Brazilian Journal of Microbiology 45, 1, 193-197 (2014)
ISSN 1678-4405

Short Communication

Screening of endoglucanase-producing bacteria in the saline rhizosphere of *Rhizophora mangle*

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Submitted: February 3, 2012; Approved: September 9, 2013.

Abstract

In screening the culturable endoglucanase-producing bacteria in the rhizosphere of Rhizophora *mangle*, we found a prevalence of genera *Bacillus* and *Paenibacillus*. These bacteria revealed different activities in endoglucolysis and biofilm formation when exposed to specific NaCl concentrations, indicating modulated growth under natural variations in mangrove salinity.

Key words: Bacillus, *Paenibacillus*, ecological behavior.

Mangrove ecosystems have been described as an environment with a particular combination of characteristics, mainly related to salinity and anaerobiosis (Al-Sayed *et al.*, 2005; Lawson, 2011), where a single microbial community composition may reside (Dias *et al.*, 2009a, 2011b; Peixoto *et al.*, 2011; Santos *et al.*, 2011). Thus these ecosystems are promising for screening organisms with possible biotechnological applications (Holguin *et al.*, 2001). The most common plant species found in mangroves is *Rhizophora mangle* (Schaeffer-Novelli *et al.*, 2000), which have a particular rhizosphere system, where bacteria must withstand the environment in order to promote plant growth.

Along these lines, it is important to consider the bacteria's ability to promote organic matter degradation, providing nutrients for the host plant. Thus, functionality in such an ecosystem makes screening for cellulose-degraders organisms interesting, since such organisms may be producers of important biotechnological enzymes, such as endoglucanases (Cunha *et al.*, 2005). Dias *et al.* (2009) stressed the presence of such an enzymatic arsenal in mangrove-inhabiting microorganisms and its important application in biotechnological tools. Here we present a survey on the culturable bacterial community that advantageously have the ability to produce endoglucanase when found in the rhizosphere of *R. mangle*, and link this process with salinity level and biofilm formation.

The mangrove rhizosphere samples used were obtained from lateral and aerial *R. mangle* roots (nutrition roots) sampled in two mangroves located in the State of São Paulo (Brazil); one located in a well-preserved area (in the city of Cananéia, 22°42'01" S, 46°58'58" W), and another located in an oil-contaminated site (city of Bertioga, 23°53'80" S and 46°12'46" W). For each sample, roots immersed in the sediment (depth of 10 to 15 cm) were removed and stored in plastic bags and brought to the laboratory (4 °C during transport for an average of 12 h). The rhizosphere was considered soil adhered to roots, avoiding liquefied/muddy soil.

Approximate 1-g aliquots of rhizosphere were diluted into 9 mL of sterilized saline solution (0.9% NaCl) and homogenized under agitation (1 h at 150 rpm). The resulting suspensions were used to plate in solid Nutrient Agar (NA®BBL) medium. The plates were incubated at 28 °C for seven days and the number of bacterial colonies was deter-

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mined. Abundance and diversity of groups of colonies was obtained from rhizosphere samples by estimating Log values of 8.07 and 7.53 cfu.g^{-1} from rhizosphere of plants collected in Cananéia and Bertioga, respectively.

Endoglucolytic isolates were selected according to the methodology described by Theather and Wood (1982). Briefly, culture plates containing 1% carboxymethylcellulose (CMC) were used for culturing bacteria. After incubating at 28 °C during 4 days, plates were covered with Congo Red solution (1%) for 15 min and then washed with NaCl 5 M. Endoglucolytic activity was observed as formation of clear zones around the colonies. Isolates classified as positive for endoglucanase production were subjected to enzymatic activity quantification by spectrophotometry, as described by Wirth and Ulrich (2002). This approach resulted in a selection of 30 endoglucanase-producers (out of 129 colonies screened), of which 22 were isolates from Cananéia and 8 from Bertioga. Endoglucolytic activity quantification revealed isolates 39a and 60a (both from samples collected in the Cananéia mangrove) to have the highest endoglucanase production rates, with enzymatic indexes (halo/colony diameter) of 2.0 for both strains, and quantification values of 0.641 and 0.426 (absorbance at 600 nm), respectively.

The 30 selected isolates were identified by fatty acid methyl ester (FAME-MID), as described by Dias *et al.* (2009), using the database TSBA60 as a model. The order Bacillales was most common (except for two Actinobacteria isolates and two that remained unidentified). The isolates from Cananéia were identified as *Paenibacillus* sp., *P. macerans*, *P. lentimorbus, B. pumilus*, *B. subtilis*, and *Streptomyces* sp., and the isolates from Bertioga were identified as *P. macerans, B. pumilus, B. sphaericus*, and *Curtobacterium flaccumfaciens*.

Bacteria from the genera *Bacillus* and *Paenibacillus* have been reported to be associated with mangrove plants by Holguin *et al.* (2001), who verified phosphate solubilized by *B. athrophaeus* and *P. macerans* associated with *Avicennia marina*. Another study verified *Bacillus* spp. isolates from rhizosphere of *R. mangle* and *Laguncularia racemosa* that were able to degrade polycyclic aromatic hydrocarbons (PAH) (Maciel-Souza *et al.*, 2006). Here, we describe the abundance of bacteria belonging genus associated with the rhizosphere of the most abundant tree species in mangroves (*R. mangle*), and present their ecological role of endoglucolytic activity.

One isolate (39a - identified as *B. subtilis*), selected for its high endoglucanase yield, was further analyzed to determine the role of salinity variation on endoglucanase production and bacterial biofilm formation. This was done by recreating the conditions in *R. mangle* rhizosphere, where salinity is variable over time. The 39a isolate was cultivated in Nutrient Broth (NB®BBL) medium supplemented with NaCl at the following concentrations: 3% (0.5 M), 5% (0.8 M), 7% (1.2 M), 10% (1.7 M), 15% (2.5 M), and 20% (3.4 M). Bacterial development was monitored by spectrophotometry (at 550 nm) at 2, 4, 10, 16, 24, and 48 h after inoculation.

Assessing the cell development indicates that saline concentrations modulate bacterial reproduction; where the log was observed after two hours of culture in the medium without NaCl, and at higher NaCl concentrations (7, 10, and 15% NaCl), the log phase is observed only after four hours after inoculation. Culturing at 20% NaCl demonstrated that, in liquid medium, this amount of salt has an inhibitory effect on bacterial growth. The literature states that organisms affiliated with the class Bacillales are salt-tolerant, being able to grow at 24% NaCl (Pooja and Mugeraya 2011; Ventosa *et al.*, 1998). It is important to note that the ability to grow under high saline conditions represents an important feature in the interaction between bacteria and *R. mangle*, which is also a halotolerant plant due to the presence of subterranean roots that can filter the water prior to uptake (Fruehaf, 2005).

Additionally, cultures of the 39a isolate in solid medium were used to determine changes in the fatty acid methyl ester profiles when cells are cultivated under distinct NaCl concentrations of (using the same concentrations but analyzed 48 h after plating). The results from both cultivation approaches (solid and liquid) revealed similar results, where among the 11 FAMEs detected, one shifted in abundance in solid culture (aiC_{15}), and two changed in abundance when cells were exposed to NaCl in liquid medium (aiC_{15} and nC_{16}) (Table 1). Changes were always related with the proportional increase of such FAMEs with NaCl concentration. The genus *Bacillus* was predominant in terminally methyl-branched saturated *iso* and *anteiso* fatty acids, with carbon chains varying between 12 and 17 carbons (Kaneda, 1977). The structural changes of membranes indicate that their structures are responsive to salinity changes, possibly preventing NaCl from entering, consequently protecting cells from leaking osmolites. This result corroborates data from Nicolaus *et al.* (2001), who proposed that increased salinity leads to increased internal osmotic pressure.

Other than cell development and FAMEs profiles, endoglucolytic activity was also quantified when bacteria were grown at different salinity levels, where the highest value was obtained in the medium with 7% NaCl, followed by a steep decrease in NaCl concentration when at 10%. The lowest endoglucanase yield by cells cultivated in higher NaCl concentrations could be related to the lower cell count per gram of medium (reducing halos and quantification), or due to a cell response to salt presence. As shown below, the increase in salt concentration also led increased biofilm formation. Thus, the high abundance of exopolysacharides (EPS) in biofilms could be related to a lower endoglucanase yield by biofilm-protected cells.

Moreover, colonies of this isolate growing at different saline concentrations were subjected to scanning elec-

Table 1 - Membrane Fatty acids (in percentage) of *Bacillus subtilis* (isolate 39a) cultivated under distinct salinity levels (percentage of NaCl).

NaCl (%)	Fatty acids									
Solid medium	iC13	iC14	nC14	iC15	aiC15	iC16	nC16	iC17	aiC17	nC18	aiC19
0%	0.09	0.89	0.49	9.86	20.45	3.01	16.38	5.39	5.79	0.34	0.00
3%	0.22	1.42	0.50	14.55	41.74	3.40	7.89	8.96	14.82	0.78	0.17
5%	0.21	1.36	0.48	12.86	40.22	3.60	8.31	9.11	14.35	1.48	1.26
7%	0.14	1.00	0.44	10.33	37.39	3.21	11.60	9.97	15.40	2.79	0.31
10%	0.00	0.60	0.40	7.50	41.18	2.68	12.99	7.91	20.14	2.47	0.00
Liquid medium	iC13	iC14	nC14	iC15	aiC15	iC16	nC16	iC17	aiC17	nC18	aiC19
0%	0.00	1.24	0.00	9.15	31.15	6.15	8.18	9.25	14.44	0.52	0.00
3%	0.00	1.43	0.00	9.65	40.64	6.56	7.67	11.53	19.57	1.02	0.00
5%	0.00	1.58	0.58	8.45	39.47	6.05	10.29	10.34	19.02	2.00	0.41
7%	0.00	1.58	0.60	6.51	41.64	6.31	12.40	7.57	18.51	2.62	0.00
10%	0.00	0.00	0.00	5.96	46.66	4.95	15.75	5.65	21.03	0.00	0.00

iC indicates: iso chains.
aiC indicates: anteiso chains.

tron microscopy. In order to properly prepare the material, samples were obtained from solid cultures, and fixed for 2 h at 28 °C under vacuum (760 mm Hg) and using 2.5% glutaraldheide and 0.2 M cacodilate buffer, pH 7.2. Next,

Figure 1 - Biofilm formation and cellular modifications in strain 39a (*Bacillus subtilis*) cultured under distinct salinity levels (percentage of NaCl).

samples were rinsed in the same buffer and dehydrated in increasing ethanol series. A final dehydration was performed in acetone, prior to drying at critical point. Samples were then stacked in stubs and covered by a thin gold layer. The samples were observed under high-resolution with a model Leo 982 GEMINIDMS (Zeiss and Leica, Germany). The scans revealed the different biofilm formation when NaCl was added to the medium (Figure 1), possibly suggesting that salinity does drive the biofilm formation in the *R. mangle* rhizosphere (Figure 1). Additionally, morphological modifications to bacterial cells were identified as a response to salinity adaptation. These modifications are probably caused by accumulated solutes within cells, which can interfere in the osmotic equilibrium, and consequently in protein activity (Sleator and Hill, 2001). The images revealed the presence of endospores in cells growing at 3 and 5% salt concentrations, while at higher NaCl concentrations (10 to 20%), other cellular modifications were observed, such as in cellular elongation, as described by Zahran, (1997).

In addition to endospore formation and cellular elongation, another modification in cellular physiology was biofilm formation, which was found to be related to increased NaCl concentrations. In this case, biofilms would act in improving the microorganisms' ability to survive under saline stress conditions, where the biofilm matrix layer covering cells may act as a barrier against to osmotic changes (Nichols *et al.*, 2005). Such a barrier constituted by microbial biofilm is mainly composed of EPS, which is a compound partially degraded by endoglucanase activity. Assuming that, it can be inferred that regulation of free-living or biofilm-forming *Bacillus* spp. in the *R. mangle* rhizosphere is partially driven by saline concentration in the mangrove sediment. This would explain why such bacteria are abundant in the rhizosphere of this plant, and could also show the regulatory mechanism that enables them to have an advantage in niche colonization.

In summary, this study explored the endoglucanase-producing bacteria in the rhizosphere of the typical mangrove plant *R. mangle*, identifying them to the genus *Bacillus* and *Paenibacillus*, which are important players in degrading cellulose-like materials. In addition, the salinity level modulated the composition of cells membrane in the 39a isolate and interfered in this bacteria's ability to produce endoglucanases and form biofilms. Thus this mechanism is indicated as a key factor in the ecology of such organisms from this niche.

Acknowledgments

This study was supported by a grant from the State of São Paulo Research Foundation (FAPESP/BIOTA 2004/13910-6). Also, A.C.F. Dias (2008/54013-8) received a graduate fellowship. We also thank the support from the Oceanographic Institute (IO, USP, São Paulo), especially Dr. Ricardo P. Menghini and João Luis da Silva for their support in mangrove expeditions and samplings.

The authors declare that they have no conflict of interest.

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