Biosurfactants synthesized by endophytic Bacillus strains as control of Moniliophthora perniciosa and Moniliophthora roreri

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Introduction

Cocoa is an agricultural, economic commodity considered essential to millions of people worldwide. More than 6 million smallholders cultivate cocoa trees, and more than 40 million people globally depend on cocoa production as their primary income source (Wickramasuriya and Dunwell, 2018). Ecuador is one of the world’s leading cocoa bean growers, producing almost 300,000 MT in a calendar year (Vega and Beillard, 2015). Worldwide, cocoa bean production is severely limited by diseases that have caused a reduction in annual yield of approximately 20%, although the percentage loss may vary across different production regions (Ploetz, 2016). Witches’ broom (Moniliophthora perniciosa) and frosty pod rot (Moniliophthora roreri) are two of the world’s most dangerous cocoa diseases. (Evans, 1981; McElroy et al., 2018). Although they have a narrow geographic distribution and are usually non-lethal diseases, they impact yields by rendering beans in affected pods commercially useless (Ploetz, 2016). Ecuador and Perú are among the top 10 cocoa-producing countries where devastating losses could occur if these phytopathogens were to spread (Evans, 2016; Ploetz, 2016).

The most common strategy for the control and prevention of both diseases is based on copper-based fungicides. (Bateman et al., 2005). Pesticides are known to produce declines in valuable natural resources, including biodiversity loss, soil depletion, and damage inflicted on people and the environment (Le Mire et al., 2016; Lópe–Morales and Salaya–Domínguez, 2017), but nowadays more eco-friendly alternatives are available and have already been implemented. This change has encouraged the use of endophytic bacteria such as biocontrol agents receiving attention as an alternative strategy for the application of synthetic fungicides.

In cocoa crops, the potential of endophytic bacteria and fungi for controlling certain cocoa diseases has been previously reported (Mejía et al., 2008; Melnick et al., 2011). For instance, many Bacillus strains have been extensively studied as they produce various antifungal lipopeptides [LPs], including surfactin, iturin, and fengycin that can elicit relevant antimicrobial properties to inhibit the growth of several phytopathogenic fungi (Torres et al., 2016). Thus, this study aimed to evaluate, under in vitro conditions, the potential antifungal activity of biosurfactant compounds synthesized by cocoa endophytic strains to inhibit the mycelial growth of M. roreri and M. perniciosa.

Materials and Methods

Isolation of endophytic strains and preliminary screening of their antifungal activity

Endophytic strains were isolated from disease-free cocoa cultivars, presenting high numbers of flowers and...
healthy pods. Cocoa cultivars (75 fine flavour National variety and 75 CCN51) were located at two plantations in Naranjal (2°40'35.2" S, 79°38'21.2" W, altitude of 25 m. a.s.l.) and Balao (2°51'51.8" S, 79°37'20.8" W, altitude of 10 m. a.s.l.), Guayas—Ecuador. Bacterial strains were isolated using a protocol previously described by Silva et al. [2012]. Before bacterial isolation, a brief disinfection procedure was carried out to discard epiphytic bacteria. Leaves were washed in running tap water to remove dust, then a 1 cm² piece was cut and sterilized in 70 % ethanol for one min, followed by immersion in 2 % sodium hypochlorite for 1 min and then washed in sterile water for 1 min. Subsequently, leaf tissue was macerated in a 0.85 % NaCl solution and plated on nutrient agar. After 24 h of incubation at 30 °C, morphologically distinct colonies were isolated and purified. The presence of Bacillus—like colonies was confirmed using standard methodologies (i.e. Gram’s staining, motility and phenotypic characteristics). Pure cultures were cryopreserved at –80 °C in Luria Bertani broth [LB, 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter] supplemented with 20 % (v v⁻¹) of glycerol.

Bacterial isolates were screened for their antifungal activity against M. roreri and M. perniciosa. The pathogens were first cultured on fresh Potato Dextrose Agar (PDA) for 10 days at 28 °C, then 5 mm disks containing mycelia and agar of M. roreri and M. perniciosa were aseptically removed. The disks were placed in the center of PDA plates and with an inoculating loop, the isolated bacteria exhibiting typical characteristics of Bacillus were streaked on the opposite side of the Petri dish. The plates were then incubated for seven days at 28 °C and their ability to inhibit fungal growth was observed by the formation of inhibition zones; all tests were performed in triplicate.

Morphological and genetic identification of endophytic bacterial strains

Strains exhibiting antagonistic activity were selected for further characterization. Aliquots of 10 µL from each endophytic isolate were grown in 250 mL flasks containing 100 mL of LB broth. The flasks were incubated at 30 °C and 150 rpm for 24 h or till the mid-log phase had been reached. For colony formation, 2 µL of the liquid culture was inoculated in triplicate onto plates containing Lysogeny broth–glycerol–manganese [LBGM, LB plus 1 % [v v⁻¹] glycerol, 0.1 mM MnSO₄, and 1.5 % agar per liter] solid medium. Endophytic strains were grown on LBGM solid medium as it strongly promotes biofilm formation, sporulation, and pigment production [Shemesh and Chai, 2013]. The plates, incubated at 30 °C for 72 h prior to analyzing the bacterial colony patterns, were photographed daily to observe differences in pattern formation between the isolates.

Genomic DNA of the isolates was extracted using a slightly modified protocol as the one described by [Ausubel et al., 2003]. The Universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (CGGTTACCTTGTTACGACTT) were used to amplify the 16S SSU rRNA gene. Reactions were carried out in 20 µL containing 20 ng of genomic DNA, 0.4 µM of forward and reverse primers, 0.4 µM of reverse primer, 400 µM of dNTPs, and 0.4 U of Taq DNA polymerase. Polymerase chain reaction (PCR) conditions started with an initial denaturation at 96 °C for 5 min, followed by 35 cycles of denaturation at 96 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min. After a final extension at 72 °C for 3 min, the amplicons were analyzed by electrophoresis on 2 % (w v⁻¹) agarose gels. The PCR products were purified and sent for sequencing at Macrogen Inc. (Seoul, South Korea), and all sequences were deposited in the NCBI database under the following accession numbers: SUB8562136 [DS03], SUB8562600 [DS07], SUB8562609 [DS10], SUB8562620 [DS18], SUB8562685 [DS23], SUB8563474 [DS31], SUB8563654 [DS34], SUB8563688 [DS50]. Sequence alignment and classification were performed using the SINA v1.2.11 aligner tool [Quast et al., 2013] and the ARB SILVA SSU NR 99 reference database [Release 132] [Pruesse et al., 2012]. The sequence “Search and Classify” option was used to classify DS strains with the least common ancestor (LCA) method based on SILVA’s taxonomies. Default parameters were used with the only exception that the Bacteria variability profile was selected under advanced alignment parameters. For computing and reconstruction of the phylogenetic tree, the FastTree program was chosen with the GTR model and Gamma rate model options activated. The neighbors mentioned above and the default parameters were used. Additionally, the endophytic strains were screened for genes (Table 1) reported in the synthesis of lipopeptide biosurfactants using specific primers in order to determine the relationship between antifungal and surface activity compounds. PCRs were carried out in 20 µL reaction mixtures containing 10 µL of GoTaq® Green Master Mix, 0.4 µL of each primer, 3.6 µL of ultrapure water and 1 µL of template DNA (20 ng of bacterial genomic DNA). The reactions were generated under the following cycling conditions: initial denaturation at 95 °C for 15 min; 40 cycles of 95 °C for 1 min, 55 °C or 52 °C for 1 min, and 72 °C extensions for 1.5 min; and a final extension at 72 °C for 7 min. A total of 5 µL of each amplification reaction was analyzed by electrophoresis in a 1.5 % agarose gel stained with SYBR Safe.

Biosurfactant production and crude extract recovery

Biosurfactant production was evaluated using two different culture media: Potato dextrose broth (PDB) and Basal Mineral Media (BMM, 20 g. glucose; 8.5 g
Table 1 – Primer sequences used for the amplification of lipopeptide–related nonribosomal peptide–synthetase genes in DS strains.

| Antibiotic     | Gene(s) | Primers                                          | Sequence               | Polymerase chain reaction (PCR) product size |
|----------------|---------|--------------------------------------------------|------------------------|---------------------------------------------|
| Bacyllomicin D | bamD    | ITUD–F1                                           | TTGAAYGTCAAGYGCSCTTTT  | 482 bp                                      |
|                |         | ITUD–R1                                           | TGCMAAATGAAGGCGCGTG    |                                             |
|                | bamC    | BMC–F1                                            | AGTAAATGAGCCGCAATC     | 957 bp                                      |
|                |         | BMC–R1                                            | CCTCTCTCTGCACACTAGAG   |                                             |
| Baciysin       | bacD    | BACD–F1                                           | GCACAGATGGAAATCTGAAG   | 688 bp                                      |
|                |         | BACD–R1                                           | GAAAATATGCCCAAAATG     |                                             |
| Ericin         | eriB    | SPAB–ERIB–F1                                      | GCACAGATGGAAATCTGAAG   | 688 bp                                      |
|                |         | SPAB–ERIB–R1                                      | GAAAATATGCCCAAAATG     |                                             |
| Fengycin       | fenB    | FENC–F1                                           | CCAATGGCACTGAAAG       | 749 bp                                      |
|                |         | FENC–R1                                           | GCACTGGCACTGAAAG       |                                             |
|                | fenC, fenA, fenE | FENC–F1                                      | CCAATGGCACTGAAAG       | 820 bp                                      |
|                |         | FENC–R1                                           | GCACTGGCACTGAAAG       |                                             |
| Iturin         | ituD    | ITUD–F1                                           | TTGAAYGTCAAGYGCSCTTTT  | 482 bp                                      |
|                |         | ITUD–R1                                           | TGCMAAATGAAGGCGCGTG    |                                             |
|                | ituC    | ITUC–F1                                           | CCCCTCTGGTCAAGTGAAATA  | 594 bp                                      |
|                |         | ITUC–R1                                           | TTGGTAAAGCTCGATGTC     |                                             |
| Mersacidin     | mrsA    | MRSA–F1                                           | CCAATGGCACTGAAAG       | 688 bp                                      |
|                |         | MRSA–R1                                           | GCACTGGCACTGAAAG       |                                             |
| Mycosubtilin   | fenF    | ITUD–F1                                           | TTGAAYGTCAAGYGCSCTTTT  | 482 bp                                      |
|                |         | ITUD–R1                                           | TGCMAAATGAAGGCGCGTG    |                                             |
|                | mycC    | MYCC–F1                                           | CCCCTCTGGTCAAGTGAAATA  | 1026 bp                                     |
|                |         | MYCC–R1                                           | TTGGTAAAGCTCGATGTC     |                                             |
| Sublancin      | sunT    | SUNT–F1                                           | CCAATGGCACTGAAAG       | 974 bp                                      |
|                |         | SUNT–R1                                           | CCAATGGCACTGAAAG       |                                             |
| Subtilin       | spaB    | SPAB–ERIB–F1                                      | GCCACAGATGGAAATCTGAAG  | 688 bp                                      |
|                |         | SPAB–ERIB–R1                                      | GAAATATGCCCAAAATG     |                                             |
|                | spaC, spaS | SPAC–F1                                       | GCCACAGATGGAAATCTGAAG  | 460 bp                                      |
|                |         | SPAC–R1                                           | GCCACAGATGGAAATCTGAAG  |                                             |
| Subtilosin     | albF    | ALBF–F1                                           | TGAATGGCACTGAAATCTGAAG | 888 bp                                      |
|                |         | ALBF–R1                                           | AGGCGGTAYGTTGCTGWATCT  |                                             |
|                | albA    | ALBA–F1                                           | TGAATGGCACTGAAATCTGAAG | 625 bp                                      |
|                |         | ALBA–R1                                           | AGGCGGTAYGTTGCTGWATCT  |                                             |
| Surfactin      | srfA    | SRFA–F1                                           | AGGCGCACTGAAATCTGAAG  | 626 bp                                      |
|                |         | SRFA–R1                                           | AGGCGCACTGAAATCTGAAG  |                                             |
|                | sfp     | SFP–F1                                            | ATGAGATTACAGGAATTTA    | 675 bp                                      |
|                |         | SFP–R1                                            | ATGAGATTACAGGAATTTA    |                                             |

NaNO₃; 4 g KH₂PO₄; 5.7 g Na₂HPO₄; 0.21 g MgSO₄·7H₂O; 7.10⁻⁴ g CaCl₂; 0.01 g FeSO₄·7H₂O; 1 g yeast extract per liter. 250 mL Erlenmeyer flasks containing 100 mL of each medium were inoculated with 2 % (v/v⁻¹) of adjusted initial inoculums of 2.0 McFarland standard inoculums. The flasks were incubated for 24 h at 30 °C and 110 rpm in an orbital shaker. Next, the samples were centrifuged for 20 min at 4 °C and 3900 × g. The cell–free supernatant surface activity was evaluated by applying the oil drop assay. In this technique, 200 µL of the cell–free supernatant was added to a Petri dish containing a thin film of crude oil, the formation of clear zones on the oil surface was recorded. The cell–free supernatants were then used to measure the surface tension with a manual force tensiometer. The instrument was calibrated against water (γ ≈ 72 mN m⁻¹) to ensure accuracy over the entire range of surface tension. Surface tension distilled water (72 mN m⁻¹) was used as a negative control, and an uncultured medium was used to compare and determine the surface tension reduction.
produced by each strain ( Coronel–León et al., 2015 ). In addition, to obtain the biosurfactant crude extract ( BCE ) the bacterial cells were removed from the culture medium by centrifugation at 8,000 × g, for 15 min at 4 °C. Next, the cell–free supernatants were subjected to acid precipitation using concentrated hydrochloric acid ( HCl ) until pH 2 was reached, and BCE was left overnight at 4 °C. The BCE was subsequently collected by centrifugation for 20 min at 4 °C, and 11,000 × g) and washed twice with acid distilled water ( pH 2 ) to eliminate any impurities. The BCE was dried in an oven at 40 °C to constant weight and then quantified by gravimetry ( g L − 1 ) ( Coronel–León et al., 2016a ).

**Antifungal activity of biosurfactant compounds against M. perniciosa and M. roreri**

Antifungal activity was determined using the poisoned food method ( Imhof et al., 2003 ). Different concentrations of BCE (0.3 – 500 mg L − 1 ) were added to tubes containing 15 mL of molten PDA; the tubes were then mixed thoroughly and poured into Petri dishes. When the molten PDA was solidified, 5 mm discs of M. perniciosa and M. roreri were placed in the centre of the Petri dishes. Plates without biosurfactant compounds ( only PDA ) were used as positive controls, and each assay was performed in triplicate.

The fungal colonies’ diameter was measured after seven days of incubation at 28 °C, and the mean diameters were calculated. Inhibition of fungal growth was estimated by measuring the fungal colony’s diameter and expressed as a percentage of the inhibition of fungal growth divided by the growth obtained in the control plates. On the other hand, in the case of the BCE a morphological comparison of the pathogen’s hyphae in the contaminated medium was used to evaluate the biosurfactant effect. The pathogen’s hyphae were fixed with scotch tape on a drop of cotton blue on a slide. The structures were observed under a microscope and compared with the forms in the water control.

**Results**

**Isolation of endophytic bacterial strains and preliminary screening of their antifungal activity**

Endophytic strains were isolated from Naranjal and Balao cocoa plantations located on the Ecuadorian coast, where fine flavour National and CCN51 cocoa varieties are commonly cultivated. One hundred and fifty trees exhibiting subtle morphological differences were monitored, 78.4 % of the trees showed no signs of infection and 21.6 % showed disease symptoms. In total, 251 endophytic bacteria were isolated exclusively from disease–free cultivars presenting high numbers of flowers and healthy pods. All isolates were tested against M. roreri and M. perniciosa to determine their potential antifungal activity ( Figure 1 ).

Only 51 bacterial isolates exhibited promising antifungal activity by being able to inhibit the mycelial growth of M. roreri and M. perniciosa. In this context, the in vitro test showed two pools of strains with sufficient antibiosis capacity; in the first group, the isolates DS03, DS07, DS18, DS23, DS31, DS34, and DS50 produced a zone of inhibition against M. roreri by not allowing the pathogen hyphae to develop normally. Out of this first pool strains DS03 and DS34 were the most efficient with an inhibition zone of 3.67 ± 0.20 cm. In the second group, strains DS50, DS03, DS23 and DS31 also inhibited M. perniciosa but to a lesser extent. Isolates DS50 and DS03 showed an inhibition zone of 3.30 ± 0.20 cm. Finally, it was found that strains DS07, DS18 and DS19 did not exert any inhibitory effect against M. roreri, although medium antifungal activity against M. perniciosa was detected ( Figure 2 ).

**Morphological and genetic identification of endophytic bacterial strains**

The preliminary screening performed to determine the potential antifungal activity of the isolates to inhibit the mycelial growth of M. roreri and M. perniciosa, allowed for the identification of seven promising bioactive
bacterial strains [DS03, DS23, DS31, DS34, DS50, DS07 and DS18]. These strains were subjected to further studies to confirm their morphotypes and phylogenetic position. In order to elicit the patterned growth of the endophytic isolates, two high–nutrient agars were chosen [LB broth and LBGM solid medium]. In this study, three out of the five morphotypes were reported on. Strains DS03 and DS07 developed the B morphotype, which is characterized by presenting typical compact round colonial growth with rough edges; strain DS18 seemed to exhibit a tip–splitting, tree branch growth, characteristic of the T morphotype; and strain DS23 looked like a spiral vortex (SV) morphotype (Figure 3). The morphotypes of the strains DS31 and DS50 could not be identified using LBGM solid medium as they did not exhibit any apparent pattern growth [Di Franco et al., 2002; Rudner et al., 1998; Shemesh and Chai, 2013].

In addition, the seven strains were genetically characterized using the 16S SSU rRNA gene sequence, generating sequences of 1,381bp, except for DS18 which presented problems during the sequencing service and only developed a 789bp sequence length. Due to issues during the 16S rRNA gene sequencing sample DS18 was excluded from further genetic analysis to avoid undesirable results. The 26 unique strains sequence matches obtained from the SINA “Search and classify,” and 6 DS sequences [DS18 was excluded] were aligned using the SINA aligner to compare and identify differences within the sequences. The phylogenetic tree generated by the SILVA platform is shown in Figure 4. The phylogenetic analysis showed the six isolated strains DS03, DS07, DS23, DS31, DS34, and DS50 clustered together. All reports showed the same alignment scores for the 16S rRNA sequences of the six DS endophytic strains and *Bacillus velezensis*, *B. amyloliquefaciens* and *B. subtilis* groups, showing the high genetic similarity of the *Bacillus subtilis* species complex. Thus, it can be challenging to define the species or specific strain we were working with solely based on the 16S rRNA gene sequence. In addition to the 16S rRNA gene sequences, there are other gene sequences (gyrA, gyrB, rpoB, polC, groEL) that can be used for the comparison and discrimination of the *Bacillus* strains, although they were not considered for this study and a phenetical approach was selected.

**Biosurfactant production and crude extract recovery**

To determine the relationship between antifungal and surface activity, the amplified 16S rRNA gene sequence (~1400 bp) was used to do a Blast. The analysis of the sequences revealed a high level of similarity between the different *Bacillus* species. The screening of genes involved in the biosynthesis of *Bacillus* spp. antimicrobial peptides showed that all the evaluated strains had ten genes in common (bamD, bamC, bacD, bacAB, fenB, ituB, ituC, ituD, fenF, and albF). Although most genes were present in all strains, the genes fenC, fenA, and fenE were not detected in strain DS34. Gene mycC was not amplified in strains DS03 and DS08. Finally, genes eriB, eriSa, mrsA, mrsM, sunT, spaB, spaC, spaS, alba, srfA and sfP were not detected in any of the isolates. Table 2 depicts the presence and distribution of genes per strain.

A significant group of peptides with antimicrobial activity from *Bacillus* genus are microbial surfactants or biosurfactants; for this reason, the capacity of the strains to synthetize biosurfactants was first tested using the oil drop assay. All tested drops of the selected

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**Figure 3** – Observed patterns of colonies grown on Lysogeny broth–glycerol–manganese (LBGM) medium. Endophytic strains morphotypes on nutrient rich, soft agar plates, from top to bottom pictures A and B depict a typical growing pattern of morphotype B, picture C seems to exhibit a morphotype T, and picture D, apparently, looks like a spiral vortex (SV) morphotype.
strains collapsed in this assay whereas non–surfactant–containing drops [water] remained stable. The surface tension measurements of the cell–free supernatant of each strain in different medium are shown in Table 3. All strains decreased the surface tension on the cell–free cultures when a mineral medium (BMM) was used. The surface tension reduction by each strain was more than 20 mN m⁻¹ after 24h culture, whereas the control medium’s surface tension was 60.0 ± 2.0 mN m⁻¹. The value of the reduction in surface tension was around 31.0 ± 2.0 mN m⁻¹ for all strains (cell–free supernatant).

On the other hand, the supernatants from PDB cultures showed no further surface tension reduction. The next step was to determine the antifungal activity of all strains.

**Antifungal activity of biosurfactant compounds against M. perniciosa and M. roreri**

The minimum inhibitory concentration (MIC) of the biosurfactant compounds ranged between 35 and 70 mg L⁻¹ for M. roreri and M. perniciosa. The quantitative test for antagonistic activity showed that after seven days of incubation, the vegetative mycelium of M. roreri did not grow on concentrations above 35 mg L⁻¹. Similarly, the fungal disc of M. perniciosa showed no signs of growth at 70 mg L⁻¹ concentration for all seven DS strains evaluated. However, below this concentration, a slight development of mycelial structures was observed. In general, BCE showed antagonistic activity against M. roreri and M. perniciosa across a broad concentration in a dose–response manner (Figure 5). Based on these results and those shown in Figure 2, isolate DS03 had the most efficient antagonism against both cocoa pathogens and was, therefore, selected for further studies.

Cellular damage inflicted by BCE (35 mg L⁻¹) against both phytopathogenic fungi was evaluated through *in vitro* tests. The hyphae in M. roreri and M. perniciosa showed degradation, granulation and fragmentation originating from the action of the biosurfactant compounds secreted by isolate DS03 as seen in Figure 6. The most compelling evidence for the production of vesicles by the fungus was observed in M. perniciosa as a consequence of exposure to the bioproduct, which was evident from the deformation of the fungal hyphae. This result strongly suggests the biological control effect that this strain exerted against the phytopathogen, *M. perniciosa*.
In the present study, 150 cocoa trees (75 National and 75 CCN51 cultivars) located at two plantations in Naranjal and Balao, Guayas–Ecuador were monitored. It was found that 78.4% of the trees showed no signs of Witches’ broom nor frosty pod rot diseases and 21.6% showed disease symptoms. In total, 251 endophytic bacteria were isolated from disease–free cultivars presenting high numbers of flowers and healthy pods. Thus, we reasoned that trees colonized by endophytic microorganism might have been protected against phytopathogen infection. There is growing evidence indicating that beneficial microorganisms can suppress the growth of phytopathogens through a number of mechanisms including direct inhibition of pathogen growth by producing antibiotics, toxins, hydrogen cyanide and hydrolytic enzymes (chitinases, proteases and lipases).

Discussion

In the present study, 150 cocoa trees (75 National and 75 CCN51 cultivars) located at two plantations in Naranjal and Balao, Guayas–Ecuador were monitored. It was found that 78.4% of the trees showed no signs of Witches’ broom nor frosty pod rot diseases and 21.6% showed disease symptoms. In total, 251 endophytic bacteria were isolated from disease–free cultivars presenting high numbers of flowers and healthy pods. Thus, we reasoned that trees colonized by endophytic microorganism might have been protected against phytopathogen infection. There is growing evidence indicating that beneficial microorganisms can suppress the growth of phytopathogens through a number of mechanisms including direct inhibition of pathogen growth by producing antibiotics, toxins, hydrogen cyanide and hydrolytic enzymes (chitinases, proteases and lipases) which can degrade virulence factors or pathogen cell–wall components (Whipps, 2001). It has been suggested that endophytic microorganisms living in cocoa trees control the growth of pathogens by secreting extracellular compounds such as antimicrobial peptides and biosurfactants. To the best of our knowledge, this represents the first report of cocoa endophytic Bacillus synthesizing bioactive compounds with antifungal and surfactant properties that have been applied to control under in vitro conditions, the mycelial growth of M. roreri and M. perniciosa, the causal

![Figure 5 - Antifungal activity of the biosurfactant crude extract obtained from the strain DS03 to inhibit the mycelial growth of Moniliophthora roreri and Moniliophthora perniciosa. The control without biosurfactant effect was included.](image-url)
agents of frosty pod rot and Witches' broom diseases, respectively. Antagonistic assays showed that seven out of the 51 endophytic strains evaluated inhibited mycelial growth in both pathogens; these results suggested that these endophytes may be able to synthesize bioactive lipopeptides with antifungal properties.

Morphological analysis suggested that the endophytic strains belong to the genus *Bacillus*. Further genetic analysis using the small ribosome subunit gene sequence confirmed these results; nonetheless, the 16S rRNA gene sequencing did not provide enough evidence to discriminate between *Bacillus* species as most of them exhibited almost 99% similarity to other *Bacillus* species as shown in Figure 4. The phylogenetic tree suggests that the endophytic bacterial strains belong to the *B. subtilis* species complex, and even more specifically to the "operational group *B. amyloliquefaciens*", as described by Fan et al. (2017). According to these results, the *B. velezensis* and *B. amyloliquefaciens* analysed species appear to be the most genetically related species to the endophytic DS strains studied and are consistent with the idea that our cocoa isolated endophytic strains belonged to the "operational group *B. amyloliquefaciens*". According to Fan et al. (2017), the members of the "operational group *B. amyloliquefaciens*" are distinguished from *B. subtilis* by their ability to synthesize nonribosomal lipopeptides such as bacillomycin D or iturin A. Based on the results shown in Table 2 from the amplicon detection of genes involved in lipopeptides’ biosynthesis, all of the seven DS strains were positive in the detection of two nonribosomal peptide–synthetase genes corresponding to *bamC* and *bamD*. These results corroborate our earlier observations, which suggested the DS strains belong to the "operational group *B. amyloliquefaciens*".

Furthermore, a second morphological analysis clearly showed differences between the DS strains, discarding the duplication of results (Figure 3). The colony formation assays allowed us to compare patterns of bacteria growing on agar plates. Differences shown in bacteria multicellularity morphology could have different causes: wider spreading in the plate due to effective use of nutrients or coordination of biochemical activities [Shapiro, 1998]. It has long been known that in natural environments, bacteria most commonly grow as organized multicellular populations that form complex pattern. [Shapiro, 1998]. These patterns are characterized by organized and readily identifiable geometric and predictable morphologies [morphotypes], which can be observed on solid and semi-solid surfaces [Rudner et al., 1998]. It has been noted that *B. subtilis* growing either on nutrient–poor hard agar surfaces or on nutrient-rich soft agar, exhibit characteristic growth patterns. Certain authors have proposed that this pattern formation might occur because the colony tries to alter its shape to survive in adverse environments [Ben–Jacob et al., 1994; Shapiro, 1998], i.e. where high humidity percentages are prevalent. Currently, five morphotypes have been described for *B. subtilis* strains which were grown under demanding agar conditions [up to 4 % agar concentration] and low levels of nutrients [Ben–Jacob et al., 1994]. Although it was previously thought that these features were specific to *B. subtilis* morphotypes, they have been observed in several other Bacilli strains, which suggest the presence of universal growing patterns within this genus [Salhi and Mendelson, 1993]. These findings made evident that the *Bacillus* species complex cannot be simply understood by comparing the 16S rRNA sequence and it is somewhat necessary to apply other identification methods [Fritze,
2004). Thus, the nonribosomal peptide synthetase (NRPS) genes were analyzed so as to characterize the endophytic strains due to their high frequency as antibiotic compounds and surfactant activities produced by Bacillus [Romero et al., 2007].

Moreover, NRPS molecular analysis helps us to understand the antagonistic mechanism of endophytic strains [Ongena and Jacques, 2008]. The seven Bacillus sp., strains selected were found to have sequences related to the genes responsible for producing fengycin, iturin, bacillomycin D, bacilysin, mycosubtilin and subtilisin antimicrobial peptides [Table 2]. It has been reported that Bacillus sp., may produce a variety of antimicrobial peptides under complex regulation influenced by environmental conditions and the presence of competing organisms [Perez et al., 2017; Benitez et al., 2011].

The analyses carried out on the endophytic strains suggest their ability to produce extracellular compounds with antifungal activity. The potential of Bacillus sp., to produce a wide variety of metabolites with antibacterial and antifungal activities has been described elsewhere (Shoda, 2000). Some of these bacteria [B. subtilis, B. pumilus, B. amyloliquefaciens and B. licheniformis] have been commercialized and applied to the control of crop diseases [Haas and Défago, 2005]. Bacillus genus is an impressive producer of lipopeptides, which are found in microbial surfactants. In view of this, our study demonstrates that the seven DS strains had the capacity to produce biosurfactants due to a decrease in the surface activity of water (72 mN m⁻¹ to 30 mN m⁻¹). The antifungal activity shown by the BCE tested, suggested that different antimicrobial molecules could be produced, including lipopeptides. The genus Bacillus is well known for producing cyclic lipopeptide families with biosurfactants properties such as surfactins, iturins, fengycins and kurstakins [Jacques, 2011]. Biosurfactants have been reported to exhibit antibiotic, fungicide, insecticide, antiviral and antitumor activities. Biosurfactant lipopeptides produced by B. subtilis such as bacillomycin [Besson et al., 1977], iturin A [Yu et al., 2002], mycosubtilin [Peypoux et al., 1986], lichenysin [Grangemard et al., 1999], and fengycin [Kim et al., 2004] have been used as ecological alternatives to synthetic agrochemicals. Plaza et al., (2013) reported that a strain of B. subtilis growing on molasses produced surfactin, a biosurfactant with antifungal activity against Botrytis cinerea, Sclerotinia sclerotiorum, Colletotrichum gloeosporioides, and Phoma complanata, achieved more than 40 % of growth inhibition. Other authors have also studied the antimicrobial potential of biosurfactants produced by B. subtilis [Ghribi et al., 2012; Melnick et al., 2011]; they reported that the evaluated strain was able to suppress the growth of Penicillium, Aspergillus, Rhizopus, Alternaria, Puccinia, Peronospora, Candida, Staphylococcus, Enterococcus and Klebsiella. Additionally, Mnif et al. (2015) reported that the lipopeptide mixture exhibited strong antifungal activity against Rhizoctonia solani and Rhizoctonia bataticola with an MIC of 4 mg mL⁻¹ and 0.04 mg mL⁻¹, respectively. Toral et al. (2018) explored the high antifungal activity against B. cinerea of a patented strain of Bacillus that produces lipopeptides; these compounds are thought to trigger antioxidant activity in fruit. It is evident that the information published about lipopeptides and their effects against phytopathogens considers them as a mixture of molecules. Therefore, the production of various lipopeptides by a single strain could be advantageous as a synergistic effect may result. Simultaneous production of certain lipopeptides in many B. subtilis strains [Tsuge et al., 2005] such as iturin A and surfactin [Ahimou et al., 2000] has also been reported. However, the co-production of three or more lipopeptide antibiotics is unusual [Perez et al., 2017]. Iturins and fengycins are mainly catalogued as antifungal lipopeptides. Thus, the presence of these genes in the Bacillus sp., DS strains can explain the potent antifungal activity shown in the dual–culture assays.

Furthermore, lipopeptides from the families of fengycins and surfactins can act as inducers of a systemic resistance response through stimulation of the plant cell’s immune response (Ongena and Jacques, 2008). This phenomenon has been demonstrated in other edible cultivars such as beans and tomatoes. As mentioned above, the cocoa trees from where the Bacillus strains were collected did not show disease symptoms, even though they were collected from a farm with high incidences of M. roreri and M. perniciosa infection. The lack of Witches’s broom and frosty pod rot symptoms on these trees could be explained by the induction of systemic resistance caused by the presence of the Bacillus sp., DS endophytic strains.

In order to analyse the antifungal mechanism of the BCE, M. roreri and M. perniciosa, two microorganisms particularly problematic for the cocoa industry in Ecuador, were selected. The degree of metabolic activity is one of the parameters used to evaluate an antimicrobial compound’s effect: the lower the metabolic activity, the greater the chance of surviving the exposure to an antimicrobial agent [Coronel–León et al., 2016 b]. With this in mind, the cellular effect of exposure to BCE was assessed. The cocoa endophytic strain DS03 showed the highest antifungal activity; this strain caused both phytopathogens’ swelling hyphae due to biosurfactant reaction over the fungus cell wall. In connection with this, Coronel–León et al. (2016c) mentioned that the mechanism of action lipopeptides from Bacillus was based on osmotic perturbation, including ion–pore formation; according to this information, results suggest that liquid accumulated in the periphery of the hyphae due to increased permeability of the cell membrane [Figure 6]. These results are consistent with those published by Mnif et al. (2015) who described the loss of the granulation and fragmentation of hyphal mycelia of the pathogenic fungiRalstonia bataticola when treated with antifungal metabolites produced by B. subtilis SPB1.

This study demonstrated that the endophytic strain DS03 exhibited strong antifungal activity against the cocoa phytopathogens M. roreri and M. perniciosa. These results are important as they provide the basis for future studies where the antifungal activity of the biosurfactants will be tested under greenhouse conditions to corroborate
the in vitro results. In addition, it is essential to note that in this study we used the crude extract biosurfactants which can be considered an economical alternative to be applied in agricultural fields and result in the reduction in phytopathogen incidences.

Conclusion

The endophytic strain isolated from healthy cocoa leaves, named isolate DS03, exhibited strong antifungal activity under in vitro conditions against the cocoa phytopathogens M. roreri and M. perniciosa. Scientific evidence has been provided to demonstrate that biosurfactant crude extract inhibits the growth of these pathogens. Thus, this strain may become a potential biological control agent that might reduce the incidence of frosty pod and Witches’ broom disease in cocoa farms.

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Authors’ Contributions

Conceptualization: Coronel-León, J.R. Data acquisition: Serrano, L.; Sosa Moreno, A.; Sosa Del Castillo, D. Data analysis: Serrano, L.; Sosa Moreno, A.; Bonilla, J.; Coronel-León, J.R. Design of methodology: Serrano, L.; Sosa Del Castillo, D.; Coronel-León, J.R. Writing and editing: Serrano, L.; Sosa Moreno, A.; Sosa Del Castillo, D.; Bonilla, J.; Romero, C.A.; Galarza, L.L.; Coronel-León, J.R. Optimizing the production of the biosurfactant lichenysin and its application in biofilm control. Journal of Applied Microbiology 120: 99–111.

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Biosurfactants to control phytopathogens

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