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Enzyme and Microbial Technology 27 (2000) 749–754

ENZYME and MICROBIAL TECHNOLOGY

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Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity

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Received 26 June 2000

Abstract

The synthesis of extracellular molecules such as biosurfactants should have major consequences on bacterial adhesion. These molecules may be adsorbed on surfaces and modify their hydrophobicities. Certain strains of *Bacillus subtilis* synthesize the lipopeptides, which exhibit antibiotic and surface active properties. In this study the high-performance liquid chromatography (HPLC) analysis of the culture supernatants of the seven *B. subtilis* strains, showed that the lipopeptide profile varied greatly according to the strain. Among the three lipopeptide types, only iturin A was produced by all *B. subtilis* strains. Bacterial hydrophobicity, evaluated by the water contact angle measurements and the hydrophobic interaction chromatography, varied according to the strain. Two strains (ATCC 15476 and ATCC 15811) showing extreme behaviors in term of hydrophobicity were selected to study surfactin and iturin A effects on bacterial hydrophobicity. The two lipopeptides modified the *B. subtilis* surface hydrophobicity. Their effects varied according to the bacterial surface hydrophobic character, the lipopeptide type and the concentration. Lipopeptide adsorption increased the hydrophobicity of the hydrophilic strain but decreased that of the hydrophobic. Comparison of lipopeptide effects on *B. subtilis* surface hydrophobicity showed that surfactin was more effective than iturin A for the two strains tested.

Keywords: Bacillus subtilis; Lipopeptide; Surface hydrophobicity

1. Introduction

Microbial compounds which exhibit pronounced surface activity are classified as biosurfactants. Microbial biosurfactants include a wide variety of surface active compounds, such as glycolipids, lipopeptides, polysaccharide-protein complexes, phospholipids, fatty acids, and neutral lipids [1]. Biosurfactants consisting of distinct hydrophilic and hydrophobic moieties, are easily biodegradable and thus are particularly suited for environmental applications [2,3].

Among the many classes of biosurfactants, lipopeptides are particularly interesting because of their high surface activities and antibiotic potential. *Bacillus subtilis* is recognized as a potent agent for the biologic control of plant diseases. This aptitude results from the production of lipopeptides (surfactin, iturin and fengycin classes), which exhibit antibiotic [4,5] and surface active properties [6,7].

Studies of *B. subtilis* surface properties showed that microbial adhesion was effected by the harvesting time, by the roughness and topography of the substrata and by the morphology of bacterial cells [8], but little is known about the effect of substances produced by these microorganisms on their surface properties.

Various macromolecular compounds such as polysaccharides, proteins and lipopolysaccharides seem to be involved in bacterial adhesion mechanisms [9–11]. Indeed, extracellular polysaccharides strengthened the bacteria binding to surfaces. Outer membrane proteins were required for the early stages of adhesion to surfaces. Lipopeptides may influence the adhesiveness of *B. subtilis* by adsorbing onto bacterial surface. Indeed, it has been reported that lipopeptides produced by *Serratia marcescens* and *Serratia rubidaea,* respectively serawettins and rubiwettins, exhibit a wetting activity, which is involved in the surface colonization by bacteria [12]. Similarly, lipopeptides produced by *Bacillus subtilis* especially surfactins showed strong surface-active properties including interfacial tension reduction and micellisation [13], as well as foaming properties [14].

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0141-0229/00/$ – see front matter
PII: S0141-0229(00)00295-7

In this paper the types of lipopeptides produced by *B. subtilis* strains were, first, determined by the high-performance liquid chromatography (HPLC) analysis. The influence of the lipopeptide type and concentration on *B. subtilis* surface hydrophobicity was evaluated by water contact angle measurements and hydrophobic interaction chromatography.

2. Material and methods

2.1. Bacillus subtilis *strains and growth conditions*

Seven *Bacillus subtilis* strains were used: ATCC 7058, ATCC 12432, ATCC 12695, ATCC 15129, ATCC 15476, ATCC 15561, and ATCC 15811. Bacterial cells were grown for 48 h at 30°C with shaking (130 rpm) in 868 medium (10 g/liter peptone, 10 g/liter yeast extract and 20 g/liter glucose dissolved in distilled water). Bacterial cells were harvested and washed three times in demineralized water by centrifugation for 10 min at 9820× g and 25°C with a centrifuge Beckman J2–21 (rotor model JA-14).

2.2. *Extraction and analysis of lipopeptides produced by* B. subtilis *strains*

B. subtilis cells were grown for 72 h at 30°C with shaking (200 rpm) in a medium optimal for lipopeptide production, containing 30 g/liter peptone, 20 g/liter saccharose, 7 g/liter yeast extract, 1.9 g/liter KH_2PO_4, 0.001 mg/liter $CuSO_4$, 0.005 mg/liter $FeCl_3.6H_2O$, 0.004 mg/liter Na_2MoO_4, 0.002 mg/liter KI, 3.6 mg/liter $MnSO_4.H_2O$, 0.45 g/liter $MgSO_4$, 0.14 mg/liter $ZnSO_4.7H_2O$, 0.01 mg/liter H_3BO_3, and 10 mg/liter $C_6H_8O_7$. The pH was adjusted to 7 with 5 M NaOH before the sterilization [15]. This culture was used to inoculate a second medium which was grown for 72 h at 30°C with shaking (200 rpm). Lipopeptide extraction has been described by Razafindralambo et al. [16]. Briefly, the culture medium was centrifuged at 11000× g for 25 min at 4°C to remove the cells. The supernatant (500 mg of dry material) was applied to Bond Elut C_{18} cartridge (0.25 g/ml) from Analytichem International (Harbour City, CA, USA). The cartridge, which retained lipopeptides, was rinsed successively with 20 ml of water and 40 ml of 50% aqueous methanol, and finally lipopeptides were eluted from the cartridge with 20 ml of methanol. The eluate was evaporated and the crude extract was dissolved in 0.5 ml methanol. Lipopeptide types were determined by analytical RP-HPLC (Chromspher 5 μm C_{18} column, 0.46 × 25 cm, Chrompack, Middelburg, The Netherlands). The following conditions were used: flow rate of 1 ml/min, acetonitrile/H_2O/TFA 0.05% as mobile phase.

2.3. *Contact angle measurements*

Initial hydrophobicity and effects of lipopeptides on *B. subtilis* strains were determined by water contact angle measurements on bacterial lawns deposited on membrane filter as described by Busscher et al. [17]. Briefly, the seven bacterial strains were suspended in demineralized water for studying the initial hydrophobicity. The two selected strains (ATCC 15476 and ATCC 15811), showing extreme behaviors in term of hydrophobicity, were suspended at pH 6.8 for 60 min in different concentrations of surfactin and iturin A ranging from 4 to 100 μg/liter. Bacterial suspensions were filtered on a cellulose acetate membrane filter (pore diameter 0.45 μm; Gelman). The filters carrying the bacterial lawns (more than 10^8 cells/mm^2) were then placed in a Petri dish on 1% (weight/volume) Agar layer containing 10% (volume/volume) glycerol and stored for 2 h to homogenize the moisture content. The filters were dried in the air for 60 min in order to obtain relatively stable contact angles. At least three separate filters, from three different cultures were used for each bacterial strain. Water droplets (10 μl) were applied at ten different places at 25°C for each filter. Water contact angles were measured with an Erma Contact Angle Meter G1 (Krüss, Germany). Values obtained were the means of 30 measurements (n = 30).

2.4. *Hydrophobic interaction chromatography (HIC)*

The test consists in measuring the amount of cells retained by a hydrophobic gel as described by Mozes and Rouxhet [18]. Washed bacteria were suspended in 10 mM of PBS solution (PBS) containing 0.87 g/liter K_2HPO_4, 0.68 g/liter KH_2PO_4 and 8.77 g/liter NaCl (pH 6.8). The two selected strains (ATCC 15476 and ATCC 15811) were suspended for 60 min in 100 mM $NaHCO_3$ containing different concentrations of surfactin and iturin A ranging from 4 to 100 μg/liter, for studying lipopeptide effects on bacterial hydrophobicity. Hydrophobic interaction chromatography was performed in small columns, prepared from Pasteur pipettes (ϕ = 5 mm) containing 0.6 ml of phenyl sepharose CL-4B or sepharose CL-4B (Pharmacia, Sweden), which served as a non-hydrophobic control, according to Clark et al. [19]. Gels were equilibrated with 3 ml of the PBS solution. 0.1 ml of bacterial suspensions (1×10^8 cells/ml) was applied to the gel and eluted with 3 ml of the same PBS solution. The absorbance of the eluate was measured at 600 nm. The percentage of cells retained only by hydrophobic interaction was determined by the difference between values obtained with phenyl sepharose CL-4B and sepharose CL-4B. At least two different cultures of bacterial cells were used and four measurements were done for each culture (n = 8).

3. Results

Table 1 shows the profile of lipopeptides assessed by HPLC analysis of the culture supernatants of the seven *B. subtilis* strains. All strains produced at least one lipopeptide type. From these results, three groups may be pointed out as

Table 1
Lipopeptide types produced by *Bacillus subtilis* strains

B. subtilis strains	Lipopeptides		
	Surfactin	Iturin A	Fengycin
ATCC 7058	−[a]	+[b]	−
ATCC 12432	+	+	−
ATCC 12695	+	+	+
ATCC 15129	+	+	+
ATCC 15476	−	+	−
ATCC 15561	−	+	−
ATCC 15811	+	+	−

[a] Unable to produce this type of lipopeptide.
[b] Production of this type of lipopeptide.

a function of the number and the type of lipopeptides synthesized. The first group includes the strains ATCC 12695 and ATCC 15129 producing the three lipopeptide types (surfactin, iturin A and fengycin). The second group is relative of the strains ATCC 12432 and ATCC 15811 producing surfactin and iturin A. The last group includes the strains ATCC 7058, ATCC 15476 and ATCC 15561 only producing iturin A. Among the three lipopeptide types, only iturin A was produced by all *B. subtilis* strains.

Fig. 1 presents bacterial hydrophobicity, assessed respectively by the water contact angle measurement (Fig. 1a) and the hydrophobic interaction chromatography (Fig. 1b). It appears that the bacterial surface hydrophobicity varies according to the strain. The variations are more marked for the case of results obtained with HIC method. However, the strains ATCC 12695 and ATCC 15811 are the most hydrophobic whatever the method used. The strains ATCC 15476 and ATCC 15561 are hydrophilic also in the two cases.

The strains ATCC 15476 and ATCC 15811 respectively hydrophilic and hydrophobic were selected to study the influence of lipopeptides on bacterial surface hydrophobicity. Fig. 2 presents the effects of surfactin on bacterial hydrophobicity evaluated respectively by the water contact angle measurement (Fig. 2a) and the hydrophobic interaction chromatography (Fig. 2b). The water contact angle and the percentage of bacteria retained by the hydrophobic gel change after treatment with surfactin. When bacterial cells are treated with increasing surfactin concentrations ranging from 4 to 100 μg/liter, an increase of the water contact angle and the percentage of cells retained by the gel is obtained for ATCC 15476 (compared to the controls). The strain ATCC 15476 becomes the most hydrophobic strain. In contrast, when the hydrophobic strain ATCC 15811 is treated under similar conditions, water contact angle values and the percentage of cells retained by the gel decrease. The strain ATCC 15811 becomes the most hydrophilic strain.

Fig. 3 presents effects of iturin A on bacterial hydrophobicity evaluated respectively by the water contact angle measurement (Fig. 3a) and the hydrophobic interaction chromatography (Fig. 3b). In the case of bacteria treated under similar conditions with increasing iturin A concentrations ranging from 4 to 100 μg/liter, the same observations are obtained, i.e. the strains ATCC 15476 (hydrophilic) and ATCC 15811 (hydrophobic) become respectively, the most and the less hydrophobic. However, the surfactin effect is more marked than that of the iturin A.

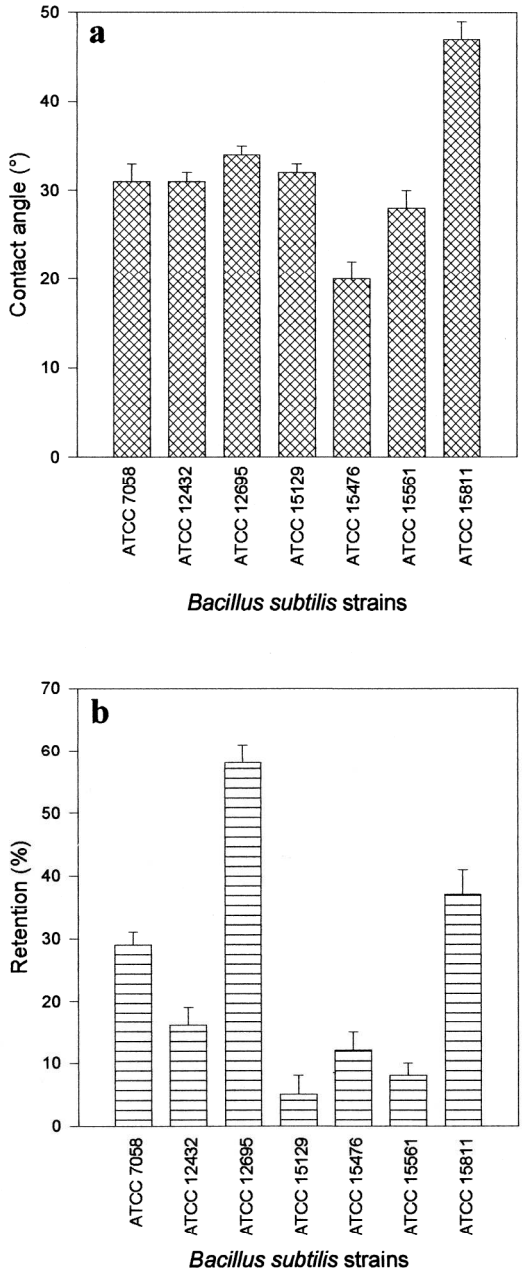

Fig. 1. *B. subtilis* surface hydrophobicity determined by: (a) water contact angle measurement, (b) hydrophobic interaction chromatography.

4. Discussion

Bacillus subtilis ability to synthesize lipopeptides is not dependent on bacterial hydrophobicity. However, after their

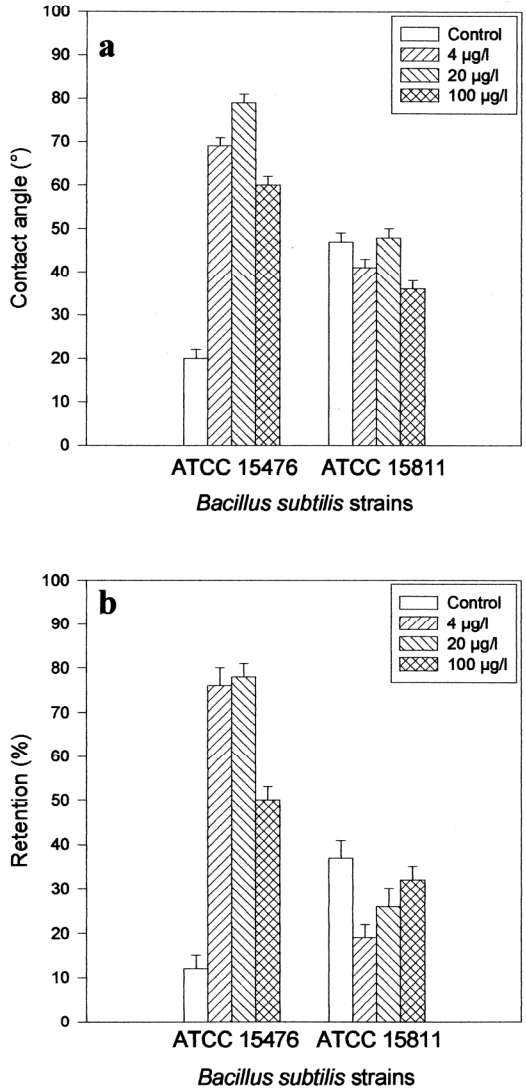

Fig. 2. Water contact angle (a) and hydrophobic interaction chromatography (b) of *B. subtilis* strains ATCC 15476 and ATCC 15811 treated with surfactin at different concentrations.

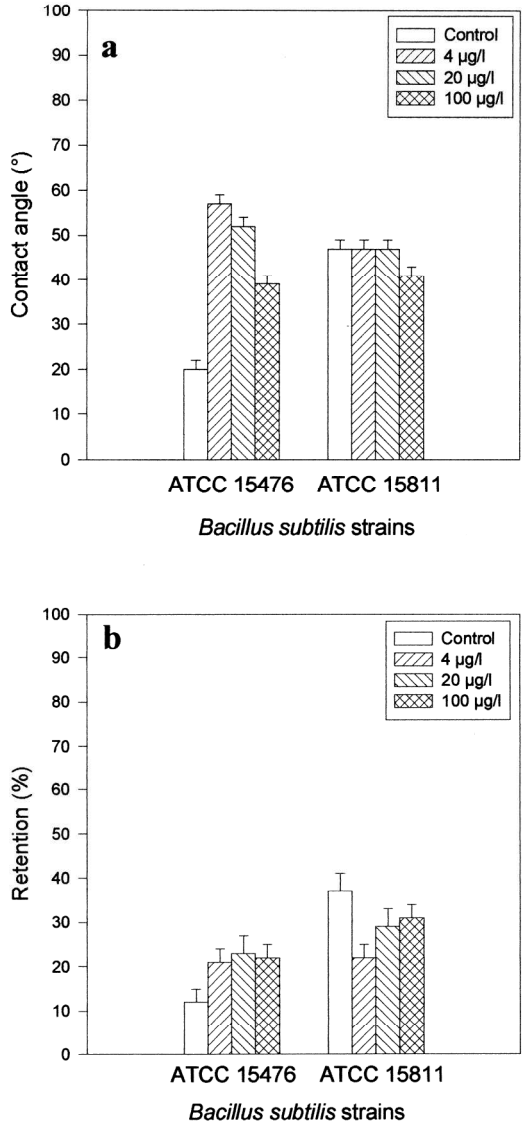

Fig. 3. Water contact angle (a) and hydrophobic interaction chromatography (b) of *B. subtilis* strains ATCC 15476 and ATCC 15811 treated with iturin A at different concentrations.

excretion in extracellular medium, lipopeptide molecules adsorb on *Bacillus subtilis* and induce changes of the cell surface hydrophobicity illustrated by alterations of the water contact angle and the percentage of cells retained by the hydrophobic gel. The hydrophobicity alterations suggest the important role of lipopeptide molecules to perform in *B. subtilis* adhesion mechanisms onto various surfaces by hydrophobic interactions. The fact that, the surfactin effect is more marked than that of iturin A, is not surprising since surfactin is able to cover more space than iturin A because of its larger molecular area [20]. The lipopeptide effect on the cell surface hydrophobicity arises from the amphiphilic structure of such compounds by considering the peptide cycle and hydrocarbon chain as polar head and apolar tail, respectively. Surfactins are cyclic lipopeptides containing seven residues of α-amino acids and one residue of a β-hydroxy fatty acid [21] (Fig. 4a). Iturins are constituted by a cyclic heptapeptide like surfactins, but contain a β-amino fatty acid as the lipidic part [22] (Fig. 4b). The fatty acid chain of lipopeptides varies from 13 to 17 carbon atoms [23,24]. The results could be explained by the orientation of the peptide cycle and the fatty acid chain in relation with the hydrophobic or hydrophilic character of the bacterial surface. Indeed, with the hydrophilic bacterial surface, lipopeptide molecules are probably oriented in such a way that the peptide cycles, as polar heads, are adsorbed onto the surface and the hydrocarbon chains are exposed to the surrounding medium. Hence, the bacterial surface becomes more hydrophobic. The orientation is inverted for the hydrophobic surface, i.e. the peptide cycles are exposed to the surrounding medium, whereas hydrocarbon chains adsorb onto the surface. Therefore, the hydrophobic strain becomes more hydrophilic after lipopeptide adsorption. Our results show

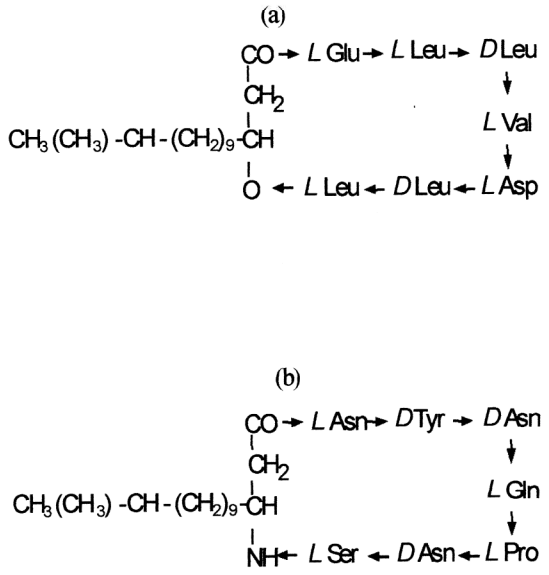

Fig. 4. Examples of lipopeptide primary structures: (a) surfactin, (b) iturin A.

that biosurfactants produced by microorganisms are able to modify bacterial surface hydrophobicity, which is involved in the adhesion. Manne et al. [25] proposed a adsorption model for ionic surfactant molecules at the graphite-aqueous solution interface. In this model, at low concentration (~10% of critical micelle concentration (CMC)), molecules were adsorbed with their alkane chains extended on the substrate plane. They reported that the alkane chain gradually was desorbed with an increase of the concentration. At concentrations near the CMC, the surfactant molecules were oriented perpendicular to the substrate plane, with the hydrophilic headgroups in contact with the aqueous phase. This model could explain the influence of lipopeptide concentration on bacterial hydrophobicity observed in our results. The spatial organization of lipopeptide molecules may be influenced by their concentration and the surface environment. Gallet et al. [26] have used computer simulation to predict the surfactin conformation at a hydrophobic/hydrophilic interface. In their model, the peptide ring is positioned in the plane of the interface with the two acidic chains close to each other and protruding in the aqueous phase. The β hydroxy fatty acid chain folded to interact mainly with two hydrophobic amino acids (Leu 2 and Val 4). The same conclusions have been reported by Deleu et al. [27]. They have studied, by atomic force microscopy (AFM) and X-ray photoelectron spectroscopy (XPS), the spatial organization (miscibility, molecular orientation) of mixed surfactin/phosphatidylcholine monolayers transferred on mica. They have shown that the polar heads of the two compounds were in contact with the mica with the fatty acid chain folded to interact with the amino acids. Biologic activity of lipopeptides is due to their interactions with biomembranes. Indeed, surfactin is able to penetrate in a modelized phospholipid monolayer. Consequently, bacterial surface is complex and can not be assimilated as an inert and plane surface.

5. Conclusions

Lipopeptide profile and bacterial hydrophobicity vary greatly within the strains. Among the three lipopeptide types only iturin A was produced by all *B. subtilis* strains. Biosurfactants produced by the microorganisms are able to modify bacterial surface hydrophobicity and consequently, microbial adhesion to solid surfaces. This is revealed by lipopeptide effect, which alters the *Bacillus subtilis* surface hydrophobicity. Their effect depends on the initial bacterial hydrophobicity, as well as on the lipopeptide type and concentration. Lipopeptides enhance or decrease the bacterial surface hydrophobicity following that the surface is less or more hydrophobic. Surfactin is more efficient than iturin A in modifying the *B. subtilis* surface hydrophobic character.

It could be expected from these results that lipopeptides excreted by *B. subtilis* play an important role in adhesion of such microorganism to fruits and plants. This aspect appears essential in association with the antifungal properties of lipopeptides involved in the biologic control of plant diseases.

Acknowledgments

The support of the National Foundation for Scientific Research (FNRS), of the Federal Office for Scientific, Technical and Cultural Affairs (Interuniversity Poles of Attraction Program), and of the European Union (BIO4-CT950176) is gratefully acknowledged.

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