Oceanobacillus iheyensis strain HTE831 was isolated from deep sea mud (1,050 m) from the Iheya Ridge near Okinawa, Japan, in 1998 (Lu et al., 2001). This organism is an alkaliphilic and extremely halotolerant Bacillus-related species. Its genome has been completely sequenced and the presence of genes required for adaptation to these extreme environments was demonstrated (Takami et al., 2002). Many proteins potentially associated with roles in the regulation of intracellular osmotic pressure and pH homeostasis were shown to be coded in its 3.6 Mb genome (Takami et al., 2002). Later on, Takaki et al. (2004) identified six kinds of new insertion sequences, a group II intron, and an incomplete transposon in the genome of strain HTE831. However, surprisingly, no other strains belonging to this species were described in the literature until 2006, when Barbosa et al. studied the Gram-positive spore-forming community found associated with plants in tropical soils. Halotolerant and facultatively alkaliphilic spore-forming Gram-positive bacteria were isolated from the rhizosphere and non-rhizosphere soils of Blutaparon portulacoides (St. Hill.) Mears (Amaranthaceae). This plant first colonized embryo dunes and backshores of Southwestern Atlantic Ocean beaches (Farias and Flores, 1989).

Different isolates were characterized phenotypically and analyzed by 16S rRNA gene sequencing and members of the species O. iheyensis were recovered (Barbosa et al., 2006a). Macroscopically, colonies of strains L4, L18, S1, S4, S14 and S18 are circular, creamy white, and slightly transparent. They are all strictly aerobic, catalase positive, nitrate negative, Voges-Proskauer (VP) negative, casein positive and starch hydrolysis negative. These six strains are able to grow in LB medium containing 0.5 to 20% NaCl and on pH varying from 5.7 to 9.5. They all formed a monophyletic branch with O. iheyensis strain HTE831 based on their 16S rRNA gene sequences (DQ058363–DQ058368; Barbosa et al., 2006a). Therefore, on the basis of the results of this investigation, different O. iheyensis strains isolated from B. portulacoides became available for further studies.

Hence, it is now reasonable to consider that strains of O. iheyensis are not restricted to extreme environments and may be spread in different habitats in the world. The study of O. iheyensis strains may lead us to a better understanding of life in saline and/or alkaline environments. New strains of O. iheyensis may also offer a potential source for the discovery of bioactive compounds with industrial value, such as antibiotics, biosurfactants and exopolysaccharides (Margesin and Schinner, 2001). However, no selective medium is
available for the isolation of O. iheyensis strains and biochemical identification is time consuming. Therefore, we describe in this study a PCR detection technique based on the 16S rRNA gene that can successfully be used for the identification of strains suspected to belong to O. iheyensis. Furthermore, this approach can also be used for the detection of this species directly in the environment, at least in sand samples.

The bacterial strains used in this study, their origins and growth characteristics are presented in Table 1. All Oceanobacillus and Halobacillus strains were grown in LB medium (tryptone 1%, yeast extract 0.5%) supplemented with 10% NaCl and incubated for 24–48 h at 32°C. Bacterial cultures were aerobically in LB/NaCl 10% slants at room temperature and at −80°C in LB/NaCl 10% medium containing 20% glycerol.

Table 1. Bacterial strains used in this study and specificity of PCR system based on 16S rRNA gene of Oceanobacillus iheyensis.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains</th>
<th>PCR product (486 bp)</th>
<th>Growth conditionsa</th>
<th>Origin/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oceanobacillus iheyensis</td>
<td>S1, S4, S14, S18</td>
<td>+</td>
<td>LB/NaCl 10%, 32°C</td>
<td>Non-rhizosphere soil; Barbosa et al. (2006a)</td>
</tr>
<tr>
<td></td>
<td>L4, L18</td>
<td>+</td>
<td>LB/NaCl 10%, 32°C</td>
<td>washed roots of Blutaparon portulacoides; Barbosa et al. (2006a)</td>
</tr>
<tr>
<td>O. picturae</td>
<td>L6, L15</td>
<td>−</td>
<td>LB/NaCl 10%, 32°C</td>
<td>washed roots of Blutaparon portulacoides; Barbosa et al. (2006a)</td>
</tr>
<tr>
<td>Halobacillus sp.</td>
<td>L7</td>
<td>−</td>
<td>LB/NaCl 10%, 32°C</td>
<td>washed roots of Blutaparon portulacoides; Barbosa et al. (2006a)</td>
</tr>
<tr>
<td>H. blutaparsonensis</td>
<td>M9</td>
<td>−</td>
<td>LB/NaCl 10%, 32°C</td>
<td>macerated roots of Blutaparon portulacoides; Barbosa et al. (2006b)</td>
</tr>
</tbody>
</table>

a LB medium consists of tryptone 1% and yeast extract 0.5% supplemented with 10% NaCl.

were incubated for 3 days at 32°C. The different isolates were characterized phenotypically as described by Barbosa et al. (2006a).

Genomic DNAs were extracted from all bacterial strains by using the protocol described by Seldin and Dubnau (1985). The total microbial community DNA was extracted directly from the sand samples and also from the enrichment cultures using FastPrep Spin kit for soil DNA (BIO 101 Systems, CA, USA). DNA preparations were visualized after electrophoresis in a 0.8% agarose gel in 1 × TBE buffer (Sambrook et al., 1989) and DNA concentration was measured in a GenQuant apparatus (Pharmacia Biotech, Cambridge, England).

The Oceanobacillus iheyensis HTE831 16S rRNA gene sequence available in GenBank (http://www.ncbi.nlm.nih.gov/) was compared to different halophilic/halotolerant bacterial 16S rRNA gene sequences by using Clustal X (Thompson et al., 1997). Those sequences were chosen based on the phylogenetic tree presented by Barbosa et al. (2006a). After alignment, two sequences were manually found as potential specific regions for O. iheyensis (Table 2): a 15-mer forward primer—OCEANO 203F: 5’-TCATCCTCCTGATGAG-3’—and a 20-mer reverse primer—O689R: 5’-CTCTTCTGACCTCAGTTCC-3’. Both primers were further analyzed by BLAST-N (Altschul et al., 1997) to search for homologous nucleotide sequences in the GenBank database.

The conditions used for amplification with both primers designed in this study were one cycle of 94°C (30 s) and 25 cycles of 94°C (1 min), 53°C (1 min 30 s) and 72°C (1 min 30 s). A final extension step was run for 5 min at 72°C and the reaction tubes were then cooled.
to 4°C. The 25 μl PCR reaction mix contained 10 mmol L⁻¹ Tris HCl, 50 mmol L⁻¹ KCl, 2.5 mmol L⁻¹ MgCl₂, 0.1% Triton X-100, 1/50 (v/v) dimethyl sulfoxide (DMSO), 0.4 mmol L⁻¹ dNTPs, 0.4 μmol L⁻¹ of each primer, 1.25 U of Taq DNA polymerase and 1 μl (about 50 ng) of the DNA extract. Negative controls (without DNA) were run in all amplifications. The PCR products were analyzed by electrophoresis in a 1.4% agarose gel in 1× TBE buffer.

The PCR product of the 16S rRNA gene obtained from the sand sample (Praia da Reserva) was cloned using the pGEM-T easy vector according to the instructions of the manufacturer (Promega, Madison, WI, USA). After transformation of Escherichia coli
other environments (at least in 2001). For the molecular detection of this species in is more ubiquitous than previously thought (Lu et al., 2006a), two strains of *O. picturae* and two strains of the related genus *Halobacillus* (Table 1). Using the conditions described here for the PCR reaction, only the strains of *O. iheyensis* showed the expected 486 bp-product (Fig. 1).

To estimate the sensitivity of the PCR amplification system for the detection of *O. iheyensis* in the environment, PCR reactions were performed using DNA extracted directly from the sand samples and from the samples pre-enriched in LB medium supplemented with 15% NaCl. No PCR product was obtained when the DNA extracted directly from the different beaches was used as a template. Conversely, when the DNA obtained from the enrichment cultures was used, the expected product could be visualized in Restinga de Jurubatiba and Praia da Reserva sand samples (Fig. 1). This fact could indicate that the population of *O. iheyensis* is low in these two sand samples (below the limit of detection for the PCR technique) or also very low or non-existent in the other beaches. To confirm the sequence of the PCR product, it was cloned in pGEM-T and seven clones were sequenced. All of them were affiliated with *O. iheyensis*, showing similarities varying from 99 to 99.8% (GenBank—accession numbers EU159894–EU159900).

To determine the detection limit of the PCR amplification system for *O. iheyensis* cells in sand, an experiment was performed with different inoculum densities of strain S4 applied to sand obtained in Praia da Reserva in microcosms. Cell inoculum densities exceeding 10^6 cells per g of sand were needed for the prompt detection via PCR, whereas uninoculated sand did not produce a PCR amplification product (Fig. 2). In previ-
Detection of *Oceanobacillus iheyensis*

Rosado et al. (1996) introduced either vegetative cells or spores of *Paenibacillus durus* at different concentrations (10^2 to 10^6 per g of dry soil) in Flevo silt loam soil and Vollú et al. (2003) introduced vegetative cells (10^2 to 10^6 per g of dry soil) of *P. macerans* in Cerrado soil (dark red latosol with clayey texture) for short-term experiments aimed at studying the sensitivity of the PCR detection method based on the 16S rRNA gene developed for *P. durus* and *P. macerans*, respectively. Inoculum exceeding 10^2 (*P. durus*) and 10^3 (*P. macerans*) cells or spores per g of soil were detected via PCR in both studies. The same level of specificity was obtained by Briglia et al. (1996) for the detection of *Mycobacterium chlorophenolicum* in soil.

Although the limit of sensitivity obtained in our study was low when compared to those studies, probably because of the different physicochemical characteristics of the soils used, the applicability of the method is evident for the detection of *O. iheyensis*. Further studies on the method developed here should be conducted in other environments, in order to enable natural detection of *O. iheyensis* populations. Primers specific for other spore-forming bacteria, such as *Clostridium* sp. (Van Dyke and McCarthy, 2002) and *Bacillus* sp. (Wu et al., 2006) have already been developed and are being used for this purpose.

Finally, to determine the applicability of our system for the identification of isolates supposed to belong to *O. iheyensis*, various colonies with different morphologies and only one colony with characteristics typical of *O. iheyensis*—circular, creamy white, and slightly transparent; casein positive and starch hydrolysis negative—were chosen from the LB plus 10% NaCl inoculated plates with the sand obtained from the three beaches in Rio de Janeiro and the two beaches in Bahia, for further amplification with primers OCEANO 203F and O689R. Only the strain (denominated AC10) isolated from Praia da Reserva in Arraial do Cabo and...
presenting characteristics typical of *O. iheyensis* showed the expected amplification product (data not shown). Hence, the method developed not only detects *O. iheyensis* in sand, but it can also be used in taxonomic studies for the quick identification of strains suspected to be *O. iheyensis*.

Acknowledgments

This work was supported by grants from the National Research Council of Brazil (CNPq) and FAPERJ.

References


