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### ► To cite this version:

Ariane Théâtre, Carolina Cano-Prieto, Marco Bartolini, Yoann Laurin, Magali Deleu, et al.. The surfactin-like lipopeptides from *Bacillus* spp.: natural biodiversity and synthetic biology for a broader application range. *Frontiers in Bioengineering and Biotechnology*, 2021, 10.3389/fbioe.2021.623701 . hal-03158419

**HAL Id: hal-03158419**

**<https://inria.hal.science/hal-03158419v1>**

Submitted on 3 Mar 2021

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**The surfactin-like lipopeptides from *Bacillus* spp.: natural biodiversity and synthetic biology for a broader application range**

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37 **Keywords:** surfactin, lipopeptide, *Bacillus* spp, biosurfactant, nonribosomal peptide  
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## 47 **Abstract**

48 Surfactin is a lipopeptide produced by several *Bacillus* species and identified for the first time in  
49 1969. At first, the biosynthesis of this remarkable biosurfactant was described in this review. The  
50 peptide moiety of the surfactin is synthesized using huge multienzymatic proteins called  
51 NonRibosomal Peptide Synthetases. This mechanism is responsible for the peptide biodiversity of the  
52 members of the surfactin family. In addition, on the fatty acid side, fifteen different isoforms (from  
53 C12 to C17) can be incorporated so increasing the number of the surfactin-like biomolecules. The  
54 review also highlights the last development in metabolic modelling and engineering and in synthetic  
55 biology to direct surfactin biosynthesis but also to generate novel derivatives. This large set of different  
56 biomolecules leads to a broad spectrum of physico-chemical properties and biological activities. The  
57 last parts of the review summarized the numerous studies related to the production processes  
58 optimization as well as the approaches developed to increase the surfactin productivity of *Bacillus* cells  
59 taking into account the different steps of its biosynthesis from gene transcription to surfactin  
60 degradation in the culture medium.

61

## 62 **1 Introduction**

63 Surfactin was firstly isolated in 1968 by Arima et al. as a new biologically active compound produced  
64 by *Bacillus* with surfactant activities, leading to its appellation. Its structure was elucidated firstly  
65 through its amino acid sequence (Kakinuma, Hori, et al., 1969) and then its fatty acid chain (Kakinuma,  
66 Sugino, et al., 1969). Surfactin was thus characterized as a lipopeptide composed of a heptapeptide  
67 with the following sequence: L-Glu1-L-Leu2-D-Leu3-L-Val4-L-Asp5-D-Leu6-L-Leu7, forming a  
68 lactone ring structure with a  $\beta$ -hydroxy fatty acid chain. Bearing both, a hydrophilic peptide portion  
69 and a lipophilic fatty acid chain, surfactin is of amphiphilic nature, leading to exceptional biosurfactant  
70 activities and diverse biological activities.

71 Surfactins are actually considered as a family of lipopeptides, sharing common structural traits with a  
72 great structural diversity due to the type of amino acids in the peptide chain and the length and isomery  
73 of the lipidic chain (Ongena et al., 2008). More than one thousand variants can potentially be naturally  
74 synthesized. This remarkable biodiversity mainly results from their biosynthetic mechanism.

75 This review is composed of 4 main sections. At first, a detailed description of the biosynthesis  
76 mechanisms will allow to understand origin of the biodiversity. Secondly, the diversity of variants will  
77 be seen, as well as its enhancement possibilities. Thirdly, the link between surfactin's varying structure  
78 and its properties and activities will be described. Lastly, the production process and its optimisation  
79 will be discussed, either for the whole surfactin family or for specific variants.

80

## 81 **2 Biosynthesis of surfactins**

### 82 **2.1 Peptide moiety**

83 Surfactins, as most of the cyclic lipopeptides (CLPs), are not synthesized ribosomally, but rather by  
84 specialized systems, termed non-ribosomal peptide synthetases (NRPSs). NRPSs are multimodular  
85 mega-enzymes, consisting of repeated modules. A module is defined as a portion of the NRPS that  
86 incorporates one specific amino acid into a peptide backbone. The order of the modules is usually co-  
87 linear with the product peptide sequence. Each module can in turn be dissected into the following three  
88 domains: the adenylation (A) domain, the thiolation (T) domain ("syn. peptidyl-carrier protein (PCP)-  
89 ") and the condensation (C) domain (Marahiel et al., 1997; Roongsawang et al., 2011). The A-domain

90 recognizes, selects and activates the specific amino acid of interest (Dieckmann et al., 1995). Taking  
91 into account the 3D-structures of several adenylation domains and their active site, several tools have  
92 been set up to correlate the amino acid residue present in this active site and their substrate specificity.  
93 A NRPS code was so defined that it is based on 8 amino acid residues from the active site (Stachelhaus  
94 et al., 1996; Rausch et al., 2005). The activated amino acid is hereby covalently bonded as a thioester  
95 to the flexible 4'-phosphopantetheinyl (4'-Ppant) arm of the T-domain. The 4'-Ppant prosthetic group  
96 is 20 Å in length and can swing from one to another adjacent catalytic centre. Exactly this flexibility  
97 enables the transfer of the activated amino acid substrate to the C-domain, which catalyzes in turn (i)  
98 the formation of a peptide bond between the nascent peptide and the amino acid carried by the adjacent  
99 module and allows afterwards (ii) the translocation of the growing chain to the following module.  
100 Various functional subtypes of the C domain have been described. For example, an <sup>L</sup>C<sub>L</sub> domain  
101 catalyzes the formation of a peptide bond between two L-amino acids while a <sup>D</sup>C<sub>L</sub> domain between a  
102 L-amino acid and a growing peptide ending with a D-amino acid (Rausch et al., 2007). The first  
103 module (A-T module) is considered the initiation module, while the subsequent (C-A-T) modules are  
104 defined as elongation modules. After several module-mediated cycles of peptide extension, the  
105 complete linear intermediate peptide is released by the terminal thioesterase (TE) domain which, often,  
106 catalyzes an internal cyclization (Marahiel et al., 1997; Trauger et al., 2000). Besides the above  
107 mentioned domains, the NRPS assembly line can furthermore comprise additional optional domains,  
108 which catalyze modifications of amino acid building blocks e.g. their epimerization (E-domains)  
109 (Süssmuth et al., 2017). The lipid moiety of surfactins and most of the microbial lipopeptides is  
110 introduced directly at the start of the biosynthesis. The initiation module features a C-A-T- instead of  
111 a classic A-T-structure (Sieber et al., 2005; Bloudoff et al., 2017). It contains a special N-terminal C-  
112 domain, termed C-starter (C<sub>S</sub>) domain and is in charge of the linkage of a CoA-activated β-hydroxy  
113 fatty acid to the first amino acid. The activated fatty acid stems foremost from the primary metabolism  
114 (Figure 1).

115 Three decades ago, the biosynthetic gene cluster (BGC) of the CLP surfactin was described in parallel  
116 by different research groups (Nakano et al., 1988; Cosmina et al., 1993; Fuma et al., 1993; Sinderen et  
117 al., 1993). The structural genes were identified in *B. subtilis* and are formed by the four biosynthetic  
118 core NRPS genes *srfAA*, *srfAB*, *srfAC* and *srfAD* (Figure 1) which code together for a heptamodular  
119 NRPS assembly line. The three-modular enzyme SrfAA contains N-terminally the typical C<sub>S</sub>-domain  
120 of CLP-BGCs and acylates the first amino acid Glu1 with various 3-OH-fatty acids stemming from  
121 primary metabolism. The peptide is subsequently extended in a co-linear fashion by the elongation  
122 modules of SrfAA, SrfAB and SrfAC to yield a linear heptapeptide (FA-L-Glu1-L-Leu2-D-Leu3-L-  
123 Val4-L-Asp5-D-Leu6-L-Leu7). The inverted stereochemistry can be readily attributed to the presence  
124 of E-domains in modules M3 and M6 and <sup>D</sup>C<sub>L</sub> domains in modules M4 and M7 (Figure 1). Finally, the  
125 TE domain of SrfAC releases the lipopeptide and performs the macrocyclization between Leu7 and  
126 the hydroxy-group of the 3-OH fatty acid. Notably, SrfAD consist solely of a second TE-domain, which  
127 represents rather a supportive repair enzyme and is able to regenerate misprimed T-domains during  
128 NRPS assembly (Schneider et al., 1998; Schwarzer et al., 2002; Yeh et al., 2004).

129 Beside the structural NRPS genes, the surfactin BGC comprises one built-in and several adjacent  
130 accessory genes encoding e.g. transporters and regulatory proteins (MiBIG Accession No:

131 BG0000433). Amongst these, we would like to further highlight the genes *sfp*, *ycxA*, *krsE*, *yerP* and  
132 *comS*, which are particularly related with the production yield of surfactin.

133 Sfp represents a phosphopantetheinyl transferase (PPTase) and is located approximately 4 kb  
134 downstream of the *srf* BGC. The T-domain of an NRPS is, upon its expression, not directly active but  
135 rather exists nascent in its non-functional apo-form. For full functionality, the flexible 4'-Ppant arm  
136 needs to be fused to the T-domain. The latter process is mediated by the PPTase Sfp, thereby converting  
137 all T-domains of the surfactin BGC into their active holo form (Quadri et al., 1998; Mootz et al., 2001).  
138 This fact makes Sfp indispensable for the production of surfactin (Tsuge et al., 1999). For example, in  
139 the reference strain, *Bacillus subtilis* 168, the *sfp* locus is truncated and therefore non-functional, which  
140 abolishes in turn surfactin production. However, the production can be restored by the transfer of a  
141 complete *sfp* locus (Nakano et al., 1988, 1992).

142 Further important genes in the context of surfactin production are genes encoding transporters which  
143 are efflux pumps. From a physiologically point of view, the pumps avoid intracellular surfactin  
144 accumulation and constitute an essential self-resistance mechanism (Tsuge et al., 2001). In particular  
145 since surfactin inserts into biomembranes and at higher concentration causes membrane disruption. An  
146 ecological rationale for transporters could be that surfactin is extracellularly at the correct site where it  
147 can exert its beneficial activity. So far, three transporters have been identified in Bacilli, that are  
148 involved in surfactin efflux, i.e. YcxA, KrsE and YerP. It has been demonstrated that the separate  
149 overexpression of the corresponding genes enhanced release rates of surfactin (Li et al., 2015) by 89%,  
150 52% and 145%, respectively.

151 Finally, the surfactin BGC exhibits a unique peculiarity on the genetic level, in bearing a co-encoded  
152 regulatory gene, termed *comS* inside itself (D'Souza et al., 1994). It is located in the open reading frame  
153 of the NRPS gene *srfAB* (Hamoen et al., 1995), more precisely within the A-domain of module 4  
154 (Figure 1). ComS is on the one hand involved in the positive regulation of the genetic competence of  
155 the cell (Liu et al., 1998) and on the other hand part of the quorum sensing system *comQXPA* (Ansaldi  
156 et al., 2002; Schneider et al., 2002; Auchtung et al., 2006) which in turn regulates surfactin production.  
157 Beyond this brief explanation, for an excellent overview about the role of ComS, the reader is referred  
158 to a review, written by Stiegelmeier and Giddings (Stiegelmeier et al., 2013). Since the production  
159 yield is coupled with the presence and functionality of ComS in the coding region of *srfAB*, the genetic  
160 engineering of the surfactin synthetase in this region requires special attention.

161

## 162 2.2 Fatty acid chain synthesis

163 Since fatty acid biosynthesis plays a critical role in surfactin production, and strongly determines its  
164 activity and properties, in this section we briefly summarize this central metabolic pathway and the  
165 subsequent steps leading to the modification and activation of the fatty acyl-CoA precursor.

166 All organisms employ a conserved set of chemical reactions to achieve the *de novo* Fatty Acid (FA)  
167 biosynthesis, which works by the sequential extension of the growing carbon chain, two carbons at a  
168 time, through a series of decarboxylative condensation reactions (Wakil et al., 1983) (Figure 2). This  
169 biosynthetic route proceeds in two stages: initiation and iterative cyclic elongation. The acetyl-CoA  
170 carboxylase enzyme complex (ACC) performs the first committed step in bacterial FA synthesis to  
171 generate malonyl-CoA through the carboxylation of acetyl-CoA (Marini et al., 1995; Tong, 2013). The  
172 malonate group from malonyl-CoA is transferred to the acyl carrier protein (ACP) by a malonyl-

173 CoA:ACP transacylase (FabD) (Serre et al., 1994, 1995; Morbidoni et al., 1996). The first reaction for  
174 the synthesis of the nascent carbon chain comprises the condensation of malonyl-ACP with a short-  
175 chain acyl-CoA (C2–C5) catalyzed by a 3-keto-acyl carrier protein synthase III (FabH). Acetyl-CoA is  
176 used as a substrate for the synthesis of straight-chain FA, while branched-chain fatty acids (BCFA)  
177 arise from isobutyryl-CoA, isovaleryl-CoA and methylbutyryl-CoA priming substrates. These  
178 precursors derive, from the catabolism of the branched-chain amino acids valine, leucine and  
179 isoleucine, respectively. The crucial branched-chain  $\alpha$ -keto acid decarboxylase (BKD) complex  
180 catalyzes the decarboxylation of  $\alpha$ -keto acids to generate the corresponding branched-chain acyl-CoA  
181 primers (Willecke et al., 1971; Kaneda, 1991; Lu et al., 2004). The substrate specificity of FabH plays  
182 a determining role in the branched/straight and even/odd characteristics of the fatty acid produced. *B.*  
183 *subtilis* possesses two FabH isoenzymes, FabHA and FabHB, both of which preferentially utilize  
184 branched-chain acyl-CoA primers (Choi et al., 2000). Therefore, BCFA are the main components of  
185 phospholipids, where iso-C15:0, anteiso-C15:0, iso-C16:0, iso-C17:0, and anteiso-C17:0 represent the  
186 major FA found in *Bacillus* species (Kaneda, 1969; Kämpfer, 1994). The pattern of the BCFA can be  
187 modified by environmental conditions such as temperature (Graumann et al., 1999).

188 Next, the keto-acyl-ACP product of FabH condensation enters the elongation/reducing cycle of the  
189 fatty acid synthase II (FAS-II). There, the keto group is reduced by the NADPH dependent  $\beta$ -ketoacyl-  
190 ACP reductase (FabG) to give  $\beta$ -hydroxy-acyl-ACP. The  $\beta$ -hydroxyacyl-ACP intermediate is then  
191 dehydrated to *trans*-2-enoyl-ACP by a 3-hydroxyacyl-ACP dehydratase (FabZ). Then, the cycle is  
192 completed by an enoyl-ACP reductase, which reduces the double bond in *trans*-2-enoyl-ACP to form  
193 acyl-ACP (Fujita et al., 2007). *B. subtilis* possesses two enoyl-ACP reductases (FabI and FabL) with  
194 opposite preferences for the NADPH or NADH cofactor (Heath et al., 2000).

195 In all the successive steps of FA elongation, the acyl-ACP intermediate and malonyl-ACP are the  
196 substrates of FabF condensing enzyme (3-oxoacyl-ACP-synthase II) that elongates the growing acyl  
197 chain and initiate each new round of the cycle (Schujman et al., 2001). Finally, the acyl-ACPs of the  
198 proper chain length are substrates of acyltransferases involved in cell membrane phospholipid  
199 synthesis. Alternatively, some structurally specific FA are not integrated in the cell membrane  
200 phospholipids. Those modified FA could be, under specific environmental or growth conditions,  
201 channelled into secondary metabolic pathways. They are then a of specialized molecules, as it is the  
202 case of lipopeptides.

203 Once the long chain FA is synthesized, the next steps needed for surfactin biosynthesis involves the  
204 production of the 3-hydroxy-acyl-coenzyme A (CoA) substrates. Youssef et al., based on *in vitro*  
205 assays, suggested that acyl 3-hydroxylation occurs prior to CoA ligation (Youssef et al., 2011). These  
206 authors reported that YbdT, a cytochrome P450 enzyme, catalyzes the hydroxylation of the FA  
207 precursors to be incorporated in the lipopeptide biosynthetic pathway (Youssef et al., 2011).  
208 Cytochrome P450 are monooxygenases capable of introducing an oxygen atom into FA and in other  
209 lipidic and non-lipidic molecules. The *B. subtilis* genome contains eight genes coding for cytochrome  
210 P450 enzymes (Hlavica et al., 2010). *In vitro*, high-performance liquid chromatography (HPLC) and  
211 gas chromatography–mass spectrometry analyses demonstrated that the recombinant *ybdT* gene  
212 product hydroxylates myristic acid in the presence of H<sub>2</sub>O<sub>2</sub>, to produce  $\beta$ -hydroxymyristic acid and  $\alpha$ -  
213 hydroxymyristic acid (Matsunaga et al., 1999). Furthermore, a *ybdT* mutant strain of *B. subtilis*

214 OKB105 produces biosurfactants with only 2.2% of 3-hydroxylated C14, while the 97.8% contained  
215 non-hydroxylated FA with chain lengths of C12, and C14–C18 (Youssef et al., 2011) and are thus linear.  
216 Finally, the surfactin synthetase assembly line can be initiated in presence of a CoA-activated FA  
217 (Steller et al., 2004). Fatty acids are converted into their corresponding acyl-CoA derivative by fatty  
218 acyl CoA ligases (FACS). Of the four putative FACS identified in homology searches in the genome  
219 of *B. subtilis*, two of them, LcfA and YhfL, were characterized *in vitro* to be involved in surfactin  
220 production. HPLC-MS based FACS activity assays indicated that LcfA and YhfL catalyze the thioester  
221 formation with CoA and various FA substrates (3-OH C8, 3-OH C10, C12 and C14). All four single  
222 mutants in the FACS homolog genes, *lcfA*, *yhfL*, *yhfT* and *yngI*, decreased surfactin production by 38%  
223 - 55%, compared with the wild-type levels. Interestingly, a quadruple mutant in the FACS did not  
224 completely abolish surfactin biosynthesis, such strain still presents 16% surfactin production,  
225 compared with the levels produced by the wild-type strain. This observation suggests that other non-  
226 canonical FACS are present in *B. subtilis* or that other pathways, such as transthioation from ACPs to  
227 CoA, could be involved in providing the fatty acyl moiety.  
228 The hydroxylated and CoA activated FA derivative is finally transferred onto the surfactin synthetase  
229 assembly line, in a reaction performed by the N-terminal condensation (Cs) domain, that is as  
230 mentioned above responsible for the lipoinitiation mechanism. *In vitro*, the recombinant dissected C  
231 domain, catalyzed the acylation reaction using glutamate-loaded PCP domain and 3-OH-C14-CoA as  
232 substrates (Kraas et al., 2010).

233

### 234 3. Variants of surfactin

235 The surfactin biosynthesis mechanism previously described is responsible for the high biodiversity of  
236 surfactin-like molecules. In addition, the assembly line machinery of surfactin synthetases can be easily  
237 modified by synthetic biology in order to increase this biodiversity. Both aspects will be developed in  
238 the following chapter.

239

#### 240 3.1. Natural variants

241 Three main peptide backbones and the NRPSs responsible for their biosynthesis, produced by different  
242 *Bacillus* species, have been so far described in literature: surfactin as previously described from *B.*  
243 *subtilis*, *B. amyloliquefaciens*, *B. velezensis* and *B. spizizeni* amongst others, pumilacidin from *B.*  
244 *pumilus* (Naruse et al., 1990) and lichenysin from *B. licheniformis* (Horowitz et al., 1990). Compared  
245 to surfactin, pumilacidin has a leucine in position 4 instead of a valine, as well as an isoleucine or a  
246 valine in position 7 instead of a leucine. Lichenysin differs from surfactin by a change in the first amino  
247 acid residue: a glutamine (Gln) instead of a glutamic acid (Figure 3).

248 This first biosynthetic diversity in surfactin is increased by the promiscuous specificity of adenylation  
249 domains of modules 2, 4 and 7 of surfactin synthetases which are able to accept L-Leu, L-Val or L-Ile  
250 amino acids residues as well as L-Ala for module 4. Similarly low levels of specificity have been  
251 observed for lichenysin (Peypoux et al., 1991; Bonmatin et al., 2003).

252 Based on all these results, it appears that the aspartic acid in position 5, as well as the D-Leucine in  
253 position 3 and 6 are present in all the members of the surfactin family. The only mention of an  
254 asparagine (Asn) for lichenysin (Yakimov et al., 1995) was quickly refuted by the same author after  
255 the use of fast atom bombardment mass spectrometry (Yakimov et al., 1999). The specificity of M3

256 and M6 could result from (i) an enzyme of the assembly line machinery such as the epimerisation  
257 domain which could accept only leucine as substrate, (ii) from the specificity of the adenylation domain  
258 or (iii) from the specificity of the involved condensation domains.

259 The changes in the peptide chain are not the only source of diversity in the surfactin family. As  
260 mentioned before, surfactin is a heptapeptide linked to a fatty acid chain. Regarding this chain, the  
261 length of it can vary from 12 to 17 carbons atoms, mainly being C14 and C15.

262 Another change in this lipid chain is its isomery, it can have a linear, *n*, configuration, but it can also  
263 be branched, iso and anteiso. Anteiso can only be in an uneven carbon chain length, while iso can be  
264 found in all chain lengths (odd and even-numbered carbon chain). These derivatives can be mainly  
265 explained by the promiscuity of the C<sub>s</sub>-domain present in module M1 towards its relaxed substrate  
266 specificity.

267 Finally, natural linear surfactins (Figure 3) have been also identified in the culture supernatant of  
268 *Bacillus* strains (Gao et al., 2017). The molecular mechanism responsible for this linearization is not  
269 yet known. It could result from an incomplete efficacy of TE domain which could release some  
270 surfactin without cyclization or from enzymatic or chemical degradation of cyclic surfactin.

271 In addition, heterologous enzymes are also capable to catalyze linearization. An *in vitro* study showed  
272 the linearisation effect of a purified V8 endoprotease from *Staphylococcus aureus* (Grangemard et al.,  
273 1999). Furthermore, an *in vivo* study demonstrated that *Streptomyces* sp. Mg1 produces, as a  
274 mechanism of resistance, an enzyme that hydrolyses surfactin into its linear form (Hoefler et al., 2012).  
275 Surfactin methyl ester was observed in the supernatant of *Bacillus subtilis* HSO121 (Liu et al., 2009),  
276 and a methylated product of surfactin with a valine in position 7 was discovered in the supernatant of  
277 a *Bacillus* mangrove bacteria strain (Tang et al., 2007). This change was also discovered in the  
278 supernatant of *Bacillus licheniformis* HSN221 with surfactin and lichenysin methyl esters (Li et al.,  
279 2010) and in the culture medium of *Bacillus pumilus* through surfactin methyl ester (Zhuravleva et al.,  
280 2010).

281

### 282 **3.2.Synthetic and biosynthetic variants**

283 In addition to the natural surfactins seen before, synthetic variants can be obtained through chemical  
284 modifications or genetic engineering of the NRPS. This leads to new forms or to a controlled  
285 production of a specific form. Reasons for structural changes are manifoldly given, foremost to reduce  
286 the toxicity of surfactin, but also to optimize its biological activities or to increase its water solubility.  
287 Esterification can be achieved through chemical treatment with alcohol, reacting with the Asp-β- and/or  
288 Glu-γ-carboxyl group, producing monoester and/or diester-surfactin (Figure 3).

289 For example, reaction of surfactin with *n*-hexyl alcohol lead to mono- and *di*-hexyl-surfactin, with 2-  
290 methoxyethanol to mono- and *di*-2-methoxy-ethyl-surfactin (Shao et al., 2015). Amidation through a  
291 reaction with alcohol and then NH<sub>4</sub>Cl was also observed (Morikawa et al., 2000). Esterification and  
292 amidation of aspartic and glutamic acid eliminate the negative charge of those amino acid residues,  
293 creating an even greater diversity in the surfactin family because of the charge change that they bring  
294 and thus the modification in surfactin biological and surfactant properties.

295 Linearization of the cyclic surfactin previously mentioned as a natural process can also be obtained by  
296 chemical alkaline treatment (Figure 3) (Eeman et al., 2006).



297 In addition to those chemical modifications of surfactin naturally produced, synthetic forms can be  
298 chemically produced (Figure 3). Liquid phase techniques have been used at first (Nagai et al., 1996)  
299 but, because of the many steps and the purification of intermediates needed, it was replaced with a  
300 quicker solid phase peptide synthesis (SPPS) technique. Different forms of surfactins have been  
301 produced, such as standard surfactin, but also analogues with a change in the amino acid sequence,  
302 such as an epimerisation (D-Leu2), a change in charge (Asn5) and the switch of two residues (Asp4-  
303 Leu5) (Pagadoy et al., 2005). Linear surfactin was also produced, as well as linear with an amidated  
304 carboxy-terminus function (Dufour et al., 2005). Finally, the fatty acid chain length was likewise  
305 changed, with C10 and C18 (Francius et al., 2008). However, due to the complexity of the production,  
306 these lipopeptides are intended only for research use.

307 As said before, in addition to the chemical changes, the genetic engineering can be also applied to the  
308 genes coding for the NRPS, in order to modify the structure of surfactin. The generation of novel  
309 derivatives by rational design can hereby be achieved by site directed mutagenesis, module- insertion,  
310 deletion and substitution (Alanjary et al., 2019). Application of the site directed mutagenesis technique,  
311 an A-domain specificity of an NRPS module shift from L-Glu to L-Gln and from L-Asp to L-Asn at  
312 position 5 in modules 1 and 5 was accomplished, respectively (Eppelmann et al., 2002).

313 Concerning the concept of module substitutions, particularly the Marahiel group showed in a ground  
314 breaking way from the mid 90s onwards the feasibility of module swaps which allowed single or  
315 multiple variations concerning all seven amino acids (Stachelhaus et al., 1995, 1996; Schneider et al.,  
316 1998; Eppelmann et al., 2002). As a practical aspect, beside the gain in basic research knowledge, for  
317 several modified surfactins, such as Cys7-surfactin, a decreased hemolytic activity was observed.  
318 Furthermore, ring contracted surfactin derivatives were obtained by deletion of complete NRPS  
319 modules. In this way, the corresponding knockouts yielded hexapeptidic surfactin congeners,  
320 individually lacking Leu2, Leu3, Asp5 and Leu6. Notably, the  $\Delta$ Leu2  $\Delta$ Leu3 and the  $\Delta$ Leu6 surfactin  
321 variants showed a reduced toxicity towards erythrocytes and enhanced antibacterial activities, while  
322 the  $\Delta$ Asp5 surfactin exhibited an even higher inhibitory ability for Gram positive bacteria, but kept the  
323 hemolytic capabilities of the native surfactin (Mootz et al., 2002; Jiang et al., 2016). However, each  
324 genetic manipulation mentioned above resulted in a significant decrease in the production yield.  
325 Nevertheless, these studies showed the feasibility and moreover demonstrated in an encouraging way  
326 that the surfactin scaffold can be fine-tuned concerning its intended activity and its undesired side  
327 effects.

328 Very recently, the Bode group revolutionized the concept of module swapping. It includes the finding  
329 that C-domains have to be subdivided into a  $C_{\text{Donor}}$  ( $C_D$ ) and  $C_{\text{Acceptor}}$  ( $C_A$ ) portion and that both are  
330 amino-acid specific (Bozhüyük et al., 2019). This redefines nowadays the borders of an exchange unit.  
331 Instead of a classic A, A-T or C-A-T domain swap, it is preferable to exchange a  $C_D$ -A-T- $C_A$  domain  
332 unit (Figure 4). The huge advantage of these findings is that peptide-variants can be generated by  
333 genetic engineering at a much higher success rate and without any production loss. The technique will  
334 be an incentive to modify highly bioactive structures, such as surfactin. The exchange units can be  
335 derived from other Bacilli or codon-optimized from other bacterial genera. Particularly, in combination  
336 with synthetic biology, in future numerous genetically-engineered modifications can be envisioned:  
337 beside the exchange of amino acids, ring contractions by module deletion and ring expansions, by  
338 addition of an exchange unit, can be generated, respectively (Figure 4). Since peptides, containing D-

339 configured amino acids are less prone to degradation, the change of the absolute configuration by  
340 insertion of epimerization domains could lead to derivatives that are less prone to enzymatic  
341 degradation. Furthermore, since the biotechnological production of surfactin always results in the  
342 production of complex mixtures, e.g. varying in the fatty acid portion, it would be desirable to produce  
343 surfactin with a more defined lipid moiety. For this purpose, the biobrick-like exchange of the C<sub>Donor</sub>-  
344 portion of the C<sub>S</sub>-domain could lead to the incorporation of the desired 3-OH fatty acid. Finally, it can  
345 be also envisioned to modify the surfactin NRPS assembly line even further, e.g. by introduction of  
346 catalytic domains which drive intramolecular cyclization-, N-methylation-, hydroxylation- and redox-  
347 reactions.

348

#### 349 **4. Structure and properties relationship**

350 Surfactins and surfactin-like molecules are amphiphilic molecules with a polar part mainly constituted  
351 by the two negatively charged amino acid residues Glu and Asp (in native surfactin) and an apolar  
352 domain formed by the lateral groups of aliphatic amino acid residues (mainly Leu) and the fatty acid  
353 chain. This amphiphilic structure is responsible for its attractive physico-chemical properties as well  
354 as its various biological activities.

355

##### 356 **4.1. Surfactin structure and its influence on physico-chemical properties and biological activities**

357 The amphiphilic structure of surfactins leads to strong surface activity, i.e. their capacity to reduce the  
358 surface/interfacial tension and to self-assembly in nanostructures, and the presence of negative  
359 charge(s). Thus, they display as physico-chemical properties foaming (Razafindralambo et al., 1998;  
360 Fei et al., 2020), emulsifying (Deleu et al., 1999; Liu et al., 2015; Long et al., 2017; Fei et al., 2020)  
361 and dispersing properties, solid surface wetting and surface hydrophobicity modification performance  
362 (Ahimou et al., 2000; Shakerifard et al., 2009; Marcelino et al., 2019; Fei et al., 2020), and chelating  
363 ability (Mulligan et al., 1999; Grangemard et al., 2001; Eivazihollagh et al., 2019). This strong surface  
364 activity leads to detergent applications (Zezzi do Valle Gomes et al., 2012), but they also show  
365 promising perspectives of applications in the environmental sector to enhance oil recovery in oil-  
366 producing wells (Liu et al., 2015; Joshi et al., 2016; Long et al., 2017; de Araujo et al., 2019; Alvarez  
367 et al., 2020; Miyazaki et al., 2020), to increase the biodegradation rate of linear and aromatic  
368 hydrocarbons (Wang et al., 2020), and for metal removal from soil or aqueous solutions (Zouboulis et  
369 al., 2003; Eivazihollagh et al., 2019). Very recently, it was also suggested that surfactin can effectively  
370 demulsify waste crude oil (Yang et al., 2020). Their emulsifying property also confers them a potential  
371 of application in the food and cosmetics area for the product formulation (Mnif et al., 2013; Varvaresou  
372 et al., 2015; Zouari et al., 2016) as well as in the pharmaceutical area for the formulation of stable  
373 microemulsion drug delivery systems (Ohadi et al., 2020).

374 The variations in the molecular structure of the peptidic part and/or of the hydrocarbon chain greatly  
375 impact their physico-chemical properties. In term of self-aggregation behavior, the critical micellar  
376 concentration (CMC) value decreases with a longer fatty acid chain (CMC Surfactin C15 = 20  $\mu$ M;  
377 CMC surfactin C14 = 65  $\mu$ M; CMC surfactin C13 = 84  $\mu$ M in Tris-HCl pH 8) (Deleu et al., 2003; Liu  
378 et al., 2015). It also decreases with the presence of a methyl ester on the Glu residue (Grangemard et  
379 al., 2001) or the replacing of the Glu residue by a Gln as in lichenysin (Grangemard et al., 2001;  
380 Bonmatin et al., 2003). On the contrary, the linearization of the peptide cycle (CMC linear surfactin

381 C14 = 374  $\mu$ M in Tris pH 8.5) (Dufour et al., 2005) and the presence of a Leu4 instead of the Val4 as  
382 in pumilacidin (de Araujo et al., 2019) increase it. Different self-assembled nanostructures like sphere-  
383 like micelles, wormlike micelles and unilamellar bilayers coexist with larger aggregates in aqueous  
384 solution depending on the surfactin concentration, pH, temperature, ionic strength and metal ions (Zou  
385 et al., 2010; Taira et al., 2017; Jahan et al., 2020). These parameters can induce conformational changes  
386 in the secondary structure of the cyclic peptide moiety and thereby affect the shape and the packing  
387 parameter of surfactin (Jahan et al., 2020).

388 The capacity of surface tension reducing is also influenced by the molecular structure of surfactin.  
389 Depending of environmental conditions, lichenysin is or not more efficient than surfactin to reduce the  
390 surface tension (in Tris pH 9.4  $\gamma_{cmc}$ =35 and 37 for lichenysin and surfactin respectively and in NaHCO<sub>3</sub>  
391 pH 9.4  $\gamma_{cmc}$ =30 and 29 for lichenysin and surfactin respectively) (Grangemard et al., 2001), while  
392 pumilacidin is less (de Araujo et al., 2019). Linearization of the peptide cycle lessens this capacity (34  
393 mN/m in Tris pH 8.5). Nevertheless, the replacing of carboxyl group by a sulfo methylene amido group  
394 leads to a complete loss of activity (Bonmatin et al., 2003). The chain length but also the branching  
395 type also impact the surface tension. A longer chain is more efficient and the normal configuration is  
396 more active than the iso one which is more powerful than the anteiso (Yakimov et al., 1996).

397 The effect of the chain length on the foaming properties does not follow this trend as it was shown that  
398 a lipidic chain with 14 carbon atoms provides surfactin with best foaming properties compared to that  
399 with 13 or 15 carbon atoms (Razafindralambo et al., 1998).

400 Lichenysin was also demonstrated to be a better divalent cation chelating agent than surfactin  
401 (Grangemard et al., 2001). This effect is assigned to an increase accessibility of the carboxyl group to  
402 the cation in the case of lichenysin (Habe et al., 2018). The complexation of divalent cations with the  
403 lipopeptide in a molar ratio of 2:1 for lichenysin leads to the formation of an intermolecular salt bridge,  
404 stronger than the intramolecular complexation in a 1:1 ratio with surfactin (Grangemard et al., 2001;  
405 Habe et al., 2018).

406 Globally speaking, the few studies focused on the structure-properties relationships of surfactin family  
407 emphasize three main facts. The first is that the unique feature of the peptide loop provides surfactin  
408 with a fascinating molecular behavior at interfaces (Liu et al., 2020). Furthermore, the peptide cycle  
409 linearization leads to a structural distortion of the molecule reducing or annihilating its surface active  
410 power. The second fact is that the surface activity of surfactin is dictated by the interplay of  
411 hydrocarbon chain and peptide sequence (Liu et al., 2020). The more distant and distinct the polar and  
412 apolar domains are, the stronger the surface active power is. The last fact is that the charges of the polar  
413 part also play a primordial role in the physico-chemical properties. A monoanionic surfactin is more  
414 efficient than a dianionic one, due to a reduced repulsive effect between the molecules at the interface.  
415

416 The remarkable physico-chemical properties of surfactin are also responsible for their biological  
417 activities which, in most of the cases, involve perturbation or disruption of membrane integrity. It was  
418 demonstrated for haemolytic (Kracht et al., 1999; Dufour et al., 2005), antibacterial (Bernheimer et al.,  
419 1970), antiviral (Yuan et al., 2018; Johnson et al., 2019) and antimycoplasma (Vollenbroich et al., 1997)  
420 activities of surfactin as well as its ability to inducing systemic resistance in plant (Ongena et al., 2007,  
421 2008). Some of those activities leading to promising results in the agricultural field (Chandler et al.,  
422 2015; Loiseau et al., 2015). But surfactin was also characterized for anti-inflammation (Takahashi et

423 al., 2006; Zhao et al., 2017), anti-sepsis (Hwang et al., 2007), anti-tumor (Wu et al., 2017) and  
424 immunomodulatory (Park et al., 2009) activities for which another target than membranes is involved.  
425 A synergistic effect has been observed between surfactin and other lipopeptides. The addition of  
426 surfactin at an inactive concentration to iturin increase its haemolytic activity (Maget-Dana et al.,  
427 1992). The combination of surfactin and fengycin lead to a decrease in disease in tomato and bean  
428 plants (Ongena et al., 2007). Furthermore, while surfactin has no effect against fungi, it has been shown  
429 to enhance the biological activities of other lipopeptides against fungi and oomycetes (Deravel et al.,  
430 2014; Tanaka et al., 2015; Desmyttere et al., 2019).

431

#### 432 **4.2. Use of molecular modelling for mechanism of action investigation**

433 Molecular modelling methods are powerful theoretical tools to investigate structure functions  
434 relationship of surfactin and its mode of action. Docking and Molecular Dynamic (MD) simulations  
435 have been used in various studies involving surfactin for the characterization of diverse properties to  
436 predict activities and domains of applications.

437 For membrane interactions, Hypermatrix (Brasseur et al., 1987), was used to simulate the interaction  
438 of surfactin with a membrane monolayer in order to determine the lipid specificity for insertion and  
439 membrane destabilization. It was shown that surfactin interacts specifically with 1,2-  
440 dipalmitoylphosphatidylcholin (DPPC) localized at the DPPC/1,2-dioleoyl-sn-glycero-3-  
441 phosphocholine (DOPC) domain boundaries (Lins et al., 1995; Deleu et al., 2003, 2013).

442 For medical applications, the interaction of surfactin with the amyloid  $\beta$ -peptide (A  $\beta$  42) has been  
443 studied with MD simulation and docking experiments (with GROMACS (Abraham et al., 2015) and  
444 AutoDock (Morris et al., 2009) respectively).

445 Further investigations have shown that surfactin binds protofibrils by forming a stable hydrogen bond  
446 with residues involved in salt bridges responsible of amyloid aggregation and plaques stability (Verma  
447 et al., 2016). Another docking investigation, employing Swiss Dock (Lien Grosdidier et al., 2011), has  
448 shown that surfactin binds favorably via hydrogen bonds to porcine pancreatic lipase and inhibits its  
449 activity, which could lead to a novel and potent body weight reducer for obesity control (Meena et al.,  
450 2018).

451 Beside these investigations on monomeric surfactin interacting with potential targets, MD simulations  
452 proved to be an efficient tool to study molecular assemblies. A surfactin monolayer at the air-water  
453 interface was studied under various interfacial concentrations. It was shown that packed structures are  
454 formed via intra- and inter-molecular hydrogen bonds, stabilizing the  $\beta$ -turn structure of the peptide  
455 ring, favouring the  $\beta$ -sheet domain organization and hydrophobic contacts between molecules Another  
456 simulation was applied to study the self-assembly of surfactin in water and more particularly the  
457 structural organization of the micelles (Lebecque et al., 2017). Micelles were pre-formed with PackMol  
458 (Martinez et al., 2009) and were simulated to analyse their behavior. The optimal aggregation number,  
459 i.e.20, predicted by this approach is in good agreement with the experimental values. Two parameters  
460 were analysed, the hydrophilic ( $\phi$ )/hydrophobic ( $\rho$ ) surface and the hydrophobic tail hydration  
461 (Lebecque et al., 2017). A higher  $\phi/\rho$  surface ratio means a more thermodynamically favorable  
462 organization of the hydrophilic and hydrophobic domains, but steric and/or electrical repulsions  
463 between polar heads have also to be considered. For surfactin, it was shown that the  $\phi/\rho$  surface  
464 ratio undergoes a decrease for the largest micelles of surfactin because they have to rearrange

465 themselves to reach a more favorable organization. The low value of apolar moieties hydration  
466 observed for surfactin micelles is due to the very large peptidic head that efficiently preserves  
467 hydrophobic tails from contact with water. The Coarse Grain (CG) representation MARTINI (Marrink  
468 et al., 2007) (grouping atoms into beads to speed up the simulation process) was similarly applied to  
469 analyse the structural properties and kinetics of surfactin self-assembly in aqueous solution and at  
470 octane/water interface (Gang et al., 2020). With complementary MD of a pre-formed micelle and a  
471 monolayer, the authors showed that their CG model is in agreement with atomistic MD and  
472 experimental data, for micelle self-assembly and stability, as well as for the monolayer. Furthermore,  
473 this study allows the development of a set of optimized parameters in a MARTINI CG model that could  
474 open further investigations for surfactin interaction with various biofilms, proteins or other targets of  
475 interest with a better sampling than atomistic MD.

476

## 477 **5. Production**

478 This last part of this review is dedicated to the improvement of the production of surfactin like  
479 compounds. It will first consider the techniques for the identification and the quantification of these  
480 lipopeptides and then focus on strain, culture conditions and bioprocess optimization. Not to forget,  
481 the purification process allows for a greater recovery of the surfactin produced and lower the losses.

482

### 483 **5.1. Identification and quantification of surfactin and its variants**

484 In order to discover new natural variants or verify the production of synthetic ones, the identification  
485 is an important process. The first surfactin structure elucidation was made through hydrolysis of the  
486 peptide and fatty acid chain into fragments, their identification and alignment (Kakinuma, Sugino, et  
487 al., 1969). However, with the continuous innovations of analytical-chemical techniques such as mass  
488 spectrometry MS/MS (Yang, Li, et al., 2015), nuclear magnetic resonance (NMR) (Kowall et al., 1998)  
489 and Fourier transform IR spectroscopy (FT-IR) (Fenibo et al., 2019), the analysis of new variants can  
490 be determined quicker and without hydrolysis. While FT-IR provides the functional groups, NMR leads  
491 to a complete structural characterization of the compounds but requires completely purified products  
492 at the level of mg quantities. Mass spectrometry does not enable the differentiation of compounds  
493 having the same mass (such as leucine and isoleucine for example), nor the type of fatty acid chain  
494 (linear, iso or anteiso), but provides the global mass and the peptide moiety primary sequence.

495 An overview of surfactin's dosage techniques can be found in Table 1. The first ones rely on surfactin's  
496 amphiphilic nature, so that its production can be detected through its surfactant activity.

497 Indirect methods, such as emulsification measure, haemolytic activity (blood agar plate) or cell surface  
498 hydrophobicity can be used. However, the correlation between those activities and surfactant activity  
499 has been refuted. Youssef et al. (Youssef et al., 2004) does not recommend the use of blood agar lysis  
500 as a screening method. Therefore, direct methods to measure the surface activity, such as interfacial  
501 tension measurement, drop shape analysis, drop collapse assay or oil spreading should be used (Youssef  
502 et al., 2004). Newer techniques have been developed the last few years for a rapid detection and  
503 quantification, based on colour shifts or fluorescence.

504 The first colour shift approach is based on the higher affinity of a mediator, initially forming a complex  
505 with a colour indicator, for surfactin and thus the release of the colour indicator in the solution (Yang,  
506 Yu, et al., 2015). The fluorescence technique is based on the same principle, but with fluorescein instead

507 of a colour indicator (Heuson et al., 2018). This leads to a more sensitive and stable procedure.  
508 However, another colour shift approach has been developed based only on the interaction between  
509 bromothymol blue solution and lipopeptides (Ong et al., 2018). However, since they are not specific  
510 for surfactin, the best and most sensitive quantification method is still the use of reversed phase HPLC-  
511 UV or MS (Geissler et al., 2017). This method also allows the discrimination between the various  
512 homologues of the surfactin family. Indeed, the molecules are separated based on their hydrophobic  
513 properties, giving a shorter retention time for lipopeptides with a leucine in position 7 and a longer  
514 retention time for lipopeptides with a valine in position 7. The separation is also based on the fatty acid  
515 chain, the shorter the fatty acid chain length is, the shorter the elution time is (Dhali, 2016).  
516 Furthermore, the production capacity of a micro-organism can be discovered through PCR, with  
517 primers specific to the surfactin biosynthesis genes (*sfp* and *srf*) (Mohammadipour et al., 2009) or  
518 genome sequencing. However, these methods do not reflect the real lipopeptide production, since only  
519 the presence of the genes is observed. RT-PCR allows the detection of the transcribed genes, but does  
520 not allow to reflect the post-transcriptional modifications.

521

## 522 **5.2.Optimisation of surfactin production**

523 In order to enhance the surfactin production, in addition to fermentation optimization, the genetic  
524 engineering of the producing strains is of great significance. It was already covered in the past by other  
525 teams (Hu et al., 2019) and will be more developed here.

526 A first strategy would be to allocate more resources of the cell to surfactin biosynthesis by suppressing  
527 different cellular processes. It was successful with the plipastatin operon disruption (Coutte, Leclère,  
528 et al., 2010) or biofilm formation related genes (Wu et al., 2019). However, a strain with a 10 % genome  
529 deletion, comprising genes for plipastatin, bacilysin, toxins, prophages and sporulation, had a lower  
530 surfactin production (Geissler et al., 2019). Then, concerning surfactin production itself, the strategy  
531 can take place at different stages of the surfactin cell production: at the transcription level by promoter  
532 substitution or modification of the transcriptional regulatory genes of *srfA* operon, at the level of  
533 surfactin synthesis by increasing the precursor availability, during the molecule's excretion and finally  
534 during its degradation (Figure 5).

535

### 536 **5.2.1. Transcription**

537 As seen before, surfactin NRPS is coded by four genes, *srfA-A*, *srfA-B*, *srfA-C* and *srfA-D*, that are  
538 controlled by the  $P_{srf}$  antoinducible promoter, triggered by signal molecules from a quorum sensing  
539 pathway. Studies were performed to exchange this promoter with inducer-specific or constitutive ones.  
540 It emerged that a replacement with a constitutive promoter in a weak surfactin producer strain leads to  
541 an increase in the production, but that the opposite effect is observed for strong surfactin producers  
542 (Willenbacher et al., 2016). However, the use of novel artificial inducible promoters leads to an increase  
543 in surfactin production of more than 17 times (Jiao et al., 2017).

544 In addition to the promoter, transcriptional regulatory genes also control the expression of the NRPS  
545 genes. The cell density dependent quorum sensing system plays a regulatory role in many pathways in  
546 *Bacillus*, and among others in the regulation of the *srfA* operon. Ohsawa et al. (Ohsawa et al., 2006)  
547 showed that the inhibition of the ComQXP quorum sensing locus lead to a decrease in the expression

548 of *surfA* genes and Jung et al. (Jung et al., 2012) showed that the overexpression of ComX and PhrC  
549 increases the production of surfactin.

550 In addition to the quorum sensing system itself, regulators also impact the *surfA* operon, the quorum  
551 sensing system or even other mechanisms that indirectly impact surfactin. There are positive regulators  
552 such as PerR (Hayashi et al., 2005) and negative regulators such as CodY (Coutte et al., 2015), Rap  
553 (Hayashi et al., 2006), SinI (López et al., 2009) and Spx (Zhang et al., 2006).

554

### 555 **5.2.2. Increasing precursor supply of NRPS by feeding or metabolic engineering**

556 Modifying media and fermentation condition is a strategy to overproduce the lipopeptide precursors as  
557 well as to favor the production of certain isoforms. For example it was seen that the feeding of leucine  
558 as 50% of the nitrogen source lead to an increase in specific surfactin production of three times (Coutte  
559 et al., 2015). Another strategy is the application of rational metabolic engineering approaches such  
560 as: (i) blocking competitive pathways for building blocks, as well as, those pathways that consume  
561 products; (ii) pulling flux through biosynthetic pathways by removing regulatory signals; and (iii) by  
562 overexpressing rate-limiting enzymes.

563

#### 564 **5.2.2.1. Amino acids precursors**

565 One way to develop this metabolic engineering approach is to use knockout of genes which negatively  
566 influence the intracellular pool of amino acids precursors. To implement the knock-out of gene which  
567 negatively influence the intracellular pool of amino acid precursor, their metabolic pathways have to  
568 be modelled as a reaction network taking into account the regulation processes.

569 Firstly, the various pathways involved in the metabolites needed for the amino acid production should  
570 be addressed. In this research for compounds from the glycolysis that influence the amino acid  
571 production, pyruvate is interesting from multiple points of view. It is the entry point of the Krebs cycle  
572 through its conversion into acetyl-CoA, but it is also used as a substrate for the production of amino  
573 acids that compose the surfactin. Indeed, pyruvate is converted into valine and leucine. Furthermore,  
574 the production of isoleucine is made through threonine and pyruvate. The Krebs cycle also contributes  
575 to the amino acid production, with oxoglutarate and oxaloacetate, they belong to the metabolism of  
576 aspartic and glutamic acid. Secondly, the various enzymes that regulates metabolite production should  
577 be addressed. The search can also go a level above, with the regulators and promoters of those enzymes,  
578 such as pleiotropic regulators CodY or TnrA (Dhali, 2016). Lastly, the transporters of the amino acid  
579 precursors can be addressed. Indeed, the amino acid can be transported into the cell from the  
580 environment.

581 Wang et al. (Wang et al., 2019), showed that the knockout of *murC*, *yrpC* and *racE*, negative regulators  
582 involved in the metabolism of glutamate, lead to an increase in surfactin production. The choice of  
583 those knock-outs can also be directed by methods from computational biology, to narrow them down  
584 and reduce the laboratory time needed.

585 Some prediction methods are based on formal reasoning techniques based on abstract-interpretation  
586 (Niehren et al., 2016). This is a general framework for abstracting formal models that is widely used  
587 in the static analysis of programming languages. Formal models are reaction networks with partial  
588 kinetic information with steady state semantics define systems of linear equations, with kinetic  
589 constraints, that are then abstracted. Here, the methods were to be developed further, so that they could

590 be applied to reaction networks rather than other kinds of programs. This approach has been used for  
591 the branched chain amino acids (leucine, valine and isoleucine) that mainly compose the surfactin  
592 peptide chain (Coutte et al., 2015).

593 The quite complex metabolic pathway of leucine production from threonine and pyruvate was  
594 modelled, by rewriting the informal model from SubtiWiki (Coutte et al., 2015) into this formal  
595 modelling language, while adding and adapting some reactions. It selected gene knock-outs that may  
596 lead to leucine overproduction, for which some of them an increase in surfactin production in *Bacillus*  
597 *subtilis* 168 was observed after experimental verification (Dhali et al., 2017).

598 Since single gene deletion is successful, multiple gene deletion must be the next aim. To be able to  
599 perform various deletions and/or insertions in the same strain, a markerless strategy is required. Various  
600 strategies can be performed such as temperature sensitive plasmid, pORI vectors, auxotrophy based  
601 methods, but also the *cre/lox* system (Yan et al., 2008), the pop-in pop-out technique (Tanaka et al.,  
602 2013) and the CRISPRi technology (Wang et al., 2019).

603

#### 604 **5.2.2.2.Fatty acid precursors**

605 As mentioned, fatty acids are one of the crucial components of surfactin, and modifications of this part  
606 of the molecule, such as length and isomerism, demonstrated to impact on the physicochemical  
607 properties and on the biological activity of lipopeptides (Dufour et al., 2005; De Faria et al., 2011;  
608 Henry et al., 2011; Liu et al., 2015; Dhali et al., 2017). Different metabolic engineering strategies were  
609 applied to improve surfactin production, in terms of the branched-chain fatty acid supply included: (i)  
610 enhancing the branched-chain  $\alpha$ -ketoacyl-CoA supply (Dhali et al., 2017; Wang et al., 2019; Wu et al.,  
611 2019); (ii) enhancing malonyl-ACP synthesis (Wu et al., 2019); (iii) overexpressing the whole fatty  
612 acid synthase complex (Wu et al., 2019); and (iv) pulling substrates flux towards surfactin biosynthesis  
613 by enhancing *stfA* transcription (Jiao et al., 2017; Wu et al., 2019).

614 Another study showed that the overexpression of the *bkd* operon produces less surfactin, besides being  
615 detrimental for cell growth (Wu et al., 2019). As the BKD complex requires lipoylation for its  
616 dehydrogenase activity, this enzyme competes with other lipoic acid dependent complexes (pyruvate  
617 dehydrogenase complex (PDH), 2-oxoacid dehydrogenase, acetoin dehydrogenase and the glycine  
618 cleavage system), generating a suppression of cell growth and, eventually, of surfactin production. By  
619 overexpressing the enzymes responsible for lipoic acid synthesis (*lipA*, *lipL* and *lipM*) (Christensen et  
620 al., 2011; Martin et al., 2011), this suppressive effect is reversed. The competitive lipoylation process  
621 between BKD and other lipoic acid dependent complexes is eliminated (Wu et al., 2019) and thus  
622 generates a higher production of surfactin with respect to the parental strain.

623 A further pathway, targeted to modification, represents the malonyl-ACP synthesis. Acetyl-CoA is  
624 converted into malonyl-CoA through the activity of ACC (*accDABC*). Thus, overexpression of these  
625 genes in combination with that of *fabD*, the malonyl-CoA:ACP transacylase, has been reported to  
626 increase the levels of surfactin production (Wu et al., 2019). Furthermore, these authors applied  
627 systematic metabolic engineering in *B. subtilis* 168 to construct surfactin hyperproducer strains. Other  
628 successful interventions related to FA biosynthesis have also been described. The simultaneous  
629 overexpression of most FAS II coding genes; *fabH* and *fabGZIF* (Runguphan et al., 2014) and  
630 expression of the *E. coli tesA* thioesterase (Steen et al., 2010), to “pull” through the pathway. The



631 combination of the mentioned interventions, in an already modified *B. subtilis* 168 chassis, further  
632 improved surfactin production by 220% (Wu et al., 2019).

633 Acetyl-CoA, is a key intermediate metabolite, which is not only used for surfactin biosynthesis, but  
634 fundamentally for cell growth and proliferation. Acetyl-CoA is generated from pyruvate by PDH;  
635 overexpression of enzymes of the glycolytic pathway and the KO of genes coding for enzymes  
636 associated with the acetyl-CoA consumption are common strategies to increase the supply of this key  
637 intermediate. Wu et al. (Wu et al., 2019) showed that the simultaneous overexpression of the PDH  
638 genes and that of the glycolysis enzymes produce an increase in biomass but not a significant increase  
639 in the levels of surfactin. However, if these interventions were combined with the  
640 overexpression/deregulation of the *urf* gene cluster, the surfactin production could be further improved  
641 to 12.8 g/l, achieving a 42% (mmol surfactin/mol sucrose) of the theoretical yield.

642

### 643 5.2.2.3. Directed biosynthesis of surfactin

644 Due to the non-specificity of some adenylation domains, the proportion of natural variants of surfactin  
645 can be modified through the feeding of certain amino acids as the nitrogen source in the culture  
646 medium. In the peptide moiety, this only affects L amino acid residues located in position 2, 4 and 7,  
647 and with a greater variation in position 4. Indeed, the feeding of valine leads to an increase of valine in  
648 position 7 (Menkhaus et al., 1993), the feeding of isoleucine (Ile) leads to the apparition of isoleucine  
649 in position 2 and/or 4 (Grangemard et al., 1997) and the feeding of alanine (Ala) lead to a surfactin  
650 with alanine in position 4 (Peypoux et al., 1994). Also, the culture medium can also influence the  
651 proportion of surfactin variants with different acyl moieties. For example, Liu et al. (Liu et al., 2015)  
652 found that the strain *B. subtilis* BS-37 has lower surfactin titers with higher proportions of C15-  
653 surfactin when grown in LB compared with glucose medium. Another team analysed the influence of  
654 amino acid residues on the pattern of surfactin variants produced by *B. subtilis* TD7 (Liu et al., 2012).  
655 The  $\beta$ -hydroxy fatty acid in surfactin variants was C15>C14>C13>C16, when no amino acid was added  
656 in the culture medium. On the other hand, when Arg, Gln, or Val was added to the culture medium, the  
657 proportion of surfactins with even  $\beta$ -hydroxy fatty acid chain significantly increased; whereas the  
658 addition of Cys, His, Ile, Leu, Met, Ser, or Thr significantly enhanced the proportion of surfactins with  
659 odd  $\beta$ -hydroxy fatty acid. Some of these results can be explained by the mode of biosynthesis of  
660 branched fatty acids, the precursors of which are branched chain amino acids (Kaneda, 1991). Thus,  
661 valine feeding enhances the proportion of iso variants with even fatty acid chains, while leucine and  
662 isoleucine feeding enhances the proportion of uneven iso or anteiso fatty acids chains respectively (Liu  
663 et al., 2012).

664 Modification of the variant pattern can also be obtained by genetic engineering of precursor pathways.  
665 As previously mentioned, increasing the branched chain 2-ketoacyl-CoAs intermediates is one of the  
666 strategies used for enhancing the synthesis of surfactin. The deletion of gene *codY*, which encodes a  
667 global transcriptional regulator and negatively regulates the *bkd* operon lead to a 5.8-fold increase in  
668 surfactin production in *B. subtilis* BBG258 with an increase by a factor 1.4 of the amino acid valine in  
669 position 7 instead of leucine (Dhali et al., 2017). On the other hand, Wang et al. (Wang et al., 2019),  
670 using CRISPR interference (CRISPRi) technology, were able to repress the *bkdAA* and *bkdAB* genes  
671 of the *bkd* operon; provoking a modest improvement in surfactin concentration, but a significant change  
672 in the proportion of the nC14 component. Similar results were observed in *B. subtilis* BBG261, a

673 derivative *lpdV* mutant strain, where the interruption of this 2-oxoisovalerate dehydrogenase of the  
674 BKD complex led to higher percentage of the nC14 isoform (52,7% in the *lpdV* mutant in comparison  
675 with the 21,2% of the control strain) (Dhali et al., 2017).

676

### 677 **5.2.3. Excretion**

678 The excretion of surfactin is another important step for its overproduction. Even if, as mentioned  
679 before, surfactin can insert itself in the membrane of the cell, the transmembrane efflux is mediated by  
680 protein transporters.

681 As mentioned before, thanks to its amphiphilic structure, surfactin can interact with the membrane of  
682 the cell. Under or at the CMC, the surfactin can insert itself in the membrane, and above the CMC it  
683 can even solubilize it (Deleu et al., 2003, 2013). However, it was hypothesised by Tsuge et al. that the  
684 gene *yerP*, homolog to the RND family efflux pumps, is involved in the surfactin efflux (Tsuge et al.,  
685 2001). Later, Li et al. (Li et al., 2015) showed that the overexpression of three lipopeptide transporters,  
686 dependent on proton motive force, *YcxA*, *KrsE* and *YerP* lead to an increase in surfactin export of 89%,  
687 52% and 145% respectively.

688 Those studies are promising and the efflux proteins need to be further investigated to fully understand  
689 the excretion of surfactin.

690

### 691 **5.2.4. Degradation**

692 Lastly, the importance of surfactin degradation should not be underestimated. Indeed, a decrease in  
693 surfactin concentration of 59% and 73% has been observed during the fermentation process (Nitschke  
694 et al., 2004; Maass et al., 2016), leading to the presence of degradation mechanisms by the cell  
695 themselves.

696 Three hypotheses are considered by the different teams observing this phenomenon. Since that, for  
697 different mediums with the same carbon content, the surfactin decrease happened at the same time, it  
698 could be that surfactin is used as a carbon source after glucose depletion. Or, since the decrease  
699 happened at the same surfactin concentration, that it is degraded because of its possible inhibitory effect  
700 at higher concentration (Maass et al., 2016). It was also shown that the surfactin decrease is linked to  
701 the increase in protease activity in the culture medium and thus the produced enzymes could be  
702 involved in this degradation (Nitschke et al., 2004).

703 As for the excretion, this degradation process was seldomly researched but could greatly influence the  
704 surfactin production.

705

## 706 **5.3. Culture medium and conditions**

707 Landy culture medium, based on glucose and glutamic acid, is one the main culture medium usually  
708 used for surfactin production. Furthermore, some studies have been performed to ameliorate it (Jacques  
709 et al., 1999; Akpa et al., 2001; Wei et al., 2007; Ghribi et al., 2011; Huang et al., 2015; Willenbacher et  
710 al., 2015).

711 However, another type of approach for the culture medium is rising. Indeed, the use of cheap substrate  
712 such as waste or by-products from the agro-industrial field is more and more researched (De Faria et  
713 al., 2011; Gudiña et al., 2015; Moya Ramírez et al., 2015; Paraszkiwicz et al., 2018), since this

714 approach enables a sustainable production of surfactins. The recent review of Zanotto et al. develops  
715 specifically this approach (Zanotto et al., 2019).

716 Concerning the fundamental parameters of culture condition, a pH of 7 and a temperature of 37°C leads  
717 to a higher production rate (Ohno et al., 1995a). However, when up-scaling from a flask culture to a  
718 larger scale, the main challenge in surfactin production appears. Indeed, the agitation rate and  
719 oxygenation of the culture medium play an important role in the production (Hbid et al., 1996; Guez  
720 et al., 2008; Ghribi et al., 2011). As surfactin is a surfactant and thus increases the stability of a gas-  
721 liquid dispersion, this agitation leads to the abundant production of foam. Nonetheless, even if this  
722 foam production is often considered as a drawback, it can be used with the appropriate reactors as an  
723 advantage to easily recover surfactin.

724

#### 725 **5.4. Production processes**

726 For an overproduction of surfactin, the addition of a solid carrier to an agitated liquid culture can  
727 enhance surfactin production by stimulating cell growth and by promoting a biofilm formation. Yeh et  
728 al. (Yeh et al., 2005) added activated carbon, agar and expanded clay, observing a 36 times increase  
729 with activated carbon.

730 Nonetheless, as mentioned before, due to the high foam generation in surfactin production, classical  
731 stirred reactors are not optimal for this bioprocess. Indeed, adding antifoam to the culture medium has  
732 many drawbacks. Antifoams may have a negative effect on cell growth and are costly, but even more,  
733 they have to be eliminated during purification. Thus, multiple strategies can be applied: (i) to use this  
734 foam production to its advantage or (ii) to reduce or avoid foam production.

735 For the first strategy, the foam fractionation method consists in a continuous removal of the foam from  
736 a liquid agitated culture to a sterile vessel. So, this removal is a first purification step and by the  
737 continuous extraction avoids any possible feedback inhibition from the products (Cooper et al., 1981;  
738 Davis et al., 2001). However, the foam can carry a part of the culture medium and cells out and thus  
739 decrease the production. For the second strategy, a rotating disk bioreactor was used by Chtioui et al.  
740 (Chtioui et al., 2012) where a biofilm formation occurs on a rotating disk in a liquid medium. The  
741 process is simple and can easily be upscaled, but the oxygen transfer is quite low and thus not optimal  
742 for surfactin production.

743 *Bacillus* biofilm formation capacity can also be used in other type of biofilm reactors such as packed  
744 bed reactors, where the liquid medium recirculates on a packing in the reactor (Zune et al., 2016). The  
745 purification is easily performed, but the biofilm growth is difficult to control because it depends on the  
746 liquid distribution in the packing. Recent studies have considered the genetic engineering of the  
747 bacterial cells to modify their biofilm formation ability or their filamentous growth in order to enhance  
748 their adhesion on the packing (Brück et al., 2019, 2020).

749 A membrane reactor allows for a bubbleless oxygen transfer through a membrane between the air and  
750 the culture medium. Furthermore, a first surfactin purification can be made through ultrafiltration  
751 coupled to the fermentation (Coutte, Lecouturier, et al., 2010). However, there is a surfactin adsorption  
752 on the membrane and they can be costly when upscaled.

753 Lastly, a solid medium can be used with solid state fermentation that avoids the mechanical stirring of  
754 liquid cultures and thus the foam production. It represents a simple process but with parameters more

755 difficult to control than in a liquid culture. However, many waste and by-products used as novel  
756 substrate are in a solid state and could thus be used without pretreatment (Ohno et al., 1995b).  
757 Most studies are performed on the enhancement of one of the steps of the production process, but some  
758 studies are performed to decrease the costs in a large scale production (Czinkóczy et al., 2020).

759

## 760 **5.5.Purification**

761 The purification process is a major step in the surfactin production and depends on the fermentation  
762 process used. Linked to the techniques mentioned before, foam can be recovered during the  
763 fermentation and lead to 70 % of recovery (Davis et al., 2001; Willenbacher et al., 2014). For a  
764 fermentation process with the surfactin in the liquid medium, acid precipitation, linked to the negative  
765 charge of surfactin, is the oldest and more common used technique. It can lead to a high recovery rate,  
766 but has a low purity (55 %) and is the only technique that cannot be continuously coupled to the  
767 production. Solvent extraction can also be used alone but it is mostly coupled with acid precipitation  
768 to enhance the purity (Kim et al., 1997; Geissler et al., 2017). One of the most common type of  
769 purification, membrane filtration, can especially be used for surfactin through its micelle forming  
770 ability above its critical micelle concentration. The aggregated molecule is larger and thus can be  
771 retained by membranes with a MWCO of 10 to 100 kDa (Jauregi et al., 2013) with recovery rates and  
772 a purity above 90 % depending on the applied membrane. Furthermore, hybrid methods have been  
773 successfully employed, i.e. precipitation before filtration (Chen et al., 2007), which facilitated the  
774 process or increased the final purity.

775 The techniques mentioned above are mostly used for the extraction of surfactin from the culture  
776 medium. Some uses of surfactin require a higher purity that can be obtained with the following  
777 methods. The physico-chemical properties of surfactin can be used through its adsorption on resin or  
778 active charcoal (Liu et al., 2007), leading to variable recovery rates and purity. Chromatographic  
779 derived methods can also be used to get a better purity and to separate individual variants or isoforms  
780 of the lipopeptide (Smyth et al., 2010). Reverse phase chromatography, based on hydrophobic  
781 interactions, is the most common technique employed.

782

## 783 **6. Conclusions**

784 With the improved genetic toolbox which is now available, a larger and more diverse chemical space  
785 of the surfactin scaffold can be generated and explored. This endeavour will create novel surfactin  
786 derivatives with improved, specialized or expanded biological activities. And even if this molecule's  
787 potential applications range is already broad and reaches different industrial sectors, it may be  
788 enhanced with those novel compounds. However, despite the advancements in surfactin production, its  
789 production cost is still withholding it for a widespread commercial use in low added-value applications.

790

## 791 **Author contribution**

792 The literature review and manuscript writing were performed by AT, CC, MB, YL, MD, JN, SG, AA,  
793 HG, HG and PJ, insights were provided by MA, and MM. In addition, AT and PJ have co-ordinated  
794 and synthesized the different contributions. All authors have read and agreed to the published version  
795 of the review.

796

797 **Funding**

798 This work was founded by the ERACoBioTech program (BestBioSurf project) and the European  
799 INTERREG Va SmartBioControl project.

800

801 **Acknowledgements**

802 We thank Edwin Foekema and Tinka Murk (Marine Animal Ecology group of Wageningen University)  
803 for their insights on this manuscript. We thank Andrew Zicler for his help in the figure design.

804

805 **Conflict of interest**

806 PJ is a co-founder of Lipofabrik and Lipofabrik Belgium and a member of the scientific advisory  
807 board of both companies.

808 MHM is a co-founder of Design Pharmaceuticals and a member of the scientific advisory board of  
809 Hexagon Bio.

810

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1392

1393 **Table 1 Techniques for detection and/or quantification of lipopeptide production**

Technique	Advantages	Disadvantages
Blood agar lysis	Ease of use	Not specific and not reliable
Drop collapse	Ease of use	Not specific
Oil spreading	Ease of use, better prediction than drop collapse	Not specific
Surface tension measurement	Ease of use, reliable	Not specific
Color shift	Ease of use, high-throughput	Not specific
HPLC-UV	Can discriminate the different lipopeptides if standard, quantification possible	Expensive equipment
LC-MS	Discriminates the different lipopeptides	Expensive equipment
PCR or genome sequencing	Production capacity measurement	Observes only genes
RT-PCR	Production capacity measurement	Observes only gene transcription

1394

*Figure 1: TOP: The surfactin biosynthetic gene cluster. Structural NRPS genes are indicated in red. The regulatory gene comS, which is co-encoded in SrfAB is indicated in purple. Bottom: Classic module and domain architecture of SrfAA-SrfAD.*

1395 *Figure 2: Biochemical steps for the formation of fatty acid and their channeling to surfactin*  
1396 *biosynthesis. The first step of fatty acid synthesis involves the production of malonyl-CoA by the*  
1397 *acetyl-CoA carboxylase complex (ACC). The malonyl-CoA-ACP transacylase, FadD, transfers*  
1398 *the malonyl groups to the acyl carrier protein (ACP) to produce malonyl-ACP. FabH,*  
1399 *condensates the malonyl-ACP and a priming acyl-CoA substrate to produce the first new C-C*  
1400 *bond. The keto group of the  $\beta$ -ketoacyl-ACP is completely reduced by the reducing enzymes of*  
1401 *the cycle, FabG, FabZ, FabI, and then the condensing enzyme FabF initiates a new round of*  
1402 *elongation of the growing carbon chain utilizing malonyl-ACP. The acyl-ACP product is*  
1403 *primarily channeled to PL biosynthesis or alternatively to surfactin biosynthesis. For this, at*  
1404 *least two additional biochemical steps are required, a hydroxylation of a free FA by YbdT and*  
1405 *its activation by an ACS.*

1406

1407 *Figure 3 : Natural and synthetic variants of surfactin. The natural variants can be obtained through*  
1408 *specific strains, the non specificity of the adenylation domain or the first condensation domain,*  
1409 *a non cyclization or a linearization and through the genetic engineering of the NRPS. The*

1410 *synthetic variants can be obtained through a chemical modification of a natural product or*  
1411 *through total chemical synthesis. The first three molecule naturally produced are surfactin*  
1412 *produced by *B. subtilis* and others, pumilacidin from *B. pumilus* and lichenysin from *B.**  
1413 *licheniformis.*

1414  
1415 *Figure 4 : TOP: Re-defined module and domain architecture of SrfAA-SrfAD with dissected C*  
1416 *subdomains. The new module definition  $C_A-A-T-C_D$  is indicated in light green. BOTTOM:*  
1417 *Examples of biobrick-like exchanges and deletions using a synthetic biology concept. The*  
1418 *resultant changes in the molecule are indicated in red. R represents the rest of the fatty acid*  
1419 *moiety, which has numerous possibilities regarding chain length, degree of saturation and*  
1420 *branching.*

1421  
1422 *Figure 5 : Steps involved in the overproduction of surfactin in Bacillus, from the gene expression to*  
1423 *the degradation. The main steps are in purple, the yellow arrow represent hypothetical*  
1424 *reactions.*  
1425