Journal of Biotechnology 224 (2016) 14–17

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Short communication

Substitution of the native *srfA* promoter by constitutive P_{veg} in two *B. subtilis* strains and evaluation of the effect on Surfactin production

Judit Willenbacher[a,*], Teresa Mohr[a], Marius Henkel[b], Susanne Gebhard[c], Thorsten Mascher[d], Christoph Syldatk[a], Rudolf Hausmann[b]

[a] *Institute of Process Engineering in Life Sciences, Section II: Technical Biology, Karlsruhe Institute of Technology (KIT), Engler-Bunte-Ring 3, 76131 Karlsruhe, Germany*
[b] *Institute of Food Science and Biotechnology (150), Section Bioprocess Engineering (150k), University of Hohenheim, Garbenstr. 25, 70599 Stuttgart, Germany*
[c] *Milner Centre for Evolution, Department of Biology & Biochemistry, University of Bath, Claverton Down, BA2 7AY Bath, United Kingdom*
[d] *Institute of Microbiology, General Microbiology, Technische Universität Dresden, Zellescher Weg 20b, 01062 Dresden, Germany*

ARTICLE INFO

Article history:
Received 4 November 2015
Received in revised form 25 February 2016
Accepted 2 March 2016
Available online 4 March 2016

Keywords:
Surfactin
srfA operon
Promoter exchange
Bacillus subtilis
Quorum sensing
Biosurfactant

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.

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 *Bacillus 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

* Corresponding author.
E-mail addresses: judit.willenbacher@kit.edu (J. Willenbacher), teresa.mohr@student.kit.edu (T. Mohr), marius.henkel@uni-hohenheim.de (M. Henkel), s.gebhard@bath.ac.uk (S. Gebhard), thorsten.mascher@tu-dresden.de (T. Mascher), christoph.syldatk@kit.edu (C. Syldatk), rudolf.hausmann@uni-hohenheim.de (R. Hausmann).

http://dx.doi.org/10.1016/j.jbiotec.2016.03.002
0168-1656/

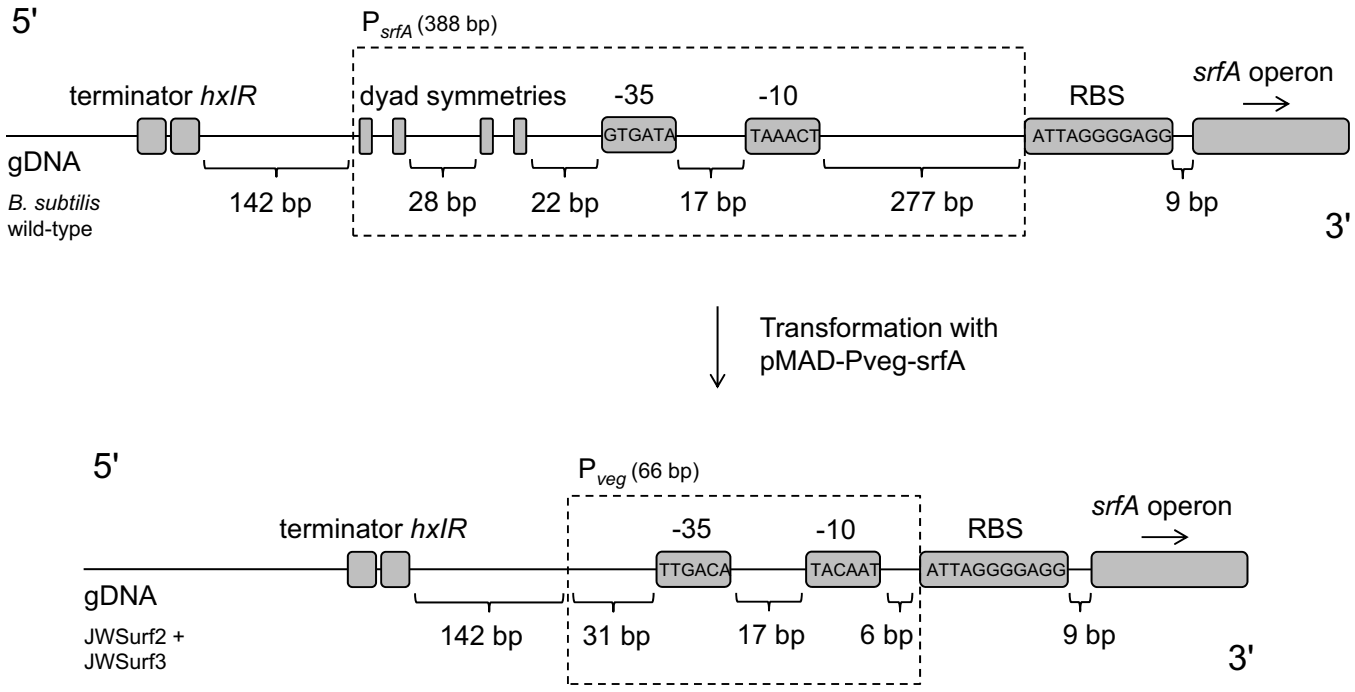

Fig. 1. Scheme of the promoter exchange upstream of the *srfA* operon on *B. subtilis* gDNA. **gDNA *B. subtilis* wild-type** Illustrated is the original composition upstream of the *srfA* operon which exhibits the native promoter P_{srfA} (388 bp). This quorum sensing regulated promoter displays dyad 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.

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 *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 1486 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 to 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 (Fig. 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 Fig. 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 concentra-