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Surfactin production is not essential for pellicle and root-associated biofilm development of *Bacillus subtilis*

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surfactins produced in these conditions [13]. Surfactin, as its name suggests, is an extremely powerful biosurfactant, and thus helps bacteria move on solid surface [6,14-18]. These molecules are abundantly produced when B. subtilis colonizes plant roots, and they elicit the induce systemic resistance in plants [19-22].

A strong link between biofilm formation and surfactin production was suggested for different Bacillus species. Mutations in the surfactin synthesis operon were reported to cause partial to severe biofilm defect in B. velezensis FZB42 and B. amyloliquefaciens UMAF6614 [23,24]. Non-surfactin producer strains of UMAF6614 were also impaired in phylloplane colonization [25]. Under specific laboratory growth conditions (i.e. exponentially growing cells inoculated into lysogeny broth medium), surfactin was shown to trigger biofilm formation in B. subtilis via a pore-forming activity, which causes intracellular potassium leakage sensed by KinC that in turn activate the genetic pathway responsible for biofilm formation [26]. This “quorum-sensing like” activity was demonstrated in the model strain NCIB3610. Similarly to reports in B. velezensis and B. amyloliquefaciens, a surfactin deletion mutant of B. subtilis 6051 was shown to be defective for biofilm formation and root colonization [19]. However, a different study showed that biofilms formed by B. subtilis tomato rhizoplane isolates had comparable dry weight among wild-type and surfactin mutants [27]. Finally, deletion of sfp, which is known to be involved in the production of surfactin since it encodes for a 4-phosphopantetheinyl transferase that activates the peptides carrier protein domains from the NRPS machinery, impairs biofilm formation in B. subtilis 3610 [28]. Since sfp mutation is defective for the synthesis of all NRP-derived molecules (surfactin, bacillibactin, plipastatin and bacillaleine), this impair in biofilm formation could be due to a defect in other biosynthetic pathways than surfactin [14]. These conflicting reports and recent results in our laboratories lead us to revisit the importance of surfactin for biofilm formation of B. subtilis in vitro and on planta.

2. Material and methods

2.1. Strains, media, and chemicals

Strains used in the study are listed in Table S1. For routine growth, cells were propagated on lysogeny broth (LB; Luria-Bertani or Lenox broth) medium. When necessary, antibiotics were used at the following concentrations: MLS (1 μg mL⁻¹ erythromycin, 25 μg mL⁻¹ lincomycin); spectinomycin (100 μg mL⁻¹); chloramphenicol (5 μg mL⁻¹) and kanamycin (10 μg mL⁻¹). New B. subtilis isolates were obtained from 5 sampling sites in Germany and Denmark (see Table S1 for coordinates) by selecting for spore formers in the soil. Soil samples were mixed with 0.9% saline solution, vortexed on a rotary shaker for 2 min, incubated at 80 °C for 25 min and serially diluted on LB medium solidified with 1.5% agar [29]. Highly structured colonies were targeted and isolation of B. subtilis strains was confirmed using 16S sequencing followed by whole genome [30]. These new isolates are all naturally competent allowing easy genetic modification using standard protocol for B. subtilis [31]. New isolates and their srfAC:spec derivatives (srfAC:spec marker was transferred from DS1122 [32]) were labeled with constitutively expressed gfp from Pyhpenpap using phygFP plasmids that integrates into the amyE locus [33].

All solvents used for HRMS and chromatography were VWR Chemicals LC-MS grade, while for metabolites extraction the solvents were HPLC grade (VWR Chemicals). Surfactin standard was purchased from Sigma-Aldrich (Cat. No. S3523).

2.2. Culture conditions

For pellicles assays, cells were pre-grown for 2 h and diluted 1:100 in 3 mL LB 3 times, and at the last incubation cells were grown until they reach an OD₆₀₀ between 0.3 and 0.6. OD₆₀₀ was then adjusted at 0.3 with LB, and 13.5 μL were used to inoculate 1 mL of medium in a 24-well plates. Media used for these experiments were MSgg [28] and MSgg + Pectin (MS: 5 mM Potassium phosphate buffer pH7, 0.1 M Mops pH7, 2 mM MgCl₂, 0.05 mM MnCl₂, 1 μM ZnCl₂, 2 μM thiamine, 700 μM CaCl₂, 0.2% NH₄Cl, 0.5% cellobiose and 0.5% pectin (Sigma)). Incubation was done at 30 °C. For pellicles assays of recent B. subtilis soil isolates and its mutant derivatives, three to four colonies were inoculated in 3 mL LB and incubated at 37 °C with shaking at 225 rpm for 4 h. The OD₆₀₀ was adjusted to 1.5 and 1% inoculum of the pre-grown culture was used to seed bacterial biofilms in MSgg [28] or MOLP [34] medium at 30 °C. For colony biofilms, one colony was inoculated in 3 mL LB and rolled for 3 h at 37 °C. The culture was adjusted to an OD₆₀₀ of 1, then 2 μL were spotted on solidified (1.5% agar) MSgg medium.

Col-0 A. thaliana ecotype (subspecies) was used throughout the study. For Fig. 3, seeds were surface-sterilized with 70% ethanol followed by 0.3% sodium hypochlorite (v/v) and germinated on Murashige and Skoog medium (Sigma) containing 0.7% agar and 0.05% glucose in a growth chamber at 25 °C. Root colonization assays were performed using MSNg (MSN supplemented with 0.05% glycerol) as described in Ref. [35]. For Fig. 4, Arabidopsis seeds were surface sterilized using 2% (v/v) sodium hypochlorite with mixing on an orbital shaker for 20 min and then washed five times with sterile distilled water. The seeds were placed on pre-dried Murashige and Skoog (MS) basal salts mixture (2.2 g L⁻¹ Sigma) containing 1% agar in an arrangement of approximately 20 seeds per plate at a minimum distance of 1 cm. After 3 days of incubation at 4 °C, plates were placed at an angle of 65° in a plant chamber (containing five Narva LT 30W T8/865 cool daylight COLOURPLUS lux on each shelf) with a light regime of 16 h light (24 °C)/8 h dark (21 °C). After 6 days, homogenous seedlings ranging 0.8–1.2 cm in length were selected for root colonization assay. Seedlings were transferred into 48-well plates containing 270 μL of MSNg medium [35] per well. The wells were supplemented with 30 μL of exponentially growing bacterial culture diluted to OD₆₀₀ = 0.2. The sealed plates were incubated at a rotary shaker (90 rpm) at 30 °C for 18 h. After the incubation, plants were washed three times with MSNg to remove non-attaching cells and then transferred to a glass slide for imaging using CLSM.

2.3. Beta-galactosidase assays

From pellicle biofilm assays, spent medium was cautiously removed from the wells. The pellicle was then collected in 1 mL of Z-buffer (40 mM NaHPO₄; 60 mM Na₂HPO₄; 1 mM MgSO₄; 10 mM KCl) and transferred in a 1.5 mL tube. The suspensions were sonicated with 1 s pulses (30% power) for 10 s total to break the biofilms, and OD₆₀₀ was measured. Then, 2-mercaptoethanol (final concentration of 38 mM) and freshly prepared lysozyme in Z-buffer (final concentration of 20 μg mL⁻¹) were added. Suspensions were incubated for 30 min at 30 °C, diluted and 100 μL of an ONPG solution (4 mg mL⁻¹ in Z-buffer with 38 mM of 2-mercaptoethanol) were added. 250 μL of Na₂CO₃ 1 M were added when solutions started turning yellow, and the reaction time was recorded. The A₄₂₀₅ₙm and OD₅₅₀ₙm were measured for each solution, and the Miller Units were calculated using: Miller Units = 1000 x ([(A₄₂₀₅ₙm - 1.75 x OD₅₅₀ₙm)]/Tmin x Vml x OD₆₀₀).

2.4. Microscopy

To visualize bacteria on root surfaces for Fig. 3, seedlings were examined with a Zeiss Axio Observer Z1 microscope equipped with a 20X/0.8 Plan-Apochromat objective, and whole root pictures were taken with a Zeiss AxioCam 506 mono. Fig. 3A presents representative images of the various mutants and times of colonization. The fluorescence signal was detected using a YFP filter (ex: 500/20, em: 535/30) and a GFP filter for autofluorescence of the root (ex: 436/20, em: 480/40). All images were taken at the same exposure time, processed identically for compared image sets, and prepared for presentation using Zeiss Zen 2.0 software. Each image is representative of at least 12 root colonization assays performed in three independent experiments. Quantification was
performed using CellProfiler 3.0 (cellprofiler.org) [36].

For Fig. 4., the washed plant roots were transferred to microscope slides and gently sealed with cover slips. Plant root colonization was analysed with a confocal laser scanning microscope (TCS SP8 (Leica) equipped with an argon laser and a Plan-Apochromat 63x/1.4 Oil objective). Fluorescent reporter expression was performed at 488 nm for green fluorescence, while the emitted fluorescence was recorded at 520/23 nm. Single-layer images were acquired and processed with the software Imaged (National Institutes of Health). Each image is representative of 2 root colonization assays performed in two independent experiments.

2.5. Chemical extraction of secondary metabolites from bacterial cultures

Bacterial strains were cultured on M5gg agar plates for 3 days at 30 °C. An agar plug (6 mm diameter) of each bacterial cultures was transferred to a vial and extracted with 1 mL of isopropanol:ethyl acetate (1:3, v/v) with 1% formic acid. The vials were placed in an ultrasonic bath at full effect for 60 min. Extracts were then transferred to new vials, evaporated to dryness under N2, and re-dissolved in 300 μL of methanol for further sonication over 15 min. After centrifugation at 13,400 rpm for 3 min, the supernatants were transferred to new vials and subjected to ultrahigh-performance liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) analysis.

2.6. UHPLC-HRMS analysis

UHPLC-HRMS was performed on an Agilent Infinity 1290 UHPLC system equipped with a diode array detector. UV–visible spectra were recorded from 190 to 640 nm. Liquid chromatography of 1 μL extract was performed using an Agilent Poroshell 120 phenyl-hexyl column (2.1 × 150 mm, 2.7 μm) at 60 °C with acetonitrile and H2O, both buffered with 20 mM formic acid, as mobile phases. Initially, a linear gradient of 10% acetonitrile in H2O to 100% acetonitrile over 15 min was employed, followed by isocratic elution of 100% acetonitrile for 2 min. The gradient was returned to 10% acetonitrile in H2O in 0.1 min, and finally isocratic condition of 10% acetonitrile in H2O for 2.9 min, all at a flow rate of 0.35 mL/min. MS detection was performed in positive ionization on an Agilent 6545 QTOF MS equipped with an Agilent Dual Jet Stream electrospray ion source with a drying gas temperature of 250 °C, drying gas flow of 8 L/min, sheath gas temperature of 300 °C, and sheath gasflow of 12 L/min. Capillary voltage was set to 4000 V and nozzle voltage to 500 V. MS data processing and analysis were performed using Agilent MassHunter Qualitative Analysis B.07.00.

2.7. Genome re-sequencing

Genomic DNA of 3610, SSB46 and MT529 were isolated using Bacterial and Yeast Genomic DNA kit (EURx). Re-sequencing was performed on an Illumina NextSeq instrument using V2 sequencing chemistry (2 × 150 nt). Base-calling was carried out with “bcl2fastq” software (v.2.17.1.14, Illumina). Paired-end reads were further analysed in CLC Genomics Workbench Tool 9.5.1. Reads were quality-trimmed using an error probability of 0.05 (Q13) as the threshold. Reads that displayed ≥80% similarity to the reference over ≥80% of their read lengths were used in mapping. Quality-based SNP and small In/Del variant calling was carried out requiring ≥10 × read coverage with ≥25% variant frequency. Only variants supported by good quality bases (Q ≥ 30) on both strands were considered.

3. Results

3.1. Absence of surfactin has no effect on pellicle formation of NCIB 3610

To assess the importance of surfactin production for biofilm development, pellicle formation, a biofilm on the air-medium interface was first examined in liquid biofilm-inducing medium, i.e. M5gg and MSNc − pectin. M5gg induces biofilm formation via iron availability and glutamate, while pectin, a plant-derived polysaccharide, is the main environmental cue inducing biofilm formation in MSNc + pectin [35]. Since both media present different cues for the bacterial cells, and that pectin was shown to strongly induce surfactin production [37], importance of this molecule for biofilm formation could vary according to the medium used. As shown in Fig. 1A, deletion of srfAA, and consequently absence of surfactin, does not visibly affect pellicle formation in either liquid media. Importantly, a strain deleted for sfp, which is defective for synthesis of all NRPS-derived molecules, is also able to form pellicle in both media.

Importantly, the 3610 srfAA deletion strain used here was newly created (harbouring a kanamycin resistant gene) and did not match the pellicle formation phenotype of the originally published laboratory stock, 3610 srfAA::erm (SSB46 [28]), the latter showing an important delay in pellicle formation (Fig. S1). When the srfAA::erm marker was re-introduced into 3610 by SPFI phage transduction, the newly obtained srfAA::erm strain (MT529) displayed comparable pellicle development to 3610 and srfAA::kan strains. Consequently, the genomes of 3610, SSB46, and the newly created MT529 strains were re-sequenced. In addition to the srfAA::erm mutation, SSB46 strain contained six point mutations that did not exist in the ancestral 3610 or the re-created MT529 strain (see Table S2). However, deletion mutants of the SNP harbouring genes combined with srfAA::kan did not recapitulate the important defect observed with SSB46 strain (Fig. S1), suggesting that the mutation causing the defect is not a loss-of-function or that certain combination of SNPs are responsible for the observed phenotype of SSB46 strain.

Importance of surfactin and sfp for activation of the two main operons involved in biosynthesis of the extracellular matrix, i.e. tapA-sipW-tasA and epsA-O, was further examined using transcriptional lacZ fusions. As shown in Fig. 1B and D, absence of surfactin or deletion of sfp have little to no effect on tapA transcription, and slightly decreases epsA-O transcription in M5gg. In MSNc pectin, absence of surfactin actually increases tapA and epsA-O transcription (Fig. 1C), which also correlates with the more vigorous aspect of pellicles (see Fig. 1A). In the same medium, absence of sfp impairs transcription of both biofilm operons. In summary, in liquid media sfRAA or sfp deletion has only mild impacts on pellicle biofilm formation in B. subtilis 3610.

3.2. Deletion of sfp and srfAA alters colony structure

Biofilm strength can also be evaluated using the complex architecture of colony biofilms growing on solid biofilm-inducing media. Since surfactin is a biosurfactant [17,38], its absence might influence colony expansion [17]. Indeed, as shown in Fig. 2A, colonies of sfRAA show less spreading on solid M5gg and MSNc pectin, but are still very wrinkly. These wrinkles are likely composed mostly of proteinaceous (Tasa) fibres, since expression of Peps is drastically reduced by absence of surfactin, while Peps is not affected (Fig. 2B). Interestingly, the sfp mutant produces small, flat colonies in both media. This strain also has significantly reduced LacZ activity for both biofilm reporters (Peps and Peps) and media, which correspond to the flat phenotype of the colonies. While calculation of the miller units includes normalization for cell number (OD600), this lack of cell growth and incapacity to reach the cell density required for biofilm formation.

Colonies morphology results on solid media clearly show that sfRAA and sfp deletion lead to phenotypes, indicating that in the latter absence of other molecule(s) synthesized via NRP machinery also impacts biofilm formation. Thus, we examined deletion mutants for bacillibactin (dhbA-F), plipastatin (papB) and bacillicain (pksL) (Fig. S2). The mutant defective for B. subtilis siderophore bacillibactin showed small, almost featureless colonies on both media, suggesting an important role for iron-acquisition molecules in this process. The double sfRAA dhbA-F deletion recapitulated the sfp phenotype, suggesting that on solid media, both molecules are required for robust biofilm formation.
3.3. Surfactin is not required for root colonization by B. subtilis

In a natural environment, surfactin production is triggered by contact with plant roots few hours before biofilm formation [37]. Thus, we evaluated the importance of surfactin for root colonization of A. thaliana seedlings, using the system described in Ref. [35]. B. subtilis root colonization was monitored using a PtapA-yfp reporter, allowing us to identify cells actively forming a biofilm on roots. Since absence of surfactin might only delay, instead of inhibit, root colonization, different time points after inoculation were examined. As shown in Fig. 3A, there was no apparent difference in the root colonization patterns and capacities of WT and srfAA cells. We validated these observations by imaging...
whole roots and determining the ratio of YFP expression/root area, which gives us a quantitative measurement of colonization. Indeed, while colonization somewhat varied from one seedling to another, overall there was no significant difference between WT and srfAA root colonization at any time points.

### 3.4. Surfactin is dispensable for pellicle and plant-associated biofilm formation in recent soil B. subtilis isolates

To address the generality of lack of surfactin production on pellicle formation ability, we tested pellicle biofilm development of 6 newly isolated B. subtilis strains recovered from soil samples. As the essentiality of surfactin production for pellicle development has been demonstrated on MOLP medium for B. amyloliquefaciens (previously identified as B. subtilis) UMAF6614 [24], pellicle formation was followed both on MSgg and MOLP liquid media that revealed no observable difference between wild-type and their surfactin mutant derivatives (Fig. 4). Additionally, plant colonization was indistinguishable between the wild-type and srfAC::spec strains (Fig. 4). Finally, to demonstrate the surfactin production ability of these new B. subtilis strains, the isolates were inoculated to MSgg medium and UHPLC-HRMS analysis was performed on isopropanol:ethyl acetate extracts of the agar medium below the colonies. Chemical analysis of the extract along with a standard demonstrated that each and every isolates produced surfactin, but not their srfAC::spec derivatives (Fig. S3).

### 4. Discussion

The promiscuous role of secondary metabolites to function as info chemicals has been previously proposed [39–41]. The B. subtilis produced surfactin has been reported to lead to induction of biofilm development under non-biofilm inducing conditions [26]. Our results highlight that under biofilm inducing conditions, on liquid or solid biofilm-promoting MSgg and MSNg media, biofilm development of B. subtilis 3610 and other newly isolated strains does not require surfactin. Production of both matrix components actually appeared more efficient in MSNg pectin in absence of surfactin, which could be due to the metabolic burden of producing an important NRP [42,43]. In solid media, srfA and sfp mutants display strikingly different phenotypes than WT. In both cases, colony diameter is smaller, stressing the need for surfactin to disperse on a surface [17,18]. Intriguingly, absence of surfactin had a stronger impact on eps than on tapA transcription, suggesting that surfactin and/or colony spreading might be involved in regulating exopolysaccharides production on solid surface. This regulation would be independent from SinR and AbrB, which act identically on both operons [44].

Similarly to pellicle biofilm formation in liquid media, various srfA mutants colonize plant roots with an efficacy identical to WT cells. Surfactin production is stimulated by plant polysaccharides such as pectin, as is biofilm formation [35,37]. Thus, our observations suggest that while surfactin production precedes biofilm formation upon contact between cells and roots, both processes are somewhat independent. They also would have independent roles; biofilms favouring root attachment and surfactin production, triggering the induced systemic resistance.

Our results show that for B. subtilis, surfactin production is not required for robust biofilm formation, which is in contradiction with many reports for surfactin requirement in various Bacilli [23,24,45]. In many of these reports however, the species or the strain examined also produce an iturin, bacillomycin, which is not the case for B. subtilis 3610 or the newly isolated B. subtilis strains [30]. Of note, Luo et al. showed that a srf mutant of Bacillus spp. 916 produces weaker pellicles in liquid medium, and flat colonies on solid MSgg. However, in this case deletion of srf also strongly impairs production of bacillomycin L, which is also
required for strong biofilm establishment and rice leaves colonization by *Bacillus* spp. [45]. Thus, requirement of surfactin for biofilm formation and plant colonization is likely species- or strain-specific in *Bacillus*, and might depend on the presence of iturin production in these strains. Nevertheless, the importance of surfactin production by PGPR strains of *Bacillus* is primarily for the anti-microbial potential and systemic resistance induction by this multi-functional secondary metabolite.

**CRediT authorship contribution statement**

**Maude Thérien**: Conceptualization, Investigation, Methodology, Writing - review & editing. **Heiko T. Kiesewalter**: Investigation, Methodology, Writing - review & editing. **Emile Auria**: Investigation, Validation. **Vincent Charron-Lamoureux**: Investigation, Writing - review & editing. **Akos T. Kovács**: Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition. **Pascale B. Beauregard**: Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biofilm.2020.100021.

**References**


