Enhancing surfactin production by using systematic CRISPRi repression to screen amino acid biosynthesis genes in *Bacillus subtilis*

Congya Wang†, Yingxiu Cao†, Yongping Wang, Liming Sun and Hao Song*

**Abstract**

**Background:** Surfactin is a cyclic lipopeptide that is of great industrial use owing to its extraordinary surfactant power and antimicrobial, antiviral, and antitumor activities. Surfactin is synthesized by a condensation reaction in microbes, which uses fatty acids and four kinds of amino acids (l-glutamate, l-aspartate, l-leucine and l-valine) as precursors. Surfactin biosynthesis could be improved by increasing the supply of fatty acids; however, the effect of the regulation of amino acid metabolism on surfactin production was not yet clear.

**Results:** In this study, we aimed to improve surfactin production in *B. subtilis* by repressing the genes on the branch metabolic pathways of amino acid biosynthesis using CRISPRi technology. First, 20 genes were inhibited individually, resulting in 2.5- to 627-fold decreases in transcriptional level as determined by RT-qPCR. Among the 20 recombinant strains, 16 strains obtained higher surfactin titres than that produced by the parent BS168NU-Sd strain (the surfactin production of BS168NU-Sd with only dCas9 but no sgRNA expression was 0.17 g/L). In particular, the strains in which the *yrpC*, *racE* or *murC* genes were inhibited individually produced 0.54, 0.41, or 0.42 g/L surfactin, respectively. All three genes are related to the metabolism of l-glutamate, whose acylation is the first step in the surfactin condensation reaction. Furthermore, these three genes were repressed in combination, and the strain with co-inhibition of *yrpC* and *racE* produced 0.75 g/L surfactin, which was 4.69-fold higher than that of the parent strain. In addition, the inhibition of *bkdAA* and *bkdAB*, which are related to the metabolism of l-leucine and l-valine, not only improved surfactin production but also increased the proportion of the C14 isoform.

**Conclusions:** This study, to the best of our knowledge for the first time, systematically probed the regulatory effect of increasing the supply of amino acids on surfactin production. It provided an effective strategy and a new perspective for systematic studies on surfactin and other amino acid-derived chemicals.

**Keywords:** Surfactin, CRISPR interference, Amino acids, *Bacillus subtilis*
ligase [11]; the biosynthesis of four kinds of amino acids; and the assembly of surfactin, in which seven amino acids are assembled successively onto fatty acyl-CoA via surfactin synthase, which is encoded by the \textit{srfA} operon and regulated by cell density signal and phosphopantetheinyltransferase (PPTase) Sfp. Previous studies mainly focused on the third part, and surfactin biosynthesis was enhanced by increasing the expression level of \textit{srfA}. For example, Jiao et al. [8] and Sun et al. [12] enhanced surfactin production by replacing the original constitutive promoter of \textit{srfA} (\textit{PsrfA}) with stronger inducible Pg3 or Pspac promoters. Others achieved \textit{srfA} overexpression by upregulating the quorum sensing system \textit{ComQXPA} [11, 13–15] or downregulating the negative factors [13, 14, 16–19]. Dhali et al. [20]. enhanced the production to 1556 ± 123 mg of surfactin per g dry weight of cell biomass by knocking out the global regulation factor \textit{codY}, which had negative effects on \textit{srfA} expression. Regarding the precursor supply, recent research suggested that increasing the supply of fatty acid precursors could greatly enhance the surfactin titre to ≈ 4.9 g/L in a flask using modified synthetic medium by overexpressing the genes involved in the fatty acid biosynthesis pathway [17]. On the other hand, amino acids are also essential precursors for surfactin biosynthesis. Thus, increasing the amino acid supply might also have positive effects on surfactin production and could be achieved by decreasing the metabolism flux in the branch pathways for amino acid biosynthesis [21–23]. However, no such study had previously been performed.

Recent advances in the field of synthetic biology are expediting our ability to regulate metabolic pathways and enhance the synthesis of target chemicals. In particular, the advent of clustered regularly interspersed short palindromic repeat interference (CRISPRi), which requires only the dCas9 enzyme, which is defunct in endo-nucleolytic activity because of point mutations in the RuvC and HNH domains, and a custom single guide RNA (sgRNA) that conveys the DNA binding specificity to dCas9 [24], enabling the rapid repression of gene transcription. The CRISPRi system has been successfully used to improve the biosynthesis of target chemicals by decreasing the metabolic flux of branch pathways. Lv et al. [25] downregulated the expression of \textit{sad}, \textit{sucC}, \textit{sucD}, \textit{sdhA}, and \textit{sdhB} simultaneously via CRISPRi to regulate the carbon flux from succinate synthesis pathways to 4-hydroxybutyrate (4HB) biosynthesis and increased the 4HB content in the poly((3HB-co-4HB)) to 18.4 mol%. Wu et al. [23] silenced the expression of \textit{ppsA}, \textit{eno}, \textit{adhE}, \textit{mdh}, \textit{fumC}, \textit{sdhA}, \textit{sucC} and \textit{citE} simultaneously by CRISPRi and enhanced the malonyl-CoA concentration 2.3-fold. CRISPRi was also used to improve the production of polyhydroxyalkanoates [26], fatty alcohols [27], shikimic acid [28], O-methylated anthocyanin [29], and poly-β-hydroxybutyrate [30].

In this study, we tried to enhance surfactin production by increasing the supply of amino acids. First, we incorporated the surfactin biosynthesis pathway into \textit{BS168NU} by expressing the exogenous \textit{sfp} gene (\textit{BS168NU-S}). Second, we constructed an effective CRISPRi system, as demonstrated by transcriptional repression measured by RT-qPCR. Third, the expression of 20 genes, which were selected from the branch metabolic pathways of amino acids, was perturbed individually by CRISPRi. Among the 20 recombinant strains, 16 recombinant strains gained increased surfactin production. In particular, the strains in which \textit{yrpC}, \textit{racE} or \textit{murC} was inhibited enhanced surfactin production to 0.54, 0.41 or 0.42 g/L, respectively. Then, the three genes were further repressed in combination. The results indicated that the strains with co-inhibition of \textit{yrpC} and \textit{racE} obtained the highest production, which was directly related to l-glutamate metabolism, whose acylation was the first step of surfactin assembly. This work revealed that engineering the amino acid metabolism is an efficient strategy to enhance the production of surfactin.
### Materials and methods

#### Genes, strains and plasmids

The sfp gene derived from *Bacillus amyloliquefaciens* DSM7 was synthesized by Genewiz (Suzhou, China), and the sequence is shown in Additional file 1: Table S1. All the wild-type and recombinant bacterial strains are listed in Table 1, and all the plasmids used in this work are listed in Table 2. *E. coli* Trans T1 was purchased from TransGen Biotech (Beijing, China) and used for gene cloning and plasmid construction. BS168 NU, constructed by our laboratory, was the starting strain for the construction of the strains for surfactin production. The pJMP1 and pJMP2 plasmids were purchased from Beijing Zhongyuan Heju Economic and Trade Co., Ltd.

#### Media and bacterial growth

Luria–Bertani medium (LB, 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) was used for *E. coli* and *B. subtilis* growth, and solid agar was used for the growth of *E. coli* and *B. subtilis* colonies.

The following materials were used to obtain competent cells: 10× Spizizen (10× Spizizen: 20 g/L (NH4)2SO4, 183 g/L K2HPO4, 60 g/L KH2PO4, 12 g/L sodium citrate), GMI medium (5 mL of GMI: 500 μL of 10× Spizizen, 100 μL of 2% casein acids hydrolysate, 100 μL of 5% yeast, 100 μL of 40% glucose, 5 μL of 20% MgSO4·H2O, 50 μL of 0.5% l-tryptophan and 4.14 mL of H2O) and GMMII medium (5 mL of GMI: 500 μL of 10× Spizizen, 50 μL of 0.1% tryptophan and 4.31 mL of H2O).

The surfactin production fermentation medium of the *B. subtilis* strains comprised 70 g/L sucrose, 1 g/L yeast extract, 25 g/L NaNO3, 0.333 g/L KH2PO4, 1 g/L Na2HPO4·12H2O, 0.15 g/L MgSO4·7H2O, 7.5 mg/L CaCl2, 6 mg/L MnSO4·H2O, and 6 mg/L FeSO4·7H2O (pH 7.0). The seed culture solution of *B. subtilis* strains was

| Name | Genotype | Source/references |
|------|----------|-------------------|
| *E. coli* Trans T1 | F-q80(lacZ)ΔM15ΔlacX74hsdR(reC, mrm)ΔrecA1398endA1tonA | TransGen Biotech |
| *B. subtilis* 168 | trpC2, ΔaraR:Para-neo, Δωpp | Laboratory stock |
| BS168NU | trpC2, ΔaraR:Para-neo, Δωpp | Laboratory stock |
| MK3-MEP2-m | BS168NU, ΔydeO:PlapS-menA, ΔydeO::P43-dxr-DN*-cat-araR | Laboratory stock |
| BS168NU-S | BS168NU, ΔydeO::P43-sfp | This work |
| BS168NU-Sd | BS168NU-S derivative, ΔlacA-Pxyl-dCaS9 | This work |
| mmgA | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>mmgA</sup> | This work |
| yhfS | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>yhfS</sup> | This work |
| asnB | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>asnB</sup> | This work |
| asnH | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>asnH</sup> | This work |
| asnO | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>asnO</sup> | This work |
| nadB | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>nadB</sup> | This work |
| ykrD | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>ykrD</sup> | This work |
| racX | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>racX</sup> | This work |
| pyrB | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>pyrB</sup> | This work |
| pyrC | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>pyrC</sup> | This work |
| purF | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>purF</sup> | This work |
| glmA | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>glmA</sup> | This work |
| yprC | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>yprC</sup> | This work |
| racE | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>racE</sup> | This work |
| glmS | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>glmS</sup> | This work |
| ybcM | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>ybcM</sup> | This work |
| murD | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>murD</sup> | This work |
| murC | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>murC</sup> | This work |
| bkdAA | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>bkdAA</sup> | This work |
| bkdAB | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>bkdAB</sup> | This work |
| H1 | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>H1</sup>, Pveg-sgRNA<sup>loxP</sup>, Pveg-sgRNA<sup>loxP</sup>, Pveg-sgRNA<sup>loxP</sup> | This work |
| H2 | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>H2</sup>, Pveg-sgRNA<sup>loxP</sup>, Pveg-sgRNA<sup>loxP</sup>, Pveg-sgRNA<sup>loxP</sup> | This work |
| H3 | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>H3</sup>, Pveg-sgRNA<sup>loxP</sup>, Pveg-sgRNA<sup>loxP</sup>, Pveg-sgRNA<sup>loxP</sup> | This work |
| H4 | BS168NU-Sd derivative, ΔamyE::Pveg-sgRNA<sup>H4</sup>, Pveg-sgRNA<sup>loxP</sup>, Pveg-sgRNA<sup>loxP</sup>, Pveg-sgRNA<sup>loxP</sup> | This work |
grown for 12 h in LB medium. On the next day, 500 μL of seed culture solution was inoculated into a test tube containing 5 mL of fermentation medium with an appropriate concentration of xylose inducer. The cell densities and surfactin concentrations were measured simultaneously at the required time points. All strains were cultured at 37 °C with shaking at 220 rpm. Whenever required, antibiotics were added to the medium at the following concentrations: 100 μg/mL ampicillin for \textit{E. coli}; 6 μg/mL chloramphenicol, 10 μg/mL erythromycin and/or 15 μg/mL neomycin for \textit{B. subtilis}.

**Competent cells and translation**

Competent cells were obtained as described by Anagnostopoulos and Spizizen [31]. A single colony of the receptor strain was picked up in a test tube with 5 mL of GMI medium and cultured for 14–16 h at 37 °C with shaking at 200 rpm. Then, 500 μL of broth was transferred into another test tube containing 4.5 mL of GMI medium and cultured for 4.5 h at 37 °C with shaking at 200 rpm to achieve mid-late logarithmic growth of the bacteria. Then, 750 μL of culture was transferred into another test tube containing 4.25 mL of GMII medium and cultured for 1.5 h at 37 °C with shaking at 240 rpm to obtain competent cells.

If plasmids were added, they were mixed and placed 1 h at 37 °C statically. Then, the cells were cultured for 1–1.5 h at 37 °C with shaking 240 rpm. Then, the EP tube was centrifuged for 2 min at 13,000 rpm. After removing 900 μL of the supernatant, the cells were suspended in the remaining 100 μL of supernatant, and the transformants were coated on a LB solid plate for screening. After 12–18 h of incubation at 37 °C, single colonies were obtained for further screening of transformants.

**Marker-free gene modification**

The method of marker-free gene modification used here for \textit{sfp} gene expression was derived from Liu et al. [32]. In the chromosome BS168NU, the \textit{araR} locus was replaced by the counter-selective marker cassette (\textit{Para-neo}) through double crossover homologous recombination,
enabling the colonies of BS168NU to grow on an Nm-resistance plate. Then, the selective marker cassette (CR), which was constituted by the Cm-resistance gene (cat) and the araR gene, and the recombination fragment (upstream fragment U, downstream fragment G and homologous recombination fragment D) were integrated into the target locus in the order UDCRG. The expression of the Nm-resistance gene (neo) was repressed by the transcriptional repressor AraR, so the colonies were selected on a Cm-resistant plate. Finally, the selective marker cassette was evicted by single-crossover with homologous recombination fragment D, and the colonies were selected on an Nm-resistance plate.

**sgRNA design [33]**

The first CCN was identified as the protospacer adjacent motif (PAM) sequence in the non-template strand, and the following 12 bp were regarded as the seed region. To avoid off-target effects, we searched the genome of *B. subtilis* 168 for the 15 bp specificity region consisting of the 12 bp 'seed' region of the sgRNA and 3 bp (CCN) PAM in the genome to rule out additional potential binding sites. If other nonspecific targets were found, the next CCN was selected as the PAM sequence, and the previous step was repeated. Then, the 20 bp after the PAM sequence were regarded as the sgRNA base-pairing region. The secondary structure of the RNA derived from the sgRNA base-pairing region and the dCas9 handle region was predicted using the UNAFold web server. If the RNA was predicted to form the correct hairpin structures, the sgRNA base-pairing region included in the corresponding primer would be synthesized, and the primer was named xxx-F, in which xxx was the name of the matching gene. If the correct hairpin structures were not formed or the strains could not grow, the next CCN was selected as the PAM sequence, and the previous steps were repeated until an appropriate sgRNA was found. The sequence of the dCas9 handle region is GTTTTA GAGCTAGAAATAGCAAGTTAAAAATACGGCTAGT CCG. All primers used in this work were synthesized by Genewiz (Suzhou, China) and are listed in Additional file 2: Table S2.

**Plasmid construction**
The single gene inhibition plasmids and the multiple gene expression plasmids were constructed from the plasmid pJMP2. For the single gene interference plasmids, the different sgRNA fragments were amplified from pJMP2 by corresponding primers, digested with Acc65I and Pmel, and then ligated into the pJMP2 backbone that was digested with BsrGI and Pmel. For the multiple gene interference plasmids, the different sgRNA fragments were amplified from pJMP2 by the corresponding primers and assembled by the BM Seamless Cloning Kit, which was purchased from Biomed (Beijing, China), as shown in Fig. 5a.

**RT-qPCR assay**
The total RNAs of different strains were extracted from the 24 h fermentation broth using the RNAprep Pure Cell/Bacteria Kit. Reverse transcription was performed with the total RNAs as the templates using the FastQuant RT Kit (with gDNase). The transcription levels of genes were detected by quantitative real-time PCR (qRT-PCR) with a Roche LightCycler 480. The relative transcription level of the target gene was quantified by the 2−ΔΔCT method using the ccpA gene as the internal control and BS168NU-Sd as the calibrator. Each qPCR run was performed in a 20 μL volume containing 10 μL of 2× SuperReal PreMix Plus, 0.6 μL of each primer, 1 μL of template cDNA and 8.2 μL of RNase-free H2O. The amplification conditions were as follows: preheating at 95 °C for 5 min followed by 40 cycles of 95 °C for 5 s, 55 °C for 20 s, and 72 °C for 20 s. Three technical replicates were carried out for each target gene. All reagents for qPCR were from TianGen Biotech (Beijing) Co., Ltd.

**Quantification of surfactin by RP-HPLC**
One millilitre of fermentation broth was centrifuged for 10 min at 5000×g, and the supernatant was removed to a clean 1.5 mL centrifuge tube. The insoluble substances were removed by filtration through a 0.2 μm filter membrane (Jinlong, Tianjin, China). The concentration of surfactin was analysed by a Waters 2695 HPLC system composed of an autosampler and an UV detector (Waters, USA). A 10 μL aliquot was injected into a Symmetry C18 column (5 μm, 250 ×4.6 mm) (Waters, USA) to separate the surfactin isoforms. A surfactin standard (purity ≥ 98%, Sigma-Aldrich Trading Co. Ltd., Shanghai, China) was used to confirm the identity of the fractions. The mobile phases were 10% water and 90% methanol, containing 0.1% trifluoroacetic acid (TFA) with a total flow rate of 1.0 mL/min, and the chromatograms were detected at 205 nm with a column temperature of 30 °C. The total concentration of surfactin was calculated from the total peak area of four surfactin isoforms according to the concentration standard curve obtained by using Sigma surfactin.

**Results and discussion**

**Establishment of surfactin biosynthesis in *B. subtilis* 168**
The wild-type *B. subtilis* 168 strain (BS168NU) is incapable of synthesizing surfactin since there is a termination codon in the middle of the *sfp* gene, which encodes the phosphopantetheinyl transferase (PPTase) that plays an essential role in surfactin synthesis [35]. Thus, we
integrated the complete \textit{sfp} gene into the genomic \textit{ydeO} site of the wild-type \textit{B. subtilis} 168 strain, which generated the recombinant BS168NU-S strain. The surfactin production by the BS168NU-S strain was 0.45 g/L, which was determined 24 h after inoculation in 5 mL of semi-defined fermentation medium with sucrose as the carbon source (Additional file 3: Figure S1).

**Construction of the CRISPRi system and the relative expression level analysis**

Many efforts have been made to enhance surfactin production, including increasing the supply of fatty acid [17] and overexpressing surfactin synthase [8, 9, 12, 17]. Here, we attempted to improve surfactin biosynthesis by increasing the supply of amino acid precursors, which was achieved by inhibiting the branch pathways of amino acid biosynthesis. Twenty genes on the branch pathways of amino acid biosynthesis were selected, including \textit{mmgA}, \textit{yhfS}, \textit{nadB}, \textit{yhdR}, \textit{asnB}, \textit{asnH}, \textit{asnO}, \textit{racX}, \textit{pyrB} and \textit{pyrC} (on the branch pathways of l-aspartate biosynthesis), \textit{purF}, \textit{glnA}, \textit{yrpC}, \textit{racE}, \textit{glmS}, \textit{ybcM}, \textit{murD} and \textit{murC} (on the branch pathways of l-glutamate biosynthesis), and \textit{bkdAA} and \textit{bkdAB} (on the branch pathways of l-valine and l-leucine biosynthesis) (Fig. 1b). First, these genes interfered individually with the CRISPRi system. Previous studies indicated that in \textit{B. subtilis} 168, the expression of dCas9 by the \textit{Pxy} promoter could achieve higher inhibition efficiency than the expression of dCas9 by other promoters [36, 37]. Thus, in this study, dCas9 was expressed under the control of the inducible \textit{Pxy} promoter and was integrated into the \textit{lacA} site of the \textit{B. subtilis} genome. The sgRNA was expressed by the constitutive \textit{Pveg} promoter and integrated into the \textit{amyE} site of the genome (Fig. 2a). To evaluate the silencing efficiency of the CRISPRi system in \textit{B. subtilis}, the transcription levels of the 20 genes were detected by quantitative real-time PCR (qRT-PCR) with a Roche LightCycler 480. The cDNAs were obtained by reverse transcription with the total RNA extracted from 24 h fermentation broth as the template. The relative transcription level of the target gene was quantified by the $2^{-\Delta\Delta CT}$ [34] method using the \textit{ccpA} gene as the internal control and BS168NU-Sd as the calibrator. The results proved that the CRISPRi system effectively inhibited gene expression in \textit{B. subtilis} 168 (Fig. 2b). However, the repression efficiencies of different genes varied. The transcription repression efficiency of \textit{mmgA}, \textit{yhfS}, \textit{yrpC}, \textit{murC} and \textit{bkdAA} genes ranged from 2.5- to 7.2-fold. The transcription levels of other genes all exhibited over tenfold repression. In particular, significant decreases in the transcriptional levels of \textit{asnH}, \textit{nadB}, \textit{yhdR}, \textit{pyrC} and \textit{bkdAB} genes (over 150-fold) were observed, which was consistent with a previous study [37].

**Effect of single gene inhibition on surfactin production**

First, BS168NU-Sd was constructed as the control by integrating the \textit{dCas9} gene into the \textit{lacA} site of the BS168NU-S genome without the sgRNA cassette. Surfactin production and cell growth were determined 24 h after inoculation in 5 mL of semi-defined fermentation...
medium with the addition of 4 g/L xylose inducer (Fig. 3a, b). The surfactin titre of BS168NU-Sd was only 0.17 g/L, which was lower than that of BS168NU-S (0.37 g/L). This negative impact on surfactin production resulted from the toxicity of the highly expressed dCaS9 protein [38]. Despite this effect, however, the individual inhibition of 16 among the 20 selected genes could increase the production of surfactin. In particular, when the gene tyrC, racE or murC was inhibited, the surfactin production was increased to 0.54, 0.41 or 0.42 g/L, respectively. The murC-inhibited strains also had the highest surfactin production per OD$_{600}$ of 0.083 g/L/OD$_{600}$ (Additional file 4: Figure S2). These three genes are related to the consumption of l-glutamate [39] (Fig. 1b), whose acylation is the first step in surfactin assembly.

To evaluate the differences over time, the surfactin production and cell biomass of BS168NU-Sd, tyrC, racE and murC were measured 0 h, 6 h, 12 h, 18 h, and 24 h after inoculation (Fig. 3c, d). The growth of the tyrC- and racE-inhibited strains was greater than that of the parent BS168NU-Sd strain throughout the fermentation (Fig. 3c). YrpC and RacE are MurI-type glutamate racemase [40], which not only catalyses the conversion of l-glutamate to d-glutamate [39] but also is a DNA gyrase inhibitor [40, 41]. Overexpression of these MurI-type glutamate racemases caused growth retardation [40–42] because of the repression of DNA gyrase activity, which plays an essential role in DNA supercoiling and cell replication [43, 44]. Thus, it was predicted that the inhibition of tyrC and racE could increase the growth rate, and the
results supported this assumption, as shown in Fig. 3c. However, although the growth of the murC-inhibited strain had a longer lag period and slightly less final biomass than BS168NU-Sd, both the maximum growth rate and the volumetric productivity were higher than those of BS168NU-Sd (Fig. 3c and Table 3). Usually, high productivity is expected to lower cell growth, since cells might redirect energy from growth to production. However, the phenomena that engineered strains could show both higher biomass and higher production were also observed in previous studies on surfactin biosynthesis [8, 45] and might be caused by a higher central metabolism or higher substrate utilization rate in the engineered strains. The three engineered strains indeed used more sucrose than BS168NU-Sd, as shown in Additional file 5: Figure S3.

The mmgA- and yhfS-inhibited strains also showed clear increases in cell biomass. The genes mmgA and yhfS encode the acetyl-CoA C-acetyltransferase, which catalyses acetoacetyl-CoA biosynthesis from acetyl-CoA. The inhibition of mmgA and yhfS could reduce the consumption of acetyl-CoA, a key intracellular intermediate metabolite, which is not only used for surfactin biosynthesis but also benefits cell growth and proliferation [46].

In contrast, the strain with inhibition of pyrB or pyrC, which catalyses the conversion of L-aspartate to uracil, produced negligible amounts of surfactin. These results were consistent with previous research showing that the disruption of pyrB and pyrC decreased surfactin production [47], which indicated that high inhibition of these genes might be harmful to surfactin biosynthesis. Thus, we decreased the repression intensity by using the leaked expression of the dCas9 protein by the Pxyl promoter [37]. As expected, the pyrB- or pyrC-inhibited strains exhibited surfactin production of 0.34 or 0.36 g/L when no xylose was added (Additional file 6: Figure S4), which were much higher values than that obtained when dCas9 was induced by 4 g/L xylose (Fig. 3a). The results indicated that sometimes the gene repression efficiency needs to be delicately regulated to improve the biosynthesis of desired products.

### Table 3 The production, volumetric productivity and yield of the parent strains and the recombinant strains with racE, murC, yrpC, H1, H2, and H3

| Strains     | Target genes | Titre (mg/L) | Volumetric productivity (mg/L/h) | Yield on carbon (mmol/mol sucrose) |
|-------------|--------------|--------------|----------------------------------|-----------------------------------|
| BS168NU-Sd  | –            | 166.88       | 6.95                             | 0.80                              |
| BS168NU-S   | –            | 372.30       | 15.51                            | 1.75                              |
| racE        | racE         | 405.83       | 16.91                            | 1.93                              |
| murC        | murC         | 421.58       | 17.57                            | 1.98                              |
| H3          | racE, murC   | 478.69       | 19.95                            | 2.26                              |
| yrpC        | yrpC         | 541.57       | 22.57                            | 2.55                              |
| H2          | yrpC, murC   | 566.41       | 23.60                            | 2.69                              |
| H1          | yrpC, racE   | 751.90       | 31.33                            | 3.54                              |

**Inhibition of bkdAA and bkdAB changed the composition of surfactin**

Surfactin is a mixture in terms of the length of the fatty acid carbon chain and the structural content of the peptide moiety. Razafindralambo et al. [48] found that the foaming capacity and quality of C14 surfactin were higher than those of C13 and C15 surfactin. The inhibition of bkdAA or bkdAB not only increased surfactin production compared with that of BS168NU-Sd but also significantly changed the proportion of each component of surfactin [20]. The proportion of C14 in the bkdAA- or bkdAB-inhibited strain increased from 25.7% (BS168NU-S) or 18.7% (BS168NU-Sd) to 82.4% or 85.1%, respectively (Fig. 4, and Additional file 7: Figure S5) [49]. These results might occur because the inhibition of bkdAA and bkdAB not only increased the accumulation of L-leucine and L-valine, but also decreased the synthesis of iso-C13 and iso-C15 fatty acids by interrupting the bkd operon (lpdV, bkdAA, bkdAB and bkdB genes). The bkd operon catalyses the synthesis of iso-C13 and iso-C15 fatty acids using L-leucine as the precursor [49] (Additional file 8: Figure S6).

**Fig. 4** Effect of inhibiting bkdAA and bkdAB on the composition of surfactin. Cn represents the carbon chain lengths of β-hydroxy fatty acids, where n is the number of carbon atoms. The amino acid sequence of the heptapeptide is Glu-Leu-Leu-Val-Asp-Leu-Leu, except for C14-2, which has the sequence Glu-Val-Leu-Leu-Asp-Leu-Val.
Multiple gene inhibition further improved surfactin production

According to the results of single gene inhibition, the three genes (yrpC, racE and murC) that most significantly increased surfactin production were selected, and their coherent co-inhibition effects on surfactin production were further studied. The sgRNA plasmids for multiple gene inhibition were constructed as shown in Fig. 5a. The surfactin production and the cell growth of the recombinant strains with multiple gene inhibition were also determined 24 h after inoculation in 5 mL of semi-defined fermentation medium with 4 g/L xylose inducer (Fig. 5b). The results indicated that the simultaneous co-inhibition of two genes, i.e., yrpC and racE, yrpC and murC, or racE and murC, further increased surfactin production to 0.75, 0.57 or 0.48 g/L, respectively (as shown in the histogram of Fig. 5b). The surfactin production per OD<sub>600</sub> of these double-gene inhibited strains
was also increased compared to that of strains with the inhibition of a single gene (Fig. 5c). The co-inhibition of yrpC and racE produced the highest titre of surfactin (0.75 g/L). Unlike murC, the genes yrpC and racE directly participate in the consumption of l-glutamate, and thus the co-inhibition of yrpC and racE might cause a higher accumulation of l-glutamate. However, further inhibition of the three genes led to a surfactin titre of only 0.07 g/L, which was lower than that of single gene inhibition. This result may be caused by the significant deterioration in cell growth upon co-inhibition of these three genes (as shown in the scatter of Fig. 5b) [47].

In summary, we improved the capacity of surfactin production in B. subtilis by repression of the genes on the branch metabolic pathways of the biosynthesis of 4 amino acid precursors for surfactin synthesis. The production, volumetric productivity and yield of the 6 recombinant strains with the clearest improvement in surfactin synthesis are presented in Table 3. The surfactin production and volumetric productivity of the yrpC- and racE- inhibited strain were 4.51-fold that of BS168NU-Sd, and the yield on sucrose was 4.43-fold that of BS168NU-Sd. The surfactin production, volumetric productivity and yield of this recombinant strain on sucrose were also more than 2 times higher than those of BS168NU-S.

Conclusions
To the best of our knowledge, this study is the first to systematically investigate the regulatory effect of increasing the supply of amino acids on surfactin production. First, we constructed an efficient CRISPRi system in B. subtilis, which was proven by transcriptional repression as measured by RT-qPCR. Then, 20 core genes on the branch metabolic pathways of four amino acid biosynthesis pathways (i.e., l-glutamate, l-aspartate, l-leucine and l-valine) were individually inhibited by CRISPRi in BS168NU-S. The inhibition of 16 genes increased surfactin production. Among them, the inhibition of yrpC, racE or murC, which are related to l-glutamate metabolism, increased surfactin production most significantly, with titres of 0.54, 0.41 or 0.42 g/L, respectively. Furthermore, the three genes were repressed in combination, and the strain with co-inhibition of yrpC and racE produced the highest surfactin titre of 0.75 g/L (the specific productivity and yield were 31.33 mg/L/h and 3.54 mmol/mol sucrose, respectively). This work indicated that inhibition of the branch pathways through the CRISPRi system to increase the amino acid precursor supply is an effective strategy for improving surfactin production in B. subtilis.

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Authors’ contributions
CYW, YXC and HS conceived the project, CYW performed the experiments; CYW, YXC and HS wrote the manuscript. HS and YXC supervised the project. All authors read and approved the final manuscript.

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Availability of data and materials
Data will be made available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors read and approved the final manuscript.

Competing interests
The authors declare no competing financial interest.

Additional files

Additional file 1: Table S1. The sequences of sfp and dCas9.
Additional file 2: Table S2. Primer sequences used in this study.
Additional file 3: Figure S1. Construction of the surfactin production B. subtilis strain BS168NU-S. (A) Schematic diagram of the double crossover homologous recombination for construction of the recombinant B. subtilis strain BS168NU-S. Phosphopantetheinyl transferase (PPTase), which plays an essential role in surfactin synthesis, is encoded by the sfp gene. However, sfp in the wild-type B. subtilis strain (BS168NU) is inactive due to a termination codon in the middle of the gene sequence. We thus integrated the sfp gene under the control of the P43 promoter into the ydeO site of the BS168NU genome using double crossover homologous recombination and the Spizizen transformation approach. (B) Surfactin production by the B. subtilis strains BS168NU and BS168NU-S 24 h after inoculation in 5 mL of semi-defined fermentation medium without xylose inducer.

Additional file 4: Figure S2. The surfactin production per OD600 of parent strains and with CRISPRi-based single gene interference in the BS168NU-Sd strain 24 h after inoculation in 5 mL of the semi-defined fermentation medium.

Additional file 5: Figure S3. Residual sucrose concentrations of BS168NU-Sd, yrpC, racE and murC strains during fermentation.

Additional file 6: Figure S4. Surfactin production of the BS168NU-S, BS168NU-Sd, pyrB, and pyrC strains in medium without xylose inducer.

Additional file 7: Figure S5. HPLC analysis of surfactin. Peak-1 contains a C14-β-hydroxy fatty acid chain and a Glu-Leu-Leu-Val-Asp-Leu-Leu peptide. Peak-2 contains a C15-β-hydroxy fatty acid chain and a Glu-Leu-Val-Asp-Leu-Leu peptide. Peak-3 contains a C14-β-hydroxy fatty acid chain and a Glu-Val-Leu-Asp-Leu-Leu peptide. Peak-4 contains a C15-β-hydroxy fatty acid chain and a Glu-Leu-Val-Asp-Val-Leu peptide.

Additional file 8: Figure S6. Schematic diagram of the biosynthesis of iso-C13 and iso-C15 fatty acids using l-leucine as a precursor.

iso-C13 and iso-C15 fatty acids using l-leucine as a precursor.
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