Substitution of the Native srfA Promoter by Constitutive $P_{\text{veg}}$ 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
possibly limits overall Surfactin yields in contrast to expression from a constitutive promoter.

Two earlier studies have investigated Surfactin yields after promoter exchange in front of the srfA operon (Coutte et al. 2010; Sun et al. 2009). The studies were conducted with different Surfactin producer strains and substitute promoter sequences and provided inconsistent results. Sun et al. (2009) reported 10-fold enhanced Surfactin yields after replacement of \( P_{srfA} \) with \( P_{spac} \), an IPTG-inducible hybrid promoter originating from \( B. subtilis \) bacteriophage SP01 and \( E. coli \) lac operon. In contrast, Coutte et al. (2010) obtained lower Surfactin concentrations after \( P_{srfA} \) exchange against \( P_{repU} \), a constitutive promoter originating from the replication gene \( repU \) of \( Staphylococcus aureus \) plasmid pUB110. These findings motivated us to analyze promoter replacement in two different Surfactin producer strains, but using the same promoter, \( P_{veg} \). This is one of the strongest, constitutive promoters of \( B. subtilis \), and originates from the vegetative gene \( veg \) (Radeck et al. 2013; Lam et al. 1998).

The aim of this study was to first construct this markerless promoter exchange upstream of the srfA operon in a modest and a strong Surfactin producer strain of \( B. subtilis \). The resulting strains should then be analyzed with regard to the Surfactin yields before and after this substitution. Our initial hypothesis predicted a general enhancement of Surfactin yields after decoupling the Surfactin synthesis from quorum sensing control, based on a continuous transcription initiated by a constitutive promoter. The shuttle-vector pMAD (Arnaud et al. 2004) was chosen for the purpose of a markerless promoter exchange, as this vector allows an efficient allelic replacement in gram-positive bacteria, introducing two flanks homologous to the Bacillus gDNA. For the vector construction, three different DNA fragments had to be designed and amplified. First, an upstream flank (700 bp) homologous to the region upstream of the natural promoter \( P_{srfA} \) (including the gene \( hxlR \), 362 bp), second, a
newly designed promoter region (including the sequence of *Bacillus* endogenous *Pveg*, 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 *Psrfl* (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. *Pveg* was integrated upstream of the *srfA* operon (with concomitant loss of the original promoter *Psrfl*) 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 *Psrfl* and successful integration of *Pveg* 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\(^\top\) 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\(^\top\)) 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\(^\top\), 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 \(P_{spa}\) 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 Mycosubtilin 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 $\text{E}\sigma^\text{A}$ 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$^\text{T}$ 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
Table 1 Comparison of the Surfactin concentrations after promoter exchange in various *B. subtilis* strains, as reported by Sun et al. (2009), Coutte et al (2010) and the current study.

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