Citation for published version:
Willenbacher, J, Mohr, T, Henkel, M, Gebhard, S, Mascher, T, Syldatk, C & Hausmann, R 2016, 'Substitution of the native *srfA* promoter by constitutive P*veg* in two *B. subtilis* strains and evaluation of the effect on surfactin production', *Journal of Biotechnology*, vol. 224, pp. 14-17. https://doi.org/10.1016/j.jbiotec.2016.03.002

DOI:
10.1016/j.jbiotec.2016.03.002

Publication date:
2016

Document Version
Peer reviewed version

Link to publication

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Download date: 08. Jul. 2024

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

3

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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.

51 **Keywords** Surfactin, *srfA* operon, promoter exchange, *Bacillus subtilis,* quorum
52 sensing, biosurfactant
53
54 Surfactin is one of the most promising biosurfactants due to its diverse possible
55 employments and strong surface activity (Peypoux et al. 1999). The industrial
56 application of Surfactin is limited which mostly originates from low product yields and
57 complex process set-ups to handle the severe foaming during cultivation. The
58 continuous improvement of fermentation processes may eventually solve difficulties
59 due to foaming. However, to achieve higher product yields it will also be necessary to
60 establish genetically modified Surfactin producer strains which could significantly
61 enhance the productivity per cell.
62 The biosynthesis is regulated by the quorum sensing system of *B. subtilis* which
63 crosslinks Surfactin synthesis, competence and sporulation in a complex network of
64 pheromones and pleiotropic regulators (Soberón-Chávez and Jacques 2011).
65 *B. subtilis* continuously secretes ComX which accumulates in the culture broth. Upon
66 reaching a certain cell density at the onset of stationary phase, the membranous
67 histidine kinase ComP is activated and phosphorylates the transcription factor ComA
68 (two-component system ComP/ComA). Activated ComA thereafter induces the
69 transcription of the *srfA* operon (Nakano et al. 1991), which contains the four open
70 reading frames *srfA-A*, *srfA-B*, *srfA-C* and *srfA-D*. However, the concentration of
71 activated ComA inside the cell is strongly influenced by several regulators belonging
72 to the Rap and Phr peptide family, and transcription of the *srfA* operon is also affected
73 by important regulators like CodY, DegU and AbrB (Soberón-Chávez and Jacques
74 2011). As a consequence of quorum sensing control the initiation of Surfactin synthesis
75 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 *hxlR*, 362 bp), second, a

102 newly designed promoter region (including the sequence of *Bacillus* endogenous P_{veg},
103 followed by the native ribosome binding site of *srfA* with the native spacing to the *srfA*
104 start codon, 186 bp), and third a flank homologous to the region downstream of the
105 original P_{srfA} (part of the first open reading frame of the *srfA* operon: *srfA-A*, 700 bp).
106 The separate fragments were fused to each other by overlap-extension PCRs, and the
107 1,486 bp DNA fragment was inserted into pMAD by ligation. Subsequently, the
108 resulting vector, pMAD-Pveg-srfA, was incorporated by transformation into *B. subtilis*
109 cells.
110 To compare the effect of promoter exchange on two strains with different Surfactin
111 production levels, *B. subtilis* strains 3A38 and DSM 10^T were chosen. Strain 3A38,
112 which exhibits enhanced capability for the uptake of exogenous DNA and originates
113 from *B. subtilis* type strain NCIB 3610 (purchased from the BGSC, *Bacillus* Genetic
114 Stock Center in Ohio, USA; Konkol et al. 2013) produces only small amounts of
115 Surfactin, whereas DSM 10^T, a wild-type and *B. subtilis* type strain (purchased from
116 DSMZ, *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*,
117 Braunschweig, Germany) is a naturally strong Surfactin producer. P_{veg} was integrated
118 upstream of the *srfA* operon (with concomitant loss of the original promoter P_{srfA}) in
119 both strains, according the published procedure (Arnaud et al. 2004). This approach
120 resulted in the transformed strains JWSurf2, originating from *B. subtilis* 3A38, and
121 JWSurf3, descending from *B. subtilis* DSM 10^T. The loss of P_{srfA} and successful
122 integration of P_{veg} was verified by sequencing the upstream region of the *srfA* operon
123 (Figure 1, see supplemental material for original and modified DNA sequences).
124 To analyze the Surfactin production before and after promoter replacement, shake
125 flask cultivations were conducted. The time courses of cell dry weight (CDW) and
126 Surfactin concentration (HPLC analytic as described in Willenbacher et al. 2014) are
127 displayed in Figure 2. Results were reproducible in two independent experiments, each

128 time employing two time-displaced inoculated shake flasks per strain for continuous
129 display of CDW and Surfactin concentrations. All strains exhibited a rather similar cell
130 growth. Maximal values differed between 1.5 g/L and 2.3 g/L CDW. In contrast,
131 maximal Surfactin concentrations varied greatly between the different strains. Wild-
132 type *B. subtilis* 3A38 produced 0.07 g/L Surfactin whereas *B. subtilis* DSM 10^T was
133 able to produce 0.62 g/L Surfactin. Interestingly, JWSurf2 (descending from *B. subtilis*
134 3A38) achieved a maximal value of 0.26 g/L Surfactin during cultivation, showing that
135 introduction of the constitutive P_{veg} promoter did indeed strongly increase Surfactin
136 production in this strain. In contrast, JWSurf3 (originating from *B. subtilis* DSM 10^T)
137 reached a maximal value of 0.04 g/L Surfactin, which was considerably lower than the
138 product concentration in its isogenic parent strain. In summary, promoter exchange did
139 have significantly different effects on Surfactin production of the *B. subtilis* strains 3A38
140 and DSM 10^T, although the strains are genetically closely related and the sequences
141 of both the native and the introduced promoters were identical.
142 Our data clearly showed that exchanging the native *srfA* promoter for the constitutive
143 P_{veg} could significantly increase Surfactin production in a strain with only low native
144 production of the compound. This is in good agreement with the previous study by Sun
145 et al. (2009), who achieved a similar 10-fold increased production by exchange of P_{srfA}
146 against P_{spac} in a weak Surfactin producer (Table 1). Interestingly, we found that
147 introduction of the same promoter into an already strong Surfactin producer had the
148 opposite effect, drastically reducing Surfactin production. This is similar to the results
149 reported by Coutte et al. (2010) where the introduction of constitutive P_{repU} also led to
150 a reduction of Surfactin concentrations (Table 1). These findings are astonishing since
151 P_{repU} is evidently a strong promoter that enhanced Mycosutilin and Iturin yields after
152 replacement of the natural promoters in front of the corresponding operons (Leclère et
153 al. 2005; Tsuge et al. 2001). The ambiguous results during the current study indicate

154 that natural strong Surfactin producer strains regulate Surfactin biosynthesis in a
155 different (and obviously more efficient) way compared to low Surfactin producer
156 strains. Furthermore, it can be concluded that the introduction of a constitutive
157 promoter in strong Surfactin producer strains disrupts the natural delicate adjustments
158 of quorum sensing regulated Surfactin biosynthesis. As a consequence, Surfactin
159 concentrations are significantly reduced after introduction of a constitutive promoter
160 that circumvented the natural regulation. Our results strongly suggest that the
161 regulatory cascades controlling Surfactin biosynthesis need to be studied in much
162 greater depth. Especially regulation differences between strong and minor Surfactin
163 producer strains need to be better understood to facilitate the establishment of a
164 Surfactin overproducing strain. Finally, further aspects, such as translation, protein
165 folding, and secretion of Surfactin, should also be taken to account. These steps could
166 also strongly influence the final concentration of Surfactin and are therefore important
167 for the final identification of the Surfactin synthesis bottleneck.
168 In summary, the current study indicates that Surfactin synthesis is differently regulated
169 in strong and minor Surfactin producer strains or at least differently integrated into the
170 quorum sensing network. In minor Surfactin producer strains it is evidently useful to
171 substitute the native promoter for a strong promoter to enhance Surfactin yields. In
172 contrast, *Bacillus* strains producing rather high amounts of Surfactin will not further
173 increase Surfactin yields after introduction of a strong constitutive promoter. In fact,
174 this modification drastically reduced Surfactin productivity. Further investigations of the
175 cascades regulating Surfactin biosynthesis will possibly facilitate identifying the
176 bottleneck of Surfactin biosynthesis. Such studies will be key to ultimately overcoming
177 the present limitations in Surfactin yields.
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179

180 **Competing interests**

181 The authors declare that they have no competing interests.

182

183 **Authors' contributions**

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

193

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248 Figure legends

249 **Figure 1** Scheme of the promoter exchange upstream of the *srfA* operon on *B. subtilis*
250 gDNA. **gDNA *B. subtilis* wild-type** Illustrated is the original composition upstream of
251 the *srfA* operon which exhibits the native promoter P_{srfA} (388 bp). This quorum sensing
252 regulated promoter displays dyad symmetries (responsible for ComA binding), -35 and
253 -10 box and a large DNA sequence for binding of transcription regulators (277 bp).
254 This is followed by the RBS and small gap (9 bp) upstream of the *srfA-A* start codon.
255 Upstream of the native promoter is the gene *hxlR* located, featuring a rho-independent
256 transcription terminator (142 bp upstream of P_{srfA}). **gDNA of JWSurf2 and JWSurf3**
257 The region upstream of *srfA-A* exhibits after transformation with pMAD-Pveg-srfA and

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

262

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

271

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

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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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