Brazilian Journal of Microbiology 45, 2, 627-631 (2014)
ISSN 1678-4405

Short Communication

Effects of different osmolarities on bacterial biofilm formation

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Submitted: March 14, 2013; Approved: September 9, 2013.

Abstract

Biofilm formation depends on several factors. The influence of different osmolarities on bacterial biofilm formation was studied. Two strains (*Enterobacter* sp. and *Stenotrophomonas* sp.) exhibited the most remarkable alterations. Biofilm formation is an important trait and its use has been associated to the protection of organisms against environmental stresses.

Key words: bacteria, biofilm formation, NaCl, D-sorbitol.

Introduction

Biofilm is defined as a matrix of microbial population adherent to each other and/or to surfaces and interfaces. Extracellular compounds, such as carbohydrates, proteins and even DNA (Branda *et al.*, 2005; Costerton *et al.*, 1995), compose this matrix. Biofilm, besides its role in supporting cells against physical forces, also helps in their survival against stress conditions such as the effect of antimicrobial compounds (Park and Fuqua, 2004). It also protects its constituents against plants defense mechanisms when attacked by pathogens (Walker *et al.*, 2004), helps the colonization of phytopathogens by blocking the flow of nutrients (Newman *et al.*, 2003) and protects against desiccation and other types of environmental stresses (Danhorn and Fuqua, 2007; Monier and Lindow, 2003). Biofilm formation is triggered by unfavorable external conditions that modify the expression of several genes. Biofilm, in turn, alters the microenvironment of its inhabitants that leading to alteration of gene expression and maturation of biofilm and so on (Jefferson, 2004). In the present study we tested whether different osmolarities influence bacterial biofilm formation.

Soil samples were obtained from the rhizosphere of *Cereus jamacaru*, a native cactus from the Caatinga biome of Northeast of Brazil. Bacteria were isolated in Tryptone Soya Agar (TSA) (10%) medium and nine strains were tested for biofilm formation. Bacterial genomic DNA was extracted according to Sunnucks and Hales (1996). The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using universal primers 1492R (5-TAC GGY TAC CTT GTT ACG ACT-3) and 27F (5-GAG AGT TTG ATC CTG GCT CAG-3). Samples were purified using 1.5 μL of an enzyme mix containing the ratio of 2 μL of Fast-Ap (Fermentas) for 0.8 μL of Exonuclease I (Fermentas). The samples were submitted for 15 min to 37 °C and then 5 min at 80 °C. Sequencing was achieved using ABI 3500 Genetic Analyzer (Applied Biosystems). Phylogenetic relationship based on partial 16S rRNA gene sequence of bacterial strains was performed with comparison to EZ-Taxon database (Kim *et al.*, 2012). Alignment was constructed using ClustalW (Thompson *et al.*, 1994), followed by clustering using Neighbor-Joining distance and Jukes-Cantor model (Jukes and Cantor, 1969) with a *bootstrap* analysis of 1,000 replicates (Felsenstein, 1985) in Mega 5.01 (Tamura *et al.*, 2011).

The method used to detect the formation of biofilm was based on the methodology described by O'Toole and Kolter (1998) with modifications. The method relies on the ability of cells to adhere to the walls of polypropylene 1.5 mL tubes. To each tube, 100 μL from an overnight bacterial culture was added to 900 μL of culture media Tryptone Soya Broth (TSB) (10%) supplemented with D-sorbitol and NaCl at different concentrations (0.03, 0.06, 0.3 and 0.6 M of D-sorbitol and 0.015, 0.03, 0.15 and 0.3 M of NaCl). After inoculation, the tubes were incubated at 40 °C for 72 h. After this stage, the content of each tube was aspirated with an automatic hand pipette and the tubes were washed three times with 1000 μL of sterilized distilled water in order to remove the non-adherent cells. The water was removed and the tubes were left to dry and then 1000 μL of

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0.1% crystal violet (CV) were added for 15 min. The CV was removed and the tubes were washed again three times with 1000 µL of sterilized distilled water in order to remove the excess of dye. To quantify biofilm formation 1000 µL of 95% ethanol was added to each tube to solubilize the CV-dyed tube. The absorbance was determined with a spectrophotometer (UV- visible spectrophotometer: UV-1601 PC, Shimadzu) at 560 nm. Before the addition of CV, the cells were homogenized by an automatic hand pipette to quantify bacterial growth at 600 nm. The values were obtained in absorbance - optical density (OD), being biofilm formation considered as OD_{560} and growth as OD_{600}. Biofilm formation was considered for $OD_{560} \geq 0.1$, in a scale of weak formation ($0.1 \leq OD_{560} < 0.2$) and medium formation ($0.2 \leq OD_{560} < 0.5$). The experiments were all performed in triplicate. Data were subjected to One-Way ANOVA followed by a classification of means with Tukey's test at 5%, using the software Assistat 7.6 beta (Silva and Azevedo, 2002).

The relationship among the nine strains used in this study is shown in Figure 1. Phylogenetic analysis assigned five isolates to several species of the genus *Bacillus* (clade I) and four to the Phylum Proteobacteria (clade II).

The addition of NaCl has significantly induced the growth of three strains (LMArzc55, LMArzc158 and LMArzc189). The remaining six strains grew well in all four concentrations. The addition of D-sorbitol had a greater influence than NaCl on the growth of strains. Two strains (LMArzc17 and LMArzc40) grew equally in all four concentrations. LMArzc158 grew significantly better with 0.6 M of D-sorbitol and strains LMArzc189 and LMArzc324 showed better growth with 0.3 and 0.6 M of D-sorbitol. Four strains (LMArzc55, LMarzc108, LMArzc192 and LMArzc214) were inhibited by the addition of D-sorbitol, with better growth within the two first concentrations.

Only three concentrations of D-sorbitol added to the TSB medium significantly affected biofilm formation of all isolates tested. The lowest concentration of sorbitol (0.03 M) did not affect significantly biofilm formation. However, concentrations of 0.06 M, 0.30 M and 0.6 M of D-sorbitol had a significant effect on the production of biofilm, with strain LMArzc17 exhibiting the best characteristic in all three concentrations, ranging from medium to weak formation ($OD_{560} = 0.21$, 0.12 and 0.16, respectively).

Concerning the formation of biofilm, Table 1 shows the biofilm formation by the nine strains in all conditions. With 0.015 M of NaCl, two strains (LMArzc17 and LMArzc 189) were able to form significantly more biofilm than others ($0.01 \leq p < 0.05$), with OD_{560} of 0.20 and 0.21, respectively. Only LMArzc189 formed statistically more biofilm with the addition of 0.03 M of NaCl than the other strains ($OD_{560} = 0.25$). The two last concentrations did not promote any significant difference in biofilm formation among all strains, and some of them showed a slight production or none.

In a general way, biofilm formation was influenced by different osmolarities, being more frequent in two strains: LMArzc17 and LMArzc189 and their growth and biofilm formation are shown in detail in Figure 2.

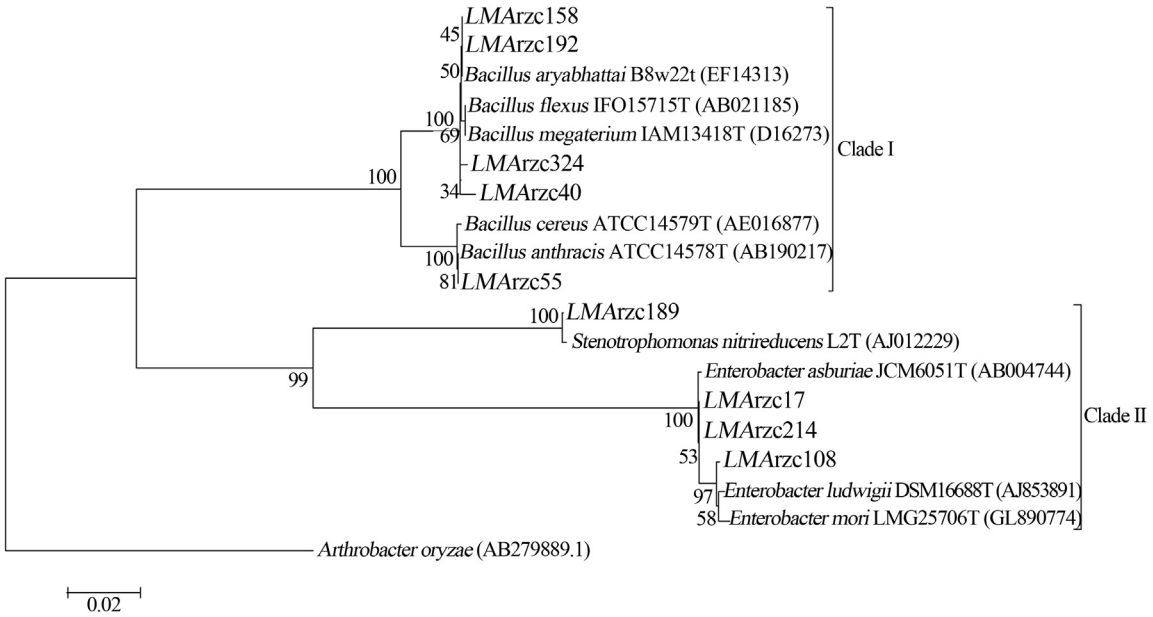

Figure 1 - Neighbor-joining tree based on 16S rRNA partial gene, with phylogenetic relationship between nine isolates belonging to Firmicutes (clade I) and Proteobacteria (clade II). The scale bar at the bottom indicates the number of differences in base composition among sequences. Bootstrap for 1,000 replicates. *Arthrobacter oryzae* was used as an outgroup.

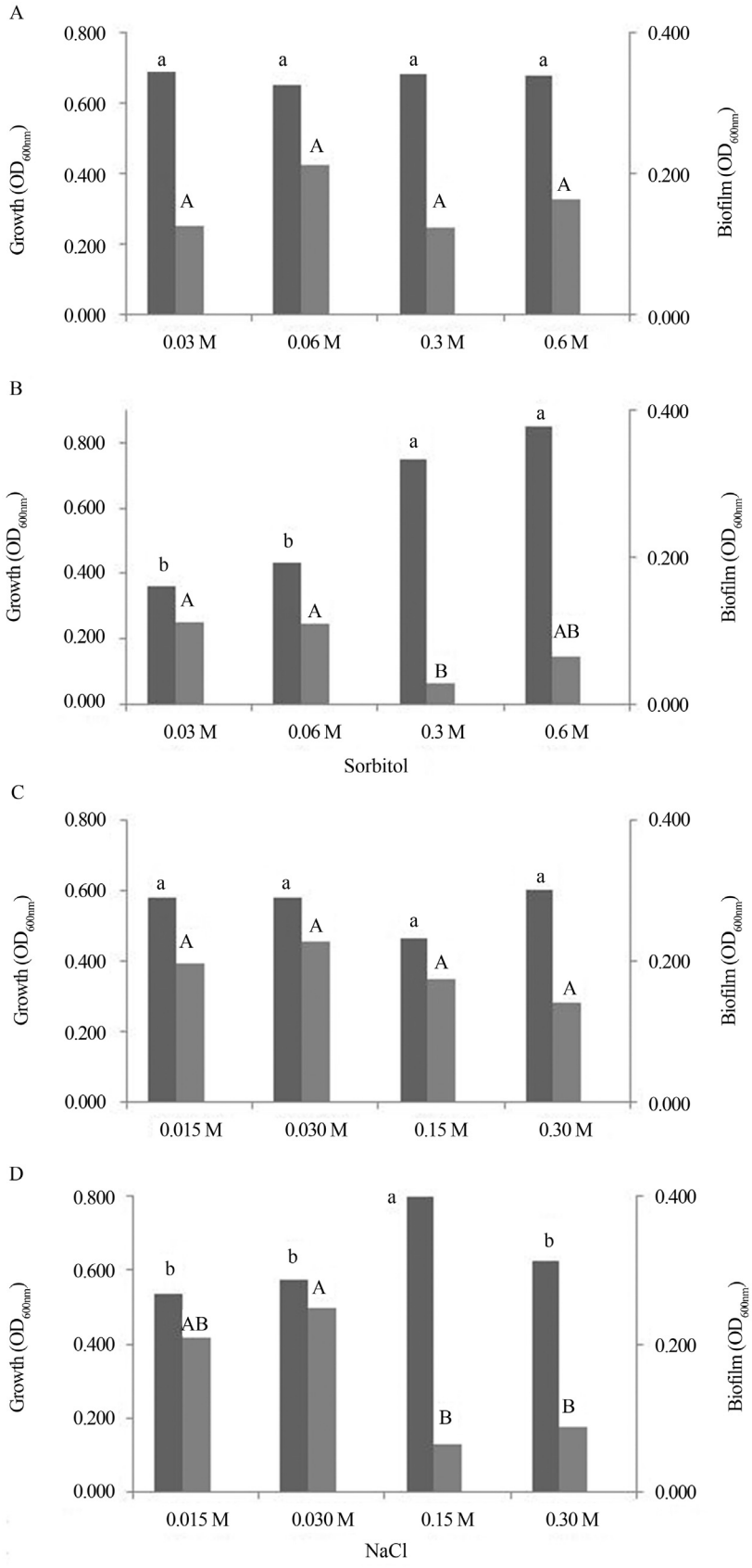

Figure 2 - Growth and biofilm formation by two strains: LMArzc17 (A and C) and LMArzc189 (B and D) with sorbitol or NaCl, both in four different concentrations. Means followed by different letters with the same size are statistically different from each other according to Tukey's test at 5%.

Table 1 - Biofilm formation as follows: weak formation ($0.1 \leq OD_{560} < 0.2$) (+), average formation ($0.2 \leq OD_{560} < 0.5$) (++), no formation (-).

Strains	D-sorbitol (M)				NaCl (M)			
	0.03	0.06	0.3	0.6	0.015	0.03	0.15	0.3
LMArzc17	+	++*	+*	+*	++*	++	+	+
LMArzc40	-	-	-	-	+	-	+	+
LMArzc55	-	+	-	-	+	-	-	-
LMArzc108	+	-	-	-	+	+	+	+
LMArzc158	-	-	-	-	-	+	-	-
LMArzc189	+	+	-	-	++*	++*	-	-
LMArzc192	-	-	-	-	-	-	-	+
LMArzc214	+	-	+	-	+	+	+	-
LMArzc324	-	-	-	-	+	+	-	+

(*) - Statistically significant differences at 5%.

Khan *et al.* (2011) suggested that the biofilm formation can be induced by certain substances. Morikawa *et al.* (2006) observed an increase in the production of biofilm by *B. subtilis* with increased concentration of Mn^{2+} and glycerol added to the culture medium. According to Hallsworth *et al.* (1998), glycerol and sorbitol when added to any medium or solution, reduces the water activity of this medium. We have observed weak to average biofilm formation by *Enterobacter* spp. and *Bacillus* spp. Biofilm formation by Enterobacteriaceae species is reported for *E. sakazakii* (Lehner *et al.*, 2005) and other species (Hurrel *et al.*, 2009).

As discussed earlier, the formation of exopolysaccharides (EPS) provides cellular protection against adverse environmental conditions and also contributes to biofilm formation (Chang *et al.*, 2007; Wai *et al.*, 1998). More recently, Seminara *et al.* (2012) observed that the production of EPS is crucial for spreading *B. subtilis* biofilm. The biofilm, in turn, may assist in root colonization during plant growth (Ramey *et al.*, 2004) and can also protect cells from nutrient deprivation, changes in pH, oxygen free radicals, antibiotics, phagocytosis (Jefferson, 2004) and water-limiting conditions (Chang *et al.*, 2007). Some EPS are highly hydrated due to the incorporation of water into its structure through hydrogen bonds, which could prevent desiccation in some biofilms (Flemming *et al.*, 2000). In this way, the verification of biofilm formation is an interesting feature because it can assist in many processes. In this study we concluded that the formation of biofilm is influenced by different concentrations of solutes added to the medium and we observed a significant production in two strains similar to *Enterobacter* sp. and *Stenotrophomonas* sp.

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