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Surfactin variants mediate species-specific biofilm formation and root colonization in *Bacillus*

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Summary

Cyclic lipopeptides (cLP) and especially surfactins produced by *Bacillus* spp. trigger biofilm formation and root colonization and are crucial for biocontrol activity and systemic resistance in plants. *Bacillus atrophaeus* 176s isolated from the moss *Tortella tortuosa* produces the cLP fengycins, iturins and surfactins, possesses antifungal activities and can protect tomato, lettuce and sugar beet against *Rhizoctonia solani* infection. In *B. atrophaeus* we identified for the first time the variant surfactin C, which differs from surfactin A produced by *B. subtilis* and *B. amyloliquefaciens* by an isoleucine instead of a leucine at position 7 of the lipopeptide backbone. The analysis of the complete surfactin gene clusters revealed that the dissimilarity is encoded in the adenylation domain of *srfC* and show that surfactin variations are distributed in a species-specific manner in bacilli. We demonstrate that the surfactin A and C with subtle structural differences have varying signal strengths on biofilm formation and root colonization and act specifically on the respective producing strain. This became evident as biofilm formation and root colonization but not swarming motility in surfactin biosynthesis mutants was restored differentially in the presence of exogenously supplemented cognate and non-cognate surfactin variants.

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Introduction

The bacterial genus *Bacillus* comprises important plant-associated strains utilized for control of plant diseases and for plant growth promotion (Ongena and Jacques, 2008). Three families of cyclic lipopeptides (cLP), surfactins, iturins and fengycins are considered as crucial components in these activities as they act as antifungal and antibacterial metabolites and have been shown to stimulate plant defense by inducing systemic resistance. Moreover, cLP have been demonstrated to play a vital role in biofilm formation and root colonization in *Bacillus subtilis* and *B. amyloliquefaciens* (Ongena *et al.*, 2007; Romero *et al.*, 2007; Ongena and Jacques, 2008).

The amphiphilic surfactins act hereby as powerful biosurfactants reducing surface tension alongside roots and facilitating bacterial swarming to nutrient rich niches (Kinsinger *et al.*, 2003; Bais *et al.*, 2004). Surfactin production is often associated with biofilm formation and subsequent root colonization and a strong correlation between defense-inducing activity and the amount of surfactin has been described (Coway *et al.*, 2015). Moreover, surfactins seem to be also involved in establishing intra- and interspecific communications between organisms thriving in the same ecological niche and are sensed by membrane disturbance recognized by the histidine kinase KinC (López *et al.*, 2009; Shah *et al.*, 2009; Oslizlo *et al.*, 2014).

Surfactins consist of a cyclic heptapeptide backbone connected via an amide and lactone bond to the carboxy- and β-hydroxy group to a fatty acid chain (variable length from C_{12} to C_{16}) respectively (Peypoux *et al.*, 1999), and their synthesis is mediated by mega-enzymes called non-ribosomal peptide synthetases (NRPS) (Finking and Marahiel, 2004; Stein, 2005). The adenylation domain, responsible for the selection and recruitment of amino acids on the peptide chain is responsible for biosynthesis of structurally diverse cLP that differ in peptide moiety (Ongena and Jacques, 2008). Variants of surfactin with the same peptide length but differences in amino acid

composition especially at position 1 and 7 have been described in *B. amyloliquefaciens, B. licheniformis, B. pumilus* and *B. subtilis*. Each of these variants contains isoforms that can vary both in length and branching point of the fatty acid chain (Ongena and Jacques, 2008; Jacques, 2011). Until now, little is known about specific roles and functions of variants and isoforms within a group of a given lipopeptide family.

Bacillus atrophaeus is a Gram-positive, non-pathogenic, sporulating bacterium distinguished from other *Bacillus* spp. by its dark pigmentation. It has been used as a surrogate for pathogenic *Bacillus anthracis* to study the spreading of its spores for safety and military aspects (Gibbons *et al*., 2011) and recently, fengycin, a novel antifungal protein and several volatile compounds have been found in the plant-associated *B. atrophaeus* CAB-1 and were shown to contribute to the suppression of cucumber powdery mildew (Zhang *et al*., 2013). However, production of other cLP in *B. atrophaeus* has not been reported so far.

In this study, we assessed the potential of *Bacillus atrophaeus* strain 176s for its capacity to produce cLP with antifungal activities and to control *Rhizoctonia solani* infection in plants and show that the surfactin variants produced in a species-specific manner are acting in their cognate form as signal for biofilm formation and root colonization.

Results

In vitro *antifungal and hemolytic activities of* B. Atrophaeus *176s*

The complete 16S rRNA gene sequence of strain 176s isolated from the moss *Tortella tortuosa* is identical to those of *Bacillus atrophaeus* strains 1942, BSS, NRS 1221A and UCMB-5137. Further *gyrA* gene sequence analysis revealed more than 99% identity to the *gyrA* genes of the *B. atrophaeus* strains. Phenotype characteristics of dark pigmentation observed on TSA plates confirmed the identity of 176s as *Bacillus atrophaeus*.

Bacillus atrophaeus 176s and its supernatants showed antifungal activity against the phytopathogens *Botrytis cinerea* ofi 501-E, *Fusarium oxysporum* ACC01, *Sclerotinia sclerotiorum* MA5092, *Rhizoctonia solani* CBS 101769 and FT1510 comparable to *B. amyloliquefaciens* FZB42 well known for its production of cLPs with antifungal activity (Supporting Information Fig. S1A). Crude cLPs extracts of *B. atrophaeus* 176s showed in liquid a minimum inhibitory concentration (MIC) against *R. solani* of 40 μg/mL comparable to a MIC of 100 μg/mL obtained with *B. amyloliquefaciens* FZB42 crude cLP extracts (data not shown), indicating a potential application in biocontrol.

The hemolytic activity of the crude cLP extracts of *B. atrophaeus* 176s was similar to commercial surfactin (Supporting Information Fig. S2A). Hemolytic activities of wild type (WT) and mutant strains of *B. atrophaeus* and *B. subtilis* published to have reduced hemolytic activities are shown in Supporting Information Fig. S2B. All tested WT strains showed hemolysis, but the *B. atrophaeus* mutant ATCC 9372-1 and the *B. subtilis* mutant OKB 105 showed no hemolysis on plate. The hemolysis mutants also displayed reduced antifungal activities. WT *B. atrophaeus* strain 1942 and WT *B. subtilis* strain OKB 105 exhibited antifungal activity, but less pronounced than *B. atrophaeus* 176s. Their natural mutants ATCC 9372-1and OKB 120 did not reveal antifungal activity (Supporting Information Fig. S1B).

Identification of the cLP based on LC-HRMS(/MS) analysis

To characterize cLP production potentially responsible for protective, antifungal and hemolytic activities, culture filtrates of *B. atrophaeus* 176s grown for 24 h, 48 h and 72 h on Landy, LB and TSB medium at 21 and 28°C were tested for their hemolytic and antifungal properties. Filtrates with the highest activity were grown on Landy at 28°C for 72 h and were subjected to LC-HRMS(/MS) analysis. As reference, *B. amyloliquefaciens* FZB42 known for its capacity to produce surfactin A, bacillomycin D and fengycin (plipastin) A and B, the fengycin and bacillomycin deficient mutant of FZB42 (AK3: ΔbmyA::EmR and Δfen::CmR) as well as commercial standards for surfactin A and iturin were used.

The analysis revealed a series of four peaks with identical mass to surfactins with a β-hydroxy fatty acid chain length from C_{13} to C_{16} in *B. atrophaeus* 176s. However, the retention times of the peaks in the extracted ion chromatograms (XICs, ± 5 ppm), were slightly shifted compared to the corresponding derivatives of different chain length of the authentic surfactin standard (Table 1). For each of the four compounds, the LC-HRMS(/MS) spectra with same precursor *m/z* exhibited the same fragmentation behavior as observed for the corresponding reference peptide (see Fig. 1 for the most intense signal at *m/z* 1022.67), which indicates an identical amino acid sequence and linkage of the β-hydroxy fatty acid as in the surfactin A standard. It should be noted however, the low collision energy fragmentation (Fig. 1) does not allow discrimination between isoleucine and leucine, D- and L-isomers or of different fatty acid isomers, which all pose potential reasons for the observed retention time shift.

LC-HRMS(/MS) analysis of *B. atrophaeus* 176s culture filtrates also showed intense peaks with identical masses (*m/z* 1449.8; 1463.8; 1477.8; 1491.8; 1505.9) to fengycin A and B and with fatty acid of a chain length from C_{15} to C_{17}. This series of peaks with the same retention time and the same fragmentation pattern indicative of fengycin A and B was also observed in *B. amyloliquefaciens* FZB42. The peaks were all missing in the mutant AK3 unable to

Table 1. Surfactins with different fatty acid chain length of culture filtrates of *B atrophaeus* 176s grown for 72 h on Landy broth and detected by LC-HRMS(/MS).

Fatty acid chain length	*m/z*	% Relative signal intensity of combined surfactins	Retention time (minutes) *B. atrophaeus* 176s	*B. amyloliquefaciens* FZB42
C_{13}	1008.66	21.9 ± 3.9%	18.9	18.2
C_{14}	1022.67	45.2 ± 1.9%	19.9	19.2
C_{15}	1036.69	31.6 ± 3.6%	20.7	19.9
C_{16}	1050.71	1.3 ± 0.3%	21.9	21.1

produce fengycins indicating that *B. atrophaeus* 176s produces the same series of fengycins as *B. amyloliquefaciens* FZB42. In addition, *B. atrophaeus* 176s culture filtrates contained low abundant of peaks with identical retention time and accurate mass (*m/z*: 1057.57; 1071.58; 1085.60) to the commercial iturin standard. In contrast, the bulk of bacillomycin D peaks observed in *B. amyloliquefaciens* FZB42 were missing in *B. atrophaeus* 176s. Together, the LC-HRMS(/MS) data indicate the production of iturins in *B. atrophaeus* 176s.

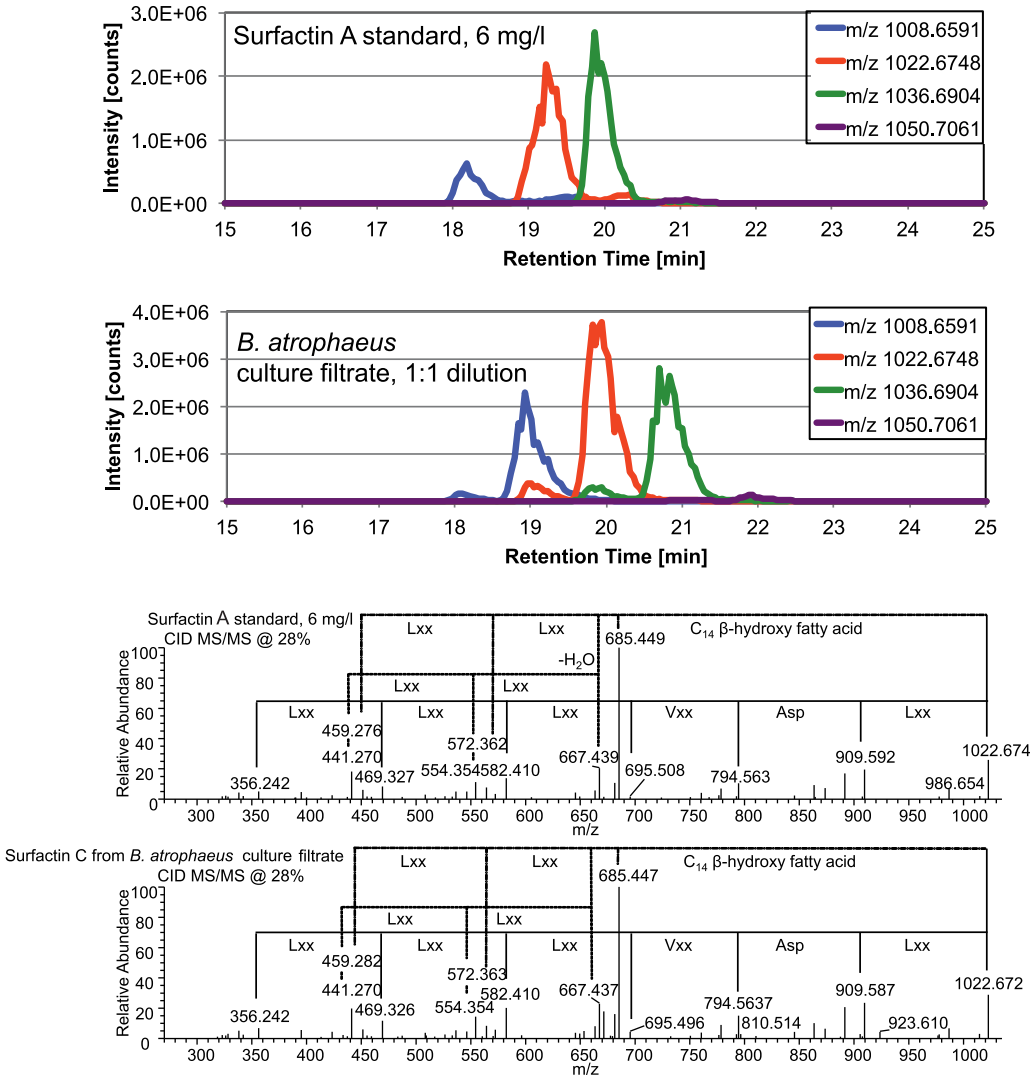

Fig. 1. Extracted ion chromatograms (XIC, ± 5ppm) and CID MS/MS spectra of the protonated molecule of the surfactin containing a C_{14} β-hydroxy fatty acid with *m/z* 1022.67 for the surfactin standard (6.67 mg/l) and a 1:1 dilution of *Bacillus atrophaeus* 176s culture filtrate.

Structure of surfactins and organization of cLP gene clusters in B. Atrophaeus *176s*

In order to determine the exact chemical structure of surfactins from *B. atrophaeus* 176s the purified surfactin (see SI Material and Methods for hemolytic and antifungal activity guided isolation, Supporting Information Fig. S3) was analyzed by NMR, which showed that the isolated compound contains a β-hydroxy fatty acid, 3 leucines and one glutamic acid, valine, aspartic acid and one isoleucine residue, clearly identified by its spin system in the TOCSY spectrum with resonances at δ 4.23 (H_α), 1.87 (H_β), 1.37 ($H_{\gamma1}$-a), 1.15 ($H_{\gamma1}$-b), 0.89 ($H_{\gamma2}$) and 0.87 (H_δ) and the corresponding carbon signals at δ 57.86 (C_α), 38.47 (C_β), 25.78 ($C_{\gamma1}$), 15.92 ($C_{\gamma2}$), and 11.84 (C_δ), respectively. Leucine and Isoleucine can clearly be distinguished by the characteristic chemical shifts of their methyl groups (Supporting Information Table S1; Supporting Information Fig. S4). Furthermore the amino acid sequence of the lipopeptide was determined by long-range crosspeaks of the amide protons of the single amino acids to the carbonyl carbons of the neighboring amino acid in the band selective hmbc spectra. By selective excitation of the carbonyl region all carbonyl resonances could be unequivocally assigned. The amino acid sequence of the compound was established as that of surfactin C, previously isolated from different *Bacillus* strains and studied by NMR spectroscopy (Baumgart *et al.*, 1991; Itokawa *et al.*, 1994; Tang *et al.*, 2007), differing from surfactin A by a leucine isoleucine substitution. The crosspeak from H-3 of the fatty acid to Ile-CO clearly locates this amino acid at position 7 in the lipopeptide sequence. Mass spectral data suggest a mixture of different chain lengths of the fatty acids (C_{13} to C_{16}), but no information on the type of the isomers (n, anteiso, or iso) could be derived from the fragmentation pattern. A more detailed analysis of the ^{13}C and hsqc spectra revealed a mixture of iso- and ante-iso isomers (Lin *et al.*, 1994; Huszcza *et al.*, 2006), whereas non-alkyl could be detected. In addition by performing a quantitative hsqc (Heikkinen *et al.*, 2003) the ratio of anteiso: iso was determined as being approx. 60: 45.

In order to understand the genetic basis for production of the lipopeptides in *B. atrophaeus* 176s, whole genome 454 pyrosequencing was performed.190,069 reads with an average length of 431 bp, reads were generated and assembled to 51 contigs encompassing size of 4.3 Mbp. A total of 9 putative gene clusters containing genes encoding NRPS, PKS, NRPS/PKS hybrids, siderophores and terpenes were predicted using antiSMASH (Weber *et al.*, 2015) and subsequently clusters were assigned to the contigs. The predicted NRPS clusters involved in the synthesis of surfactin, iturin and fengycin were subjected to BLAST and in all cases high similarities to the corresponding clusters of *B. atrophaeus* 1942 were observed. Therefore, surfactin, iturin and fengycin gene sequences of *B. atrophaeus* 1942 were used as reference sequences to assemble the corresponding *B. atrophaeus* 176s contigs to scaffolds. AntiSMASH prediction for the fengycin cluster of *B. atrophaeus* 176s and 1942 is identical to the prediction for *B. amyloliquefaciens* FZB42 and expects a sequence of the lipopeptide backbone of L-Glu-D-Orn-L-Tyr-D-Thr-L-Glu-D-Val-L-Pro-L-Gln-D-Tyr-L-Ile, which is consistent with LC-HRMS(/MS) data and plipastin (fengycin) structures both in *B. atrophaeus* 176s and in *B. amyloliquefaciens* FZB42. The predictions for the iturin clusters of *B. atrophaeus* 176s and 1942 are different to that for *B. amyloliquefaciens* FZB42 and predict a mycosubtilin structure with the oligopeptide sequence L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Ser-L-Asn in agreement with the LC-HRMS(/MS) data indicating the presence of iturins.

Due to the modular structure of the NRPS similar regions regarding *srfA* and *srfB* genes the surfactin cluster could not be unambiguously assigned with NGS data alone. A complete surfactin gene cluster was obtained by gap filling with sequences obtained from PCR with primers as indicated in Supporting Information Table S2 followed by Sanger sequencing. Overall, the complete surfactin gene cluster of *B. atrophaeus* 176s showed 97% identity to *B. atrophaeus* 1942 and 79% to *B. amyloliquefaciens* FZB42 on amino acid level. AntiSMASH analysis of the surfactin cluster of *B. atrophaeus* 176s and 1942 predicted a lipopeptide sequence of L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Ile consistent with the LC-HRMS(/MS) and NMR data and the surfactin C structure (Fig. 2, Supporting Information Table S3).

Antifungal and 176s detection assays in greenhouse

To further evaluate the biocontrol activity of *B. atrophaeus* 176s greenhouse assays with lettuce, sugar beet and tomato infected with *R. solani* were performed under greenhouse conditions. *B. amyloliquefaciens* FZB42 was used as reference strain, as it has been shown to form robust biofilms, colonize roots, support plant growth and exhibit strong biocontrol activity against *R. solani* in lettuce (Chowdhury *et al.*, 2013). The assays revealed that untreated plants of lettuce, sugar beet and tomato were susceptible to fungal infections and majority of the plants died due to damping off within seven days after *R. solani* infection (Supporting Information Fig. S5). Plants inoculated with *B. amyloliquefaciens* FZB42 and *B. atrophaeus* 176s were protected in a comparable manner. Application of both strains resulted in a higher resistance to fungal infection with reduced symptoms (Supporting Information Fig. S5A) and increased plant recovery (Supporting Information Fig. S5B).

Similarly, lettuce plants were treated with WT and their natural surfactin-deficient mutant strains of *Bacillus* and

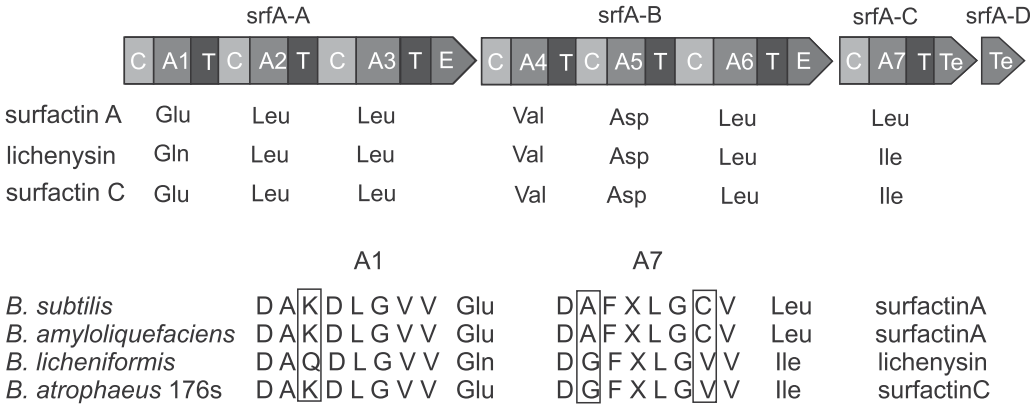

Fig. 2. The architecture of a typical surfactin synthetase gene coding for different surfactin variants.
Condensation domains (C), adenylation domains (A), thiolation domains (T) and thioesterase domains (TE) for the genes of the surfactin operons are shown. The amino acids recruited for the lipopeptide by each adenylation domain are indicated for the different surfactin variants. Dissimilarities between surfactin variants and species can be found in the active site of the adenylation domains A1 and A7 and responsible amino acids in the primary sequence are indicated in one letter code.

challenged with *R. solani* under greenhouse conditions in order to assess the effect of functional surfactin production on biocontrol activity. Two weeks post-inoculation of fungi a clear protective effect was shown by WT strains *B. atrophaeus* 1942 and *B. subtilis* OKB105 compared to the surfactin-deficient mutants *B. atrophaeus* ATCC9372 and *B. subtilis* OKB120, implying surfactin production is crucial for biocontrol activity (Supporting Information Fig. S6A and Supporting Information Fig. S6B).

Surfactin cluster sequences of *B. atrophaeus* 176s show enough differences in the primary nucleotide sequence to *B. atrophaeus* strains in the database to allow design of specific marker primers on the 3`end of the *sfrA* gene (Supporting Information Table S2). These primers allowed the specific detection of this strain in *B. atrophaeus* 176s pre-treated roots of tomato, sugar beet and lettuce, while control and *B. amyloliquefaciens* FZB42 treated plants and controls gave no specific PCR product. The amplicon was 700 bp and identity as 176s *srfA* was confirmed by Sanger sequencing.

Restoration of swarming motility and pellicle formation

To evaluate a potential specific role of the surfactin variants A and C, we examined differences in swarming motility and the pellicle formation in WT and mutant strains of *B. subtilis* and *B. atrophaeus*. The surfactin-deficient mutant ATCC9372 of *B. atrophaeus* showed slightly reduced swarming motility compared to the corresponding WT 1942 (Supporting Information Fig. S7). Also the surfactin-deficient *B. subtilis* mutant OKB120 had less swarming activity compared to its WT OKB 105, albeit less pronounced. After exogenous addition of surfactin A and surfactin C to the growth medium, both surfactins could slightly elicit swarming in the *B. atrophaeus* mutant ATCC9372 and in the *B. subtilis* mutant OKB120, comparable to levels of the corresponding WT strains (Supporting Information Fig. S7; lower panel).

In opposite to the effects on cell swarming, pellicle formation was restored in surfactin biosynthesis mutants to varying amounts depending on the exogenously supplemented surfactin. Importantly, *B. atrophaeus* mutant ATCC9372 with reduced surfactin production responded to exogenous, cognate surfactin C by forming robust pellicles, while only sparse pellicle was observed in the presence of *B. subtilis*–derived surfactin A. Similarly, exogenous, cognate surfactin A triggered robust pellicles in the mutant OKB120 of *B. subtilis*, while *B. atrophaeus*-derived surfactin C induced only sparse pellicles (Fig. 3). This indicates a compound-specific response.

Restoration of bacillus *biofilm development and root colonization by surfactin under gnotobiotic environment*

Given that the pellicle development in surfactin deficient *Bacillus* strains was restored by addition of its cognate surfactin exogenously, we further assessed whether these surfactin mutants might also restore biofilm and root colonization in lettuce plants upon addition of surfactin A or C. One week after inoculation of *Bacillus* strains, we performed live staining and examined the root system under confocal laser scanning microscope to visualize *Bacillus* biofilm and root colonization of lettuce plants grown under gnotobiotic conditions. The WT strains including *B. atrophaeus* 176s and *B. amyloliquefaciens* FZB42 were able to form clusters of cells along cell-walls and root hairs forming biofilm like structures, colonizing the roots (Fig. 4). The WT *B. subtilis* OKB105 showed reduced biofilms compared with the other WT strains, whereas the WT *B. atrophaeus* 1942 heavily colonized the root elongation

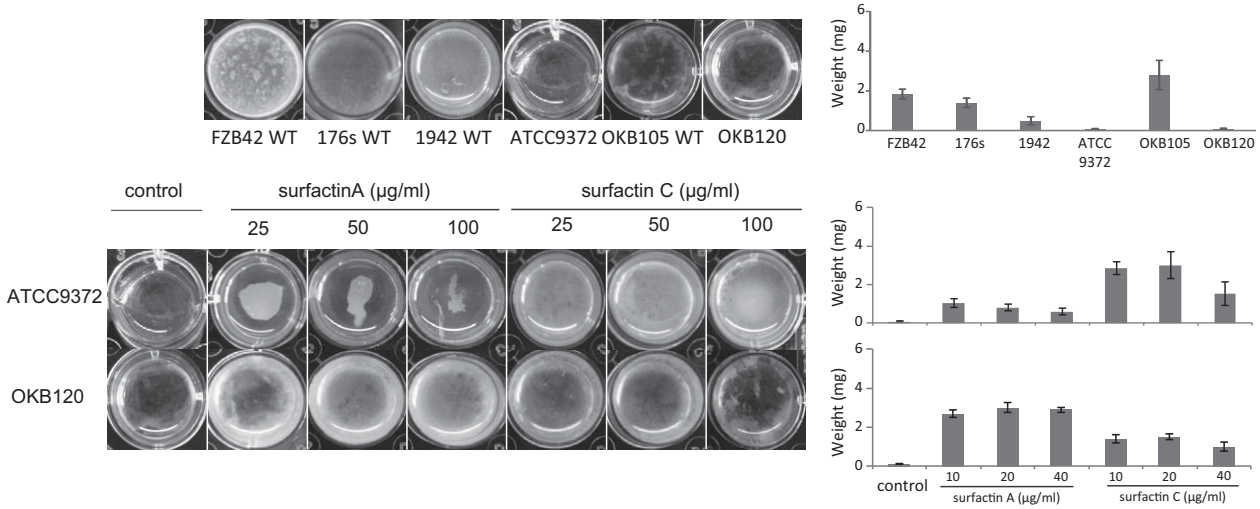

Fig. 3. Influence of surfactin variants on floating pellicle formation.
WT and mutant strains affected in surfactin biosynthesis of *B. amyloliquefaciens, B. subtilis* and *B. atrophaeus* as indicated were grown on LB medium (top and control lane) or LB supplemented with surfactin A from *B. subtilis* (bought from Sigma) and purified surfactin C from *B. atrophaeus* at different concentrations as indicated. Pellicle formation was restored to varying degrees in presence of exogenous surfactins. Bars on the right show pellicle weights determinate in triplicates with standard deviations.

zone but did not display visible biofilm like structures. In contrast, the corresponding surfactin biosynthesis mutant strains *B. atrophaeus* ATCC9372 and *B. subtilis* OKB120 showed only sporadic root hair colonization and showed no sign of biofilm development. Interestingly, addition of exogenous surfactin C but not of surfactin A to the rhizosphere soil has induced robust colonization of *B. atrophaeus* ATCC9372, whereas surfactin A supplementation resulted in increased colonization density and biofilm formation in *B. subtilis* OKB120 suggesting species-specific perception of surfactin variants in induction of biofilm development and root colonization (Fig. 4). The results of biofilm development and root colonization in lettuce plants are consistent with in vitro pellicle development assays.

Discussion

cLP play important roles in plant colonialization and biological control properties of bacilli. Here, we show that the *B. atrophaeus* 176s produces three classes of cLP including antifungal fengycins and surfactins, the latter a compound class which is crucial for biofilm formation and root colonization of plants (Bais et al., 2004; Ongena and Jacques, 2008). Interestingly, the surfactin variant from *B. atrophaeus* 176s differs from surfactins from *B. amyloliquefaciens* and *B. subtilis*. The difference is encoded in *srfC* of the surfactin biosynthesis gene cluster and species-specific surfactin variants are produced by *B. amyloliquefaciens, B. atrophaeus*, *B. licheniformis* and *B. subtilis* indicating that these surfactins may act as species-specific signaling compounds. Supplementation of *B. atrophaeus* and *B. subtilis* mutants with cognate and non-cognate surfactins suggests a specific signaling role in biofilm formation and root colonization.

We identified both gene clusters and showed production of surfactin, iturin and fengycin cLP families in *B. atrophaeus* 176s, compounds likely playingcrucial roles for the protective activities, similarly as described for *B. amyloliquefaciens* (Ongena and Jacques, 2008; Cawoy *et al.*, 2015) and for fengycin fractions of *B. atrophaeus* CAB-1 (Zhang *et al.*, 2013). While surfactins are likely responsible for hemolytic activity, the strong antifungal *in vitro* activity of *B. atrophaeus* 176s can be explained by the production of fengycins. Also co-production of the various cLP may synergistically enhance their individual activities and various lipopeptides can induce resistance in plants (Razafindralambo *et al.*, 1997; Ongena *et al.*, 2007; Romero *et al.*, 2007). Up to now, this is the first report to show the presence and co-production of three lipopeptide families and specifically of surfactin C in *B. atrophaeus* and taken together, these results imply its potential application as biocontrol agent.

Intriguingly, the surfactins from *B. atrophaeus* differs from surfactin A of *B. subtilis* and *B. amyloliquefaciens* by a Leu instead of Ile at position 7 (Koumoutsi *et al.*, 2004; Chen *et al.*, 2009a; Chen *et al.*, 2009b). The assembled surfactin NRPS cluster of *B. atrophaeus* 176s revealed 97% identity to *B. atrophaeus* 1942 and 79% to *B. amyloliquefaciens* FZB42 on amino acid level. The non-ribosomal code encoded by eight amino acids within adenylation domain allows the prediction of A-domain specificity for certain amino acids based on the primary sequence (Stachelhaus *et al.*, 1999; Jacques, 2011). Here, we confirmed that the

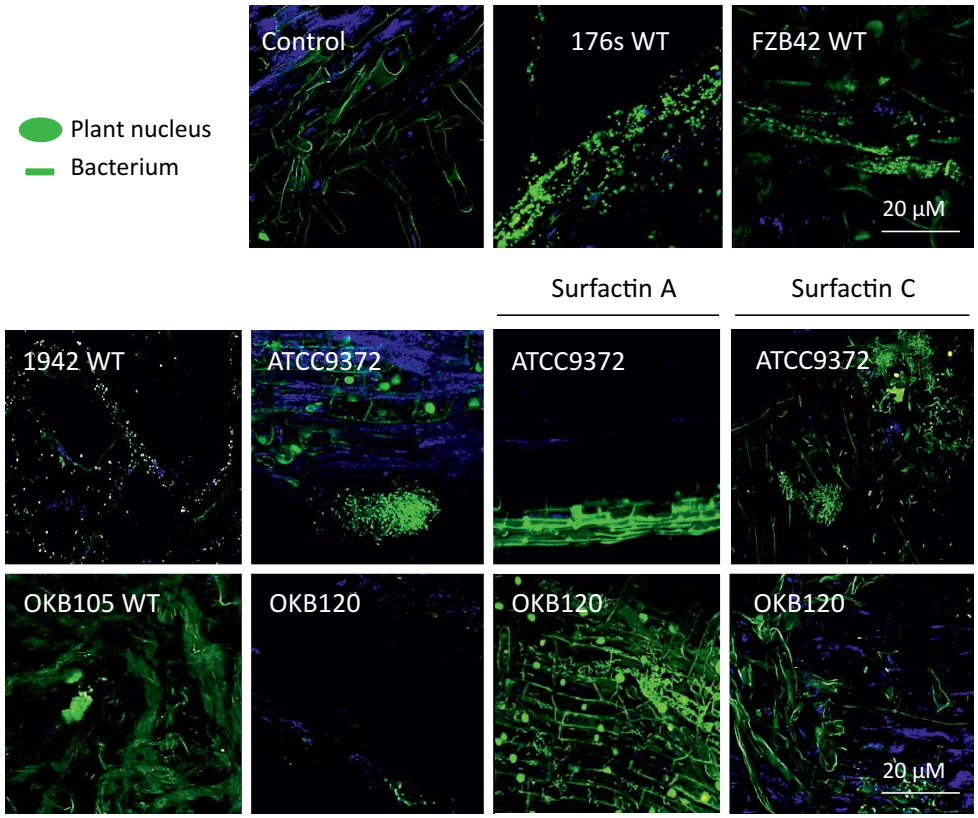

Fig. 4. Biofilm formation and root colonization of WT and surfactin biosynthesis mutant strains of *B. amyloliquefaciens*, *B. atrophaeus* and *B. subtilis*.
Bacillus cells on lettuce roots were visualized by live staining as described in methods. While WT strains were able to colonize the root and except strain 1942 form robust biofilms, the corresponding mutant strains deficient in surfactin production were able to colonize only few regions of the root with severely reduced cell numbers. Exogenous supplementation of surfactin C at a concentration of 40 μM has improved the colonization density of *B. atrophaeus* ATCC9372 compared to control and surfactin A, and vice versa in the case of *B. subtilis* OKB120 were biofilm formation was restored specifically. Pictures are representative of roots of three plants. Scale bars: 20 μm.

non-ribosomal code showed identical formation of amino acids in *B. atrophaeus* 176s and *B. amyloliquefaciens* FZB42 in the first six amino acids of the surfactin (Koumoutsi *et al*., 2004), but striking dissimilarities can be found in of the A domain of *srfC* that encompasses the module 7 (Fig. 2). We observed that substitution of an Ala by a Gly and a Cys by a Val in the primary amino acid composition of A domain seems responsible for recruiting an Ile instead of a Leu in the formation of surfactin. These results are similar to the previous findings observed in the module 7 of lichenysin operon (Jacques, 2011). In our previous study (Aleti *et al*., 2015), utilizing web based prediction tools we confirmed that the majority of the surfactin variants encoded in the genomes of bacilli and related species differed in peptide moiety particularly at position 1 and 7. It is also evident that the well-known *B. subtilis* and *B. amyloliquefaciens* species code for Leu at position 7, while *B. atrophaeus* code for Ile and *B. licheniformis* strains encode Gln and Ile at position 1 and 7, respectively (Aleti *et al*., 2015) (Fig. 2, Supporting Information Table S3). Earlier work on cLP production especially in *Bacillus subtilis* has shown that growth medium supplemented with amino acids substitute L amino acid residues (Peypoux *et al*., 1999). The variant production has been associated with the flexibility of adenylation domains, which can activate and recruit amino acids with similar structures (Shu *et al*., 2002). The results here imply that under growth conditions without specific artificial amino acid the surfactin synthetase precisely produces either surfactin A or C and this information is genetically fixed in NRPS in the form of non-ribosomal code.

Surfactin variants are likely species-specific and genetically encoded suggesting that these compounds are involved in species-specific signaling potentially resulting in different ecological behavior. Intriguingly, surfactin has been implied as a signaling molecule in microbial communication (López *et al*., 2009; Shah *et al*., 2009) and is

believed to act as a quorum sensing molecule involved in the activation of a membrane-associated sensory receptor histidine kinase (KinC). KinC activates a pathway for biofilm formation and consequently root colonization (Ongena and Jacques, 2008; López *et al*., 2009). In addition, strong biosurfactant nature of surfactin plays an intricate role on bacterial swarming to nutrient rich environments and to the biofilm expansion (Kinsinger *et al*., 2003; Bais *et al*., 2004; Angelini *et al*., 2009). In accordance with previous research (Ghelardi *et al*., 2012) our results also indicated that the surfactins influenced swarming motility and were able to enhance the phenotype of swarming motility in surfactin-deficient *Bacillus* spp mutants, particularly in *B. atrophaeus*. We could not observe any surfactin A or C specific effect, pointing to an effect due to the amphiphilic nature of the surfactins or variant unspecific signaling. Biofilm formation and root colonialization, however, was strikingly restored to varying degrees by different surfactin variants. Surfactin mutants of *B. atrophaeus* and *B. subtilis*, which are deficient in pellicle formation androot colonialization, effectively restored phenotypes only in presence of their native surfactins. The differences in the ability of *Bacillus* spp. to respond to exogenous surfactins and to form biofilms suggest the variations in the signal perception of surfactin A and C depending on the genetic background and suggest that subtle structural differences may play a role in species-specific communication. In this respect it is also interesting to note that surfactin A and C show also differences in their confirmation in solution (Itokawa *et al*., 1994) indicating that even the small difference between a Ile and a Leu moiety can have effects on the three dimensional structure of a molecule and consequently on its ability to act as a signal component.

Small signaling molecules like homoserine lactones are very well described for their quorum sensing signaling in Gram-negative bacteria and small chain length differences in the structure of these molecules can lead to substantial differences in cell signaling (Sjöblom *et al*., 2006). However, the role of native surfactins in induction of robust biofilms in *Bacillus* spp. is not well understood. In *B. subtilis* five distinct sensor kinases (Kin A-E) are known to activate the master regulator Spo0A by phosphorylation, which in turn triggers the cellular events sporulation and biofilm formation in response to several environmental and physiological cues (LeDeaux *et al*., 1995; Jiang *et al*., 2000; Vlamakis *et al*., 2013). Surfactins act as a signaling molecule selectively induce potassium leakage and known to stimulate biofilm formation upon membrane disturbance and KinC perception (López *et al*., 2008). Recently, it has also been described that the membrane-associated chaperone protein, flotillin (FloT) embedded in the membrane micro-domains of *Bacillus*, interact with KinC to promote effective binding of specific-signaling proteins (Schneider *et al*., 2015). Also host related factors might modulate biofilm formation (Beauregard *et al*., 2013) and related histidine kinases, particularly KinD, seem to be involved in sensing products released by plant roots (Chen *et al*., 2012). It remains to be seen Kin histidine kinases signaling or so far uncharacterized components are involved in variant-specific recognition of surfactins.

Experimental procedures

Bacillus and fungal strains, cultivation conditions, and in vitro *assays*

B. atrophaeus strain 176s has been isolated from surface-sterilized *Tortella tortuosa* (Pottiaceae, Bryophyta) grown in an Austrian pine forest on limestone. Detailed description on *in vitro* assays and greenhouse assays for protection against *R. solani* are presented in Supporting Information Material and Methods. The identity of the *Bacillus* strain was evaluated by Sanger sequencing of 16S rRNA (8F and 1520R primers) (Weisburg *et al*., 1991) and *gyrA* genes (gyrA-F and gyrA-R primers) (Chun *et al*., 2000), further sequenced with the primers (1520R and gyrA-R, respectively) (Supporting Information Table S1). The gene sequences of 16S rRNA and *gyrA* have been deposited under GenBank accession number KT777650 and KT777651. The reference *Bacillus* strains *B. amyloliquefaciens* FZB42, its mutant AK3 ΔbmyA::EmR, Δfen::CmR deficient in bacillomycin and fengycin biosynthesis, *B. atrophaeus* 1942 and its natural mutant ATCC9372 affected in surfactin biosynthesis as well as *B. subtilis* OKB105 and its mutant OKB120 unable to produce surfactin (Nakano *et al*., 1988; Vollenbroich *et al*., 1994) were obtained from the *Bacillus* Genetic Stock Center (BGSC) (Table 2). All bacilli were cultivated in tryptic soy broth for maintenance and DNA isolation.

Fungal strains are summarized in Table 2. For biocontrol assays, fungal mycelial plugs were placed on YMA plates and incubated at 28°C. After one day, *Bacillus* strains or their cell free supernatants were placed 2cm away from the plug and plates were evaluated after one week. MIC assays were set up in 96 microtitre plates and dilution series of extracted cLP were incubated against *R. solani* at 28°C for 3 days according to the protocol Troskie *et al*. (2012). For hemolytic assays, 1 μL overnight cultures of *Bacillus* strains, cell free supernatants and lipopeptide fractions were tested on 10% sheep blood agar plates and incubated at 28°C for 48 h. Surfactin A from *B. subtilis* (from Sigma) were included as positive control.

Analysis and purification of cLP

For cLP production, a single colony from TSA plates was used as inoculant in three different media (Landy, TSB and LB medium) and grown at different temperatures (21 and 28°C). After 24, 48 and 72 h, the growth medium was centrifuged (5 min, 14,200*g*), the supernatant was filter-sterilized and 500 μL cell free culture filtrate was then mixed with 500 μL acetonitrile (Merck, pA) for LC-HRMS(/MS) analysis on a Accela HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled to an LTQ Orbitrap XL (Thermo Fisher Scientific) equipped with an electrospray ionization (ESI)

Table 2. Bacillus WT and mutant strains used in this study.

Microbial strain	Description	Source/reference
B. atrophaeus 176s	Wild type	this study; AIT
B. atrophaeus 1942	Wild type	Gibbons et al., 2011; BGSC
B. atrophaeus ATCC9372-1	Natural surfactin biosynthesis mutant	Gibbons et al., 2011; BGSC
B. subtilis OKB105	Wild type	Nakano et al., 1988; BGSC
B. subtilis OKB120	Surfactin biosynthesis mutant, srfa::Tn917 ΩOK120	Nakano et al., 1988; BGSC
B. amyloliquefaciens FZB42	Wild type	Koumoutsi et al., 2004; BGSC
B. amyloliquefaciens AK3	Bacillomycin & fengycin biosyntheis mutant, δbmya::emr, Δfen::cmr	Koumoutsi et al., 2004; BGSC
Botrytis cinerea ofi 501-E	Wild type fungus	AIT
Fusarium oxysporum ACC01	Wild type fungus	AIT
Sclerotinia sclerotiorum MA5092	Wild type fungus	ACBR
Rhizoctonia solani CBS101769	Wild type fungus from soy bean	CBS
Rhizoctonia solani FT1510	Wild type fungus from sugar beet	ACBR

ACBR: Vienna University of Natural Resources and Life Sciences strain collection; AIT: Austrian Institute of Technology strain collection; BGSC: *Bacillus* Genetic Stock Center; CBS: Centraalbureau voor Schimmelcultures.

interface which was operated in positive ionization mode (Supporting Information Methods).

For preparative isolation, crude cLP were extracted as previously described (Vater *et al.*, 2002; Smyth *et al.*, 2010). The cLP were further purified by an adapted SPE (solid phase extraction) technique (Kinsella *et al.*, 2009; Pertot *et al.*, 2013) as described in Supporting Information Methods.

For structural analysis, the purified surfactin was analyzed by NMR on a Bruker Avance II 400 (resonance frequencies 400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C) equipped with a' 5 mm observe broadband probe head (BBFO) with z–gradients at room temperature with standard Bruker pulse programs (Supporting Information Methods). The samples were dissolved in 0.6 mL of CD3CN (99.8% D, euriso-top). Chemical shifts are given in ppm, referenced to residual solvent signals (1.94 ppm for ^{1}H, 118.26 ppm for ^{13}C).

Sequence analysis

Overnight cultures were harvested by centrifugation and DNA was isolated with phenol/chloroform and isopropanol precipitation. Five μg DNA of *B atrophaeus* 176s was subjected to pyrosequencing with a 454 SeqAnal Roche at GATC (Konstanz, Germany). Reads were assembled to contigs with the assembly package at GATC. Contigs were analyzed and annotated with RAST (Brettin *et al.*, 2015) and antiSMASH (Weber *et al.*, 2015) for the presence of contigs encoding for secondary metabolites. Contigs were assembled to scaffolds with *B atrophaeus* 1942 as reference. Contigs 4, 11, 18 and 33 contain surfactin encoding regions, which were further assembled by Sanger sequencing of the joint regions using the primers in the regions *srfA, srfB* and *srfC* (Supporting Information Table S2). The complete gene sequences of surfactin, iturin and fengycin biosynthesis clusters have been deposited in GenBank under accession numbers KP943734 to KP943745.

Swarming motility and floating pellicle formation assays

Swarming motility and biofilm formation assays were carried out in 24-well microtiter plates as previously reported (Luo *et al.*, 2015). 1 μL of cells from the overnight cultures at 28°C were inoculated on LB plates containing 20 μg/mL congo red and 10 μg/mL coomassie brilliant blue solidified by adding 0.7% agar. Following plates were incubated at 28°C for 72 h to evaluate swarming motility by measuring the diameter. For exogenous surfactin supplementation studies, appropriate dilutions of surfactin from Sigma and purified surfactin from *B. atrophaeus* were spread and air dried before the assay.

For floating biofilm formation (pellicle) studies, 1 μL of cells from the overnight cultures at 28°C were subsequently inoculated in 2 mL of LB medium containing 20 μg/mL congo red and 10 μg/mL coomassie brilliant blue in 24-well microtiter plates, next incubated at 28°C without shaking and dry weight of floating pellicle was recorded. *B. subtilis* and its mutants were grown for 48 h and other strains for 24 h. For pellicle restoration assays, surfactin from Sigma and purified surfactin from *B. atrophaeus* 176s (see below) were supplemented in LB medium at different concentrations. These experiments were repeated at least twice with triplicates.

Microscopy and bacillus *root colonization under gnotobiotic conditions*

Wild-type strains and surfactin biosynthesis mutants of *Bacillus* were tested for biofilm formation and root colonization of lettuce roots with or without exogenously added surfactin A or C. Lettuce seeds were surface sterilized in 70% ethanol for 1 min followed by a wash step with sterile distilled water and then treated in 3.5% sodium hypochlorite for 15 min prior rinsing thoroughly with sterile distilled water thrice. Seeds were then placed in plastic magenta boxes filled with 50 g of soil (1:1 v/v potting soil/sand, autoclaved twice) that had been watered with 17 mL of sterile tap water and were grown in greenhouse with photoperiod of 16 h of light and 8 h of dark. Boxes were opened under sterile laminar air flow and 2 mL of bacteria (10^{7} cells/mL in 0.85% NaCl) with or without 40μM surfactin A or C were added two weeks after germination. One week post-inoculation, plants were delicately harvested, and roots were immersed for at least 15 minutes in Eppendorf tubes containing a Syto9 solution (3 μm in PBS pH 7.2) resulting in green fluorescence of bacteria. Samples were then observed under a confocal microscope (Olympus Fluoview

FV1000 with multiline laser FV5-LAMAR-2 HeNe(G)laser FV10-LAHEG230-2). Observations were carried out with 10X, 20X or 40X objectives. X, Y, Z pictures were taken at 405, 488, 549 nm and with 40X objective and then merged (RGB) using Imaris software. Pictures were cropped due to the convolution process in the microscope. Whole pictures were sharpened and the light balance was improved to observe the image details better as seen when samples were observed in the dark under the microscope as described in Glassner et al. (2015). All experiments were repeated on 3 plants. Images presented in this publication represent the average of colonization.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. ^{1}H (400 MHz) and ^{13}C NMR spectral data for surfactin C with C-14 fatty acid in CD_3CN

Table S2. Primers used in this study

Table S3. An overview of surfactin variants. Differing Surfactin monomers at positions 1 and 7 are underlined.

Fig. S1. In vitro biocontrol assays. (A) Evaluation of antifungal activity of *B. atrophaeus* 176s against (a) *Fusarium oxysporum* ACC01, (b) *Botrytis cinerea* 501-E, (c) *Rhizoctonia solani* FT1510, (d) *Sclerotinia sclerotiorum* MA5092. (B) Antifungal activity of *B. atrophaeus* and *B. subtilis* strains against *F. oxysporum*. The WT strains of *B. atrophaeus* 176s and 1942, and the mutant ATCC9372 affected in surfactin production are shown on the left. *B. subtilis* OKB 105 and its

surfactin biosynthesis mutant *B. subtilis* OKB 120 on the right.

Fig. S2. Hemolytic activities on 10% sheep blood agar plate recorded 48 h after application. A) Hemolytic activities of WT strains *B. atrophaeus* 176s and 1942, *B. amyloliquefaciens* FZB42 and *B. subtilis* OKB 105 and lacking activity of the corresponding mutants *B. atrophaeus* 9372 and *B. subtilis* OKB 120. B) Crude cLP (500 μg) extracted from *B. atrophaeus* 176s with pronounced hemolytic activity. Surfactin from Sigma was used as a positive control.

Fig. S3. Evaluation of SPE purified cLP fractions through hemolytic and activity against *Fusarium oxysporum*. Fraction C displayed strong hemolytic activity but no antifungal activity, representing surfactins, while fraction A displayed strong antifungal activity but no hemolytic activity, indicating fengycins.

Fig. S4. Surfactin C structure shown with the dominant C-14 fatty acid chain as determined by NMR analysis. Numbering of amino acids in accordance with Table S2.

Fig. S5. Suppression of *R. solani* infection in plants pretreated with *B. atrophaeus* 176s and *B. amyloliquefaciens* FZB42. (A) Representative pictures one week after inoculation with *R. solani* strains and plants as indicated in the figure. (B) Protective effect of *B. atrophaeus* 176s and *B. amyloliquefaciens* FZB42 compared to untreated control one week after fungal inoculation. The health status of plants is indicated as H (healthy), S (symptomatic), and D (dead) 7 days after infection. The data shown here represent two independent experiments carried out in the greenhouse.

Fig. S6. Biocontrol activity in lettuce plants pretreated with WT and their surfactin biosynthetic mutants of *B. subtilis* and *B. atrophaeus* in response to *R. solani* infection under greenhouse conditions. (A) Representative pictures two week after fungal inoculation with *R. solani* as shown in the figure. (B) Protective effect of WT strains *B. atrophaeus* 1942 and *B. subtilis* OKB105 compared to their mutants impaired in surfactin biosynthesis two week after fungal inoculation. The health status of plants is indicated as H (healthy), S (symptomatic), and D (dead) 14 days post infection. The data shown here represent two independent experiments carried out in the greenhouse.

Fig. S7. Influence of surfactin variants on swarming motility. WT and mutant strains affected in surfactin biosynthesis of *B. amyloliquefaciens*, *B. subtilis* and *B. atrophaeus* were grown on LB agar plates supplemented with surfactin A from *B. subtilis* (bought from Sigma) and purified surfactin C from *B. atrophaeus* at different concentrations for 72 h. Strains are indicated on the left and concentrations of exogenous surfactins are indicated on the top. In the control lane and WT (on top) no surfactin has been added. Bars show diameter of colonies after 72 h indicative for swarming capacity. Bars signify standard deviations of three replicates.