Substitution of the Native *srfA* Promoter by Constitutive P_{veg} in Two *B. subtilis* Strains and Evaluation of the Effect on Surfactin Production

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Abstract

The genetic enhancement of Surfactin production increasingly gained attention in the last years, since relatively low product yields limit the industrial application of this biosurfactant. The natural quorum sensing regulation of the *srfA* operon (coding for the Surfactin synthetase) can reasonably be assumed to be the bottleneck of Surfactin synthesis. Therefore, the replacement of the naturally quorum sensing regulated, and herewith cell density dependent, promoter P_{srfA} against the *Bacillus subtilis* endogenous and constitutive promoter P_{veg} was hypothesized to generally enhance Surfactin yields. The markerless promoter replacement was conducted in the two *B. subtilis* Surfactin producer strains 3A38 and DSM 10^T. The promoter substitution led to an enhancement of Surfactin concentrations in the producer strain 3A38, initially producing only minor amounts of Surfactin (0.07 g/L increased to 0.26 g/L). In contrast, promoter exchange in *B. subtilis* DSM 10^T (wild type strain producing 0.62 g/L Surfactin) did not achieve an enhancement of Surfactin concentrations (detrimental reduction to 0.04 g/L). These findings implicate that Surfactin synthesis is differently regulated in minor and strong Surfactin producer strains. The hypothesized general enhancement of Surfactin yields after substitution of the native promoter was therefore not confirmed.

Keywords Surfactin, *srfA* operon, promoter exchange, *Bacillus subtilis,* quorum sensing, biosurfactant

Surfactin is one of the most promising biosurfactants due to its diverse possible employments and strong surface activity (Peypoux et al. 1999). The industrial application of Surfactin is limited which mostly originates from low product yields and complex process set-ups to handle the severe foaming during cultivation. The continuous improvement of fermentation processes may eventually solve difficulties due to foaming. However, to achieve higher product yields it will also be necessary to establish genetically modified Surfactin producer strains which could significantly enhance the productivity per cell.

The biosynthesis is regulated by the quorum sensing system of *B. subtilis* which crosslinks Surfactin synthesis, competence and sporulation in a complex network of pheromones and pleiotropic regulators (Soberón-Chávez and Jacques 2011). *B. subtilis* continuously secretes ComX which accumulates in the culture broth. Upon reaching a certain cell density at the onset of stationary phase, the membranous histidine kinase ComP is activated and phosphorylates the transcription factor ComA (two-component system ComP/ComA). Activated ComA thereafter induces the transcription of the *srfA* operon (Nakano et al. 1991), which contains the four open reading frames *srfA-A*, *srfA-B*, *srfA-C* and *srfA-D*. However, the concentration of activated ComA inside the cell is strongly influenced by several regulators belonging to the Rap and Phr peptide family, and transcription of the *srfA* operon is also affected by important regulators like CodY, DegU and AbrB (Soberón-Chávez and Jacques 2011). As a consequence of quorum sensing control the initiation of Surfactin synthesis is dependent on cell density which prevents a constant biosurfactant production and

76 possibly limits overall Surfactin yields in contrast to expression from a constitutive
77 promoter.
78 Two earlier studies have investigated Surfactin yields after promoter exchange in front
79 of the s*rfA* operon (Coutte et al. 2010; Sun et al. 2009). The studies were conducted
80 with different Surfactin producer strains and substitute promoter sequences and
81 provided inconsistent results. Sun et al. (2009) reported 10-fold enhanced Surfactin
82 yields after replacement of P_{srfA} with P_{spac}, an IPTG-inducible hybrid promoter
83 originating from *B. subtilis* bacteriophage SP01 and *E. coli lac* operon. In contrast,
84 Coutte et al. (2010) obtained lower Surfactin concentrations after P_{srfA} exchange
85 against P_{repU}, a constitutive promoter originating from the replication gene *repU* of
86 *Staphylococcus aureus* plasmid pUB110. These findings motivated us to analyze
87 promoter replacement in two different Surfactin producer strains, but using the same
88 promoter, P_{veg}. This is one of the strongest, constitutive promoters of *B. subtilis*, and
89 originates from the vegetative gene *veg* (Radeck et al. 2013; Lam et al. 1998).
90 The aim of this study was to first construct this markerless promoter exchange
91 upstream of the *srfA* operon in a modest and a strong Surfactin producer strain of
92 *B. subtilis.* The resulting strains should then be analyzed with regard to the Surfactin
93 yields before and after this substitution. Our initial hypothesis predicted a general
94 enhancement of Surfactin yields after decoupling the Surfactin synthesis from quorum
95 sensing control, based on a continuous transcription initiated by a constitutive
96 promoter. The shuttle-vector pMAD (Arnaud et al. 2004) was chosen for the purpose
97 of a markerless promoter exchange, as this vector allows an efficient allelic
98 replacement in gram-positive bacteria, introducing two flanks homologous to the
99 *Bacillus* gDNA. For the vector construction, three different DNA fragments had to be
100 designed and amplified. First, an upstream flank (700 bp) homologous to the region
101 upstream of the natural promoter P_{srfA} (including the gene *hxIR*, 362 bp), second, a

newly designed promoter region (including the sequence of *Bacillus* endogenous P_{veg}, followed by the native ribosome binding site of *srfA* with the native spacing to the *srfA* start codon, 186 bp), and third a flank homologous to the region downstream of the original P_{srfA} (part of the first open reading frame of the *srfA* operon: *srfA-A*, 700 bp). The separate fragments were fused to each other by overlap-extension PCRs, and the 1,486 bp DNA fragment was inserted into pMAD by ligation. Subsequently, the resulting vector, pMAD-Pveg-srfA, was incorporated by transformation into *B. subtilis* cells.

To compare the effect of promoter exchange on two strains with different Surfactin production levels, *B. subtilis* strains 3A38 and DSM 10^T were chosen. Strain 3A38, which exhibits enhanced capability for the uptake of exogenous DNA and originates from *B. subtilis* type strain NCIB 3610 (purchased from the BGSC, *Bacillus* Genetic Stock Center in Ohio, USA; Konkol et al. 2013) produces only small amounts of Surfactin, whereas DSM 10^T, a wild-type and *B. subtilis* type strain (purchased from DSMZ, *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*, Braunschweig, Germany) is a naturally strong Surfactin producer. P_{veg} was integrated upstream of the *srfA* operon (with concomitant loss of the original promoter P_{srfA}) in both strains, according the published procedure (Arnaud et al. 2004). This approach resulted in the transformed strains JWSurf2, originating from *B. subtilis* 3A38, and JWSurf3, descending from *B. subtilis* DSM 10^T. The loss of P_{srfA} and successful integration of P_{veg} was verified by sequencing the upstream region of the *srfA* operon (Figure 1, see supplemental material for original and modified DNA sequences).

To analyze the Surfactin production before and after promoter replacement, shake flask cultivations were conducted. The time courses of cell dry weight (CDW) and Surfactin concentration (HPLC analytic as described in Willenbacher et al. 2014) are displayed in Figure 2. Results were reproducible in two independent experiments, each

time employing two time-displaced inoculated shake flasks per strain for continuous display of CDW and Surfactin concentrations. All strains exhibited a rather similar cell growth. Maximal values differed between 1.5 g/L and 2.3 g/L CDW. In contrast, maximal Surfactin concentrations varied greatly between the different strains. Wild-type *B. subtilis* 3A38 produced 0.07 g/L Surfactin whereas *B. subtilis* DSM 10^T was able to produce 0.62 g/L Surfactin. Interestingly, JWSurf2 (descending from *B. subtilis* 3A38) achieved a maximal value of 0.26 g/L Surfactin during cultivation, showing that introduction of the constitutive P_{veg} promoter did indeed strongly increase Surfactin production in this strain. In contrast, JWSurf3 (originating from *B. subtilis* DSM 10^T) reached a maximal value of 0.04 g/L Surfactin, which was considerably lower than the product concentration in its isogenic parent strain. In summary, promoter exchange did have significantly different effects on Surfactin production of the *B. subtilis* strains 3A38 and DSM 10^T, although the strains are genetically closely related and the sequences of both the native and the introduced promoters were identical.

Our data clearly showed that exchanging the native *srfA* promoter for the constitutive P_{veg} could significantly increase Surfactin production in a strain with only low native production of the compound. This is in good agreement with the previous study by Sun et al. (2009), who achieved a similar 10-fold increased production by exchange of P_{srfA} against P_{spac} in a weak Surfactin producer (Table 1). Interestingly, we found that introduction of the same promoter into an already strong Surfactin producer had the opposite effect, drastically reducing Surfactin production. This is similar to the results reported by Coutte et al. (2010) where the introduction of constitutive P_{repU} also led to a reduction of Surfactin concentrations (Table 1). These findings are astonishing since P_{repU} is evidently a strong promoter that enhanced Mycosutilin and Iturin yields after replacement of the natural promoters in front of the corresponding operons (Leclère et al. 2005; Tsuge et al. 2001). The ambiguous results during the current study indicate

that natural strong Surfactin producer strains regulate Surfactin biosynthesis in a different (and obviously more efficient) way compared to low Surfactin producer strains. Furthermore, it can be concluded that the introduction of a constitutive promoter in strong Surfactin producer strains disrupts the natural delicate adjustments of quorum sensing regulated Surfactin biosynthesis. As a consequence, Surfactin concentrations are significantly reduced after introduction of a constitutive promoter that circumvented the natural regulation. Our results strongly suggest that the regulatory cascades controlling Surfactin biosynthesis need to be studied in much greater depth. Especially regulation differences between strong and minor Surfactin producer strains need to be better understood to facilitate the establishment of a Surfactin overproducing strain. Finally, further aspects, such as translation, protein folding, and secretion of Surfactin, should also be taken to account. These steps could also strongly influence the final concentration of Surfactin and are therefore important for the final identification of the Surfactin synthesis bottleneck.

In summary, the current study indicates that Surfactin synthesis is differently regulated in strong and minor Surfactin producer strains or at least differently integrated into the quorum sensing network. In minor Surfactin producer strains it is evidently useful to substitute the native promoter for a strong promoter to enhance Surfactin yields. In contrast, *Bacillus* strains producing rather high amounts of Surfactin will not further increase Surfactin yields after introduction of a strong constitutive promoter. In fact, this modification drastically reduced Surfactin productivity. Further investigations of the cascades regulating Surfactin biosynthesis will possibly facilitate identifying the bottleneck of Surfactin biosynthesis. Such studies will be key to ultimately overcoming the present limitations in Surfactin yields.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JW designed and cloned all vectors for transformation, created the strain *B. subtilis* JWSurf2, supervised the Master thesis of TMo, collected all data, created the graphs and figure and drafted this manuscript. TMo conducted her Master thesis under the supervision of JW, created the strain *B. subtilis* JWSurf3 and contributed the data of the shake flask experiments. MH contributed to the strategy of the experiments and designed the graphical abstract. SG and TMa provided the shuttle-vector pMAD and majorly contributed to the conception of cloning. CS and RH substantially contributed to conception and design of the conducted experiments. All authors read and approved the final version of this manuscript.

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Figure legends

Figure 1 Scheme of the promoter exchange upstream of the *srfA* operon on *B. subtilis* gDNA. **gDNA *B. subtilis* wild-type** Illustrated is the original composition upstream of the *srfA* operon which exhibits the native promoter P_{srfA} (388 bp). This quorum sensing regulated promoter displays dyad symmetries (responsible for ComA binding), -35 and -10 box and a large DNA sequence for binding of transcription regulators (277 bp). This is followed by the RBS and small gap (9 bp) upstream of the *srfA-A* start codon. Upstream of the native promoter is the gene *hxIR* located, featuring a rho-independent transcription terminator (142 bp upstream of P_{srfA}). **gDNA of JWSurf2 and JWSurf3** The region upstream of *srfA-A* exhibits after transformation with pMAD-Pveg-srfA and

markerless promoter exchange the constitutive promoter P_{veg}. The new promoter is substantially shorter (66 bp) and exhibits -35 and -10 box for recognition of $E\sigma^A$ RNA polymerases. The sequence upstream of P_{srfA} and the original RBS remained unmodified.

Figure 2 Time courses of shake flask cultivations comparing original and modified *B. subtilis* strains. Illustrated are the CDW (A, g/L) and Surfactin concentrations (B, g/L) over time. The results of the original strains *B. subtilis* 3A38 and DSM 10^T are indicated as *black square* and *black dot*, respectively, whereas results of *B. subtilis* JWSurf2 and JWSurf3 are illustrated in *grey squares* and *white dots*. Results were reproducible in two independent experiments. The figure shows data from the first experiment, employing two time-displaced inoculated shake flasks per strain to obtain a continuous presentation of CDW and Surfactin concentrations.

Supplementary Material: DNA sequences upstream of the *srfA* operon before and after transformation

283 **Table 1** Comparison of the Surfactin concentrations after promoter exchange in
284 various *B. subtilis* strains, as reported by Sun et al. (2009), Coutte et al (2010) and the
285 current study.

B. subtilis strain	Promoter	Max. concentration	Study
fmbR	P_{srfA}	0.38 g/L	Sun et al. 2009
fmbR-1	P_{spac}	3.87 g/L	
BBG111	P_{srfA}	1.50 g/L	Coutte et al. 2010
BBG113	P_{repU}	1.21 g/L	
3A38	P_{srfA}	0.07 g/L	This study
JWSurf2	P_{veg}	0.26 g/L	
DSM 10^{T}	P_{srfA}	0.62 g/L	
JWSurf3	P_{veg}	0.04 g/L	

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