig. 1C). Additionally, KEGG pathway analysis revealed that the dominant DEGs were enriched in pyrimidine metabolism and arginine and proline metabolism (Fig. 1D; Additional file 2: Table S4). Collectively, the results suggested that

| Samples          | Sequence  | Base (bp)   | Error rate (%) | Q20 (%) | Q30 (%) | GC (%) |
|------------------|-----------|-------------|----------------|---------|---------|--------|
| BJ3-2 37 °C      | 13,969,028| 1,863,773,158| 0.0114         | 98.5    | 95.93   | 46.27  |
| BJ3-2 45 °C      | 11,090,274| 1,491,854,650| 0.0116         | 98.42   | 95.7    | 47.2   |
Table 3  The DEGs of RNA-seq at 45 °C

| Gene ID  | BJ 3–2 37 °C | BJ 3–2 45 °C | log₂FC (BJ 3–2 45 °C/BJ 3–2 37 °C) | P-value | FDR   | Regulate |
|----------|---------------|---------------|-----------------------------------|---------|-------|----------|
| BSU13700 | 77.522        | 5185.634      | 6.06                              | 1.56E-16| 5.70E-13| Up       |
| BSU17820 | 0             | 6.491         | 6.04                              | 1.01E-03| 3.50E-02| Up       |
| BSU02160 | 8.142         | 289.265       | 5.13                              | 1.68E-12| 8.78E-10| Up       |
| BSU12300 | 35.418        | 1082.334      | 4.93                              | 4.74E-13| 4.13E-10| Up       |
| BSU11750 | 32.984        | 1008.288      | 4.93                              | 2.23E-12| 9.01E-10| Up       |
| BSU39580 | 12.584        | 358.188       | 4.82                              | 4.26E-10| 1.04E-07| Up       |
| BSU12310 | 12.634        | 355.103       | 4.8                               | 3.10E-12| 1.03E-09| Up       |
| BSU14569 | 53.082        | 1210.986      | 4.51                              | 1.17E-07| 1.77E-05| Up       |
| BSU00240 | 138.86        | 2344.553      | 4.08                              | 1.60E-09| 3.65E-07| Up       |
| BSU02970 | 10.84         | 146.608       | 3.75                              | 1.15E-06| 1.40E-04| Up       |
| BSU02170 | 33.435        | 435.053       | 3.7                               | 1.81E-09| 3.87E-07| Up       |
| BSU11740 | 25.23         | 323.403       | 3.67                              | 1.36E-08| 2.62E-06| Up       |
| BSU12090 | 31.566        | 349.892       | 3.47                              | 1.28E-07| 1.86E-05| Up       |
| BSU36970 | 40.022        | 429.186       | 3.42                              | 1.79E-08| 3.26E-06| Up       |
| BSU12320 | 9.033         | 90.74         | 3.31                              | 1.02E-07| 1.61E-05| Up       |
| BSU17320 | 2.032         | 20.756        | 3.29                              | 1.33E-03| 4.20E-02| Up       |
| BSU12340 | 11.731        | 114.535       | 3.28                              | 8.88E-08| 1.47E-05| Up       |
| BSU11760 | 8.632         | 81.025        | 3.22                              | 2.99E-06| 3.11E-04| Up       |
| BSU12330 | 10.765        | 97.552        | 3.17                              | 2.41E-07| 3.25E-05| Up       |
| BSU13520 | 4.768         | 33.11         | 2.77                              | 1.24E-05| 1.01E-03| Up       |
| BSU10710 | 12.019        | 81.937        | 2.76                              | 7.47E-04| 2.78E-02| Up       |
| BSU36960 | 49.193        | 320.369       | 2.7                               | 1.70E-06| 1.94E-04| Up       |
| BSU12360 | 7.415         | 48.837        | 2.7                               | 4.64E-06| 4.23E-04| Up       |
| BSU06850 | 9.309         | 60.185        | 2.68                              | 9.66E-05| 5.78E-03| Up       |
| BSU12350 | 14.441        | 90.526        | 2.64                              | 4.64E-06| 4.23E-04| Up       |
| BSU37620 | 100.13        | 605.721       | 2.6                               | 1.28E-06| 1.51E-04| Up       |
| BSU19670 | 3.952         | 23.716        | 2.56                              | 3.82E-04| 1.70E-02| Up       |
| BSU33510 | 27.25         | 151.488       | 2.47                              | 6.82E-04| 2.59E-02| Up       |
| BSU22860 | 4.692         | 25.493        | 2.42                              | 6.57E-04| 2.52E-02| Up       |
| BSU00430 | 2.886         | 15.713        | 2.4                               | 6.57E-04| 2.52E-02| Up       |
| BSU11240 | 30.261        | 155.485       | 2.36                              | 4.58E-06| 4.23E-04| Up       |
| BSU11230 | 27.363        | 133.296       | 2.28                              | 1.20E-05| 9.97E-04| Up       |
| BSU38450 | 59.481        | 281.155       | 2.24                              | 1.84E-05| 1.37E-03| Up       |
| BSU00640 | 30.035        | 135.911       | 2.17                              | 1.36E-05| 1.06E-03| Up       |
| BSU00650 | 107.821       | 484.02        | 2.17                              | 1.50E-05| 1.14E-03| Up       |
| BSU07140 | 30.763        | 135.31        | 2.13                              | 3.92E-05| 2.80E-03| Up       |
| BSU32850 | 3.438         | 15.261        | 2.12                              | 1.31E-03| 4.18E-02| Up       |
| BSU30270 | 9.623         | 41.992        | 2.11                              | 6.52E-05| 4.32E-03| Up       |
| BSU00830 | 81.386        | 319.295       | 1.97                              | 6.36E-05| 4.30E-03| Up       |
| BSU28060 | 23.9          | 93.458        | 1.96                              | 7.45E-05| 4.60E-03| Up       |
| BSU11250 | 32.143        | 123.455       | 1.94                              | 7.38E-05| 4.60E-03| Up       |
| BSU00660 | 35.405        | 134.701       | 1.92                              | 7.38E-05| 4.60E-03| Up       |
| BSU36550 | 417.195       | 1556.086      | 1.9                               | 5.17E-05| 3.63E-03| Up       |
| BSU19050 | 7.277         | 27.08         | 1.88                              | 1.08E-03| 3.68E-02| Up       |
| BSU30280 | 10.263        | 35.571        | 1.78                              | 4.72E-04| 1.96E-02| Up       |
| BSU30290 | 8.444         | 28.629        | 1.75                              | 9.44E-04| 3.34E-02| Up       |
| BSU38080 | 223.922       | 747.383       | 1.74                              | 1.76E-04| 9.59E-03| Up       |
| BSU31060 | 17.602        | 58.817        | 1.73                              | 2.31E-04| 1.16E-02| Up       |
| BSU25150 | 9.968         | 32.51         | 1.73                              | 1.03E-03| 3.53E-02| Up       |
| Gene ID  | BJ 3–2 37 °C | BJ 3–2 45 °C | log2FC (BJ 3–2 45 °C/BJ 3–2 37 °C) | P-value  | FDR       | Regulate |
|---------|--------------|--------------|-----------------------------------|----------|-----------|----------|
| BSU16740 | 18.631       | 61.488       | 1.72                              | 7.91E-04 | 2.86E-02  | Up       |
| BSU32180 | 5.357        | 17.113       | 1.66                              | 1.45E-03 | 4.43E-02  | Up       |
| BSU33500 | 42.669       | 132.323      | 1.63                              | 2.43E-04 | 1.20E-02  | Up       |
| BSU37630 | 40.825       | 124.492      | 1.61                              | 3.84E-04 | 1.70E-02  | Up       |
| BSU24580 | 6.11         | 18.56        | 1.59                              | 1.11E-03 | 3.74E-02  | Up       |
| BSU05880 | 7.189        | 21.896       | 1.59                              | 1.46E-03 | 4.45E-02  | Up       |
| BSU12069 | 6213.887     | 18553.429    | 1.58                              | 2.52E-04 | 1.22E-02  | Up       |
| BSU11780 | 41.076       | 122.185      | 1.57                              | 1.24E-03 | 4.13E-02  | Up       |
| BSU27310 | 29.935       | 87.795       | 1.55                              | 4.25E-04 | 1.81E-02  | Up       |
| BSU12050 | 125.11       | 367.154      | 1.55                              | 4.93E-04 | 1.98E-02  | Up       |
| BSU33950 | 56.006       | 161.841      | 1.53                              | 4.66E-04 | 1.95E-02  | Up       |
| BSU24580 | 6.11         | 18.56        | 1.59                              | 1.46E-03 | 4.45E-02  | Up       |
| BSU02360 | 243.519      | 638.924      | 1.39                              | 7.36E-04 | 2.77E-02  | Up       |
| BSU02350 | 68.903       | 174.218      | 1.34                              | 9.74E-04 | 3.42E-02  | Up       |
| BSU31050 | 64.173       | 161.213      | 1.33                              | 1.13E-03 | 3.79E-02  | Up       |
| BSU11220 | 71.588       | 176.107      | 1.3                               | 1.30E-03 | 4.18E-02  | Up       |
| BSU15510 | 1928.76      | 14.424       | -7.05                             | 1.22E-13 | 2.22E-10  | Down     |
| BSU15520 | 2240.655     | 18.756       | -6.89                             | 1.97E-13 | 2.40E-10  | Down     |
| BSU15500 | 1042.241     | 8.719        | -6.88                             | 5.66E-13 | 4.13E-10  | Down     |
| BSU15530 | 2030.044     | 18.342       | -6.78                             | 9.39E-13 | 5.71E-10  | Down     |
| BSU15550 | 1525.052     | 14.708       | -6.69                             | 2.47E-12 | 9.01E-10  | Down     |
| BSU15560 | 2006.169     | 21.44        | -6.54                             | 4.81E-12 | 1.46E-09  | Down     |
| BSU11990 | 94,545.251   | 1019.604     | -6.53                             | 2.08E-12 | 9.01E-10  | Down     |
| BSU15540 | 1912.399     | 23.571       | -6.34                             | 1.31E-11 | 3.69E-09  | Down     |
| BSU15490 | 268.461      | 3.331        | -6.29                             | 2.98E-10 | 7.78E-08  | Down     |
| BSU37350 | 2,666,228.43 | 69,847.529   | -5.25                             | 8.15E-09 | 1.65E-06  | Down     |
| BSU01620 | 379.143      | 10.008       | -5.23                             | 2.88E-08 | 5.01E-06  | Down     |
| BSU37770 | 3233.527     | 117.411      | -4.78                             | 1.57E-07 | 2.20E-05  | Down     |
| BSU01600 | 79.028       | 3.085        | -4.63                             | 1.29E-05 | 1.02E-03  | Down     |
| BSU40330 | 4924.789     | 203.326      | -4.6                              | 4.62E-07 | 6.02E-05  | Down     |
| BSU37760 | 2661.551     | 110.143      | -4.59                             | 4.99E-07 | 6.28E-05  | Down     |
| BSU01630 | 639.373      | 31.384       | -4.34                             | 2.85E-06 | 3.06E-04  | Down     |
| BSU40340 | 9319.344     | 465.809      | -4.32                             | 2.35E-06 | 2.60E-04  | Down     |
| BSU18310 | 796.701      | 42.378       | -4.23                             | 3.91E-06 | 3.97E-04  | Down     |
| BSU38560 | 1224.887     | 65.094       | -4.23                             | 4.36E-06 | 4.23E-04  | Down     |
| BSU40320 | 6670.251     | 373.473      | -4.16                             | 6.14E-06 | 5.47E-04  | Down     |
| BSU18300 | 795.359      | 44.677       | -4.15                             | 6.46E-06 | 5.61E-04  | Down     |
| BSU15480 | 415.79       | 23.288       | -4.15                             | 8.63E-06 | 7.32E-04  | Down     |
| BSU30560 | 3284.05      | 218.997      | -3.91                             | 2.51E-05 | 1.83E-03  | Down     |
| BSU37260 | 331.894      | 22.608       | -3.87                             | 7.70E-05 | 4.68E-03  | Down     |
| BSU37250 | 732.741      | 53.182       | -3.78                             | 6.80E-05 | 4.43E-03  | Down     |
| BSU37780 | 9186.092     | 681.619      | -3.75                             | 5.73E-05 | 3.94E-03  | Down     |
| BSU38570 | 655.683      | 48.934       | -3.74                             | 1.60E-04 | 9.18E-03  | Down     |
| BSU33240 | 100.946      | 7.784        | -3.68                             | 2.00E-04 | 1.07E-02  | Down     |
| BSU34370 | 102.087      | 7.975        | -3.66                             | 3.79E-04 | 1.70E-02  | Down     |
| BSU01610 | 274.333      | 22.027       | -3.63                             | 1.70E-04 | 9.52E-03  | Down     |
| BSU37790 | 2151.666     | 174.823      | -3.62                             | 1.20E-04 | 7.04E-03  | Down     |
the expression of genes related to various pathways differed in BJ3-2 cultured at 37 °C and 45 °C.

**The expression of rocF in BJ3-2 incubated at 37 °C and 45 °C**

Through the analysis of RNA-seq data from BJ3-2 incubated at different temperatures, we observed that several genes were involved in arginine and proline metabolism (Table 4), which are closely associated with the products of TTMP in *B. subtilis* [21], thus suggesting that these genes may affect the products of TTMP. Among them, the expression of three genes (*fadM*, *argF*, and *argD*) was up-regulated at least 1 ~ fold (log2FC), and five genes (*rocD*, *rocF*, *sped*, *rocG*, and *rocA*) was down-regulated at least 3 ~ fold (log2FC) after incubation at 45 °C. Hampel et al. suggested that the knockdown of *rocD* affects the normal growth of microorganisms [25]. Interestingly, the expression of *rocF* was down-regulated by 4.16 ~ fold (log2FC) after incubation at 45 °C (Table 4). Therefore, we selected *rocF* for further investigation and its expression in BJ3-2 incubated at different temperatures by RT-qPCR. The results showed that the dissolution curve of *rocF* was a single peak, and the amplification curve had a high degree of coincidence (Additional file 1: Fig. S3). The expression of *rocF* was low at 45 °C and high at 37 °C. Notably, the RT-qPCR results were consistent with the RNA-seq data (Fig. 2).

### Table 3 (continued)

| Gene ID | BJ3–2 37 °C | BJ3–2 45 °C | log2FC (BJ3–2 45 °C/BJ3–2 37 °C) | P-value | FDR | Regulate |
|---------|-------------|-------------|---------------------------------|---------|-----|---------|
| BSU14700 | 3806.507    | 323.198     | −3.56                           | 1.61E-04| 9.18E-03| Down    |
| BSU38580 | 682.143     | 57.556      | −3.56                           | 1.72E-04| 9.52E-03| Down    |
| BSU33430 | 364.978     | 31.714      | −3.52                           | 2.10E-04| 1.13E-02| Down    |
| BSU29020 | 7948.458    | 696.634     | −3.51                           | 2.22E-04| 1.13E-02| Down    |
| BSU32130 | 1442.649    | 126.693     | −3.51                           | 2.12E-04| 1.11E-02| Down    |
| BSU18340 | 521.503     | 45.961      | −3.5                          | 2.12E-04| 1.11E-02| Down    |
| BSU29010 | 9626.346    | 860.824     | −3.48                           | 2.55E-04| 1.22E-02| Down    |
| BSU37420 | 432.702     | 39.14       | −3.46                           | 3.05E-04| 1.43E-02| Down    |
| BSU26900 | 2580.202    | 235.026     | −3.46                           | 3.82E-04| 1.70E-02| Down    |
| BSU34360 | 506.423     | 46.282      | −3.45                           | 3.92E-04| 1.70E-02| Down    |
| BSU18320 | 483.601     | 44.598      | −3.44                           | 2.97E-04| 1.41E-02| Down    |
| BSU39380 | 2061.209    | 196.04      | −3.39                           | 3.90E-04| 1.70E-02| Down    |
| BSU33440 | 188.58      | 18.049      | −3.38                           | 5.00E-04| 1.98E-02| Down    |
| BSU06530 | 1375.189    | 132.495     | −3.37                           | 4.26E-04| 1.81E-02| Down    |
| BSU06520 | 1500.562    | 147.454     | −3.35                           | 4.81E-04| 1.97E-02| Down    |
| BSU05370 | 35.066      | 3.369       | −3.34                           | 1.53E-03| 4.61E-02| Down    |
| BSU39370 | 2356.907    | 237.199     | −3.31                           | 5.63E-04| 2.21E-02| Down    |
| BSU06460 | 851.54      | 86.678      | −3.29                           | 1.44E-03| 4.43E-02| Down    |
| BSU37370 | 5365.257    | 567.502     | −3.24                           | 7.88E-04| 2.86E-02| Down    |
| BSU18330 | 185.255     | 19.877      | −3.21                           | 9.00E-04| 3.22E-02| Down    |
| BSU06590 | 161.895     | 17.866      | −3.17                           | 1.36E-03| 4.23E-02| Down    |
| BSU26820 | 20,896.556  | 2374.192    | −3.14                           | 1.30E-03| 4.18E-02| Down    |
| BSU13190 | 1899.828    | 215.47      | −3.14                           | 1.32E-03| 4.18E-02| Down    |
| BSU06510 | 1424.683    | 164.735     | −3.11                           | 1.61E-03| 4.76E-02| Down    |
| BSU37280 | 167.252     | 19.356      | −3.1                             | 1.60E-03| 4.76E-02| Down    |

### Table 4 The DEGs in arginine and proline metabolism

| Gene   | Enzyme                        | log2FC (BJ3–2 45 °C/BJ3–2 37 °C) | P-value   |
|--------|-------------------------------|----------------------------------|-----------|
| fadM   | Proline dehydrogenase         | 2.12                             | 1.31E-03  |
| argF   | Ornithine carbamoyltransferase| 1.94                             | 7.38E-05  |
| argD   | Acetylornithine aminotransferase| 1.3                            | 1.30E-03  |
| rocD   | Ornithine aminotransferase    | −4.32                           | 2.35E-06  |
| rocE   | Arginase                      | −4.16                           | 6.14E-06  |
| sped   | 5-adenosylmethionine decarboxylase| −3.48          | 2.55E-04  |
| rocG   | Glutamate dehydrogenase       | −3.62                           | 1.20E-04  |
| rocA   | 1-pyrroline-5-carboxylate dehydrogenase| −3.75          | 5.73E-05  |
Generation of the rocF knockout strain
To reveal the role of the rocF gene, the knockout vector pUC18-HLarm-cm-HRarm of rocF was constructed and transformed into B. subtilis BJ3-2 (Additional file 1: Fig. S4). The positive clones were verified by PCR (Additional file 1: Fig. S4E). Subsequently, the positive clones were sequenced, and the results indicated that the rocF were successfully replaced by a cm fragment in the pUC18-HLarm-cm-HRarm vector (Additional file 1: Fig. S5). These results demonstrated that rocF of B. subtilis BJ3-2 was successfully knocked out and denoted by BJ3-2ΔrocF.

Characterization of the BJ3-2ΔrocF strain
To investigate the growth ability of BJ3-2ΔrocF, the growth characteristics of BJ3-2ΔrocF were assessed. As expected, the color and shape (rough surface, irregular shape, and surrounding folds) of BJ3-2ΔrocF on the plate were similar to BJ3-2 (Fig. 3A, B). In addition, the microscopic morphology of BJ3-2ΔrocF was short and rod-shaped, similar to BJ3-2. Moreover, BJ3-2ΔrocF was also purple, similar to BJ3-2, as shown by Gram staining (Fig. 3C, D). The results indicated no significant differences in the morphology and color by Gram staining were observed between BJ3-2ΔrocF and BJ3-2. Correspondingly, almost the same results were observed for the growth curves of BJ3-2ΔrocF and BJ3-2 (Fig. 3E). However, the arginase activity was significantly inhibited in BJ3-2ΔrocF compared to BJ3-2 (Fig. 4). These results suggest that knockout of rocF only contributes to the inhibition of the arginase activity, but not change the growth rate of BJ3-2.

Sensory evaluation of fermented soybeans
To assess the fermentation characteristics of BJ3-2ΔrocF in soybeans, the BJ3-2ΔrocF and BJ3-2 strains were
inoculated with soybeans at 45 °C for 72 h. The sensory evaluation of the fermented soybeans was performed by well-trained panelists on five components that included two appearance components (color and stickiness), two aroma components (ammonia and soy sauce-like aroma), and texture, with the individual scores added together to provide a total score (Additional file 2: Table S5). According to the sensory evaluation, it was found that the soy sauce-like aroma of fermented soybeans with BJ3-2ΔrocF was prominent, whereas the ammonia was decreased (Additional file 2: Table S5). The degree of browning and viscosity were decreased in BJ3-2ΔrocF and BJ3-2ΔrocF+Arg (Fig. 5A, B) (Additional file 1: Fig. S6). Moreover, the pH of the fermented soybeans with BJ3-2ΔrocF was significantly lower than that of the fermented soybeans with BJ3-2 (Fig. 5C), indicating that knockout of rocF induced changes in the pH of the fermentation environment. In addition, the OD600 values of soybean slurries fermented with BJ3-2 and BJ3-2ΔrocF were 0.518 and 0.501, respectively (Fig. 5D), suggesting that the OD600 value of the soybean slurry fermented with BJ3-2ΔrocF was significantly reduced compared to that of BJ3-2.

**rocF decreased the production of ammonia**

Following the sensory evaluation, we found that the ammonia of fermented soybeans with BJ3-2ΔrocF was decreased. Therefore, we further detected the ammonia content of the fermented soybeans by a gas detector. The results showed that the ammonia content of fermented soybeans with BJ3-2ΔrocF was 137.7 ppm, while the value was 241.6 ppm in BJ3-2, which was decreased by 43% (Table 5). The results indicated that the rocF gene affects the products of ammonia.

**rocF enhanced the contents of arginine, NH₄⁺, and TTMP in fermented soybeans**

Indeed, arginine was converted into urea and ornithine through the urea cycle [26]. Therefore, the arginine content was measured by HPLC methodologies. The arginine content of fermented soybeans with BJ3-2ΔrocF was 0.590 mg/mL, which was dramatically increased compared to those fermented with BJ3-2 (Table 5). Subsequently, the content of NH₄⁺ was detected by ion chromatography. A significant increase was obtained in fermented soybeans with BJ3-2ΔrocF (0.410 mg/kg) compared to those fermented with BJ3-2 (0.297 mg/kg) (Table 5). In addition, we also observed that the content of TTMP was increased by 8.6% in the fermented soybeans with BJ3-2ΔrocF (0.4617 mg/g) compared to those fermented with BJ3-2 (0.425 mg/g) (Table 6). Furthermore, a significantly increased content of TTMP in the BJ3-2ΔrocF treated with arginine were observed (Table 6), which indicated that rocF affects TTMP.
metabolic flow through the arginine pathway. However, the knockout of rocF resulted in slightly higher accumulations of the acetoin (Table 6). To sum up, these results indicated that the rocF gene affected the products of TTMP.

**Discussion**

*B. subtilis* is an aerobic, Gram-positive soil bacterium widely used in the food industry [6, 27, 28]. *B. subtilis* BJ3-2, used in this study, was isolated from fermented soybeans [29]. Fermented broth with BJ3-2 exhibited a prominent soy sauce-like aroma at 45 °C (Table 1). Furthermore, RNA-seq was performed on BJ3-2 (at 37 °C and 45 °C). The dominant DEGs were enriched in pyrimidine metabolism and arginine and proline metabolism (Additional file 2: Table S4). Most DEGs, including carA, pyrAa, pyrC, pyrDI, pyrDII, carB (BSU11240), pyrE, carB (BSU15520), pyrF, and pyrB, were involved in pyrimidine metabolism. The deficiency of each gene affects pyrimidine biosynthesis and ultimately leads to pyrimidine-deficient strains [30–32]. Moreover, eight DEGs (fadM, argF, argD, rocD, rocF, sped, rocG, and rocA) were enriched in arginine and proline metabolism (Table 4). Arginine metabolism is closely related to the products of TTMP, which contributes to the soy sauce-like aroma [33]. Among them, the expression of three genes (fadM, argF, and argD) was up-regulated at least 1 -fold (log2FC), and five genes (rocD, rocF, sped, rocG, and rocA) were down-regulated at least 3 -fold (log2FC) after incubation at 45 °C. The rocD was the most significantly down-regulated gene (4.32 -fold), while the knockdown of rocD resulted in arginine-deficient strains [25]. Interestingly, the rocF of BJ3-2 was down-regulated 4.16 -fold at 45 °C compared to 37 °C (Table 4), indicating its potential function in producing the soy sauce-like aroma.

Recently, studies have found that the deletion of key genes in the microorganisms may harm strain growth. For example, the knockout of the *surfactin synthetase* (srf) gene seems not to be favorable for strain growth in *B. subtilis* PB2-L [34]. However, in this study, the growth of the BJ3-2ΔrocF strain showed no significant changes in color and morphology, and growth curves compared to BJ3-2 (Fig. 3), suggesting the knockout of the rocF gene does not affect the normal growth of BJ3-2. However, the

### Table 5  Quantitative detection of arginine, NH$_4^+$, and ammonia

| Strain   | Arginine content | NH$_4^+$ content | Ammonia content |
|----------|------------------|------------------|-----------------|
|          | Concentration (mg/mL) AVG ± SD | Concentration (mg/kg) AVG ± SD | Concentration (ppm) AVG ± SD |
| BJ3-2    | 0.552 ± 0.038    | 0.288 ± 0.0074  | 245.8 ± 12.1    |
|          | 0.502 ± 0.0074  | 0.306 ± 0.0074  | 225.1 ± 12.1    |
| BJ3-2ΔrocF | 0.599 ± 0.008  | 0.396 ± 0.0104* | 135.1 ± 14.4*   |
|          | 0.579 ± 0.0074  | 0.413 ± 0.0074  | 156.4 ± 12.1    |
|          | 0.593 ± 0.0074  | 0.421 ± 0.0074  | 121.5 ± 12.1    |

* Significantly different at $P < 0.05$

### Table 6  Quantitative detection of acetoin and tetramethylpyrazine

| Strain     | Acetoin content | Tetramethylpyrazine content |
|------------|-----------------|-----------------------------|
|            | Concentration (mg/g) AVG ± SD | Concentration (mg/g) AVG ± SD |
| BJ3-2      | 55.793 ± 0.702  | 0.418 ± 0.0046              |
|            | 55.695 ± 0.702  | 0.432 ± 0.0046              |
|            | 54.76 ± 0.702   | 0.425 ± 0.0046              |
| BJ3-2ΔrocF | 55.793 ± 0.061  | 0.446 ± 0.0104*             |
|            | 55.968 ± 0.061  | 0.477 ± 0.0104*             |
|            | 55.891 ± 0.061  | 0.462 ± 0.0104*             |
| BJ3-2ΔrocF + Arg | 65.631 ± 10.493 | 0.492 ± 0.0177*             |
|            | 57.571 ± 10.493 | 0.488 ± 0.0177*             |
|            | 57.953 ± 10.493 | 0.530 ± 0.0177*             |

* Significantly different at $P < 0.05$
arginase activity was significantly inhibited in BJ3-2ΔrocF (Fig. 4), suggesting knockout of rocF only contributes to the inhibition of the arginase activity, but not change the growth characteristics of BJ3-2. Moreover, nonenzymatic browning is important in evaluating the Maillard reaction products (MRPs) [35, 36], which is affected by the reaction time, temperature, pH, solvent, and other conditions [37–40]. In the final stage of the Maillard reaction, the browning intensity is enhanced by increasing the initial pH value and other conditions [41]. In our study, the fermented soybeans with BJ3-2ΔrocF exhibited a lighter color and a slightly decreased pH (Fig. 5). These results are consistent with a number of previous studies on the Maillard reaction.

Zhao et al. reported a strong relationship between the amino acids and pyrazines [23]. In our study, the arginine content in fermented soybeans with BJ3-2ΔrocF was dramatically increased compared with BJ3-2 (Table 5), indicating the rocF gene affects arginine metabolism. In addition, we found that deletion of rocF gene changes the expression of arginine and proline metabolic pathway genes (Additional file 2: Table S4). Therefore, we speculated that rocF participates in the arginine and proline metabolic pathway and may convert arginine into urea and ornithine in the urea cycle (Fig. 6). Moreover, Rajini et al. reported that the production of TTMP is closely associated with amino acid metabolism and glycolytic pathways [12]. The amino acid metabolic pathway mainly provides a nitrogen source (NH$_3$ or NH$_4^+$), and the glycolytic pathway mainly provides the precursor compound (acetoin) for the production of TTMP [21]. In our study, the knockout of the rocF gene dramatically increased the contents of NH$_4^+$ and TTMP in fermented soybeans (Table 5, 6). Furthermore, a significantly increasing of TTMP content was also observed in the BJ3-2ΔrocF treated with arginine (Table 6). Additionally, we detected the ammonia of the fermented soybeans, and the results showed that the ammonia content was significantly reduced in BJ3-2ΔrocF, while one unexpected finding was the extent to which the soy sauce-like aroma was prominent (Additional file 2: Table S5). Thus, it is suggested that arginine could not be decomposed into urea and ornithine through the urea cycle in BJ3-2ΔrocF, which causes a reduction in the urea content. In accordance with the present results, previous studies have demonstrated that urea was degraded to ammonia by urease [42]. Collectively, we proposed a model to elucidate the potential function of rocF in changed arginine metabolic flux to enhance the TTMP yield (Fig. 6).

![Fig. 6](image.jpg)

**Fig. 6** The metabolic pathways of arginine and TTMP.
Conclusion
In this study, a comparative metabolite and transcription profiling of BJ3-2 strain at 37 °C and 45 °C showed transcriptional changes in arginine and proline metabolism, which are closely associated with the products of TTMP in *B. subtilis*. In addition, the function of *rocF* gene was further studied, and *rocF* knockout mutant (BJ3-2△rocF) was generated using homologous recombination. The results indicated that the knockout of *rocF* affects the contents of arginine, ammonia, NH$_4^+$, and TTMP in fermented soybeans. Therefore, our data provide new light into the understanding of the production of TTMP in *B. subtilis*.

Methods
Strains and vectors
The strain (*B. subtilis* BJ3-2) was isolated from fermented soybeans (Patent No. 201110023795.4; https://www.patentstar.com.cn/) [29]. The genome sequences of *B. subtilis* BJ3-2 have been submitted to NCBI (GI: CP025941). *Escherichia coli* DH5α and pUC18 vectors were purchased from TaKaRa (Dalian, China). The pH701cas9-p43 vector was acquired from Biomics Biotech (Jiangsu, China).

The collection of *B. subtilis* BJ3-2
A *B. subtilis* BJ3-2 single colony was cultured in 5 mL of liquid Luria–Bertani (LB) medium (10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L NaCl) and grown at 37 °C for 12 h under shaking at 180 rpm. The above bacterial cultures (3 mL) were reinoculated in 300 mL of liquid LB medium (1%) and divided into two groups, one of which was incubated at 37 °C, and the other was incubated at 45 °C, for 12 h under shaking at 180 rpm. Then, the cultures of the exponential growth phase were collected in a 50 mL sterile centrifuge tube and centrifuged at 5,000 g for 8 min. The sedimentation for each treatment was collected after discarding the supernatant, and the procedure was repeated three times.

Total RNA extraction
After the collection of BJ3-2, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) by following the manufacturer’s protocol. The concentration and purity of the total RNA were assessed using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The RNA integrity values (RIN) of the total RNA were evaluated using an agarose gel and an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA, USA).

RNA sequencing
The samples for each treatment were collected at least three times and pooled for RNA-seq analysis. Total RNA was extracted as described above. RNA-seq was performed by the Majorbio Technology Co., Ltd. (Shanghai, China). The 16S rRNA was used as an internal standard. Sequences of the raw data containing a small number of reads with sequencing adaptors or low-quality sequences were filtered. For accurate sequencing, we adopted a sequencing error rate distribution examination to establish the quality of sequencing data. Then, the differentially expressed genes (DEGs) were screened using the following conditions: $P$-value < 0.05 and $\log_2$ fold change (FC) ≥ 1. GO (Gene Ontology, http://www.geneontology.org/) enrichment analysis was performed to determine the DEGs that were common at 37 °C and 45 °C with opposite regulatory patterns with a threshold of $P$ < 0.05. Proteins were filtered based on their grouping into biological processes (BP), cellular components (CC), and molecular function (MF). KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) pathway enrichment analysis was performed for each DEG [43]. The raw reads and processed RNA-seq data in this work have been deposited in the NCBI Gene Expression Omnibus (GEO) database under record number GSE166082.

Reverse transcription-quantitative real-time PCR (RT-qPCR)
To detect *rocF* gene expression, the total RNA extracted above was used to synthesize the first chain cDNA using the StarScript II First-strand cDNA Synthesis Mix with gDNA Remover Kit (GenStar, Beijing) following the manufacturer’s recommendations. Then, the cDNA was diluted to 200 ng/µl, and RT-qPCR was performed using a CFX96 Touch PCR instrument (Bio-Rad, USA). The composition of the reaction mixture and conditions were the same as previously described in Zhang’s study [44]. Each RT-qPCR analysis experiment was performed three times, and the primers used in this study are listed in Table S1. 16S rRNA was used as a reference gene for expression analysis.

Homologous recombination knockout vector construction and transformation
The HLarm and HRarm correspond to the homologous left and right arms of *rocF*, whereas *cm* corresponds to the chloramphenicol gene. DEDP is a double-exchange detection primer (Additional file 2: Table S1) for assessing the success of the transformation. The HLarm and HRarm of *rocF* were amplified from the *B. subtilis* BJ3-2 genome. The *cm* was amplified from the pH701cas9-p43 vector. The primers used for amplification are listed in Table S1. HLarm, *cm*, and HRarm were
double-digested with the restriction endonucleases Sac I and BamH I, BamH I and Xba I, and Xba I and Hind III, respectively. The digested fragments of HLarm, cm, and Hrarm were sequentially connected to the pUC18 vector. Finally, the homologous recombination knockout vector pUC18-HLarm-cm-Hrarm was constructed. The recombinant plasmid was then transformed to B. subtilis BJ3-2 according to the description in the literature [45, 46]. The transformants were verified by PCR using DEDP primers (Additional file 2: Table S2). The sensory analy-
were verified by PCR using DEDP primers (Additional file 2: Table S3).

Bacterial DNA was extracted using the Bacterial DNA Kit (OMEGA, USA) for further validation. The genomic DNA of the transformants was extracted using the Bacterial DNA Kit (OMEGA, USA) following the manufacturer’s recommendations and sequenced by the Sangon Biotech Co., Ltd. (Shanghai, China).

Enzyme determination assays
For the arginase determination assays, 0.1 g of BJ3-2 and BJ3-2ΔrocF bacteria were collected into a 1.5 mL centrifuge tube, sterile PBS (pH = 7.2) was added and broken by ultrasound in an ice bath and subjected to centrifugation (8,000 g for 5 min) to obtain the supernatant. The supernatant was transferred to a new tube used for the further assay. The arginase activity in BJ3-2 and BJ3-2ΔrocF was analyzed using the MEIMIAN reagent kit (Shanghai, China) and MULTISKAN GO (Thermo Fisher Scientific, Wilmington, DE, USA) according to the manufacturer’s instructions. A series of 2.5U/L, 5 U/L, 10 U/L, 20 U/L and 40 U/L concentrations were used to generate a standard curve. Three independent experiments were performed for each sample. The assays were done at least in triplicate.

Fermentation experiments
BJ3-2ΔrocF and BJ3-2 were inoculated in 5 mL of liquid LB medium and subsequently incubated at 37 °C with shaking at 180 rpm for 12 h. The soybeans were autoclaved at 121 °C for 20 min. The above bacterial suspensions (OD600 = 0.465) of BJ3-2ΔrocF and BJ3-2 were inoculated in autoclaved soybeans (1%, v/m) and fermented at 45 °C for 72 h.

Sensory evaluation
To assess the fermentation characteristics, the BJ3-2 strain was inoculated in a liquid LB medium and incubated at 37 °C and 45 °C for 72 h. The sensory analysis of the fermented broth was evaluated by 10 trained expert sensory panelists. The sensory score was a total of 3 indicators, including soy sauce-like aroma, Chi-flavour and ammonia. The sensory analysis of fermented soybeans by different strains was evaluated by 10 trained expert sensory panelists. The sensory score was a total of 5 indicators, including color, stickiness, ammonia, soy sauce-like aroma and texture (Additional file 2: Table S3).

pH measurement
The pH test was performed according to the Chinese national standard (GB5009.237–2016). Briefly, slurry was created from the fermented soybeans (10 g) using ultrapure water (50 mL), and the pH of the slurry was measured using a standard laboratory pH meter (pH5-3C). Experiments were performed in triplicate.

OD600 detection for the color of fermented soybeans
Fermented soybeans (20 g) were weighed and placed into a beaker with 100 mL of ultrapure water. The viscoid was cleaned, and the excess water of the soybeans was absorbed. Five grams of cleaned soybeans were placed in a mortar and ground into the homogenate with a pestle. Then, 50 mL of ultrapure water was added and mixed in the above homogenate. The mixture was well stirred and centrifuged at 8,000 g for 10 min. The absorbance of the supernatant fluid was measured at OD600. Experiments were conducted in three times.

Ammonia contents of fermented soybeans
The ammonia content of the fermented soybeans was detected by a gas detector (WOST Co., Ltd., Shenzhen, China) following the manufacturer’s recommendations.

Acetoin and TTMP contents of fermented soybeans
The concentrations of acetoin and TTMP were analyzed by HPLC. In brief, 10 g fermented soybean with BJ3-2, BJ3-2ΔrocF and BJ3-2ΔrocF + Arg (treated with 0.05% arginine) were dissolved in 30 mL 60% ethanol with 0.1 g CaCl₂ and sonicated for 30 min at 25 °C (100 W, 20 kHz), and then centrifuged at 9000 rpm/min for 10 min at 4 °C. The supernatant was filtered by a 0.22 μm membrane before injection. The analysis was performed using Agilent LC1260 liquid chromatography (LC) system (California, USA), which was equipped with a flame ionization detector, a capillary column of Shim-pack GIST C18-AQ 5 μm (4.6 I.D. × 250 mm, SHIMADZU, Japan). Water with trifluoroacetate concentration of 0.05% was mixed with methanol in a ratio of 7:3 (v/v) and used as the mobile phase at a flow rate of 0.7 mL/min. The column oven was kept at 30 °C for 5 min, then programmed to 220 °C with a stepwise increase of 30 °C /min and maintained at 220 °C for 3 min. The injection volume was 5 μL.

Arginine content of fermented broth
BJ3-2ΔrocF and BJ3-2 were inoculated in liquid LB medium (1% v/v) and fermented at 45 °C for 72 h. Subsequently, 2 mL of fermented broth was collected and centrifuged at 10,000 g for 2 min. Then, the supernatant was collected and stored at 4 °C. The arginine content was analyzed using a Waters 1525 high-performance
liquid chromatography (HPLC) system equipped with an automatic injector and an ultraviolet detector (UV) at 210 nm (Waters Corp., Milford, USA), according to the description in the literature [47]. Briefly, all fermented samples were treated with potassium ferrocyanide (106 g/L) and zinc sulfate (300 g/L) and then centrifuged at 2,000 g for 8 min. Next, the supernatant was filtered through a 0.22 µm filter membrane prior to injection for HPLC analysis. Each injection volume was set to 10 µl. Chromatographic separation was achieved using a Waters Atlantis C18 column (5 µm, 4.6 × 150 mm) (Waters, USA) at 30 °C. The mobile phase of HPLC was 20 mmol/L NaH2PO4, and the flow rate was 1.0 mL/min. A calibration curve was established to quantify arginine. The experiment was performed in three biological replicates.

NH4+ contents of fermented soybeans
Fermented soybeans (20 mg) were ground into homogenate using a mortar and pestle and dissolved in 30 mL of methanesulfonic acid (MSA) (20 mM). Then, the above solution was extracted with ultrasonication for 20 min and repeated three times. All suspensions were collected and diluted to 100 mL. The mixture was filtered with a 0.45 µm filter membrane. The NH4+ content was determined with ion chromatography according to the description in the literature [48]. Briefly, the samples were detected with an LC2010PLUS (Shimadzu, Japan) equipped with an EGC eluent generator and a DS6 conductivity detector. The chromatographic system was an IonPac CS11-HC column (4.0 × 150 mm) (Waters, USA) at 30 °C. The mobile phase of HPLC was 20 mmol/L NaH2PO4, and the flow rate was 1.0 mL/min. A calibration curve was established to quantify arginine. The experiment was performed in triplicate.

Abbreviations
TTMP: Tetramethylpyrazine.; SSF: Solid-state fermentation.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12896-022-00748-4.

Additional file 1. Figure S1. Data quality control; Figure S2. Expression density distribution; Figure S3. RT-qPCR of rocF; Figure S4. Construction of the homologous recombination knockout vector; Figure S5. Sequencing of BJ3-2ΔrocF; Figure S6. Fermented soybeans with BJ3-2, BJ3-2ΔrocF and BJ3-2ΔrocF + Arg at 45 °C

Additional file 2. Table S1. Primers used in the study; Table S2. Scoring standard for sensory evaluation of BJ3-2 at 37 °C and 45 °C; Table S3. Scoring standard for sensory evaluation of fermented soybeans by different strains. Table S4. The analysis of KEGG pathway enrichment; Table S5. Sensory evaluation of fermented soybeans by different strains.

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Author contributions
LZL and WYJ conceptualized and designed the study, drafted the initial manuscript, and reviewed and revised the manuscript. ZLC, TSQ, JJ, GX and ZJ designed the data collection instruments, collected data, carried out and initial analyses, and reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets supporting the conclusions of this article are included within the article.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication
The authors provide consent for publication.

Competing interests
The authors declare no competing interests.

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References
1. Xiao ZJ, Xie NZ, Liu PH, Hua DL, Xu P. Tetramethylpyrazine production from glucose by a newly isolated Bacillus mutant. Appl Microbiol Biotechnol. 2006. https://doi.org/10.1007/s00253-006-0491-6.
2. Müller R, Rappert S. Pyrazines: occurrence, formation and biodegradation. Appl Microbiol Biotechnol. 2010. https://doi.org/10.1007/s00253-009-2362-4.
3. Fan WL, Yan X, Zhang YH. Characterization of pyrazines in some Chinese liquors and their approximate concentrations. J Agric Food Chem. 2007;55(24):9956–62. https://doi.org/10.1021/jf071357q.
4. Xiao ZB, Dai SP, Niu YW, Yu HY, Zhu JC, Tian HX, Gu YB. Discrimination of Chinese vinegars based on headspace Solid-Phase Microextraction-Gas chromatography mass spectrometry of volatile compounds and multivariate analysis. J Food Sci. 2011. https://doi.org/10.1111/j.1750-3841.
5. Meng W, Wang RM, Xiao DG. Metabolic engineering of Bacillus subtilis to enhance the production of tetramethylpyrazine. Biotechnol Lett. 2015. https://doi.org/10.1007/s10529-015-1950-x.
6. Xu Y, Jiang YF, Li XT, Sun BG, Teng C, Yang R, Xiong K, Fan GS, Wang WH. Systematic characterization of the metabolism of acetoin and its derivative ligustrazine in Bacillus subtilis under micro-Oxygen conditions. J Agric Food Chem. 2018. https://doi.org/10.1021/acs.jafc.8b00113.
7. Meng W, Xiao DG, Wang RM. Enhanced production of tetramethylpyrazine in Bacillus licheniformis BL1 by bdA disruption and 2,3-butanediol supplementation. World J Microbiol Biotechnol. 2016. https://doi.org/10.1007/s11274-015-1992-1.
8. Wang YF, Zhang XJ, Xu CJ, Zhang GX. Synthesis and biological evaluation of danchensu and tetramethylpyrazine conjugates as cardioprotective agents. Chem Pharm Bull (Tokyo). 2017. https://doi.org/10.1248/cpb.c16-00389.
9. Fadel HHM, Lotfy SN, Askar MMS, Mahmoud MG, Al-Okbi Y. Nutty-like flavor production by Corynbacterium glutamicum1220T from enzymatic soybean hydrolysate. Effect of encapsulation and storage on the nutty
flavoring quality. J Adv Res. 2018. https://doi.org/10.1016/j.jare.2018.01.003.

30. Ralli P, Srivastava AC, O’Donovan G. Regulation of the pyrimidine biosynthetic pathway in a pyrD knockout mutant of Pseudomonas aeruginosa. J Basic Microbiol. 2010. https://doi.org/10.1002/jobm.200610128.

31. Butcher BG, Chakravarty S, D’Amico K, Stooos KB, Fililartaj MJ. Disruption of the genes in Pseudomonas syringae results in reduced fitness and alters motility. BMC Microbiol. 2016. https://doi.org/10.1186/s12866-016-0819-z.

32. Yang HJ, Bogomolovna Y, McClelland M, Andrews-Polynnis H. De novo pyrimidine synthesis is necessary for intestinal colonization of Salmonella Typhimurium in chicks. PLoS ONE. 2017. https://doi.org/10.1371/journal. pone.0183751.

33. Shen YF. Type of flavor of Baijiu. Nongjiu. 2003. CNKI:SUN:NJZZ.0.2003–01–000.

34. Liu HX, Qiu XX, Zhao SM, Lu ZX, Zhang C, Bie XM. Characterization of a Bacillus subtilis surfactin synthetase knockout and antimicrobial activity analysis. J Biotechnol. 2016. https://doi.org/10.1016/j.jbiotec.2016.08.018.

35. Jalbout AF, Shipar MAJ, Trzaskowski B, Adamowicz. Formation of pyrazines in hydroxyacetaldehyde and glucose nonenzymatic browning Maillard reaction: a computational study. Food Chem. 2007. https://doi.org/10.1016/j.foodchem.2006.07.061.

36. Li ZP, Ma DX, He YY, Guo SQ, Liu FG, Liu XB. Simultaneous ultrasound and heat enhance functional properties of glycosylated lactoferrin. Molecules. 2020. https://doi.org/10.3390/molecules25235774.

37. Scaman C, Nakai S, Amini M. Effect of pH, temperature and sodium bisulfite or cysteine on the level of Maillard-based conjugation of lysoyxime with dextran, galactomannan and mannan. Food Chem. 2006. https://doi.org/10.1016/j.foodchem.2005.08.003.

38. Benjakul S, Lerttitikul W, Buer F. Antioxidant activity of Maillard reaction products from a porcine plasma protein-sugar model system. Food Chem. 2005. https://doi.org/10.1016/j.foodchem.2004.10.019.

39. Chen SL, Jin SY, Chen CS. Relative reactivities of glucose and galactose in browning and pyruvaldehyde formation in sugar/glycine model systems. Food Chem. 2005. https://doi.org/10.1016/j.foodchem.2004.09.005.

40. Ramirez-Jimenez A, Garcia-Villanova B, Guerra-Hernandez E. Effect of toasting time on the browning of sliced bread. J Sci Food Agric. 2001. https://doi.org/10.1002/1097-0045(2001)18:2<647::AID-JSFA647>3.0.CO;2-K.

41. Liu PL, Lu XM, Li NY, Zheng ZJ, Qiao XG. Characterization, variables, and antioxidant activity of the Maillard reaction in a Fructose-Histidine model system. Molecules. 2018. https://doi.org/10.3390/molecules24010056.

42. Kanamori T, Kanou N, Atomi H, Imanaka T. Enzymatic characterization of a pyrD mutant and depends on Rv2323c for growth on arginine. PLoS ONE. 2016. https://doi.org/10.1371/journal.pone.0161929.

43. Kanehisa M, Furumichi M, Sato Y, Ishiguro-Watanabe M, Tanabe. KEGG: Integrating pathways and experimental results between yeast and bacteria. Database. 2013. https://doi.org/10.1093/database/bat012.

44. Zhang LC, Zhu MK, Ren LJ, Li AZ, Chen GP, Hu ZL. The carA gene controls pyrimidine synthesis is necessary for intestinal colonization of Pseudomonas aeruginosa. J Basic Microbiol. 2010. https://doi.org/10.1002/jobm.200610128.

45. Butcher BG, Chakravarty S, D’Amico K, Stooos KB, Fililartaj MJ. Disruption of the genes in Pseudomonas syringae results in reduced fitness and alters motility. BMC Microbiol. 2016. https://doi.org/10.1186/s12866-016-0819-z.

46. Yang HJ, Bogomolovna Y, McClelland M, Andrews-Polynnis H. De novo pyrimidine synthesis is necessary for intestinal colonization of Salmonella Typhimurium in chicks. PLoS ONE. 2017. https://doi.org/10.1371/journal. pone.0183751.

47. Shen YF. Type of flavor of Baijiu. Nongjiu. 2003. CNKI:SUN:NJZZ.0.2003–01–000.