

Article

Bacillus subtilis Genome Reduction Improves Surfactin Production

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Abstract

Bacillus subtilis Δ6 is a genome-reduced strain derived from the laboratory strain 168 through deletion of six prophages and AT-rich islands. The parental and the genome-reduced strains were edited to restore the capacity to synthesize surfactin. Although the genome deletions are not directly related to surfactin biosynthesis, the Δ6 strain produces more surfactin while building lower biomass compared to the parental strain. Further editions to Δ6, such as *urfA* promoter replacement, *codY* deletion, and *comA* overexpression, were deleterious to surfactin production. The results showcase that the Δ6 is metabolically distinct from its parental strain and other surfactin-producing strains, as the gene editions made have been previously described to increase surfactin production in these strains. The Δ6 produced the highest surfactin titer, rate, and yield in LB medium enriched with glucose, compared to other commonly used media for *B. subtilis*. This work demonstrates the enhanced capacity of a genome-reduced strain to produce surfactin compared to the parental strain, as well as the metabolic changes resulting from genome engineering.

Keywords: surfactin; biosurfactant; bioproduction; *Bacillus subtilis*; genome-reduced; CRISPR editing



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1. Introduction

Genome reduction has been used to reduce complexity and heterogeneity by streamlining existing microbial genomes. Common targets are non-essential genes such as those related to mobile genetic elements, extracellular proteases, sporulation, flagella formation, and antibiotic biosynthesis [1]. A genome-reduced strain is, ideally, a simplified cell factory with comparable growth and higher productivity than its parental strain, since it saves energy with genome replication and synthesis of dispensable proteins and has the potential to redirect precursors to the synthesis of value-added compounds [2]. Genome reduction in *Bacillus subtilis* has been performed stepwise from the laboratory strain 168, starting with 8% (strain Δ6) and reaching up to 36% (strain PS38) of reduction in the size of its chromosome [1,3]. These strains exhibit some notable traits, including increased total cellular NADPH levels, superior secretion of difficult-to-produce antigens, and a good capacity to produce surfactin and acetoin [3–5].

Surfactin is a lipopeptide first isolated from *B. subtilis* in 1968 [6] and since then, it has attracted attention due to its potent surfactant activity, excellent surface activity [6,7], antimicrobial properties [8], high temperature stability, and salt tolerance [7]. However,

despite more than half a century of studies, producing surfactin on a large scale remains a challenge.

Surfactin biosynthesis, mediated by non-ribosomal peptide synthetases (NRPSs), integrates multiple metabolic pathways, encompassing the initial synthesis of precursor molecules and culminating in the assembly of the biosurfactant. Once the precursor molecules (fatty acids, NADPH, glutamate, aspartate, valine, and leucine) are available in the cell, surfactin biosynthesis is catalyzed by the modular peptide synthetases SrfAA, SrfAB, SrfAC, and SrfAD, encoded by the *srfA* operon (26 kb) [9–11]. The first three synthetases form a set of seven modules that are responsible for the incorporation of amino acid residues into the lipopeptide structure [10]. The modules have catalytic domains for adenylation, peptidyl carrier (or thiolation), condensation, epimerization, and thioesterase, which are responsible for amino acid activation, peptide chain elongation, and product release [12,13]. Surfactin biosynthesis depends on the maturation of SrfAA, SrfAB, and SrfAC subunits, which occurs through the activation of their peptidyl carrier domains by a 4-phosphopantetheyl transferase encoded by the *sfp* gene [14]. The SrfAD synthetase recycles misfolded peptidyl carrier domains [10].

Naturally, the transcription of the *srfA* operon is controlled by the P_{srfA} promoter, which is activated by the binding of the phosphorylated ComA protein associated with the sigma factor SigA [11]. ComA is a response regulator in the two-component regulatory system ComP/ComA. ComA phosphorylation is the result of a cascade effect initiated by the signal peptide ComX from the quorum-sensing system COMQXPA of *B. subtilis*, which responds to the cellular concentration in the medium [15]. Rap proteins, a family of aspartate phosphatases, are known to inhibit ComA activity. Meanwhile, Phr peptides, especially PhrC (also known as CSF for competence and sporulation stimulating factor), stimulate ComA activity by antagonizing the Rap regulators [15,16].

The P_{srfA} is also subject to repression by the CodY protein, a GTP- and branched-chain amino acid-dependent transcriptional regulator, that directly binds to the promoter region and blocks RNA polymerase activity [17]. Other regulators, such as DegU, Abh, PhoP, PerR, and Spx, can influence *srfA* operon transcription, but some of their mechanisms remain unclear [11,18–21].

Here, we evaluated the capacity of *B. subtilis* $\Delta 6$ as a surfactin producer and the effect of genome editing affecting the regulation of the *srfA* operon on surfactin production. $\Delta 6$ is a genome-reduced strain derived from *B. subtilis* 168 through deletion of two prophages (SP β , PBSX), three prophage-like regions (prophage 1, prophage 3, and *skin*), and the large polyketide synthase operon (*pks*), resulting in a 7.7% genome reduction [22,23]. The strain is genetically more stable compared to its parental strain and other natural surfactin-producer strains due to the deletion of prophages and the *BsuM* restriction-modification system [22], an important trait for industrial strains. Moreover, the deletion of the *pks* operon, which encodes polyketide synthases related to bacillaene synthesis [24], could be beneficial for surfactin production due to the elimination of competition for precursors [25]. Despite the significant genome reduction, the strain shows no significant difference in physiology, metabolic flux patterns, or genetic competence compared to the parental strain. Importantly, $\Delta 6$ maintains the same maximum growth rate as *B. subtilis* 168.

2. Materials and Methods

2.1. DNA Sequences and Cloning

All plasmids used were derived from pJOE8999 [26]. For each edition, we designed a guide sequence (GS) for the gRNA and a donor DNA sequence for double-strand break repair through homology recombination. The gRNA was designed using online tools such as the CRISPR-Cas9 guide RNA design checker (available on the IDT Integrated DNA

Technologies website) and Cas-Designer from CRISPR RGEN Tools [27], and acquired as a pair of single-stranded DNA oligonucleotides. The GS oligonucleotides were annealed and cloned into pJOE8999 using the *BsaI* restriction sites upstream of the SpCas9 gRNA scaffold sequence (sgRNA) and following usual molecular biology digestion and ligation protocols. The donor DNA consisted of two 400–800 bp homology sequences flanking the desired genome editing site. The parts of the donor DNA were PCR-amplified from genomic DNA or purchased as a synthetic DNA sequence. The parts were assembled through Golden Gate using *BsaI* restriction sites. The complete donor sequence was cloned into the plasmid using *SfiI* restriction sites. Plasmid construction was confirmed by Sanger sequencing. All the plasmids used are described in Table 1. More details about the gRNA and donor DNA sequences are provided in Appendix A (Tables A1 and A2), and sequences corresponding to the donor DNA are provided in the Supplementary Materials.

Table 1. Plasmids used in this study.

Plasmid	Characteristics	Reference
pJOE8999	Base plasmid: pUCori, rep pE194ts, <i>kanR</i> , $P_{\text{manP-cas9}}$, $P_{\text{vanP-lacPOZ'-sgRNA}}$	[26]
pEBScas9.1	pJOE8999, $P_{\text{vanP-gRNA}_1}$, <i>sfp</i> *	This study
pEBScas9.2	pJOE8999, $P_{\text{vanP-gRNA}_2}$, <i>hxlR</i> *-S0-R6- <i>srfAA</i> *	This study
pEBScas9.3	pJOE8999, $P_{\text{vanP-gRNA}_2}$, <i>hxlR</i> *-TP2- <i>srfAA</i> *	This study
pEBScas9.4	pJOE8999, $P_{\text{vanP-gRNA}_3}$, S0*- P_{srfA} -R6- <i>srfAA</i> *	This study
pEBScas9.5	pJOE8999, $P_{\text{manP-cas9}}$, $P_{\text{vanP-gRNA}_4}$, <i>hslU</i> *- <i>flgB</i> *	This study
pEBScas9.6	pJOE8999, $P_{\text{vanP-gRNA}_5}$, <i>comP</i> *- $P_{\text{srfA-comA}}$	This study

* Denotes a partial sequence of the gene, necessary for homologous recombination.

2.2. Bacterial Strains and Growth Conditions

E. coli TOP10 was used for cloning procedures, and *E. coli* JM109 was used for plasmid propagation. *B. subtilis* strains were derived from strains 168 and $\Delta 6$, both non-producers of surfactin. All bacterial strains used are presented in Table 2.

Lysogeny broth (LB) was used for cloning and transformation procedures (10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, and 10 g L⁻¹ NaCl), enriched with kanamycin (final concentration of 50 µg mL⁻¹ for *E. coli* and 5 µg mL⁻¹ for *B. subtilis*). For surfactin production, *B. subtilis* strains were cultivated in two complex media: LB and PW, and two mineral media: MM1 and MM2. PW medium is composed of 1 g L⁻¹ yeast extract, 25 g L⁻¹ NaNO₃, 0.333 g L⁻¹ KH₂PO₄, 1 g L⁻¹ Na₂HPO₄·12H₂O, 0.15 g L⁻¹ MgSO₄·7H₂O, 7.5 mg L⁻¹ CaCl₂, 6 mg L⁻¹ MnSO₄·H₂O, 6 mg L⁻¹ FeSO₄·7H₂O, pH 7.0. MM1 medium is composed of 4 g L⁻¹ KH₂PO₄, 12.2 g L⁻¹ K₂HPO₄, 3.3 g L⁻¹ (NH₄)₂SO₄, 0.123 g L⁻¹ MgSO₄·7H₂O, 2.32 mg L⁻¹ MnSO₄·4H₂O, 1.7 mg L⁻¹ ZnCl₂, 22 mg L⁻¹ ferric ammonium citrate, supplemented with tryptophan 50 µg mL⁻¹, pH 7.0. MM2 has the same composition as MM1, except that the nitrogen source (NH₄)₂SO₄ was replaced with 4.25 g L⁻¹ NaNO₃. LB medium was tested without sugar supplementation and with glucose at 1% or 2% (*m/v*). PW, MM1, and MM2 media were supplemented with glucose at 1% or 2% (*m/v*). The standard cultivation medium was LB enriched with 1% (*m/v*) glucose, unless otherwise stated. Chloramphenicol at a final concentration of 5 µg mL⁻¹ was added to the overnight pre-cultures of *B. subtilis* $\Delta 6$ derivatives.

Table 2. Bacterial strains used in this study.

Strain	Characteristics	Reference
<i>E. coli</i> JM109	recA1, endA1, gyrA96, thi-1, hsdR17(r _K [−] , m _K ⁺), mcrA, supE44, gyrA96, relA1, λ [−] , Δ(lac-proAB), F'(traD36, proAB ⁺ , lacI ^q , ΔlacZ)M15)	[28]
<i>E. coli</i> TOP10	mcrA, Δ(mrr-hsdRMS-mcrBC), Phi80lacZ(del)M15, ΔlacX74, deoR, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL(SmR), endA1, nupG	Invitrogen
<i>B. subtilis</i> ATCC 21332	Wild-type, surfactin producer	BGSCID 3A22 *
<i>B. subtilis</i> 168	<i>B. subtilis</i> Marburg 168, trpC2	BGSCID 1A1 *
<i>B. subtilis</i> Δ6	Genome-reduced <i>B. subtilis</i> 168 trpC2, Δskin, ICEBs1(0), Δprophage1, Δprophage3, ΔSPbeta, ΔPBSX, Δpks::CmR	BGSID 1A1299 *
<i>B. subtilis</i> CS01	<i>B. subtilis</i> 168 derivate <i>sfp</i> ⁺	This study
<i>B. subtilis</i> D6S01	<i>B. subtilis</i> Δ6 derivate <i>sfp</i> ⁺	This study
<i>B. subtilis</i> D6S02	<i>B. subtilis</i> D6S01 derivate <i>sfp</i> ⁺ , S0-R6- <i>srfAABCD</i>	This study
<i>B. subtilis</i> D6S03	<i>B. subtilis</i> D6S01 derivate <i>sfp</i> ⁺ , TP2- <i>srfAABCD</i>	This study
<i>B. subtilis</i> D6S04	<i>B. subtilis</i> D6S02 derivate <i>sfp</i> ⁺ , S0-P _{srfA} -R6- <i>srfAABCD</i>	This study
<i>B. subtilis</i> D6S05	<i>B. subtilis</i> D6S01 derivate <i>sfp</i> ⁺ , Δ <i>codY</i>	This study
<i>B. subtilis</i> D6S06	<i>B. subtilis</i> D6S05 derivate <i>sfp</i> ⁺ , Δ <i>codY</i> , P _{srfA} - <i>comA</i>	This study

* ID number for the strain at the *Bacillus* Genetic Stock Center (BGSC).

Cultivations for surfactin production were carried out in test tubes (23 mm diameter × 145 mm height) and 250 mL Erlenmeyer flasks containing 6 and 25 mL of culture, respectively. All bacterial strains were aerobically cultivated at 37 °C and 220 rpm.

2.3. *B. subtilis* Strain Engineering

Genome editing with CRISPR-Cas9 was carried out following a protocol adapted from [26]. *B. subtilis* cells were transformed with plasmids derived from pJOE8999 following the two-step transformation protocol [29]. Transformed cells were plated on LB enriched with 5 g L^{−1} mannose and 5 μg mL^{−1} kanamycin. The plates were incubated at 37 °C for two days. Isolated colonies were collected and streaked onto new LB plates enriched with mannose and 5 μg mL^{−1} kanamycin and incubated at 30 °C for one day. Following, single colonies were collected, streaked out on LB plates, and incubated at 42 °C (for strain 168) and 46 °C (for strain Δ6 and its derivatives) for one day for plasmid curing. The next day, plasmid curing was repeated at higher temperatures: single colonies were selected, streaked out on an LB plate, and incubated at 46 °C (for strain 168) and 50 °C (for strain Δ6 and its derivatives) for one more day. Finally, isolated colonies were tested for plasmid loss: single colonies were streaked out on both LB and LB plates enriched with 5 μg mL^{−1} kanamycin and incubated at 37 °C overnight. Colonies that grew only on the LB plates

were then tested for genome editing through colony PCR and Sanger sequencing. Positive clones were preserved at $-80\text{ }^{\circ}\text{C}$ with 20% (*v/v*) glycerol.

To eliminate the premature stop codon in the *sfp* gene and enable surfactin production, *B. subtilis* strains 168 and $\Delta 6$ were edited using pEBScas9.1, resulting in *B. subtilis* CS01 and D6S01. Strain D6S01 was further edited with pEBScas9.2 and pEBScas9.3, resulting in strains D6S02 and D6S03, respectively. Strain D6S02 was further edited with pEBScas9.4, resulting in strain D6S04. Strain D6S04 was further edited with pEBScas9.5, resulting in strain D6S05. Strain D6S05 was further edited with pEBScas9.6, resulting in strain D6S06.

2.4. Analytical Methods

Culture samples were collected periodically to determine growth and surfactin production. Culture growth was measured by optical density at 600 nm (OD_{600}) in a 96-well transparent plate filled with 200 μL samples and read in a microplate reader Tecan Infinite 200 Pro (Tecan, Männedorf, Switzerland). *B. subtilis* cell suspensions at different OD_{600} were dried out completely and weighed to correlate OD_{600} to dry weight. The conversion of OD_{600} to biomass (g L^{-1}) was calculated based on the resulting calibration curve using Equation (1):

$$\text{OD}_{600} = 1.1005 \times \text{Biomass} \left(\text{g} \cdot \text{L}^{-1} \right) - 0.0046 \quad (1)$$

where $R^2 = 0.9984$ and $0.089 \text{ g/L} \leq \text{Biomass} \leq 0.896 \text{ g/L}$.

Culture samples were centrifuged at $17,000 \times g$ for 10 min at room temperature to collect the cell-free supernatant. The reducing sugars (glucose) present in the supernatant were quantified by the method of Miller [30] modified by [31].

Surfactin was measured by HPLC after extraction using the cell-free supernatant [32]. Extraction started by adding 2 mL of chloroform/methanol (2:1) to 2 mL of culture supernatant. The mixture was vortexed for 30 s and centrifuged at $3000 \times g$ for 10 min at $4\text{ }^{\circ}\text{C}$. The lower layer was collected in a fresh tube, and the extraction process was repeated two more times with the upper layer. The fractions collected were dried out in a fume hood at room temperature overnight. Dried samples were resuspended in 2 mL of methanol 100% (HPLC grade) and filtered through a hydrophilic PTFE 0.22 μm membrane. HPLC analysis was performed on Shimadzu Scientific Instruments equipment (Kyoto, Japan) with an SPD-M20A photodiode detector, using an InfinityLab Poroshell 120 EC-C18 column ($3.0 \times 50 \text{ mm}$, $2.7 \mu\text{m}$; Agilent–Santa Clara, CA, USA). The mobile phase consisted of acetonitrile (HPLC grade) and 3.8 mM trifluoroacetic acid (TFA) at 80:20 (*v/v*), and an isocratic flow of 0.4 mL/min at $30\text{ }^{\circ}\text{C}$ [33,34]. Surfactin isoforms were detected at 200 nm and compared to the HPLC-grade standard (Merck S3523) used to build the calibration curve.

The cell-free supernatant was also used to quantify surfactin by a colorimetric method based on bromothymol blue (BTB) and cetylpyridinium chloride (CPC) [35]. The reaction was carried out on a 48-well transparent plate, adding 100 μL of sample and 800 μL of 0.1 mM CPC–BTB (buffered with 0.1 M PBS at pH 8.0). The plate was incubated for five minutes at room temperature and orbital shaking (6 mm amplitude) in the microplate reader, and the change of color was measured at 600 nm in the microplate reader. Surfactin HPLC-grade standard (Merck S3523) was used to build the calibration curve.

2.5. Data Analysis

The yields of surfactin per substrate ($Y_{P/S}$) and product per biomass ($Y_{P/X}$) were determined using Equations (2) and (3).

$$Y_{P/S} = \frac{P}{S} \quad (2)$$

$$Y_{P/X} = \frac{P}{X} \quad (3)$$

where P is the highest surfactin concentration detected in g L^{-1} , S is the consumed total reducing sugar in g L^{-1} , and X is the biomass in g L^{-1} at the highest point detected.

It is noteworthy that *B. subtilis* preferably consumes glucose, and when it is depleted, *B. subtilis* may use complex mixtures such as yeast extract (present in LB and PW media) and tryptone (LB medium) as carbon sources. Therefore, the reported yields represent values under the assumption that the reducing sugars (glucose included) were the sole carbon source.

Statistical analysis was performed using one-way ANOVA, Levene test for equality of variances, and post hoc Tukey test with 95% significance. For data with $p < 0.05$ on the Levene test, the post hoc Games–Howell test was performed with 95% significance.

3. Results

3.1. Genome-Reduced *B. subtilis* Performs Better than Its Parental Strain

Laboratory strains, such as *B. subtilis* 168, carry a mutant *sfp* gene that encodes a truncated and inactive 4'-phosphopantetheinyl transferase (Sfp). Such strains still have a functional *srfA* operon. However, the encoded non-ribosomal peptide synthases are unable to synthesize surfactin if not activated by Sfp. We edited the genomes of *B. subtilis* strains 168 and D6 to reverse the mutation and turn them into surfactin producers, resulting in strains D6S01 and CS01, respectively (Figure 1A).

Strain CS01 showed faster and higher biomass formation, reaching 2.3 g L^{-1} in 28 h compared to 1.4 g L^{-1} reached by D6S01 in the same period (Figure 1B). Surfactin concentration for both strains reached a maximum within 28 h, when D6S01 produced 1.2 g L^{-1} and CS01 produced 1.0 g L^{-1} . The difference between the strains was larger after 40 h, when CS01 degraded some of the surfactin in the culture medium, while D6S01 preserved the produced biosurfactant (Figure 1C). The strain D6S01 was slightly better at converting glucose into surfactin, reaching 0.15 g g^{-1} compared to 0.12 for CS01 (Figure 1D). The higher difference was found for the product yield per biomass, with D6S01 reaching 0.87 , twice as high as the yield reached by CS01 (Figure 1E). These findings indicate that the genome-reduced strain redirected resources from biomass to product formation, compared to the parental strain.

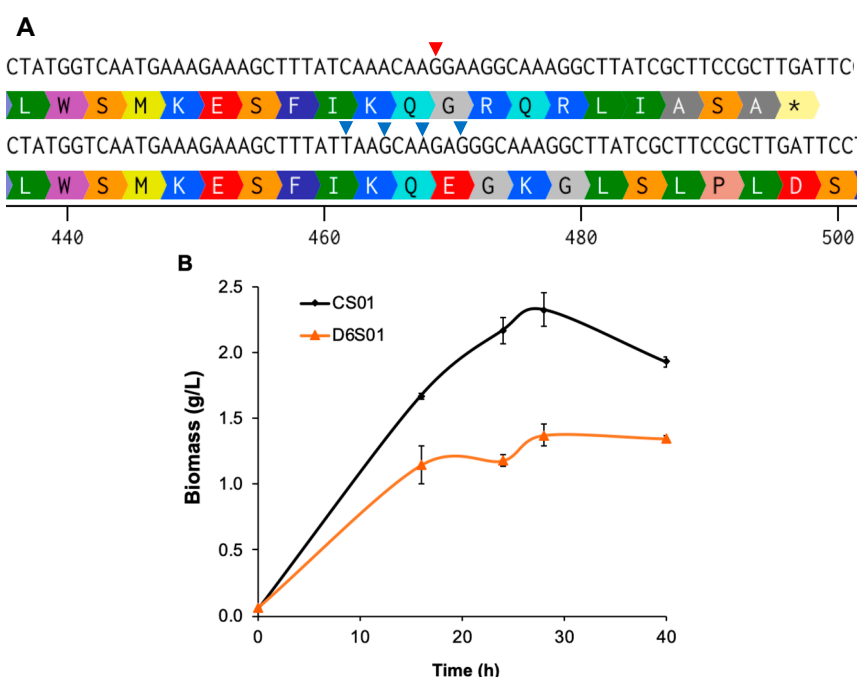


Figure 1. Cont.

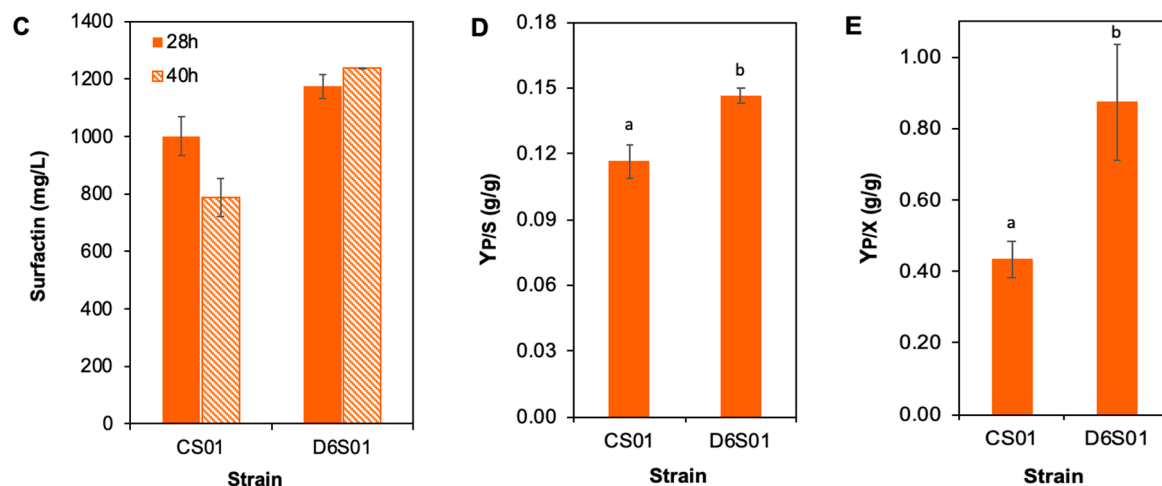


Figure 1. Surfactin production by *sfp*-edited *B. subtilis* 168 (CS01) and *B. subtilis* D6 (D6S01). (A) *sfp* gene and the 4'-phosphopantetheinyl transferase partial sequences. The upper sequence represents the inactive version of *sfp* found in both strains with a G insertion at position 469 (marked with a red triangle), causing a frame shift and creating a premature stop codon (represented as * in the amino acid sequence). The lower sequence shows the edited gene with a deletion of G-469, and silent point mutations C462T, A465G, G468A, and A471G (blue triangles). (B) Biomass formation over time. (C) Surfactin concentration in the cultures after 28 h and 40 h. (D) Surfactin yield per mass of glucose at 28 h of cultivation. (E) Surfactin yield per biomass. Data are presented as mean $\pm$ SD (n = 3). Different small letters identify statistically significant differences.

The surfactin mixture produced by the DS6S01 strain is composed of 21% C14-surfactin and 42.8% C15-surfactin. Additional peaks, potentially accounting for C16- and C17-surfactins, were also found. The total share of surfactins with 15 or longer C-chains was 58.7% of the total surfactin amount produced. This is very similar to the composition of the surfactin produced by the CS01, with 23% of C14 and 56.5% of C15 or longer C-chain surfactins.

3.2. Promoter Editing

The P_{srfA} is a strong promoter activated during the transition to the stationary phase of growth by the ComQXPA quorum-sensing system in *B. subtilis*. Previous attempts to replace the P_{srfA} showed divergent results. While some reported an increase in production [36,37], others reported a loss of surfactin biosynthesis [38,39] when the native P_{srfA} was replaced. Therefore, we decided to test three different promoter configurations: (i) replacement of the P_{srfA} with the constitutive and strong dual-promoter TP2 [40] to generate the strain D6S03; (ii) replacement of the P_{srfA} with a synthetic autoinduction device S0-R6 [41] to generate the strain D6S02; and (iii) insertion of the synthetic autoinduction device S0-R6 in addition to the P_{srfA} to generate the strain D6S04, creating a double autoinduced process to control the *srfA* genes (Figure 2A). We chose the synthetic autoinduction device S0-R6 because it is a quorum-sensing-based system like the ComQXPA- P_{srfA} , but with an earlier induction point in the first half of the exponential growth phase. All three strategies were deleterious to production. Replacing the P_{srfA} with the synthetic autoinduction device S0-R6 was the worst strategy, abolishing the production completely. Interestingly, the resources were not re-allocated to biomass formation, and the strain D6S02 built the lowest amount of biomass from all tested (Figure 2B,C). Replacing P_{srfA} with the strong TP2 promoter (D6S03) resulted in low levels of surfactin production, with resources re-allocated to the formation of biomass. Finally, we decided to use the two autoinduction processes simultaneously, in an attempt to start production earlier and continue the biosynthesis through the stationary phase. Therefore, we inserted the S0 induction module (*luxRI* genes) upstream of the

P_{srfA} and the R6 promoter downstream of the P_{srfA} (Figure 2A). The R6 promoter is only suitable for induction by the LuxR protein, while the P_{srfA} is suitable for both repression and activation processes. Therefore, the inverse order for the promoters would cause the R6 blockage by the repressors of P_{srfA} . The double-promoter strategy resulted in increased production parameters compared to the production solely driven by the S0-R6 device. However, the D6S04 only reached about half the production yield of the D6S01 strain. From these results, we inferred that the regulatory processes involved in the *srfA* transcription are important for high levels of surfactin production, and abolishing or even perturbing them is deleterious to the biosynthesis.

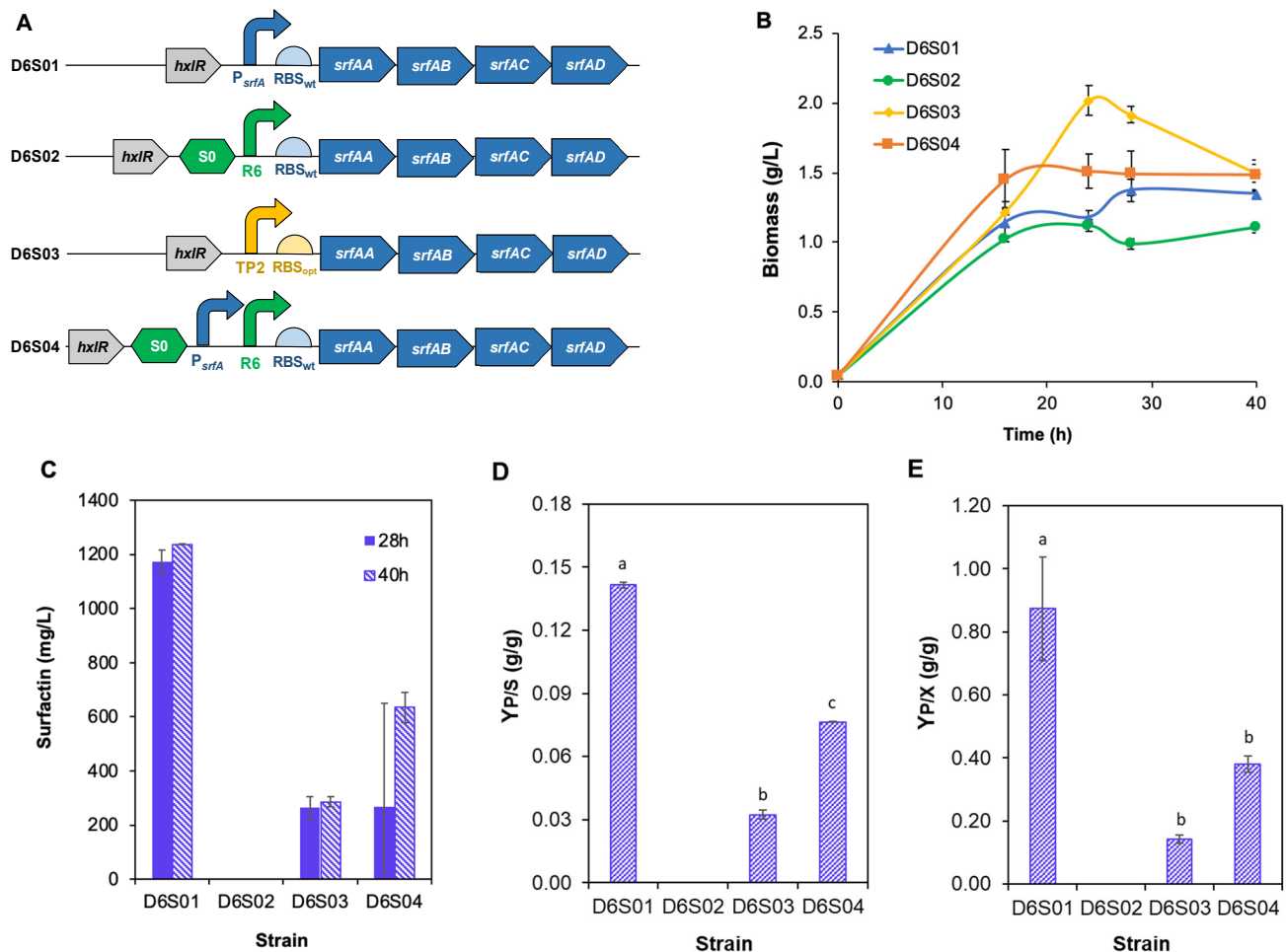


Figure 2. Production of surfactin from different promoter configurations. (A) Wild-type *srfA* operon and edited operons. The P_{srfA} was either replaced or used in combination with another promoter. (B) Biomass formation over time. (C) Surfactin concentration in the cultures after 28 h and 40 h. (D) Surfactin yield per mass of glucose. (E) Surfactin yield per biomass. Data are presented as mean $\pm$ SD, $n = 3$ (for D6S04 surfactin production, $n = 2$). Different small letters identify statistically significant differences.

3.3. Engineering Transcription Regulators

Since our efforts on promoter editing did not increase surfactin production, we turned our attention to two transcription regulators involved in the biosynthetic process, the CodY and the ComA. The two regulators have opposite effects on the P_{srfA} ; the first represses transcription, and the second activates it. CodY is a regulator that acts as a transcriptional repressor to genes involved in carbon and nitrogen metabolism, transportation of degraded extracellular macromolecules, and sporulation competence [42], some of which are involved in surfactin biosynthesis. CodY affects surfactin biosynthesis in two ways:

(i) it binds directly to the P_{srfA} promoter, inhibiting transcription [17]; (ii) it also inhibits the transcription of *ilvD*, *ybgE*, and *ilvBHC-leuABCD*, involved in the biosynthesis of branched-chain amino acids [43], and of the *bkd* operon, which is responsible for the biosynthesis of branched-chain ketoacids (precursors of branched-chain fatty acids) [44]. The ComA regulatory protein is involved in the expression of at least 89 genes in 35 operons in *B. subtilis*, acting predominantly as a transcription activator. In its phosphorylated form, ComA is responsible for directly activating the transcription from the P_{srfA} [45].

To eliminate the direct and indirect interference of the transcriptional repressor CodY in the surfactin production, we deleted *codY* in the strain D6S01 to generate D6S05 (Figure 3A). CodY deletion reduced surfactin yields by over two-fold while increasing biomass formation (Figure 3B–E). Based on this result, we hypothesized that releasing the CodY blockage could be beneficial only if transcription activation would increase at the same time. Therefore, we further edited D6S05 to add the P_{srfA} promoter between genes *comP* and *comA* in the *comQXPA* operon (strain D6S06) so that *comA* can be transcribed at an increased rate to activate the *srfA* operon upon culture transition to the stationary phase (Figure 3A). D6S06 showed a slight increase of 1.2 to 1.4-fold in surfactin yields compared to the parental strain D6S05. However, D6S06 still performs much worse than D6S01 (Figure 3C–E). These findings confirm that changing the regulatory processes involved in the *srfA* operon transcription is indeed deleterious to surfactin biosynthesis in *B. subtilis* $\Delta 6$.

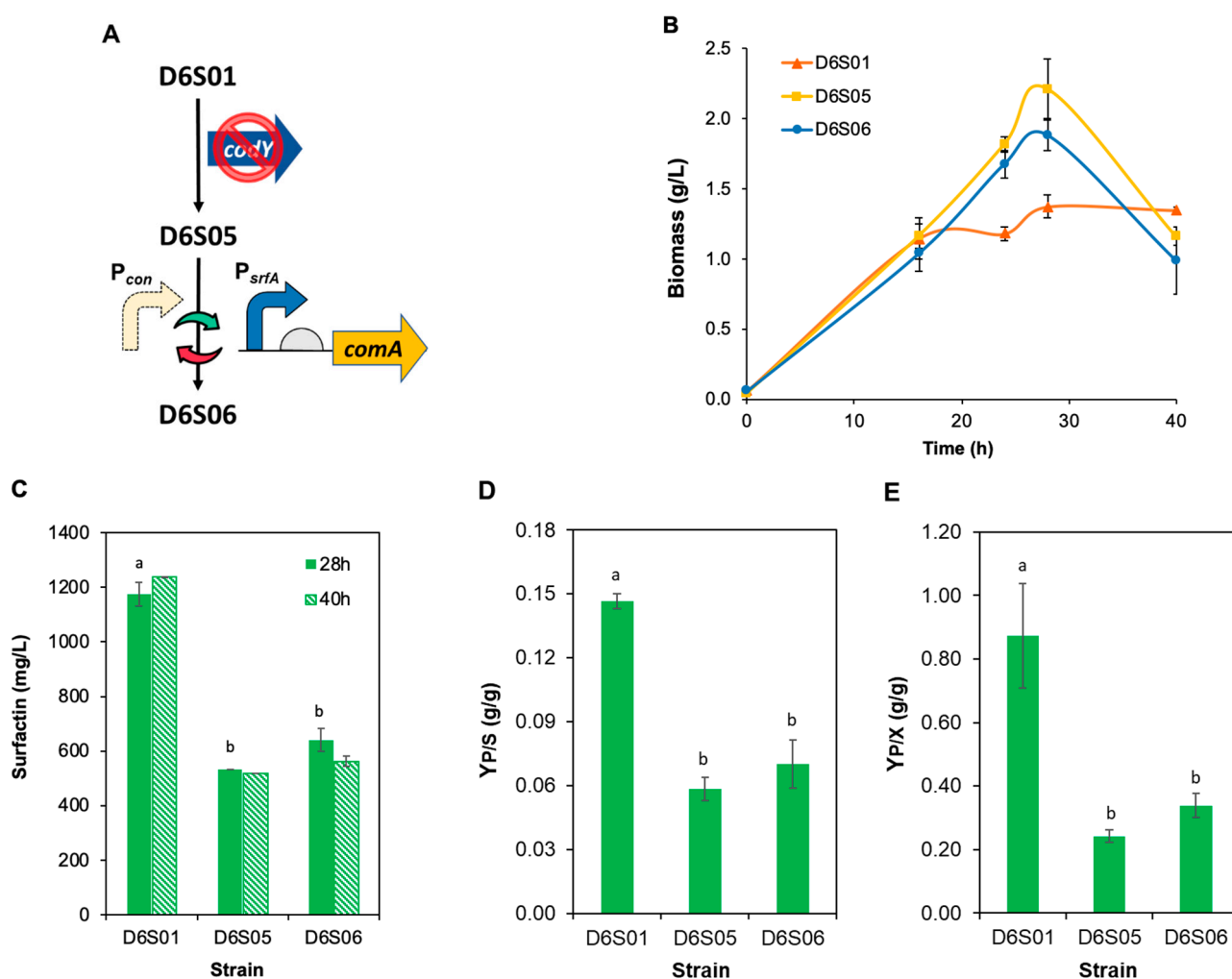


Figure 3. Production of surfactin by regulator-edited strains. (A) Edited strains. *codY* was deleted from the genome of D6S01 to generate D6S05. The P_{srfA} was inserted into the *comQXPA* operon to

drive the expression of *comA*. In this configuration, the operon transcription is still driven by the wild-type constitutive promoter (P_{con}), and *comA* is additionally transcribed from the P_{srfA} activation in the late exponential growth phase. (B) Biomass formation over time. (C) Surfactin concentration in the cultures after 28 h and 40 h. (D) Surfactin yield per mass of glucose. (E) Surfactin yield per biomass. Data are presented as mean $\pm$ SD, $n = 3$. Different small letters identify statistically significant differences.

3.4. Culture Medium Optimization

The biosynthesis of surfactin, which involves the condensation of four amino acids and a fatty acid, is subject to stringent regulation by global cellular mechanisms; consequently, surfactin yields are significantly influenced by the composition of the culture medium, among other cultivation parameters. Some ingredients, such as nitrate, have been suggested to increase surfactin production [46]. However, the combination of ingredients and the genetic background of the producer make it difficult to foresee the optimal formulation. With that in mind, we tested the strain D6S01 in three media formulations with two different glucose concentrations for each.

Mineral medium C (MM1), a commonly used defined medium for *B. subtilis* [47], supported poor growth and the worst surfactin yield when supplemented with 1% (m/v) glucose (Figure 4A). Increasing glucose supplementation to 2% (m/v) resulted in a 3.3-fold increase in biomass formation and a 2.8-fold increase in surfactin concentration (Figure 4A,B). Nonetheless, the production level was still too low. In an attempt to increase the production performance in the mineral medium, we modified its composition by replacing the nitrogen source from ammonium sulfate to ammonium nitrate (MM2). MM2 supplemented with 1% (m/v) glucose had a strong deleterious effect on growth, extending the lag phase. Surprisingly, increasing the glucose supplementation three times resulted in a 6.5-fold increase in biomass formation (Figure 4A). Although the production yield increased in MM2 compared to MM1, the gain in biomass did not translate into a proportional increase in surfactin production (Figure 4B–D). From these results, we inferred that using nitrate was beneficial, but the medium formulation was not yet ideal for surfactin biosynthesis. Therefore, we tested the PW medium, a complex medium formulated with yeast extract, sugar as a carbon source, and NaNO_3 as an additional inorganic nitrogen source, commonly used for surfactin production [35,48–50]. The PW formulation supported a 2.3 to 3.4-fold increase in surfactin yields compared to MM2 at 1% (m/v) glucose (Figure 4B–D). However, increasing glucose supplementation did not increase biomass formation or production. Since more glucose was consumed (Table A3), we suspect that carbon was lost through side products such as acetate and lactate. Finally, we tested lysogeny broth (LB). LB is widely used for routine bacterial cultivation in the laboratory, but it is not commonly used for surfactin production. Surfactin yield in LB only was still at low levels; however, supplementation with glucose strongly boosted production and growth (Figure 4A–D). At 1% (m/v), glucose supplementation resulted in surfactin yields of 1.2 g L^{-1} , 0.15 g/g of glucose, and 0.95 g g^{-1} of biomass. LB supplemented with more glucose (2%, m/v) resulted in a higher surfactin concentration; however, with no statistical difference compared to 1%. Moreover, the other yield parameters were worse for the higher glucose concentration.

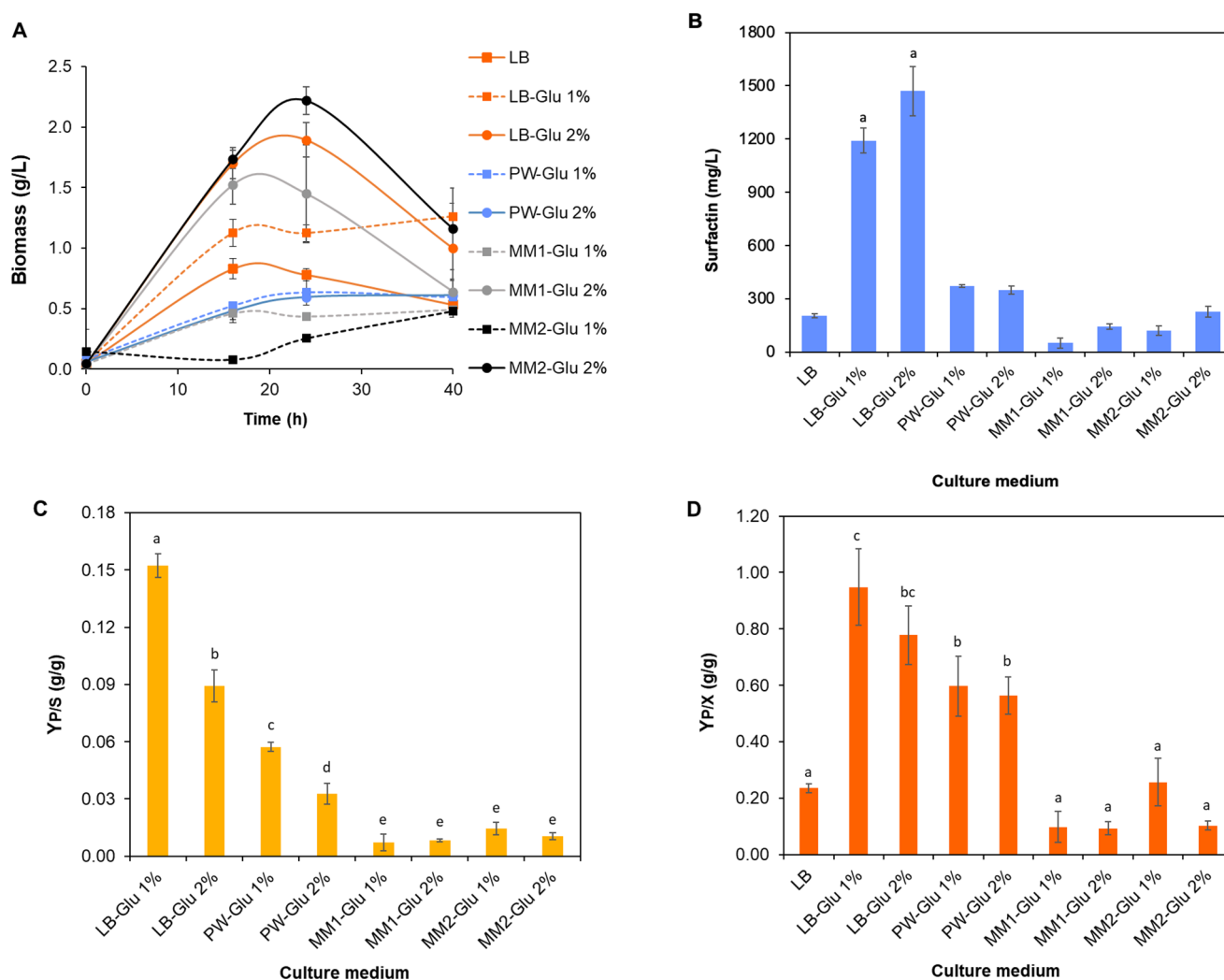


Figure 4. Production of surfactin in different media formulations. (A) Biomass formation over time. (B) Surfactin concentration in the cultures after 28 h. (C) Surfactin yield per mass of glucose. (D) Surfactin yield per biomass. Data are presented as mean $\pm$ SD, $n = 3$. Media used: LB and LB supplemented with glucose 1% or 2% (m/v), PW medium, mineral medium 1 (MM1), and 2 (MM2), also supplemented with glucose 1% or 2% (m/v). Data are presented as mean $\pm$ SD, $n = 3$. Different small letters identify statistically significant differences.

Comparing the surfactin production in LB only and LB enriched with glucose, we can evaluate the sugar contribution to the production. At 1% (m/v), glucose generated an extra 0.99 g/L of surfactin concentration, resulting in a conversion yield of 0.13 g/g, still the highest among all conditions tested.

3.5. The CPC–BTB Method Is Suitable for Interference from the Culture Medium

The surfactin quantification presented so far in this work has been measured using HPLC, as described in the Methods section. In an attempt to increase the throughput during the study, we tested the colorimetric CPC–BTB method for surfactin quantification [35]. A comparison between the CPC–BTB and the HPLC methods for culture supernatants from different strains in the same medium (Figure 5A) and different media for the same strain (Figure 5B) showed a large discrepancy. The colorimetric method only agreed with the HPLC for the strains CS01 and D6S01 cultivated in LB enriched with glucose 1% (m/v) for 28 h. For all other strains and conditions tested, we found discrepancies up to 310%. The CPC–BTB method seems to suffer interference from the medium composition, including

compounds generated during microbial growth. Therefore, it is not a reliable method for absolute surfactin measurement nor for relative comparison.

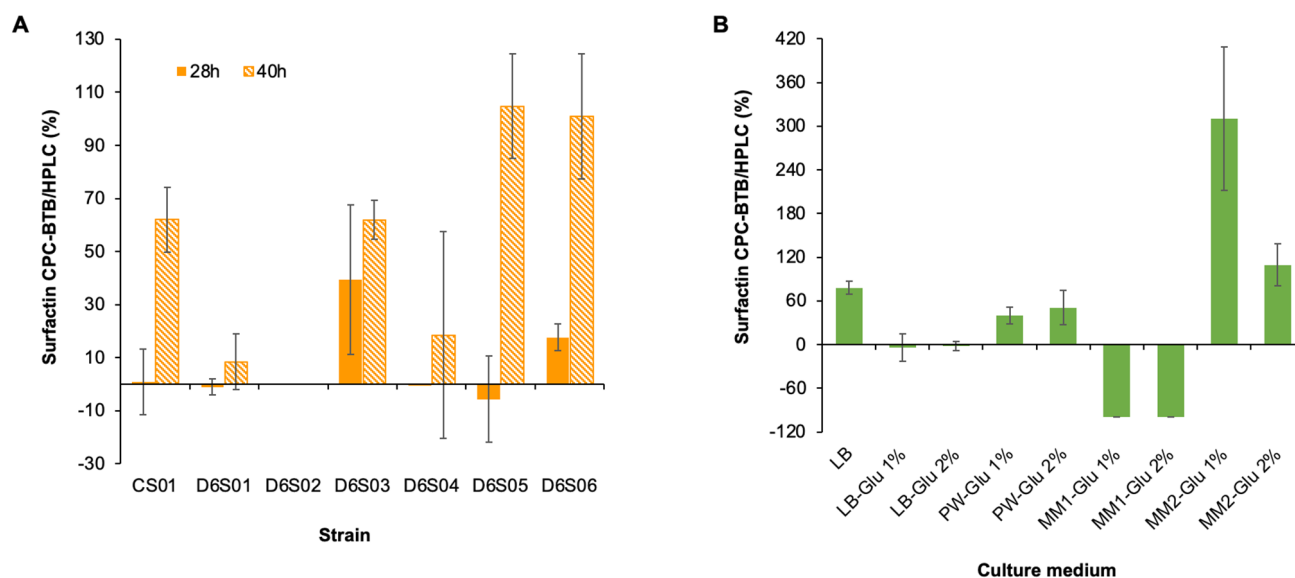


Figure 5. Comparison of quantification methods for surfactin produced using different strains (A) and different media (B). *B. subtilis* strains in (A) were cultivated in medium LB enriched with 1% (*m/v*) glucose. Strain D6S01 was cultivated in different media in (B). Data are presented as mean $\pm$ SD, *n* = 3.

4. Discussion

Genome-reduced microbes are simplified cell factories, with stable genomes and lower maintenance energy requirements compared to their parental strains [1]. Therefore, they are attractive for engineering metabolite-producing strains for industrial scale. The highly genome-reduced strain PG10, which lacks 36% of the original genome, has been demonstrated to be an efficient cell factory for proteins considered difficult to produce. The strain seems to have a higher translational efficiency than the parental strain 168, which is speculated to be a consequence of the decreased number of translatable mRNAs in the strain [51]. Indeed, basal processes such as DNA replication, transcription, and translation together require 47.5% of the resources of the translation apparatus [1]. Any relief in that cost due to genome reduction can potentially make the remaining cellular processes more efficient.

We compared the strains *B. subtilis* 168 and its genome-reduced variant $\Delta 6$ for surfactin production. Despite the reduction, $\Delta 6$ keeps all the required genes for surfactin biosynthesis. Indeed, the genome-reduced strain is more efficient for surfactin biosynthesis, yielding 0.95 g g^{-1} of biomass and 0.15 g g^{-1} of sugar using LB, and 0.13 g g^{-1} of glucose (excluding LB-supported surfactin production). The latter represents 29.5% of the maximum theoretical yield from glucose (0.44 g/g) [25]. This is a better performance than achieved for the further reduced genome strain IIG-Bs20-5-1, which yielded 0.87 g g^{-1} of biomass and 0.10 g g^{-1} of glucose [4]. The strain IIG-Bs20-5-1 has a 13% genome reduction in size, with further prophages and antibiotic biosynthesis genes deleted, along with protease and sporulation-related genes; none of these genes is directly related to surfactin biosynthesis [52]. Although high yields for $Y_{P/S}$ and $Y_{P/X}$ have been achieved for $\Delta 6$, the volumetric production is still far from the best engineered strains. A systematic engineering approach increased the production from 0.4 to 12.8 g L^{-1} using *B. subtilis* 168 *sfp*⁺ [25]. However, looking from the starting point, the first step of reversing the *sfp* mutation resulted in a much higher surfactin production from the $\Delta 6$ (1.2 g L^{-1}).

In an attempt to improve the $\Delta 6$ strain production capacity, we replaced the *surfA* promoter with a strong constitutive promoter, which was highly deleterious to the surfactin yields. Combining the wild-type promoter with another autoinduced promoter was less prejudicial, but still worse than P_{surfA} alone. A previous study reported that replacing P_{surfA} with a constitutive promoter only benefited a weak surfactin-producer strain [39]. Similarly, another study tested eight different promoters to replace P_{surfA} in a weak surfactin producer and found that three of them enhanced the surfactin biosynthesis [53].

Unable to improve surfactin biosynthesis through promoter engineering, we turned our attention to the regulators of P_{surfA} . Deletion of *codY*, a P_{surfA} repressor, has been demonstrated to increase surfactin production in different strains of *B. subtilis*, including 168 [25,54–56]. However, we could not reproduce these gains in the $\Delta 6$ strain. The other target we approached was *comA*, a P_{surfA} activator. Two other studies indicated that overexpression of this regulator is beneficial to surfactin biosynthesis [25,56]. However, $\Delta 6$ did not yield higher surfactin production after this edition. Genome reduction affects the entire cell metabolism, including carbon catabolism and amino acid biosynthesis [1]. Therefore, genome-reduced strains may not respond to genome editing in the same way as their parental strain.

Finally, we tested different culture media for surfactin production. Compared to commonly used media for production, LB enriched with glucose supported much higher surfactin yields. Interestingly, LB provides an organic source for nitrogen, but the literature correlates inorganic nitrogen, especially nitrate, with increased surfactin biosynthesis [45]. This different response to the nitrogen source reinforces our claim that the genome-reduced strain has significant differences in its basal metabolism that are not easily deduced from its genetic background, resulting from the deletions.

The genome-reduced *B. subtilis* $\Delta 6$ strain is a compelling chassis for surfactin production, yielding a C15-rich mixture of surfactins with potential application in formulations requiring high antibacterial activity [57]. This study not only elucidates its potential in surfactin biosynthesis but also highlights the significant influence of the genetic background on both product yield and the strain's responsiveness to regulatory processes.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation11090508/s1>, File S1: Supplementary material (nucleic acid sequences as a Microsoft Word file); File S2: Supplementary data (raw data as a Microsoft Excel file).

Author Contributions: Conceptualization: D.B.P. and V.F.B.Z.; Investigation: V.F.B.Z., F.d.O.B. and L.F.T.; Analysis: V.F.B.Z. and G.E.K.; Resources: F.P.P. and A.C.d.S.M.; Data curation: V.F.B.Z. and D.B.P.; Writing—original draft preparation: V.F.B.Z. and D.B.P.; Writing—review and editing: V.F.B.Z. and D.B.P.; Supervision: D.B.P.; Project administration: D.B.P.; Funding acquisition: D.B.P. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable.

Data Availability Statement: All data generated in this study are provided in Appendix A and Supplementary Materials.

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

Appendix A

Appendix A.1. gRNA Sequences

Each gRNA is composed of a guide sequence (GS), the spacer, and the scaffold sequence. The spacer and the scaffold sequences are provided by the pJOE8999 plasmid [29].

Table A1. gRNA sequences used in this study.

ID	Target	Sequence (5'-3')	Reference
GS1	<i>sfp</i>	AAAGCTTTATCAAACAAGGA	This study
GS2	P _{srfA}	AAAGATTGAACGCAGCAGTT	This study
GS3	R6	AATAGGTAAGGATAAAAGAGA	This study
GS4	<i>codY</i>	ACCCAAGGAGTTTCCCTCTG	This study
GS5	<i>comA</i>	ACTAGTGATTGATGACCATC	This study

Appendix A.2. Donor DNA Sequences

Table A2. DNA parts used in this study.

DNA Part *	Description	Reference
S0-R6	Transcription autoinduction device	[40]
TP2	Constitutive dual promoter	[39]
RBS_{opt}	Optimized RBS	[39]
P_{srfA}	Wild-type inducible promoter, amplified from the <i>B. subtilis</i> Δ6 genome	This study
RBS_{wt}	Wild-type RBS associated with P _{srfA} , amplified from the <i>B. subtilis</i> Δ6 genome	This study
DS1	Donor DNA 1 for <i>sfp</i> point mutations	This study
DS2	Donor DNA 2 for substitution of P _{srfA} for S0-R6 to control <i>srfA</i> operon expression	This study
DS3	Donor DNA 3 for substitution of P _{srfA} -RBS _{wt} for TP2-RBS _{opt} to control <i>srfA</i> operon expression	This study
DS4	Donor DNA 4 for insertion of P _{srfA} between S0 and R6 to control <i>srfA</i> operon expression	This study
DS5	Donor DNA 5 for <i>codY</i> deletion	This study
DS6	Donor DNA 6 for insertion of P _{srfA} -RBS _{wt} to control <i>comA</i> expression	This study

* DNA sequences for all donor sequences (DS) are presented in the Supplementary Materials.

Appendix A.3. Culture Medium Optimization: Reducing Sugars Quantification

Table A3. Sugar consumption and surfactin production by strain D6S01 in different media.

Medium	Initial Reducing Sugars (g L ⁻¹)	Final Reducing Sugars (g L ⁻¹)	Consumed Reducing Sugars (g L ⁻¹)	Surfactin (g L ⁻¹)
LB	0.16 ± 0.00	0.11 ± 0.02	0.04 ± 0.01	0.20 ± 0.01
LB-Glu 1%	9.26 ± 0.03	0.28 ± 0.05	7.82 ± 0.14	1.19 ± 0.07
LB-Glu 2%	26.09 ± 1.20	7.29 ± 0.45	16.47 ± 1.06	1.47 ± 0.14
PW-Glu 1%	7.83 ± 0.17	0.31 ± 0.13	6.50 ± 0.36	0.37 ± 0.01
PW-Glu 2%	23.23 ± 2.18	11.71 ± 2.30	10.82 ± 1.76	0.35 ± 0.02
MM1-Glu 1%	8.08 ± 0.04	0.54 ± 0.24	6.96 ± 0.02	0.05 ± 0.03
MM1-Glu 2%	23.76 ± 0.57	6.68 ± 0.94	17.08 ± 0.94	0.14 ± 0.02
MM2-Glu 1%	8.42 ± 0.32	0.07 ± 0.01	8.32 ± 0.04	0.12 ± 0.03
MM2-Glu 2%	24.30 ± 0.89	0.07 ± 0.02	21.79 ± 1.01	0.23 ± 0.03

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