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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Cyclic lipopeptide signature as fingerprinting for the screening of halotolerant *Bacillus* strains towards microbial enhanced oil recovery

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Abstract

Cyclic lipopeptides (CLPs) are non-ribosomal biosurfactants produced by *Bacillus* species that exhibit outstanding interfacial activity. The synthesis of CLPs is under genetic and environmental influence, and representatives from different families are generally co-produced, generating isoforms that differ in chemical structure and biological activities. This study to evaluate the effect of low and high NaCl concentrations on the composition and surface activity of CLPs produced by *Bacillus* strains TIM27, TIM49, TIM68, and ICA13 towards microbial enhanced oil recovery (MEOR). The strains were evaluated in mineral medium containing NaCl 2.7, 66, or 100 g L^{-1} and growth, surface tension and emulsification activity were monitored. Based on the analysis of 16S rDNA, *gyrB* and *rpoB* sequences TIM27 and TIM49 were assigned to *Bacillus subtilis*, TIM68 to *Bacillus vallismortis*, and ICA13 to *Bacillus amyloliquefaciens*. All strains tolerated up to 100-g L^{-1} NaCl, but only TIM49 and TIM68 were able to reduce surface tension at this concentration. TIM49 also showed emulsification activity at concentrations up to 66-g L^{-1} NaCl. ESI-MS analysis showed that the strains produced a mixture of CLPs, which presented distinct CLP profiles at low and high NaCl concentrations. High NaCl concentration favored the synthesis of surfactins and/or fengycins that correlated with the surface activities of TIM49 and TIM68, whereas low concentration favored the synthesis of iturins. Taken together, these findings suggest that the determination of CLP signatures under the expected condition of oil reservoirs can be useful in the guidance for choosing well-suited strains to MEOR.

Keywords Lipopeptides · Biosurfactant · *Bacillus* · MEOR · ESI-MS

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Introduction

Microbial enhanced oil recovery (MEOR) is an alternative tertiary oil recovery process in which microorganisms and/or their metabolites (biopolymers, gases, acids, solvents, enzymes and biosurfactants) are used to release residual oil from mature and depleted reservoirs (Sen 2008; Suthar et al. 2008).

Biosurfactants are key compounds in MEOR due to their ability to reduce the interfacial tension (IFT) between oil-rocks and oil-brine as well as to alter the wettability of porous media and to emulsify crude oil, which in turn contribute to increase the mobility of oil in the capillary network of the reservoirs (Khire and Khan 1994; Gudiña et al. 2012; Al-Wahaibi et al. 2016). Currently, there is an increased interest in biosurfactants mainly due to their environmentally friendly profile compared to petroleum-based chemical surfactants. Biological surfactants are usually more efficient in extreme conditions of temperature, salinity and pH, and present higher surface activity at lower concentrations than their synthetic counterparts (Banat et al.

2010; Cameotra et al. 2010). Furthermore, they are less toxic, more easily biodegradable, and can be produced from renewable substrates and agroindustrial residues (Mercade and Manresa 1994;Marchant and Banat 2012).

Among surfactants of bacterial origin, the lipopeptides produced by *Bacillus* species have been reported as suitable candidates for MEOR (Joshi et al. 2016). Lipopeptides are mixed molecules composed of a few amino acids attached to a fatty acid tail. Surfactin, a typical cyclic lipopeptide (CLP) produced by *Bacillus subtilis*, is composed by β-hydroxy fatty acids linked to a heptapeptide (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu). Lipopeptides are recognized by their broad-spectrum of biological activities including antimicrobial, antiviral, cytotoxic, antitumor, immunosuppressant, and surfactant (Raaijmakers et al. 2010; Mnif and Ghribi 2015). The main families of lipopeptides comprise surfactins, iturins, lichenysins, and fengycins (Roongsawang et al. 2002; Ongena and Jacques 2008; Kim et al. 2010; Stankovic et al. 2012). Representatives of iturin and fengycin are well-known by their strong antifungal activity (Vanittanakom et al. 1986; Moyne et al. 2004), while surfactin and lichenysin are mainly recognized as powerful biosurfactants (Bonmatin et al. 2003). *Bacillus* species generally synthetize a mixture of lipopeptides from different families by large multifunctional non-ribosomal peptide synthetases (NRPS) (Mootz and Marahiel 1997), generating a plethora of isoforms that differ in amino acid composition, length, and branching of the fatty acids (Li et al. 2012; Mnif and Ghribi 2015), and, therefore, in their biological properties.

For MEOR, biosurfactants can be produced in bioreactors and applied as crude formulations in oil reservoirs. Alternatively, they can be produced in situ by stimulating the native microbial community of the reservoir or by the injection of allochtonous microorganisms. Allochtonous microorganisms must tolerate the prevailing conditions in the harsh environment of reservoirs, including high temperature and salinity and low or no oxygen content (Gudiña et al. 2012). One should keep in mind that the synthesis of lipopeptides is under genetic and environmental influence, and each family of lipopeptide exhibits distinct biological activities; therefore, the composition of the lipopeptide mixtures may be crucial to the success of MEOR.

In this study, we evaluated the effect of low and high NaCl concentration on the composition and surface activity of CLPs produced by *Bacillus* strains aiming to identify candidates and mixtures of lipopeptides more suitable for MEOR application.

Methodology

Source of *Bacillus* strains

Four biosurfactant-producing bacterial strains TIM27, TIM49, TIM68, and ICA13, isolated from Timonha (TIM) and Icapuí (ICA) mangrove sediments in the Ceará State, Brazil, were evaluated in the current study. Sediment samples (0–10-cm depth) were randomly collected at low tide (0.1 m) using a cylindrical sampler (30-cm long and 10 cm in diameter) and transferred to sterile jars. The samples were kept in an ice-cooled box before being transported to the laboratory. These strains were isolated by spread plate after heating the sediments to 80 °C for 60 min (heat shock) with the purpose of selecting spore-producing *Bacillus* strains, which are known to produce CLP biosurfactants. A number of 200 strains were isolated and the strains used in the currently study were selected by their preliminary performance under high NaCl concentration and surfactant activity. The strains were stored at − 80 °C in TGE medium (composition per liter: tryptone 5 g, glucose 1 g, and yeast extract 2.5 g) supplemented with 20% (*v*/*v*) glycerol. The strain TIM49 was deposited at the Coleção de Culturas do Gênero *Bacillus* e Gêneros Correlatos in the Fundação Oswaldo Cruz, Fiocruz/CCGB (registered at WDCM with number 574) and identified with the collection number LFB-FIOCRUZ 1733.

Tolerance to NaCl

The evaluation of the isolates grown in medium containing increasing concentrations of NaCl (2.7, 66, and 100 g L^{-1}) was performed on 96-well microplates containing 100 μl of mineral medium (MM) adapted from Morán et al. (2000), with the following composition per liter: 5-g yeast extract, 1-g $(NH_4)\ 2SO_4$, 6-g Na_2HPO_4, 3-g KH_2PO_4, 0.6-g $MgSO_4 \cdot 7H_2O$, and 10-g glucose, supplemented with 0.1% (*v*/*v*) of a micronutrient solution containing the following composition per liter: 10.95-g $ZnSO_4 \cdot 7H_2O$, 5.0-g $FeSO_4 \cdot 7H_2O$, 1.54-g $MnSO_4 \cdot H_2O$, 0.39-g $CuSO_4 \cdot 5H_2O$, 0.25-g $Co(NO_3)_2 \cdot 6H_2O$, and 0.17-g $Na_2B_4O_7 \cdot 10H_2O$. Each well was inoculated with 50 μl of a pre-culture grown in the MM without NaCl for 16 h after adjustment of the optical density to 0.45 at 600 nm. The microplates were incubated at 30 °C under stirring of 150 rpm for 48 h and bacterial growth was monitored by measuring the culture optical density at 600 nm at different intervals. The assays were performed in triplicates and repeated three times.

Molecular identification

The genomic DNA of the isolates was extracted using a cetyltrimethylammonium bromide (CTAB) protocol (Warner 1996). The 16S rDNA was amplified by polymerase chain reaction (PCR) with the universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGWTCCARCC-3′) (Marchesi et al. 1998). The PCR reaction was performed in a final volume of 25 μL containing 100 ng of genomic DNA (template), 20-mM Tris-HCl (pH 8.4), 50-mM KCl, 1.5-mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, and 1.0 unit of Taq DNA polymerase

(MBI Fermentas Inc., Waltham, MA, USA). PCR reactions were carried out in a thermocycler programmed for an initial denaturation step (4 min at 94 °C) followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. Additionally, the housekeeping genes that encode the subunit B protein of DNA gyrase, a type II DNA topoisomerase (*gyrB*), and RNA polymerase β-subunit (*rpoB*) were amplified and sequenced using the primers UP1-F (5′-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAARTTYGA-3′) and UP2-R (5′-AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT-3′) for *gyrB*; and *rpoB*-F (5′-AGGTCAACTAGTTCAGTATGGAC-3′) and *rpoB*-R (5′-AAGAACCGTAACCGGCAACTT-3′) for *rpoB* (Ahaotu et al. 2013). The PCR was performed at a final volume of 50 μL containing 50 ng of genomic DNA (template), 20-mM Tris-HCl (pH 8.4), 3-mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, and 1.0 unit of Taq DNA polymerase (GoTaq, Promega, Madison, WI, USA). PCRs for the amplification of *gyrB* gene were performed in a thermocycler programmed for an initial denaturation step of 2 min at 94 °C followed by 30 cycles at 94 °C for 1 min, 66 °C for 1 min, and 72 °C for 2 min. The final extension was carried out at 72 °C for 7 min and the product cooled at 4 °C. For amplification of *rpo*B gene, the PCR reaction was performed at the following conditions: first, denaturation at 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 51 °C for 45 s, 68 °C for 50 s, and a final extension of 68 °C for 90 s.

All the PCR products were analyzed by electrophoresis on a 1% (*w*/*v*) agarose gel stained with SYBR ™ safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) and purified using the Wizard ™ SV gel and PCR clean-up system (Promega, USA).

Samples were sequenced by Macrogen (South Korea) using the following primer pairs for 16S rRNA: 27F and 1492R, 518F and 800R, and UP1-F; UP2-R for *gyr*B and *rpoB*-F; *rpoB*-R for *rpo*B genes using the ABI PRISM Bigdye™ terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The partial sequences were used to generate consensus sequences using CodonCode Aligner 5.1.1. The sequences were analyzed and compared with others deposited in GenBank (www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool—BLAST (Altschul et al. 1997). Multiple sequence alignment was performed using the ClustalW program (Thompson et al. 1994). The obtained sequences were deposited in GenBank under the accession numbers KU556321, KU556322, KU556325, KU556327, KY126819, KY126821, KY126812, KY621336, KY126805, KY126807, KY126798, and KY621337.

For construction of the phylogenetic tree, multiple sequences were aligned using the program ClustalW (Thompson et al. 1994) algorithm for each gene separately, then the alignments were trimmed and concatenated. Phylogenetic analysis was performed using Geneious® 9.1.5 software (http://www.geneious.com) (Kearse et al. 2012), and phylogenetic trees were constructed using inferences by the neighbor-joining (Saitou and Nei 1987) method with bootstrap values based on 1000 replications and by Tamura-Nei (Tamura and Nei 1993) method of estimating pairwise evolutionary distances. The phylogenetic tree was edited on MEGA7: Molecular Evolutionary Genetics Analysis version 7.0.21 for bigger datasets (Kumar et al. 2016).

Influence of NaCl on biosurfactant production and activity

The production of biosurfactant was evaluated in 500-mL Erlenmeyer flasks containing 100 mL of the MM medium supplemented with different concentrations of NaCl (2.7, 66, and 100 g L^{-1}) and an inoculum of 1×10^7 cfu mL^{-1} prepared in the same medium. The flasks were incubated for 48 h at 30 °C, under 150 rpm, and bacterial growth was monitored measuring the optical density at 600 nm. The cell-free supernatants were used to measure surface tension and emulsifying activity as well as for biosurfactant purification. Negative controls were performed with MM supplemented with 2.7, 66, or 100 g L^{-1} of NaCl without inoculum.

Measurement of biosufactants' surface tension was performed according to the Ring method described elsewhere (Gudiña et al. 2010). A Krüss K6 Tensiometer (Krüss GmbH, Hamburg, Germany) equipped with a 1.9-cm Du Noüy platinum ring was used. All measurements were performed at 20 °C. Critical micelle concentration (CMC) was calculated from the surface tension measurements of the purified biosurfactants at 20, 100, 250, and 500 mg L^{-1} prepared in ultrapure water. Measurements were performed at 25 °C, and ultrapure water was used to calibrate the tensiometer. The experiments were done in triplicate and repeated three times.

Emulsification activity was determined according to Iqbal et al. (1995) by the addition of 2 ml of kerosene to the same volume of cell-free culture supernatants in glass test tubes in triplicate. The tubes were mixed using a vortex at high speed for 2 min and subsequently incubated at 25 °C for 24 h. The stability of the emulsion was determined after 24 h and the emulsification index (E_{24}) was calculated as the percentage of the height of the emulsified layer (mm) divided by the total height of the liquid column (mm).

Biosurfactant purification

The cell-free supernatant was used to purify the biosurfactants by acid precipitation according to Pereira et al. (2013). Briefly, the supernatants were acidified with 12-M HCl to pH 2.0 and left overnight at 4 °C. Subsequently, the precipitate was collected by centrifugation (15,000 *g*, 20 min, 4 °C) and washed twice with acidified water, pH 2.0. Thereafter, the precipitate was dissolved in ultrapure water by adjusting the pH to 7.0

using 1-M NaOH. The biosurfactant solution was lyophilized and weighted.

Analysis of tensioactive compounds by electrospray ionization mass spectrometry (ESI-MS)

The samples were prepared by solubilizing the recovered biosurfactants in water and methanol (1:1) at a final concentration of 1–2 mg mL^{-1} (*w*/*v*) and the mass determination was performed using a HPLC system (Agilent Technologies 1290 series Infinity System LC, Santa Clara, USA) coupled to a Q-ToF iFunnel (Agilent Technologies 6550) mass spectrometry fitted with an electrospray ionization (ESI) source was used for LC-MS analysis. The mobile phase was 90% methanol with 0.1% of formic acid. Samples (1 μL) were directly infused and were analyzed in positive ion mode. The mass spectrometry parameters used were as follows: the spray voltage was adjusted to + 2.8 kV, the nozzle potential was adjusted to + 110 V, the N_2 desolvation flow was 6.0 L min^{-1} at 300 °C, the sheath gas temperature was set at 350 °C, fragmentor voltage was set at 100 V, and a nebulizer pressure of 30 psi was used. To ensure highest *m/z* accuracy, internal calibration was performed during the analysis using a calibration solution with an ion of reference *m/z* 922,009798 (HP-0921, hexaquis [1H, 1H, 3H tetrafluoropropoxi] phosphazine).

The software MassHunter Workstation LC-MS Data Acquisition B.05.00 was utilized to control and obtain the data. The spectra were analyzed using the software MassHunter Qualitative Analysis B.03.00 (Agilent Technologies, Santa Clara, CA, USA). The spectra were clean regarding the noise in the baseline and unrelated ions using the extraction tool of molecular entities (molecular feature extraction—MFE) and were recalibrated using the reference ion *m/z* 922,009798 (internal standard). Data acquisition was performed along the *m/z* 1000–1700 range (full mode). Two commercial lipopeptides, iturin A and surfactin from *B. subtilis* (Sigma-Aldrich, São Paulo, Brazil), were also analyzed and used as references.

The data were analyzed using heatmap clustering analysis and partial least squares—discriminant analysis (PLS-DA) modules in MetaboAnalyst 3.0 (Xia et al. 2009, 2012, 2015) and the data compared by univariate analyses utilizing normalization to sample median and Pareto scaling.

Results

Tolerance to high NaCl concentration

All four isolates from mangrove sediments were able to grow in medium containing up to 100-g L^{-1} NaCl, being classified as moderate halotolerants.

Molecular identification

The isolates were identified as belonging to the genus *Bacillus*. The near full-length 16S rRNA gene sequence was determined for all strains, and *gyrB* and *rpoB* were applied as complementary genomic markers for the identification of the strains at species level. Pairwise comparison excluding the sites with gaps on GenBank using BLAST revealed that the sequences from TIM27 and TIM49 were most related (99% identity) to *B. subtilis*, while TIM68 had 99% identity to *Bacillus vallismortis*. When these gene sequences from ICA13 were analyzed separately, they showed 100% identity with different strains of *B. subtilis* and *Bacillus amyloliquefaciens*; nevertheless, phylogenetic tree of the three concatenated sequences performed with genomes of *Bacillus* strains has inferred that it is more closely related to *B. amyloliquefaciens* (Fig. 1).

Effect of NaCl concentration on biosurfactant activities

The greatest decreases in the surface tension were observed in the supernatants of cultures grown in medium containing NaCl 2.7 and 66 g L^{-1}, except for strain ICA13. At 100 g L^{-1}, TIM49 and TIM68 reduced the surface tension of the MM from 56 to 46.5 and 35 mN/m, respectively (Fig. 2). The emulsifying activity was greater in cultures grown at NaCl 2.7 g L^{-1} reaching rates up to 56% (Fig. 3). Only TIM49 showed emulsifying activity at 66-g L^{-1} NaCl. The CMC of the purified lipopeptides varied between 20 and 100 mg L^{-1} (data not shown).

Influence of NaCl concentration on lipopeptide signatures

First, we performed FTIR (Fourier transform infrared spectroscopy) analysis that confirmed the presence of functional groups related only to lipopeptide biosurfactants in the culture supernatants (data not shown), subsequently the ESI(+)-MS confirmed that all four strains produced a mixture of surfactin, iturin, and fengycin isoforms (Supplementary Table S1). The main ions assessed were distributed in two distinct regions, one comprising surfactins (*m/z* 990–1072) and iturins (*m/z* 1043–1133) and the other corresponding to fengycins (*m/z* 1431–1505) including $[M + H]^+$, $[M + Na]^+$, and $[M + K]^+$ ions. Each ion displayed a homologous + *m/z* 14 series corresponding to a methylene group (CH_2). The detection of these biomolecules as $[M + H]^+$, $[M + Na]^+$, and $[M + K]^+$ further enhanced the complexity of the observed spectrum.

The ESI(+)-MS of commercial iturin A and surfactin (Sigma-Aldrich®, Saint Louis, EUA) was used as standards to confirm the presence of these classes within the tested samples. The identification of ions belonging to fengycin was

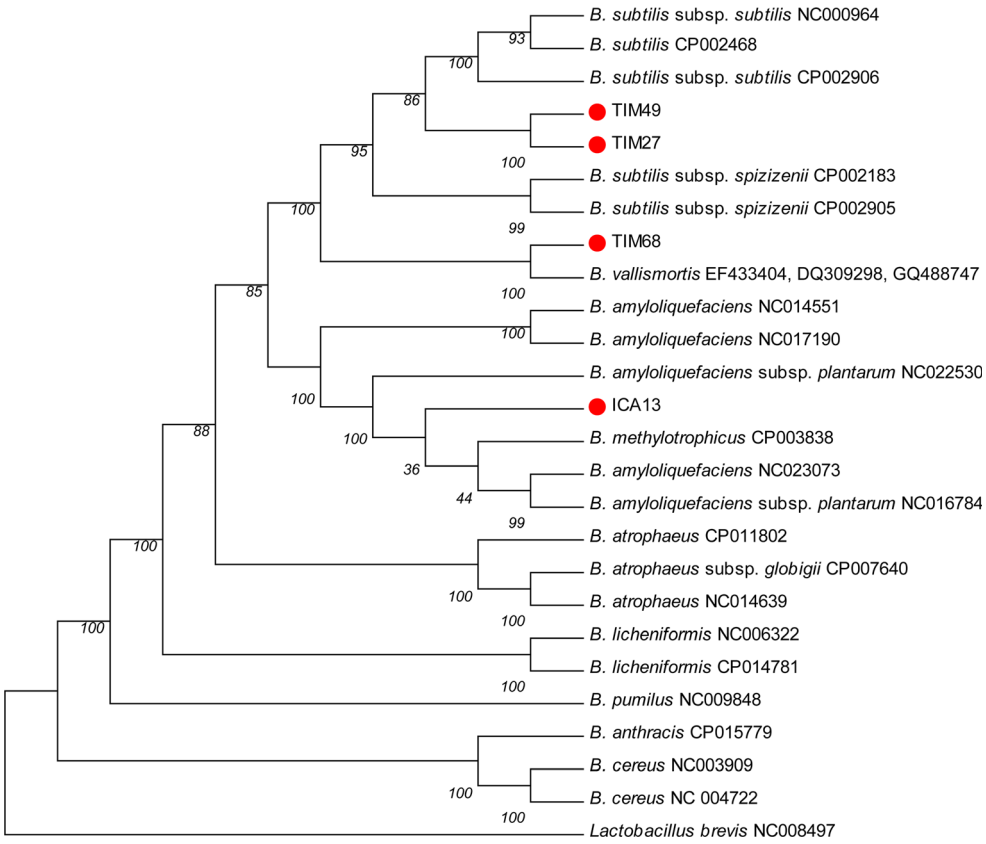

Fig. 1 Majority rule consensus tree from the 16S rRNA, *rpoB*, and *gyrB* gene sequences of the 26 *Bacillus* strains using the neighbor-joining method with Tamura-Nei distances (NJ-TN)

performed in silico using previous reports as reference (Williams et al. 2002; Vater et al. 2002; Chen et al. 2008; Stein 2008; Pueyo et al. 2009; Kim et al. 2010).

Strains ICA13, TIM27, TIM49, and TIM68 exhibited surfactin isoforms A and B, with C12 to C17 fatty acid chains. The ESI(+)-MS showed four $[M+H]^+$ ions of *m/z* 994.64, 1008.66, 1022.67, and 1036.69, which were identified as C12–C15 surfactin A or C13–C16 surfactin B, respectively, and five ions of *m/z* 1016.62, 1030.64, 1044.66, 1058.67, and 1072.69, which were attributed to the $[M+Na]^+$ ion of surfactin A (C12–C16) or B (C13–C17), respectively.

For the iturin family, all strains exhibited isoforms with C13 to C17 fatty acid chains and $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ ions. The ESI(+)-MS of strain ICA13 showed ions identified as iturins A $[M+H]^+$ of *m/z* 1043.55 (C14), 1057.52 (C15), and 1071.58 (C16) and $[M+Na]^+$ of *m/z*

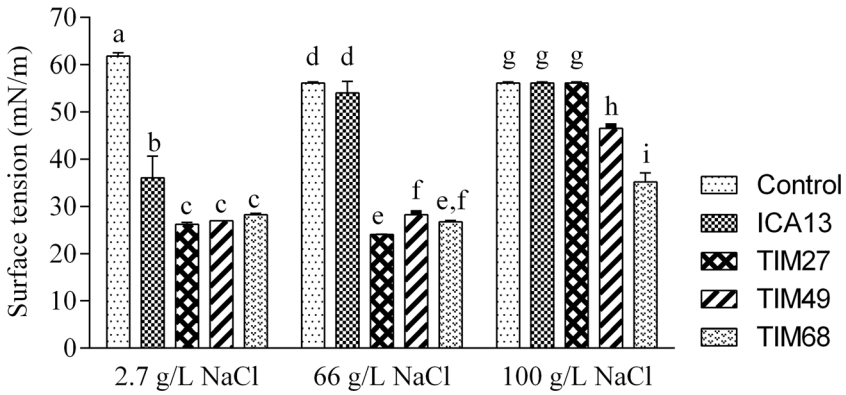

Fig. 2 Effect of NaCl concentration on the reduction of the water's surface tension by halotolerant bacterial strains cultured in mineral medium supplemented with 2.7, 66, and 100 g L^{-1} of NaCl at 30 °C under 150 rpm for 48 h. Negative control corresponds to mineral medium supplemented with 2.7, 66, or 100 g L^{-1} of NaCl without inoculum. For standard deviation calculation, strains were cultivated in triplicate and measurements were also performed in triplicate. Data were analyzed by two-way analysis of variance (ANOVA) with Tukey's post-test with the confidence intervals of 95% ($p < 0.05$) using GraphPad Prism 6.01

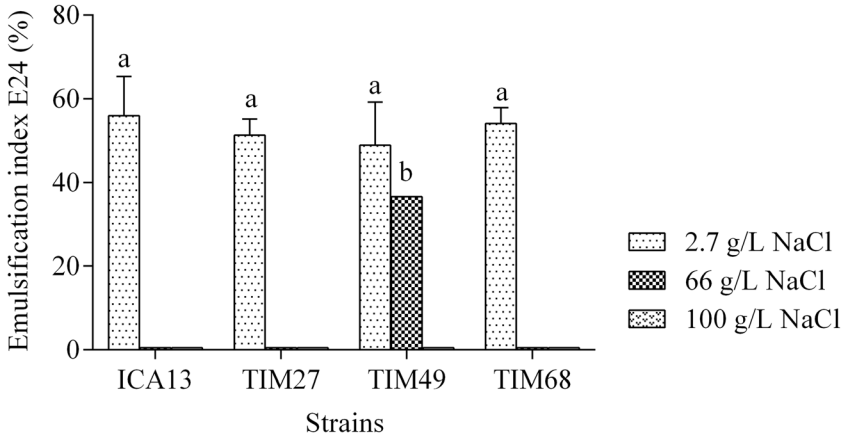

Fig. 3 Effect of NaCl concentration on the emulsification activity of a mixture of water and kerosene by halotolerant bacterial strains cultured in mineral medium supplemented with 2.7, 66, and 100 g L^{-1} of NaCl at 30 °C under 150 rpm for 48 h. For standard deviation calculation, strains were cultivated in triplicate and measurements were also performed in triplicate. Data were analyzed by two-way analysis of variance (ANOVA) with Tukey's post-test with the confidence intervals of 95% ($p < 0.05$) using GraphPad Prism 6.01

1065.54 (C14), 1079.55 (C15), 1093.56 (C16), and 1107.58 (C17). Strain TIM49 displayed ions of *m/z* 1066.64 assigned to iturin A C14 $[M + Na]^+$ or iturin A C13 $[M + K]^+$ and *m/z* of 1080.65 assigned to iturin A C15 $[M + Na]^+$ or iturin A C14 $[M + K]^+$, respectively. TIM68 displayed ions attributed to isoforms of iturin A of *m/z* 1084.58: $[M + H]^+$ (C17), $[M + Na]^+$ 1093.56 (C16), and 1106.56 (C17). This strain also displayed ions with a − 17 (Δ 0.12 ppm) *m/z* difference, that is $[M\text{-}OH]^+$ of *m/z* 1048.65 and 1076.68, which are likely fragments from *m/z* 1065.54–17 and *m/z* 1093.56–17. These ions were then assigned as $[M + Na]^+$ C14 and C16 iturin A, respectively. Strain TIM27 only displayed ions associated with isoforms of iturin A $[M + Na]^+$ of *m/z* 1107.58 (C17). ICA13 also exhibited isoforms belonging to bacillomycin L, a lipopeptide member of iturin family, with fatty acid chains consisting of C14 to C16 identified as $[M + Na]^+$ of *m/z* 1043.55 (C14), 1057.52 (C15), and 1071.58 (C16). Such ions were identified in accordance with Roongsawang et al. (2002) and Stein (2008).

All four strains produced fengycin, exhibiting the major group of $[\text{fengycin} + Na]^+$ comprising fatty acid chains from C14 to C19. Fengycins have been classified into two classes, A and B, corresponding to the presence of D-Ala or D-Val at position 6. Strains ICA13, TIM27, and TIM49 exhibited ions of *m/z* 1477.82 and 1491.83, which were identified as the $[M + H]^+$ ions of C17 fengycin A or C15 fengycin B and C18 fengycin A or C16 fengycin B, respectively. TIM27 and TIM49 also presented an ion of *m/z* 1505.85, which was attributed to $[M + H]^+$ of C19 fengycin A or C17 fengycin B. TIM27 also exhibited an ion of *m/z* 1463.80 corresponding to the $[M + H]^+$ of C16 fengycin A or C14 fengycin B. The ESI-MS of the strain TIM68 exhibited ions of *m/z* 1431.83, 1445.85, and 1461.84, which were attributed to $[M\text{-}OH]^+$ fragment ions from the ions of *m/z* 1449.9, *m/z* 1463.0, and *m/z* 1477.8, respectively (Bie et al. 2009).

Heatmap clustering analysis showed clear differences in the surfactin, iturin, and fengycin isoforms produced at high (100 g L^{-1}) and low (2.7 g L^{-1}) NaCl concentrations (Fig. 4). High NaCl concentration positively influenced the production of surfactin isoforms in TIM49, TIM68, and ICA13 and fengycin, especially in TIM27 and TIM49. fengycin peaks of *m/z* 1477.82 and 1505.85 appeared only for TIM49.

PLS-DA confirmed the clustering based on NaCl concentration and also showed that the strains ICA13, TIM27, and TIM68 grouped more closely, while TIM49 stands apart at both NaCl concentrations (Fig. 5a). Figure 5b shows the main 25 peaks retrieved by PLS-DA for classifying the lipopeptides produced at high and low NaCl concentrations.

Discussion

CLPs comprise surfactin, iturin, fengycin, and lichenysin as the major families of biosurfactant produced by *Bacillus* species (Deleu et al. 1999). Representatives from each CLP family display various surface-active properties depending on their chemical structures. Therefore, CLPs exhibit a wide range of biological activities and several studies have demonstrated their potential for applications in agriculture, medicine, and oil industry, especially for MEOR (Banat 1995; Youssef et al. 2007a; Al-Wahaibi et al. 2016).

The role of biosurfactants in MEOR derives fundamentally from their properties to reduce the interfacial tension between oil/brine and oil/rock, to emulsify oil and to cause changes in the rock's wettability, easing the flow and distribution of the oil inside the reservoir (Khire and Khan 1994).

The surface-active properties of CLPs have been widely described for purified compounds. However, for the application of CLPs in MEOR in situ, it has to be taken into account that they are synthesized by large NRPS or hybrid polyketide

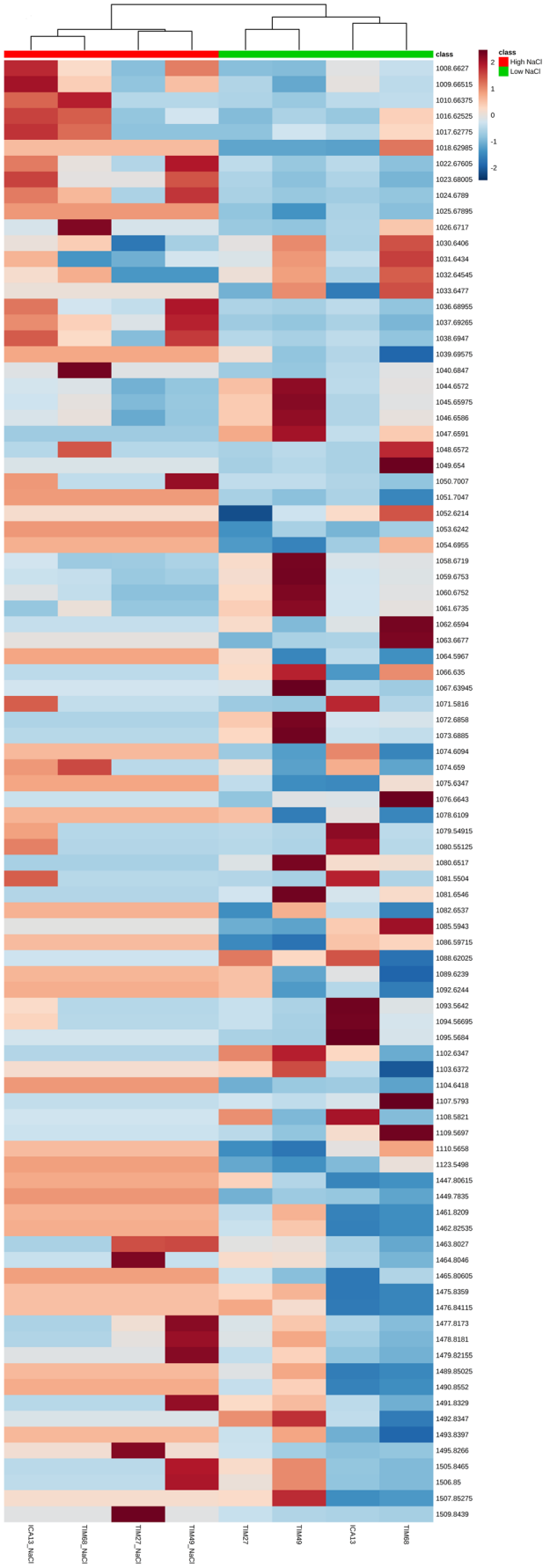

Fig. 4 Heatmap clustering analysis performed in MetaboAnalyst 3.0 using the obtained ESI(+) MS spectra of the lipopeptides produced in the presence (100 g L^{-1}) of NaCl and in the absence (2.7 g L^{-1}) of NaCl by halotolerant bacterial strains (TIM27, TIM49, TIM68, and ICA13). Analysis was performed using Euclidian distance method with ward clustering algorithm after normalization to sample median and Pareto scaling

synthases/non-ribosomal peptide synthetases (PKS-NRPS) (Walsh 2004) and that strains generally produce different lipopeptide molecules both qualitatively and quantitatively (Kowall et al. 1998; Ongena and Jacques 2008). Considering the peculiar synthesis of these compounds and the multiple genetic and environmental factors that influence it, it is reasonable to assume that the resulting CLP mixtures are highly variable and will not always be suitable for the expected biological application. Herein, we suggest that the correlation analysis between the CLP profile and its surface-active properties in the expected conditions of the reservoir can be a useful approach to identify *Bacillus* strains more suitable for MEOR.

In the current study, four bacterial strains isolated from mangrove sediments were classified as moderate halotolerants, growing in up to 1.7 M of NaCl (100 g L^{-1}) (Oren 2008). Halophilic and halotolerant bacteria are key players in MEOR, especially those producers of useful compounds to enhance oil recovery, such as biopolymers and biosurfactants (Belyaev et al. 2004; Sen 2008; Marchant and Banat 2012). When applied in MEOR in situ, bacteria are injected in the reservoirs and should grow in salinity ranging from 0.5 to 160 g L^{-1}, depending on the reservoir (Bass and Lappin-Scott 1997); therefore, the strains studied here could tolerate a broad range of reservoirs.

The selected halotolerant strains TIM27 and TIM49 were identified as *B. subtilis*, ICA13 as *B. amyloliquefaciens*, and TIM68 as *B. vallismortis*. In groups containing closely related species, such as bacilli group, the high similarity between the sequences of the 16S rRNA gene has made it impossible to distinguish individual species. Wang et al. (2007) indicated that genes encoding type II DNA topoisomerase (*gyr*B) and RNA polymerase β-subunit (*rpo*B) are more suitable phylogenetic markers than the 16S rRNA gene alone for the study of phylogenetic and taxonomic relationships at species level. In fact, in our study, the analysis of *gyr*B was decisive for the identification of *B. vallismortis* TIM68.

All four strains produced CLPs in medium containing 2.7-g L^{-1} NaCl and were able to reduce the surface tension to below 30 mN/m. The genus *Bacillus* is widely reported by its lipopeptide production as major supernatant components when cultivated in the conditions we have used (Giro et al. 2009; Pereira et al. 2013; Oliveira et al. 2013). The production of CLPs was initially confirmed by FTIR spectrum (data not shown). This technique can provide crucial information on the molecular structure of organic and inorganic components and

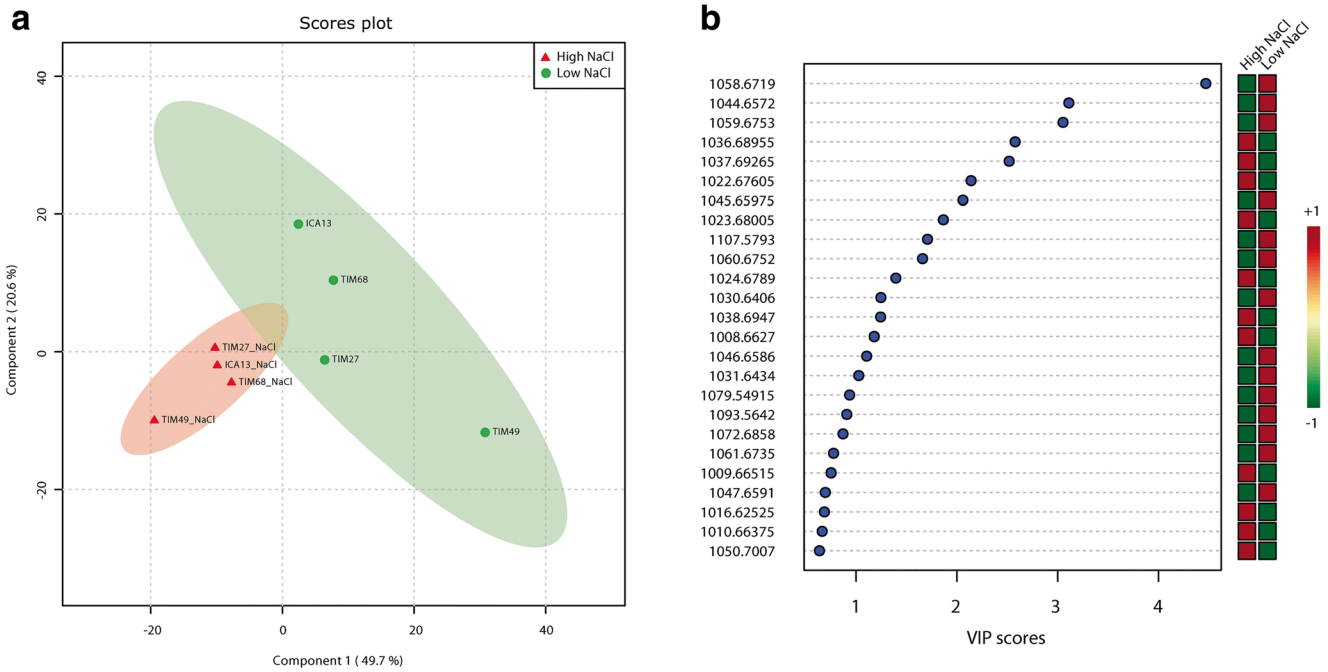

Fig. 5 **a** Score plot of the first two principal component of PLS-DA utilizing normalization to sample median and Pareto scaling. **b** Significant features identified by PLS-DA performed in MetaboAnalyst 3.0. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study

has been used extensively for chemical characterization of crude extracts (Varadavenkatesan and Murty 2013, Sousa et al. 2014). According to Mulligan (2005), good surfactants are those capable of reducing the water surface tension from 72 to 35 mN/m; thus, the strains characterized here are good biosurfactant-producers. As seen in Figs. 2 and 3, at higher NaCl concentrations, the production and surface activities of CLPs varied markedly among the strains. At 66 g L^{-1}, TIM27, TIM49, and TIM68 reduced the surface tension to 27 mN/m, but ICA13 lost activity. At 100 g L^{-1}, only *B. subtilis* TIM49 and *B. vallismortis* TIM68 reduced the surface tension to 46.5 and 35 mN/m, respectively, standing out as promising strains for MEOR.

Regarding emulsification activity, all strains lost activity at high NaCl, except for *B. subtilis* TIM49 that retained this activity at 66 g L^{-1}. In general, high salinity has an adverse impact on the efficiency of surfactants (Negin et al. 2016). Surfactin, for example, undergoes changes in its conformation and self-assembly properties with the increase of ionic strength (Knoblich et al. 1995). Therefore, TIM49 stands out as the most promising strain among the four halotolerant strains tested.

The CMC of the purified CLPs varied between 20 and 100 ppm, which is in contrast to the very high CMCs of the synthetic surfactants commonly used in oil recovery (Samanta et al. 2012). CMC corresponds to the concentration at which a surfactant solution begins to form micelles in large amounts (Hoff et al. 2001). Efficient surfactants have a low CMC, i.e., less surfactant is necessary to decrease the surface tension. In general, CMCs for biosurfactants are very low (Giro et al. 2009; Marin et al. 2015). Surfactin, one of the most potent known biosurfactants, reduces the water surface tension to 27 mN/m at 21.3 ppm (Peypoux et al. 1999). Taking into account that the purification of the evaluated CLPs involved a single acid precipitation step, their low CMCs suggest that they are efficient biosurfactants; therefore, a further purification step would probably result in lower CMC values.

The effect of NaCl concentration on the composition of the CLPs produced by the four strains was further investigated by the comparative analysis of the metabolites at 2.7- and 100-g L^{-1} NaCl by ESI(+) MS. The data showed that each strain co-produced a mixture of surfactins, iturins, and fengycins, but each one exhibited its own signature of CLPs (Fig. 4). The richness and relative abundance of isoforms from each CLP family varied significantly in both NaCl concentrations. This allowed for their ordering in two large groups based on NaCl concentration, and within each group, the strains were paired based on their similarities of isoforms (Figs.4 and 5). According to our analyses, the salt influence was higher in the *m/z* regions equivalent to surfactin (*m/z* 990–1072) and fengycin (*m/z* 1431–1505).

At 100-g L^{-1} NaCl, a significant increase of surfactins was observed in TIM68 and ICA13, a co-abundance of surfactins and fengycins in TIM49, and an increase of fengycins in TIM27. At 2.7-g L^{-1} NaCl, the synthesis of iturins was clearly favored in all strains, except in TIM27, which did not present predominance of any CLP family. The modulation of the CLP signature was also shown in *B. amyloliquefaciens* S499 living

in the tomato rhizosphere compared to laboratory conditions. Nihorimbere et al. (2012) reported that much higher proportions of surfactins than fengycins and iturins were found in tomato rhizosphere. Taking into account any practical application, the differential secretion of CLP is considered extremely relevant, since each family of CLPs displays specific biological activities (Ongena and Jacques 2008; Raaijmakers et al. 2010).

The closest phylogenetically related species, TIM27 and TIM49 as well as the most distant, TIM68 and ICA13, grouped together at both NaCl concentrations. It is noteworthy that even though the 16S rDNA, *rpo*B and *gyr*B genes have positioned the strains in different clades in the phylogenetic tree, all four strains belong to the same group of *B. subtilis* according to current classification based on a set of molecular and biochemical characteristics (Suihko and Stackebrandt 2003; Lasch et al. 2009).

Interestingly, all four strains produced only surfactin A and B isoforms, comprising chains of fatty acids ranging from 12 to 17 carbons, and seven amino acids with leucine and valine, respectively, at seventh position. As natural surfactins are mixtures of isoforms A, B, C, and D (Dae et al. 2006), the trend observed herein can be correlated to the strains' habitat specificity, since they were isolated from mangrove sediments. Price et al. (2007) showed a correlation between the CLP production profile and geographic origin in a comparative study of more than 50 *B. subtilis* and *Bacillus licheniformis* strains isolated from seven extreme habitats ranging from glacier to desert. Analysis of 100 isolates from different Brazilian mangroves is underway by our group in order to confirm the putative relationship between habitat and CLP isoforms.

All strains produced fengycin A and B isoforms and iturin A. Only ICA13 produced bacillomycin L, an iturin relative. It is well-known that *Bacillus* strains can simultaneously produce lipopeptides from different families, as well as multiple structural analogues of one particular lipopeptide (Kowall et al. 1998; Ongena and Jacques 2008). This trait is mainly due to the flexibility in amino acid selection, a common phenomenon in non-ribosomal peptide synthesis, and is influenced by innumerous genetic and environmental factors (Cooper et al. 1981; Sheppard and Mulligan 1987; Peypoux et al. 1999; Youssef et al. 2005; Cagri-Mehmetoglu et al. 2012; Oliveira et al. 2013; Zhu et al. 2013; Mnif and Ghribi 2015).

Among the strains, TIM49 produced the greatest diversity of CLPs in both concentrations of NaCl 2.7 and 100 g L^{-1}. A deeper analysis of the major peaks led to the identification of 25 major biomarkers that further contributed to differentiate TIM49 from the other strains. The peaks assigned to surfactin of *m/z* 1036.68955 and 1037.69265 (VIP score > 2.5) were the most representatives at 100-g L^{-1} NaCl, whereas the peak assigned to iturin of *m/z* 1058.6719 (VIP score > 4.5) was the major biomarker at 2.7-g L^{-1} NaCl. Furthermore, the fengycin peaks of *m/z* 1477.82 and 1505.85 were predominant in TIM49. Therefore, the synergistic effect of these surfactin and fengycin isoforms may have played a key role in the surfactant activity of TIM49 at 100-g L^{-1} NaCl. Razafindralambo et al. (1997) have shown that surfactin-C15 and iturin A-C15 molecules interact leading to the synergistic effects on surface-active properties at the air-water interface and in aqueous solution. Youssef et al. (2007b) also reported that certain mixtures of CLPs or mixtures of lipopeptide and rhamnolipid biosurfactants can be more effective in lowering the interfacial tension and to mobilize entrapped hydrocarbons. The contribution of each molecule to the overall activity is very important in order to prepare surfactant formulations more effective. In addition, knowledge about synergism between CLPs is crucial, since in most practical application mixtures rather than individual components are used.

Similarly, the peaks assigned to surfactin of *m/z* 1026.67 and 1040.68 produced by *B. vallismortis* TIM68 seem to have been the major responsible for the reduction of surface tension at 100-g L^{-1} NaCl. It is worth mentioning that there are only a few studies on lipopeptides produced by *B. vallismortis* and most of them are related to the control of plant diseases (Chakraborty et al. 2014; Park et al. 2016; Kaur et al. 2017). Thus, to the best of our knowledge, this is the first study to detail the composition of CLPs produced by a strain of *B. valllismortis* in presence of low and high NaCl concentrations.

It is important to emphasize the results assigned to *B. subtilis* TIM27 and *B. amiloliquefaciens* ICA13, which grouped with TIM49 and TIM68, respectively, at 100-g L^{-1} NaCl, but did not present relevant surfactant activity. ICA13 did not produce the surfactin isoforms assumed to be responsible for the activity of TIM68, while TIM27 secreted negligible amount of surfactins. TIM27 was also characterized by producing exclusive isoforms of fengycins, which apparently alone cannot reduce the surface tension. Halophilic or halotolerant bacteria adapt themselves by accumulating high concentration of compatible solutes (organic and inorganic) in response to external osmotic pressure (Brown 1978), but the strains respond differently. Among the functions of the compatible solutes is the modulation of individual enzymes activities (Roberts 2005), which in turn may have influenced the assembly of CLPs generating a diversity of isoforms. Our data allowed the speculation that a few specific isoforms of surfactins A and B alone or in combination with some specific fengycins drive the reduction of surface tension in *B. vallismortis* TIM68 and *B. subtilis* TIM49, respectively.

In this study, we provide experimental evidence on the main changes in the CLP composition and its relationship with the surface-active properties of bacilli growing in presence of 100-g L^{-1} NaCl concentration. We demonstrate that the analysis of CLP signature can be a fast and robust

guidance approach for choosing good surfactant producers towards application in high-saline reservoirs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Ahaotu I, Anyogu A, Njoku OH, Odu NN, Sutherland JP, Ouoba LII (2013) Molecular identification and safety of *Bacillus* species involved in the fermentation of African oil beans (*Pentaclethra macrophylla* Benth) for production of Ugba. Int J Food Microbiol 162(1):95–104. https://doi.org/10.1016/j.ijfoodmicro.2013.01.001

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25(17):3389–3402. https://doi.org/10.1093/nar/25.17.3389

Al-Wahaibi Y, Al-Hadrami H, Al-Bahry S, Elshafie A, Al-Bemani A, Joshi S (2016) Injection of biosurfactant and chemical surfactant following hot water injection to enhance heavy oil recovery. Pet Sci 13(1):100–109. https://doi.org/10.1007/s12182-015-0067-0

Banat IM (1995) Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: a review. Bioresour Technol 51(1):1–12. https://doi.org/10.1016/0960-8524(94)00101-6

Banat IM, Franzetti A, Gandolfi I, Bestetti G, Martinotti MG, Fracchia L, Smyth TJ, Marchant R (2010) Microbial biosurfactants production , applications and future potential. Appl Microbiol Biotechnol 87(2): 427–444. https://doi.org/10.1007/s00253-010-2589-0

Bass C, Lappin-Scott H (1997) The bad guys and the good guys in petroleum microbiology. Oilfield Rev 9:17–25

Belyaev SS, Borzenkov IA, Nazina TN, Rozanova EP, Glumov IF, Ibatullin RR, Ivanov MV (2004) Use of microorganisms in the biotechnology for the enhancement of oil recovery. Microbiology 73(5):590–598. https://doi.org/10.1023/B:MICI.0000044250.21076.0e

Bie X, Lu Z, Lu F (2009) Identification of fengycin homologues from *Bacillus subtilis* with ESI-MS/CID. J Microbiol Methods 79(3): 272–278. https://doi.org/10.1016/j.mimet.2009.09.013

Bonmatin J-M, Laprévote O, Peypoux F (2003) Diversity among microbial cyclic lipopeptides: iturins and surfactins. Activity-structure relationships to design new bioactive agents. Comb Chem High Throughput Screen 6(6):541–556. https://doi.org/10.2174/138620703106298716

Brown AD (1978) Compatible solutes and extreme water stress in eukaryotic micro-organisms. Adv Microb Physiol 17:181–242. https://doi.org/10.1016/S0065-2911(08)60058-2

Cagri-Mehmetoglu A, Kusakli S, van de Venter M (2012) Production of polysaccharide and surfactin by *Bacillus subtilis* ATCC 6633 using rehydrated whey powder as the fermentation medium. J Dairy Sci 95(7):3643–3649. https://doi.org/10.3168/jds.2012-5385

Cameotra SS, Makkar RS, Kaur J, Mehta SK (2010) Synthesis of biosurfactants and their advantages to microorganisms and mankind. Adv Exp Med Biol 672:261–280. https://doi.org/10.1007/978-1-4419-5979-9_20

Chakraborty J, Chakrabarti S, Das S (2014) Characterization and antimicrobial properties of lipopeptide biosurfactants produced by *Bacillus subtilis* SJ301 and *Bacillus vallismortis* JB201. Appl Biochem Microbiol 50(6):609–618. https://doi.org/10.1134/S0003683814060039

Chen H, Wang L, Su CX, Gong GH, Wang P, Yu ZL (2008) Isolation and characterization of lipopeptide antibiotics produced by *Bacillus subtilis*. Lett Appl Microbiol 47(3):180–186. https://doi.org/10.1111/j.1472-765X.2008.02412.x

Cooper D, Macdonald C, Duff S, Kosaric N (1981) Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Appl Environ Microbiol 42(3):408–412

Dae KS, Cho JY, Park HJ, Lim CR, Lim JH, Yun HI, Park SC, Kim SK, Rhee MH (2006) A comparison of the anti-inflammatory activity of surfactin A, B, C, and D from *Bacillus subtilis*. J Microbiol Biotechnol 16:1656–1659

Deleu M, Razafindralambo H, Popineau Y, Jacques P, Thonart P, Paquot M (1999) Interfacial and emulsifying properties of lipopeptides from *Bacillus subtilis*. Colloids Surfaces A Physicochem Eng. ASp 152(1-2):3–10. https://doi.org/10.1016/S0927-7757(98)00627-X

Giro MEA, Martins JJL, Rocha MVP, Melo VMM, Gonçalves LRB (2009) Clarified cashew apple juice as alternative raw material for biosurfactant production by *Bacillus subtilis* in a batch bioreactor. Biotechnol J 4(5):738–747. https://doi.org/10.1002/biot.200800296

Gudiña EJ, Teixeira JA, Rodrigues LR (2010) Isolation and functional characterization of a biosurfactant produced by *Lactobacillus paracasei*. Colloids Surf B Biointerfaces 76(1):298–304. https://doi.org/10.1016/j.colsurfb.2009.11.008

Gudiña EJ, Pereira JFB, Rodrigues LR, Coutinho JAP, Teixeira JA (2012) Isolation and study of microorganisms from oil samples for application in microbial enhanced oil recovery. Int Biodeterior Biodegrad 68:56–64. https://doi.org/10.1016/j.ibiod.2012.01.001

Hoff E, Nyström B, Lindman B (2001) Polymer–surfactant interactions in dilute mixtures of a nonionic cellulose derivative and an anionic surfactant. Langmuir 17(1):28–34. https://doi.org/10.1021/la001175p

Iqbal S, Khalid ZM, Malik KA (1995) Enhanced biodegradation and emulsification of crude oil and hyperproduction of biosurfactants by a gamma ray-induced mutant of *Pseudomonas aeruginosa*. Lett Appl Microbiol 21(3):176–179. https://doi.org/10.1111/j.1472-765X.1995.tb01035.x

Joshi SJ, Al-Wahaibi YM, Al-Bahry SN, Elshafie AE, Al-Bemani AS, Al-Bahri A, Al-Mandhari MS (2016) Production, characterization, and application of *Bacillus licheniformis* W16 biosurfactant in enhancing oil recovery. Front Microbiol 7:1–14

Kaur PK, Joshi N, Singh IP, Saini HS (2017) Identification of cyclic lipopeptides produced by *Bacillus vallismortis* R2 and their antifungal activity against *Alternaria alternata*. J Appl Microbiol 122(1): 139–152. https://doi.org/10.1111/jam.13303

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and

analysis of sequence data. Bioinformatics 28(12):1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Khire JM, Khan MI (1994) Microbially enhanced oil recovery (MEOR). Part 1. Importance and mechanism of MEOR. Enzym Microb Technol 16(2):170–172. https://doi.org/10.1016/0141-0229(94)90081-7

Kim PI, Ryu J, Kim YH, Chi Y-T (2010) Production of biosurfactant lipopeptides iturin A, fengycin and surfactin A from *Bacillus subtilis* CMB32 for control of *Colletotrichum gloeosporioides*. J Microbiol Biotechnol 20(1):138–145

Knoblich A, Matsumoto M, Ishiguro R, Murata K, Fujiyoshi Y, Ishigami Y, Osman M (1995) Electron cryo-microscopic studies on micellar shape and size of surfactin, an anionic lipopeptide. Colloids Surfaces B Biointerfaces 5(1-2):43–48. https://doi.org/10.1016/0927-7765(95)01207-Y

Kowall V, Kluge S, Franke Z (1998) Separation and characterization of surfactin isoforms produced by *Bacillus subtilis* OKB 105. J Colloid Interface Sci 204(1):1–8. https://doi.org/10.1006/jcis.1998.5558

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33(7):1870–1874. https://doi.org/10.1093/molbev/msw054

Lasch P, Beyer W, Nattermann H, Stammler M, Siegbrecht E, Grunow R, Naumann D (2009) Identification of *Bacillus anthracis* by using matrix-assisted laser desorption ionization-time of flight mass spectrometry and artificial neural networks. Appl Environ Microbiol 75(22):7229–7242. https://doi.org/10.1128/AEM.00857-09

Li X-Y, Mao Z-C, Wang Y-H, Wu Y-X, He Y-Q, Long C-L (2012) ESI LC-MS and MS/MS characterization of antifungal cyclic lipopeptides produced by *Bacillus subtilis* XF-1. J Mol Microbiol Biotechnol 22(2):83–93. https://doi.org/10.1159/000338530

Marchant R, Banat IM (2012) Microbial biosurfactants: challenges and opportunities for future exploitation. Trends Biotechnol 30(11):558–565. https://doi.org/10.1016/j.tibtech.2012.07.003

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Dymock D, Wade WG (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl Environ Microbiol 64(2):795–799

Marin CP, Kaschuk JJ, Frollini E, Nitschke M (2015) Potential use of the liquor from sisal pulp hydrolysis as substrate for surfactin production. Ind Crop Prod 66:239–245. https://doi.org/10.1016/j.indcrop.2015.01.001

Mercade ME, Manresa MA (1994) The use of agroindustrial by-products for biosurfactant production. J Am Oil Chem Soc 71(1):61–64. https://doi.org/10.1007/BF02541473

Mnif I, Ghribi D (2015) Lipopeptide surfactants: production, recovery and pore forming capacity. Peptides 71:100–112

Mootz HD, Marahiel MA (1997) Biosynthetic systems for nonribosomal peptide antibiotic assembly. Curr Opin Chem Biol 1(4):543–551. https://doi.org/10.1016/S1367-5931(97)80051-8

Morán AC, Olivera N, Commendatore M, Esteves JL, Siñeriz F (2000) Enhancement of hydrocarbon waste biodegradation by addition of a biosurfactant from *Bacillus subtilis* O9. Biodegradation 11(1):65–71. https://doi.org/10.1023/A:1026513312169

Moyne A, Cleveland TE, Tuzun S (2004) Molecular characterization and analysis of the operon encoding the antifungal lipopeptide bacillomycin D. FEMS Microbiol Lett 234(1):43–49. https://doi.org/10.1111/j.1574-6968.2004.tb09511.x

Mulligan CN (2005) Environmental applications for biosurfactants. Environ Pollut 133(2):183–198. https://doi.org/10.1016/j.envpol.2004.06.009

Negin C, Ali S, Xie Q (2016) Most common surfactants employed in chemical enhanced oil recovery. Petroleum 3:197–211

Nihorimbere V, Cawoy H, Seyer A, Brunelle A, Thonart P, Ongena M (2012) Impact of rhizosphere factors on cyclic lipopeptide signature from the plant beneficial strain *Bacillus amyloliquefaciens* S499. FEMS Microbiol Ecol 79(1):176–191. https://doi.org/10.1111/j.1574-6941.2011.01208.x

Oliveira DWF, França ÍWL, Félix AKN, Martins JJL, Giro MEA, Melo VMM, Gonçalves LRB (2013) Kinetic study of biosurfactant production by *Bacillus subtilis* LAMI005 grown in clarified cashew apple juice. Colloids Surfaces B Biointerfaces 101:34–43. https://doi.org/10.1016/j.colsurfb.2012.06.011

Ongena M, Jacques P (2008) *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol 16(3):115–125. https://doi.org/10.1016/j.tim.2007.12.009

Oren A (2008) Microbial life at high salt concentrations: phylogenetic and metabolic diversity. Saline Syst 4(1):2. https://doi.org/10.1186/1746-1448-4-2

Park K, Park Y-S, Ahamed J, Dutta S, Ryu H, Lee S-H, Balaraju K, Manir M, Moon S-S (2016) Elicitation of induced systemic resistance of chili pepper by iturin A analogs derived from *Bacillus vallismortis* EXTN-1. Can J Plant Sci:564–570. https://doi.org/10.1139/cjps-2015-0199

Pereira JFB, Gudiña EJ, Costa R, Vitorino R, Teixeira JA, Coutinho JAP, Rodrigues LR (2013) Optimization and characterization of biosurfactant production by *Bacillus subtilis* isolates towards microbial enhanced oil recovery applications. Fuel 111:259–268. https://doi.org/10.1016/j.fuel.2013.04.040

Peypoux F, Bonmatin JM, Wallach J (1999) Recent trends in the biochemistry of surfactin. Appl Microbiol Biotechnol 51(5):553–563. https://doi.org/10.1007/s002530051432

Price NPJ, Rooney AP, Swezey JL, Perry E, Cohan FM (2007) Mass spectrometric analysis of lipopeptides from *Bacillus* strains isolated from diverse geographical locations. FEMS Microbiol Lett 271(1):83–89. https://doi.org/10.1111/j.1574-6968.2007.00702.x

Pueyo MT, Bloch C, Carmona-Ribeiro AM, Di Mascio P (2009) Lipopeptides produced by a soil *Bacillus megaterium* strain. Microb Ecol 57(2):367–378. https://doi.org/10.1007/s00248-008-9464-x

Raaijmakers JM, De Bruijn I, Nybroe O, Ongena M (2010) Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiol Rev 34(6):1037–1062. https://doi.org/10.1111/j.1574-6976.2010.00221.x

Razafindralambo H, Popineau Y, Deleu M, Hbid C, Jacques P, Thonart P, Paquot M (1997) Surface-active properties of surfactin/iturin A mixtures produced by *Bacillus subtilis*. Langmuir 13(23):6026–6031. https://doi.org/10.1021/la970533u

Roberts MF (2005) Organic compatible solutes of halotolerant and halophilic microorganisms. Saline Syst 1:1–30

Roongsawang N, Thaniyavarn J, Thaniyavarn S, Kameyama T, Haruki M, Imanaka T, Morikawa M, Kanaya S (2002) Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: bacillomycin L, plipastatin, and surfactin. Extremophiles 6(6):499–506. https://doi.org/10.1007/s00792-002-0287-2

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4):406–425

Samanta A, Bera A, Ojha K, Mandal A (2012) Comparative studies on enhanced oil recovery by alkali–surfactant and polymer flooding. J Pet Explor Prod Technol 2(2):67–74. https://doi.org/10.1007/s13202-012-0021-2

Sen R (2008) Biotechnology in petroleum recovery : the microbial EOR. Prog Energy Combust Sci 34(6):714–724. https://doi.org/10.1016/j.pecs.2008.05.001

Sheppard J, Mulligan C (1987) The production of surfactin by *Bacillus subtilis* grown on peat hydrolysate. Appl Microbiol Biotechnol 27:110–116

Sousa M, Dantas IT, Feitosa FX, Alencar AEV, Soares SA, Melo VMM, Gonçalves LRB, Sant'ana HB (2014) Performance of a biosurfactant produced by *Bacillus subtilis* LAMI005 on the formation of oil/biosurfactant/water emulsion: study of the phase behaviour of

emulsified systems. Braz J Chem Eng 31(3):613–623. https://doi.org/10.1590/0104-6632.20140313s00002766

Stankovic S, Mihajlovic S, Draganic V, Dimkic I, Vukotic G, Beric T, Fira D (2012) Screening for the presence of biosynthetic genes for antimicrobial lipopeptides in natural isolates of *Bacillus* sp. Arch Biol Sci 64(4):1425–1432. https://doi.org/10.2298/ABS1204425S

Stein T (2008) Whole-cell matrix-assisted laser desorption/ionization mass spectrometry for rapid identification of bacteriocin/lantibiotic-producing bacteria. Rapid Commun Mass Spectrom 22(8):1146–1152. https://doi.org/10.1002/rcm.3481

Suihko M-L, Stackebrandt E (2003) Identification of aerobic mesophilic bacilli isolated from board and paper products containing recycled fibres. J Appl Microbiol 94(1):25–34. https://doi.org/10.1046/j.1365-2672.2003.01803.x

Suthar H, Hingurao K, Desai A, Nerurkar A (2008) Evaluation of bioemulsifier mediated microbial enhanced oil recovery using sand pack column. J Microbiol Methods 75(2):225–230. https://doi.org/10.1016/j.mimet.2008.06.007

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10(3):512–526

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22(22):4673–4680. https://doi.org/10.1093/nar/22.22.4673

Vanittanakom N, Loeffler W, Koch U, Jung G (1986) Fengycin–a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. J Antibiot (Tokyo) 39(7):888–901. https://doi.org/10.7164/antibiotics.39.888

Varadavenkatesan T, Murty VR (2013) Production of a lipopeptide biosurfactant by a novel *Bacillus* sp. and its applicability to enhanced oil recovery. ISRN Microbiol 2013:1–8. https://doi.org/10.1155/2013/621519

Vater J, Wilde C, Franke P, Mehta N, Cameotra SS (2002) Matrix-assisted laser desorption ionization–time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. Appl Environ Microbiol 68(12):6210–6219. https://doi.org/10.1128/AEM.68.12.6210-6219.2002

Walsh CT (2004) Polyketide and nonribosomal peptide antibiotics: modularity and versatility. Science 303(5665):1805–1810. https://doi.org/10.1126/science.1094318

Wang L-T, Lee F-L, Tai C-J, Kasai H (2007) Comparison of *gyrB* gene sequences, 16S rRNA gene sequences and DNA—DNA hybridization in the *Bacillus subtilis* group. Int J Syst Evol Microbiol 57(8):1846–1850. https://doi.org/10.1099/ijs.0.64685-0

Warner SAJ (1996) Genomic DNA isolation and lambda library construction. Wiley & Sons, New York

Williams BH, Hathout Y, Fenselau C (2002) Structural characterization of lipopeptide biomarkers isolated from *Bacillus globigii*. J Mass Spectrom 37(3):259–264. https://doi.org/10.1002/jms.279

Xia J, Psychogios N, Young N, Wishart DS (2009) MetaboAnalyst: a web server for metabolomic data analysis and interpretation. Nucleic Acids Res 37(Web Server):W652–W660. https://doi.org/10.1093/nar/gkp356

Xia J, Mandal R, Sinelnikov IV, Broadhurst D, Wishart DS (2012) MetaboAnalyst 2.0—a comprehensive server for metabolomic data analysis. Nucleic Acids Res 40(W1):W127–W133. https://doi.org/10.1093/nar/gks374

Xia J, Sinelnikov IV, Han B, Wishart DS (2015) MetaboAnalyst 3.0—making metabolomics more meaningful. Nucleic Acids Res 43(W1):W251–W257. https://doi.org/10.1093/nar/gkv380

Youssef NH, Duncan KE, McInerney MJ (2005) Importance of 3-hydroxy fatty acid composition of lipopeptides for biosurfactant activity. Appl Environ Microbiol 71(12):7690–7695. https://doi.org/10.1128/AEM.71.12.7690-7695.2005

Youssef NH, Simpson DR, Duncan KE, McInerney MJ, Folmsbee M, Fincher T, Knapp RM (2007a) *In situ* biosurfactant production by *Bacillus* strains injected into a limestone petroleum reservoir. Appl Environ Microbiol 73(4):1239–1247. https://doi.org/10.1128/AEM.02264-06

Youssef NH, Nguyen T, Sabatini DA, McInerney MJ (2007b) Basis for formulating biosurfactant mixtures to achieve ultra low interfacial tension values against hydrocarbons. J Ind Microbiol Biotechnol 34(7):497–507. https://doi.org/10.1007/s10295-007-0221-9

Zhu Z, Zhang J, Wu Y, Ran W, Shen Q (2013) Comparative study on the properties of lipopeptide products and expression of biosynthetic genes from *Bacillus amyloliquefaciens* XZ-173 in liquid fermentation and solid-state fermentation. World J Microbiol Biotechnol 29(11):2105–2114. https://doi.org/10.1007/s11274-013-1375-4