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ENGINEERING REGULATORY PROCESSES OF SUFACTIN BIOSYNTHESIS IN A GENOME REDUCED *Bacillus subtilis*

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ABSTRACT

Surfactin is a potent biosurfactant naturally produced by *Bacillus* spp. It can be applied in several areas of industry due to its high surface activity and biodegradability, low toxicity and antimicrobial properties. Surfactin is a cyclic lipopeptide synthetized by four non-ribosomal peptide synthetases (NRPS), which are encoded in the *srfA* operon. Transcription of the *srfA* operon is regulated directly by the quorum sensing system in *B. subtilis* and can be repressed by some global regulators. Protein ComA acts as a transcription activator when phosphorylated, and the global regulator CodY is one of the main repressors. The goal of this study was to evaluate the effect of editing these two regulators on the surfactin production and bacterial growth. Here, three genomic editions were introduced into the genome reduced *B. subtilis* 449 strain: *codY* deletion, autoinducible overexpression of *comA* through the promoter P_{srfA}, and the constitutive overexpression of *comA* through the P_{veg} promoter. The double edited strain Δ*codY*-P_{srfA}_*comA* was the best producer with 1.8-fold increase in surfactin production and no significant growth impairment compared to the parental strain.

Keywords: Surfactin. *Bacillus subtilis*. Genome editing. Gene expression regulation.

1 INTRODUCTION

Surfactin is a cyclic lipopeptide, composed of a peptide ring of seven amino acids (glutamate, leucine, aspartate and valine) linked to a 9- to 19-carbon β-hydroxy fatty acid chain, produced manly by *Bacillus* spp [1]. This biosurfactant attracts attention due to its excellent surface activity, antimicrobial properties, biodegradability, low toxicity, high temperature stability and salt tolerance [2,3]. These properties grant surfactin a wide range of potential applications in health and food industries as well as in oil recovery and bioremediation of contaminated areas [1]. However, high production costs and low yields prevent industrial scale production of surfactin [2].

Biosynthesis of surfactin in *Bacillus subtilis* is catalyzed by four non-ribosomal peptide synthetases (NRPS), SrfAA, SrfAB, SrfAC and SrfAD, which form a linear array of seven modules and each one is responsible for adding one amino acid residue to the fatty acid chain [2]. These NRPS are encoded by the *srfA* operon, regulated by the inducible promoter P_{srfA}, that responds to the quorum sensing system of *B. subtilis* [1]. The quorum sensing system triggers a series of events and ultimately phosphorylated ComA binds to the promoter P_{srfA}, contributing with SigA for activation of the transcription. Concomitantly, expression of *srfA* operon is repressed by several molecules, one of them is protein CodY, a global regulator that binds directly to the P_{srfA} promoter and blocks gene transcription. CodY is activated by high cellular concentrations of GTP and branched chain amino acids [1,2]. Studies have shown that *codY* knockout [4,5,6] and *comA* overexpression [4] can improve surfactin biosynthesis in *B. subtilis* [4].

Here, the CRISPR-Cas9 system was used for genome editing of *B. subtilis* 449, a genome reduced laboratorial strain previously converted into surfactin producer through a point mutation on the *sfp* gene. Three editions were introduced into *B. subtilis* 449 genome in order to increase surfactin production: first, the *codY* gene was deleted to engineer the 449(Δ*codY*) strain; subsequently, the *comA* gene was overexpressed either using the strong autoinducible promoter P_{srfA} or the strong constitutive promoter P_{veg}, and 449(Δ*codY*-P_{srfA}_*comA*) and 449(Δ*codY*-P_{veg}_*comA*) strains were obtained. Bacterial growth and surfactin production were evaluated for the novel strains.

2 MATERIAL & METHODS

Vectors derived from pJOE8999 [7] were used to introduce the CRISPR-Cas9 system into the *B. subtilis* strains. Vectors pJOE8999(Δ*codY*), pJOE8999(P_{srfA}-*comA*) and pJOE8999(P_{veg}-*comA*) were constructed and propagated in *E. coli* TOP10, following stablished Molecular Biology protocols. *B. subtilis* 449 was transformed with pJOE8999(Δ*codY*) to generate the 449(Δ*codY*) strain, which was further engineered, in parallel, with pJOE8999(PsrfA-*comA*) and pJOE8999(P_{veg}-*comA*) to generate 449(Δ*codY*-P_{srfA}_*comA*) and 449(Δ*codY*-P_{veg}_*comA*) strains. Genome editing with CRISPR-Cas9 system and plasmid loss followed published protocol [7]. Genome editions were confirmed by Sanger sequencing.

The four *B. subtilis* strains were cultivated in biological triplicates in test tubes containing 6 mL of PW medium [8] with 60 g/L of glucose as carbon source. The cultures were incubated for 36 h at 37°C and 220 rpm. Samples were collected at 12 h, 24 h, and 36 h for evaluation of growth through optical density at 600 nm (OD_{600}) and surfactin production with the CPC–BTB method [8].

3 RESULTS & DISCUSSION

The results did not shown difference on cell growth (Fig. 1-A) comparing *B. subtilis* strains 449, 449(Δ*codY*), 449(Δ*codY*-P_{srfA}_*comA*), and 449(Δ*codY*-P_{veg}_*comA*), none of the genome editions seems to have significantly impaired cell growth. From all four strains, 449(ΔcodY-P_{srfA}_comA) strain is the only one to show an increase in surfactin production (Fig. 1-B), reaching 961,4 ± 41,5 mg/L of surfactin after 36 h, an increase of approximately 1.8-fold compared to the other strains, which produced on average 527,1 ± 76,7 mg/L. Similarly, 449(ΔcodY-P_{srfA}_comA) has the highest cellular productivity (Fig. 1-C), where surfactin production is normalized by OD_{600}.

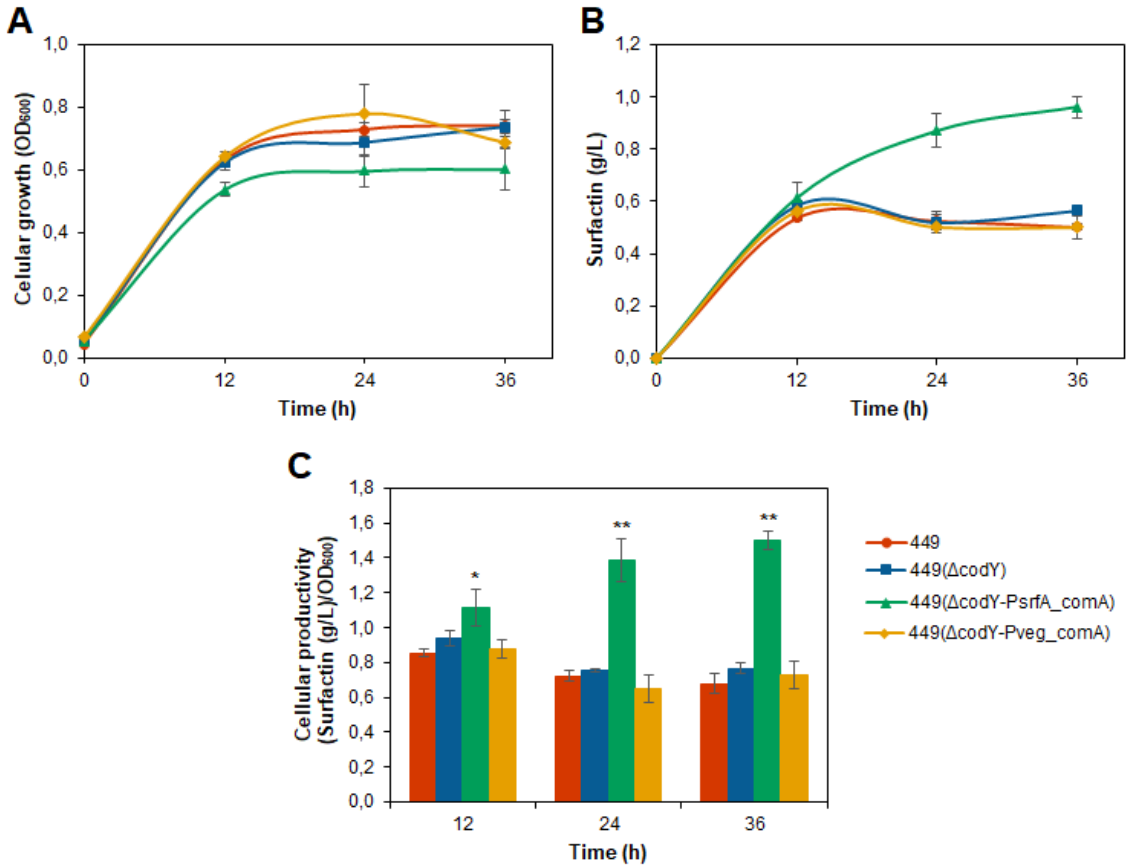

Figure 1. *B. subtilis* 449 and derivated strains cultivation in test tubes. (A) Cellular growth, (B) surfactin production, and (C) cellular productivity.

Previous study have shown that the expression vector carring an extra copy of *comA* under control of promoter P_{grac} resulted in a 1.4-fold increase on surfactin production compared to the parental strain [4], and another study demonstrated an increase of 1.1-fold on surfactin prodution with the knockout of *codY* [6].

Here, the genome editions of *codY* deletion associated with autoinducible overexpression of *comA* through strong promoter P_{srfA} increased surfactin prodution in 1.8-fold for the 449(Δ*codY*-P_{srfA}_*comA*) strain compared to parental strain 449. As expected, *comA* autoinduced expression and, consequently, surfactin prodution were induced mainly in stationary growth phase, since promoter P_{srfA} responds to quorum sensing system and requires a high cellular concentration to activate gene transcription. Moreover, it is believed that deletion of *codY* was important to remove the main repressor of *srfA* operon trancription and repressor of branched chain amino acids biosynthesis, some of which are surfactin precursors, this may have contributed for an increase in surfactin production.

It was expected that surfactin production would increase and be induced earlier in the growth curve for the 449(Δ*codY*-P_{veg}_*comA*) strain, and that it could cause cellular growth impairment due to the metabolic burden caused to the cell in consequence of the constitutive synthesis of ComA. However, neither of these results were observed.

4 CONCLUSION

The results show that the association of *codY* deletion and autoinducible expression of *comA* under control of the strong promoter P_{srfA} increases surfactin production in the 449(ΔcodY-P_{srfA}_*comA*) strain. Genome reduced strains are still new and few data are available on their cellular physiology. More tests with optimized culture conditions, such as culture medium, dissolved oxygen and pH, are necessary to reach the full potential of this strain for surfactin production.

REFERENCES

1 QI, X., LIU, W., HE, X., DU, C. 2023. Arch. Microbiol. 205. 313.
2 HU, F., LIU, Y., LI, S. 2019. Microb. Cell. Fact. 18. 42.
3 XIA, L., WEN, J. 2023. Crit. Rev. Biotechnol. 43 (7). 1111-1128.
4 SUN, J., LIU, Y., LIN, F., LU, Z., LU, Y. 2021. J. Appl. Microbiol. 131 (3). 1289-1304.
5 COUTTE, F., NIEHREN, J., DHALI, D., JOHN, M., VERSARI, C., JACQUES, P. 2015. Biotechnol. J. 10. 1216-1234.
6 WU, Q., ZHI, Y., XU, Y. 2019. Metab. Eng. 52. 87-97.
7 ALTENBUCHNER, J. 2016. Appl. Environ. Microbiol. 82. 5421–5427.
8 YANG, H., YU, H., SHEN, Z. 2015. J. Ind. Microbiol. Biotechnol. 42 (8). 1139-1147.

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