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Construction of probe of the plant growth-promoting bacteria *Bacillus subtilis* useful for fluorescence *in situ* hybridization

Luisa F. Posada [a], Javier C. Alvarez [b], Chia-Hui Hu [e], Luz E. de-Bashan [c,d,e], Yoav Bashan [c,d,e,*]

[a] Department of Process Engineering, Cra 49 #7 sur-50, Universidad EAFIT, Medellín, Colombia
[b] Departament of Biological Sciences, Cra 49 #7 sur-50, Universidad EAFIT, Medellín, Colombia
[c] The Bashan Institute of Science, 1730 Post Oak Ct., AL 36830, USA
[d] Environmental Microbiology Group, Northwestern Center for Biological Research (CIBNOR), Av. IPN 195, La Paz, B.C.S. 23096, Mexico
[e] Department of Entomology and Plant Pathology, Auburn University, 301 Funchess Hall, Auburn, AL 36849, USA

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ABSTRACT

Strains of *Bacillus subtilis* are plant growth-promoting bacteria (PGPB) of many crops and are used as inoculants. PGPB colonization is an important trait for success of a PGPB on plants. A specific probe, based on the 16 s rRNA of *Bacillus subtilis*, was designed and evaluated to distinguishing, by fluorescence *in situ* hybridization (FISH), between this species and the closely related *Bacillus amyloliquefaciens*. The selected target for the probe was between nucleotides 465 and 483 of the gene, where three different nucleotides can be identified. The designed probe successfully hybridized with several strains of *Bacillus subtilis*, but failed to hybridize not only with *B. amyloliquefaciens*, but also with other strains such as *Bacillus altitudinis*, *Bacillus cereus*, *Bacillus gibsonii*, *Bacillus megaterium*, *Bacillus pumilus*; and with the external phylogenetic strains *Azospirillum brasilense* Cd, *Micrococcus* sp. and *Paenibacillus* sp. The results showed the specificity of this molecular probe for *B. subtilis*.

1. Introduction

Bacillus sp. are commonly investigated and commercialized as plant growth-promoting bacteria (PGPB/PGPR) for several crops (Broggini et al., 2005; Idris et al., 2007; Laux et al., 2003; Ramirez and Kloepper, 2010; Zhang et al., 2010). The major difficulties in evaluation the effect of species of bacilli is that some are systematically closely related to each other and can be identify solely by detailed analysis of their 16S rRNA gene or only by other genes within species (Borriss, 2011). For example, strains of two closely related bacilli, *Bacillus subtilis* and *Bacillus amyloliquefaciens* can be either saprophytic, common PGPB, or both (Calvo et al., 2014; Kloepper et al., 2004; Pérez-García et al., 2011; Tumbarski et al., 2014). Colonization with one species of PGPB can be masked by saprophytic rhizosphere dwellers of the other species. Consequently, a failure in inoculation with a successful laboratory-proven strain cannot specifically pinpoint a deficiency in colonization capacity of the strain as a PGPB or to a technical failure in the inoculation method or to micro-environmental conditions. This genetic relatedness among bacilli creates major difficulties in building molecular detection tools, such as fluorescence *in situ* hybridization (FISH) for monitoring colonization of the PGPB after application. FISH probes specific for bacilli species are not available and the available probes are for clusters of several species of bacilli (http://probebase.csb.univie.ac.at/pb_results/2/).

Our hypothesis was that a detailed analysis of the 16s rRNA gene of closely related bacilli PGPB species in the rhizosphere will locate a unique sequence long enough for a FISH probe that is not shared by the two species. Based on this sequence, we will be able to construct an efficient probe that allows differential detection of each strain. To test this hypothesis, we tested 18 strains of bacilli and two strains that served as negative controls, using five molecular probes. We searched the GenBank for matches of the probe for other bacilli.

2. Materials and methods

2.1. Microorganisms and culture conditions

Bacillus subtilis EA-CB0575, the principal microorganism in this study, was isolated from the rhizosphere of banana plants at Urabá, Antioquia, Colombia (7°51′58.6″ N, 76°37′39.0″ W) in September 2009 and was identified using almost all of the 16s rDNA gene (1428 bp) sequenced with 8F and 1492 R primers and also with 8F and 907 R primers (performed at Macrogen, Seoul, South Korea). The strain was stored in tryptic soy broth (105,459, Merck & Co, Kenilworth, NJ) and 20% glycerol *V*/V (GenBank, accession number KC170988, Instituto Alexander von Humboldt, Collection of Microorganisms #191, Bogotá, Colombia). Strains of the following species were compared: *B. subtilis* EA-CB0015, EA-CB0575, EA-CB1121, 168, and NTC-3610; *B. amyloliquefaciens* EA-CB0158, FZB42, and EA-CB0959; *Bacillus altitudinis* EA-CB0686; *Bacillus cereus* MP1AC4 and EA-CB0131; *Bacillus*

* Corresponding author at: The Bashan Institute of Science, 1730 Post Oak Ct., AL 36830, USA.
E-mail addresses: bashan@bashanfoundation.org, ybashan@cibnor.mx (Y. Bashan).

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Table 1
Strains tested in this study.

Strain	Species	Isolation source	Reference
168	*Bacillus subtilis*	Culture of *B. subtilis* Marburg subjected to X-ray. *Bacillus* Genetic Stock Center (BGCS, Ohio University, USA): 1 *A*1.	Burkholder and Giles (1947), Zeigler et al. (2008)
AB-CD1	*Azospirillum brasilense*	*Cynodon dactylon*. American Type Culture Collection (ATCC): 29,710.	Eskew et al. (1977)
EA-CB0015	*B. subtilis*	*Musa* AAA var. Valery (banana) phyllosphere. Urabá, Colombia.	Ceballos et al. (2012)
EA-CB009	*B. pumilus*	*Musa* AAA var. Gran Enano rhizosphere. Urabá, Colombia.	Microbial collection, EAFIT. Colombia
EA-CB0131	*B. cereus*	*Musa* AAA var. Valery, rhizosphere. Urabá, Colombia.	Microbial collection, EAFIT. Colombia
EA-CB0158	*B. amyloliquefaciens*	*Musa balbisiana* rhizosphere. Urabá, Colombia.	Microbial collection, EAFIT. Colombia
EA-CB0575	*B. subtilis*	*Musa* AAA var. Valery rhizosphere. Urabá, Colombia.	Posada-Uribe et al. (2015).
EA-CB0579	*B. gibsonii*	*Musa* AAA var. Gran Enano rhizosphere. Urabá, Colombia.	Microbial collection, EAFIT. Colombia
EA-CB0686	*B. altitudinis*	*Musa* AAA var. Gran Enano rhizosphere. Urabá, Colombia.	Shivaji et al. (2006); microbial collection, EAFIT. Colombia
EA-CB0784	*B. megaterium*	*Musa* AAA var. Valery rhizosphere. Urabá, Colombia.	Microbial collection, EAFIT. Colombia
EA-CB0840	*Paenibacillus pasadenensis*	*Musa* AAA var. Valery. Urabá rhizosphere, Colombia.	Ceballos et al. (2012)
EA-CB0888	*Paenibacillus pasadenensis*	*Musa* AAA var. Valery phyllosphere. Urabá, Colombia.	Ceballos et al. (2012)
EA-CB0959	*B. amyloliquefaciens*	*Musa balbisiana* rhizosphere. Urabá, Colombia.	Ceballos et al. (2012)
EA-CB1121	*B. subtilis*	*Musa* AAA var. rhizosphere. Gran Enano. Urabá, Colombia.	Microbial collection, EAFIT. Colombia
ES4	*B. pumilus*	Cardon. La Paz, BCS, México. GenBank accession number: FJ032017	de-Bashan et al. (2010)
FZB42	*B. amyloliquefaciens*	Infested soil in sugar beet field, Brandenburg, Germany.10 A6 del BGSC (Ohio University, USA)	Fan et al. (2012), Idris et al. (2007)
M01	*Micrococcus lylae*	Mangrove bacteria. La Paz, BCS, Mexico.	Holguin and Bashan (1996)
MEG03	*B. megaterium*	Cardon. La Paz, BCS, Mexico.	Culture collection, CIBNOR, Mexico.
MP1AC4	*B. cereus*	Cardon. La Paz, BCS, Mexico.	Culture collection, CIBNOR, Mexico.
NCTC-3610	*B. subtilis*	Isolated from blood of phenylketonuria patient, assessment by Ehrenberg, 1835. ATCC: 6051.	Nakamura et al. (1999); Zeigler et al. (2008)

gibsonii EA-CB0579; *Bacillus megaterium* EA-CB0784 and BMEG03; *Bacillus pumilus* EA-CB0009 and ES4; and the external phylogenetic strains *Azospirillum brasilense* Cd, *Micrococcus* sp. M01, and *Paenibacillus* sp. EA-CB0840 and EA-CB0888 (Table 1).

Bacillus and *Paenibacillus* strains were inoculated in TSB; *A. brasilense* and *Micrococcus* sp. were inoculated in nutrient broth (105443, Merck & Co.). All cultures were incubated for 24–48 h at 150 rpm and 30 ± 2 °C.

2.2. Probe design for fluorescence in situ hybridization (FISH)

The probe sequence bsub-ss-0463-aA-22A for detecting *B. subtilis* by FISH was designed (Table 2), and the name assigned is based on probe nomenclature rules (Alm et al., 1996). It was labelled with two different fluorophores at the 5′ end: 56-FAM (fluorescein, wavelength absorption/emission 495 nm/520 nm) and Cy3 (cyanine, wavelength absorption/emission 552 nm/565 nm). A universal probe for eubacteria, an equimolar mix of probes EUB338 (Amann et al., 1990), EUB338-II, and EUB338-III (EUBMIX) (Daims et al., 1999), was used and labelled with the cyanine Cy3 at the same end. All probes were purchased (Integrated DNA Technology, Coralville, IA). Probe sequences are listed in Table 2. The working solution concentration for each probe (in TE buffer (10 mM Tris HCl, pH 7.2, 1 mM EDTA) was 50 ng μL^{-1} for FAM labelled probe and 30 ng mL^{-1} for Cy3 probe. They were stored at −20 °C in the dark.

2.3. Sample preparation and fixation

Vegetative cell cultures were centrifuged at 3260 × *g* for 15 min. Supernatants were discarded and the biomass was washed twice with 0.85% saline solution. Optical density was adjusted at 1.0 ($O.D_{600\ nm}$), corresponding to ~10^8 CFU mL^{-1} for *Bacillus*, *Paenibacillus*, and *Micrococcus* strains, and ~10^9 CFU mL^{-1} for *A. brasilense*. The counts were verified by plate count method on tryptic soy agar (#105458, Merck & Co) and nutrient agar (#105450 Merck & Co), respectively.

Pellets of Gram-positive cells were fixed with a solution of 500 μL 0.85% NaCl and 500 μL 96% ethanol at 4 °C. Pellets of Gram-negative cells were fixed in 4% paraformaldehyde (#158127, Sigma-Aldrich, St. Louis, MO) and incubated for 3 h at 4 °C. Ten microliters of Gram-positive fixed cell suspension and 4 μL of Gram-negative suspension were placed on gelatinized slides (0.1% w/v gelatine [#G2500, Sigma-Aldrich] and 0.01% w/v chromium potassium sulphate (#243361, Sigma-Aldrich) (Daims et al., 2005). Slides were dehydrated using successive 50, 80, and 96% ethanol rinsing (3 min each), then dried at 37 °C. Lysozyme (#L-7651, Sigma-Aldrich, 10 mg mL^{-1}) was applied over Gram-positive samples and incubated at 37 °C for 20 min and then dehydrated again, as described earlier and dried. Slides were stored at 4 °C until hybridization.

2.4. Fluorescence in situ hybridization (FISH)

Hybridization buffer was prepared from 360 μL 5 M NaCl, 40 μL 1 M Tris-HCl (#10812846001, Sigma-Aldrich) at pH 8.0; 2 μL 10% SDS (#L3771, Sigma-Aldrich); 700 μL deionized formamide (#F9037, Sigma-Aldrich); and 900 μL ultrapure water. The amount of formamide and water depended on the stringency of the probe. In this case, probe stringency was 35%. A solution of 40 μL of the hybridization buffer, 4 μL EUBmix probe, and 10 μL of the designed probe was added to each sample. This process was done in the dark to reduce bleaching of the fluorophore. Slides were place into 50 mL Falcon tubes with a

Table 2
Probes used in this study.

Probe	Sequence	Tm*	MW**	% G-C***	Nucleotides	Reference
bsub-ss-0463-aA-22-FAM	5′−/56-FAM/TAC CGC CCT ATT CGA ACG GTA C-3′	58.5	7192.8	54.5	22	This study
bsub-ss-0463-aA-22-Cy3	5′−/Cy3/TAC CGC CCT ATT CGA ACG GTA C-3′	58.5	7161.9	54.5	22	This study
EUB338-Cy3	5′−/5Cy3/GCT GCC TCC CGT AGG AGT-3′	59.4	5998.2	66.6	18	Amann et al. (1990), Daims et al. (1999)
EUB338 II-Cy3	5′−/Cy3/GCA GCC ACC CGT AGG TGT-3′	60.5	6007.2	66.6	18	
EUB338 III-Cy3	5′−/Cy3/GCT GCC ACC CGT AGG TGT-3′	60.5	5998.2	66.6	18	

*Tm: Melting temperature (°C), ** MW: Molecular weight (Da), *** % G-C: Guanine-cytosine percentage.

piece of paper towel impregnated with the remnant hybridization buffer (hybridization chambers), and heated in darkness in a hybridization oven (model 5430, VWR International, Radnor, PA) at 46 °C for 2 h. Afterward, slides were washed with a buffer of 1 mL 1 M Tris-HCl at pH 8.0, 500 μL 0.5 M EDTA at pH 8.0, 700 μL 5 M NaCl solution (to meet stringency of 35%), 50 mL ultrapure water, and 50 μL 10% SDS. Falcon tubes with this buffer were preheated to 48 °C and slides were put inside the tubes in a water bath for 15 min. The slides were rinsed for a few seconds with deionized water at 4 °C and then air-dried at room temperature in the dark. Experiments were independently repeated twice using two slides with two replicated samples per strain.

2.5. Visualization

Citifluor AF1 mountant (Electron Microscopy Sciences, Hatfield, PA) was applied to dried hybridized slides. Slices were visualized under oil immersion with the 100× objective on a fluorescence microscope (Olympus BX41 Tokyo, Japan) or on an Axioscope A1 (Carl Zeiss, Oberkochen, Germany) microscope equipped with filters Cy3, and FITC, for samples hybridized with the fluorochrome FAM. Sample pictures were taken with cameras (Axiocam CC5 or Evolution V) and processed with imaging software (ImagePro Plus 6.3, Media Cybernetics, Silver Spring MD or ZEN2012 Blue edition, Carl Zeiss). Two identical images were taken, each with the two probes, yielding green (for the specific probe, bsub-ss-0463-aA-22) and red (for the universal probe for eubacteria) images. The two images were combined by the software, and when the two probes hybridized with the sample, they yield a green-yellow-orange tone, depending on the intensity of the individual colour channels (de-Bashan et al., 2011). This identified the strain as *B. subtilis*. If hybridization occurred only with the universal probe for eubacteria, the images stayed red, even when combined with the green images. Some images were stained green because of interference

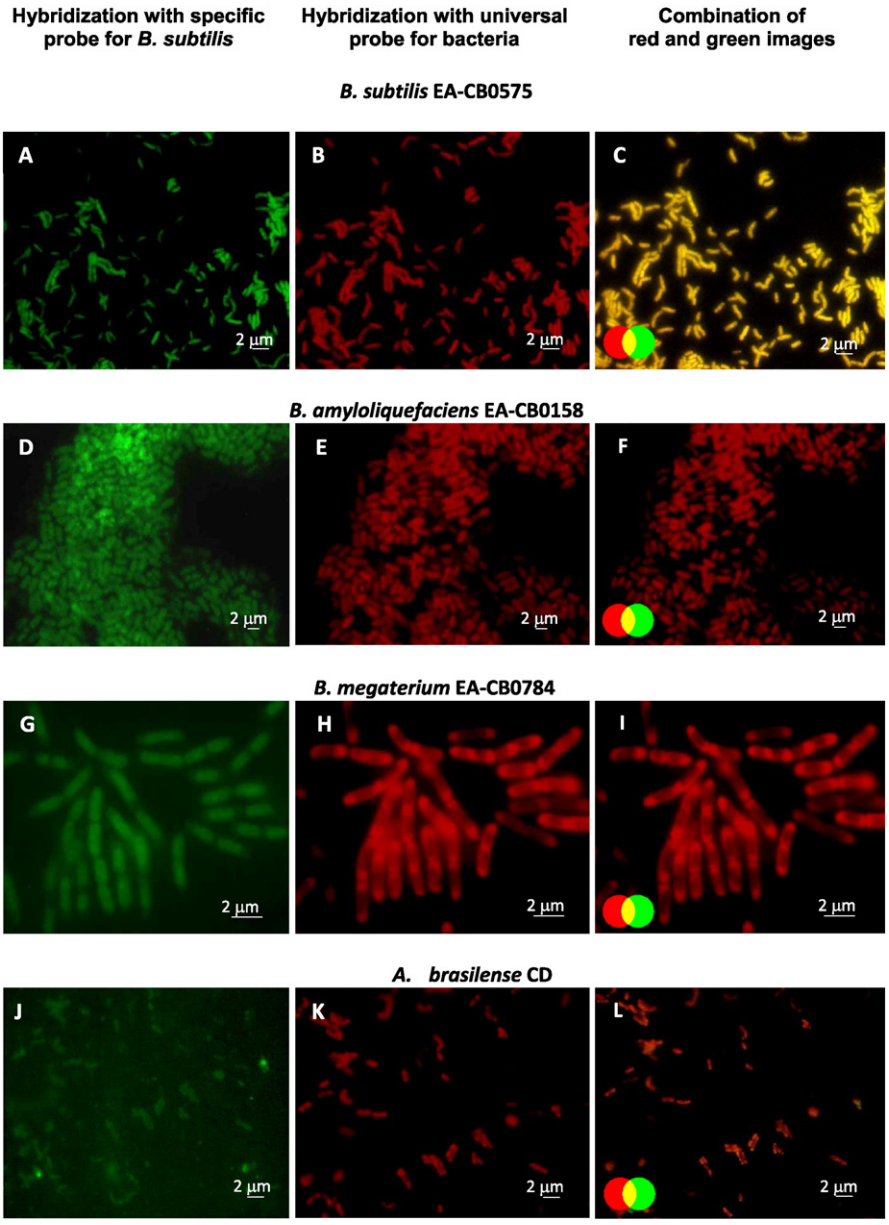

Fig. 1. FISH evaluation for *Bacillus* species: *B. subtilis*, *B. amyloliquefaciens*, and *B. megaterium*, and the phylogenetic foreign strain *Azospirillum brasilense* CD. The non-specific green signal of all strains, other than *B. subtilis*, was barely seen, which did not allow distinguishing the cells from the background. To demonstrate that the bacterial cells are present, additional artificial light was introduced in these images. Several original faded green images are presented as Fig. S5. Assays were done by duplicate, where each replicate consisted of two samples of each of the bacterial strains. Images shown are representative images.

of natural low autofluorescence of some microorganisms under fluorescent light; however, the hybridization signal was always greater (Moter and Gobel, 2000).

3. Results

For future detection and monitoring of the PGPB *B. subtilis* strain EA-CB0575 in plants, a probe based on the 16 s rRNA of the strain was designed. This was done by using nucleotides sequences differentiating *B. amyloliquefaciens* and *B. subtilis*. These two bacilli are closely related and both are PGPB and saprophytes of the same habitat (Reva et al., 2004). The selected section for the probe was between nucleotides 465 and 483 of the gene, where three different nucleotides can be identified (Fig. S1).

B. subtilis strain EA-CB0575 was positively identified by FISH by using a combination of the probe designed for this species and the universal probe for eubacteria. When green and red images (Fig. 1a, b) were combined, they yielded the distinctive yellow colour that identified the strain as *B. subtilis* (Fig. 1c). The other two *Bacillus* species, the very closely related *B. amyloliquefaciens* 158, the distant *B. megaterium* 784, and the PGPB *A. brasilense* Cd, serving as negative controls, were not recognized as *B. subtilis*. An additional 14 strains of *Bacillus* sp. and *Micrococcus* sp. (negative control) were tested. Only strains of *B. subtilis* could be identified by the developed probe, while all the other strains hybridized with the universal probes for eubacteria (Figs. S2, S3, S4).

A BLAST program search through the GenBank for potential *Bacilli*, having the same sequence of our probe, and therefore, theoretically can also hybridize with our probe, yielded 25 strains (Table 3).

4. Discussion

Successful root colonization after inoculation with any PGPB is an almost universal requisite when testing the effect of a PGPB/PGPR strain on performance of plants (Bashan et al., 2004; Kloepper and Beauchamp, 1992; Lugtenberg et al., 2001). Numerous methods were developed employing microbiological, immunological, cultural, and molecular approaches. A contemporary successful method to address this fundamental requirement is FISH (de-Bashan et al., 2010). The FISH procedure requires development of a molecular probe, and this probe should be as specific as possible for the inoculated species. Development of a probe should consider the following specific characteristics for an appropriate hybridization: (1) probe length should be a ~18–30 bp sequence of the 16s rRNA gene. Longer probes result in poor hybridization and shorter probes generate lack of specificity. (2) Base composition should be between 40–60% G-C, because different G-C percentages may produce nonspecific hybridization. (3) Probe should be a stretched sequence and not hairpin structures because these structures inhibit hybridization. (4) Probe should not have long stretches of the same nucleotide. (5) The probe should have a melting temperature of 57 °C or higher because empirical analyses of other studies indicate that this temperature enhances the probability that hybridization will be successful (Hugenholtz et al., 2002).

The size of the 16s rRNA gene shared by all eubacteria and used to identify species, while unique to every species, is too short to allow that any short nucleotide sequence is unique to a single species. Other species may share a specific sequence but differ in others; thus making them different species. As a result of this difficulty, designing a probe for detection should emphasize more in practicality and not in uniqueness because the latter may be impossible to attain. If two related species residing in the same habitat differ in a sequence, this will allow creation of a technique capable of differentiating between their populations. At the same time, this sequence may be shared by other species not residing in that environment. Thus, the existence of the sequence in these species, from a practical approach, is irrelevant and does not interfere with specific detection of the species in a specific habitat.

The family Bacillaceae is highly diverse. In 2016, it contained 52 genera and 570 species and is continuing to proliferate. *Bacilli* can be found in any environment, including the rhizosphere of plants (Earl et al., 2008). Few bacilli are also PGPB/PGPR, including the two species compared in this study (Borriss, 2011). Almost all strains found by the BLAST program of the GenBank are not known as PGPB or colonizers of the rhizosphere or have any known function in the rhizosphere, the target site of our probe. It is also doubtful if hybridization for FISH will happen in these species at all. When we tested one of these bacilli, *B. gibsonii*, by the FISH procedure, no hybridization occurred (Fig. S4).

This probe was developed for the practical purpose of distinguishing between two common PGPB in the rhizosphere. No other probes of *Bacillus* specifically for FISH are available. Some probes are available for microarrays of *Bacillus* sp. (http://probebase.csb.univie.ac.at/pb_results/2/). The hypothesis that other bacteria may have the same sequences, and perhaps can be also detected, is less relevant in this context because these bacteria do not share the same habitat as the inoculated PGPB.

In summary, this study developed a practical probe to distinguish between the PGPB *B. subtilis* and *B. amyloliquefaciens* by fluorescence *in situ* hybridization.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2016.05.029.

Table 3
List of species from the GenBank (search February 2016) that may interact with *Bacillus subtilis* 16S FISH probe developed in this study.

1.	HQ223107 *Bacillus tequilensis* 10b
2.	AB021191 *Bacillus mojavensis* IFO15718
3.	AJ831843 *Bacillus aerius* 24 K
4.	L09227 *Saccharococcus thermophilus*
5.	AF326278 *Geobacillus toebii* BK-1
6.	AF067651 *Geobacillus caldoxylolyticus* ATCC 700356
7.	X76440 *Bacillus clausii* DSM 8716
8.	X76446 *Bacillus gibsonii* DSM 8722
9.	AB021182 *Bacillus carboniphilus* JCM9731
10.	X76449 *Bacillus pseudalcaliphilus* DSM 8725
11.	AF547209 *Bacillus acidicola* 105–2
12.	AY228462 *Bacillus algicola* KMM 3737
13.	AF541966 *Bacillus hwajinpoensis* SW-72
14.	AY603978 *Bacillus taeanensis* BH030017
15.	AF483625 *Bacillus aquaemaris* TF-12
16.	AJ315075 *Bacillus decolorationis* LMG 19507 T
17.	AJ316316 *Bacillus murimartini* LMG 21005
18.	AJ880003 *Bacillus plakortidis* P203T
19.	EF422411 *Bacillus alkalinitrilicus* ANL-iso4
20.	CP000002 *Bacillus licheniformis* ATCC 14580
21.	AB271756 *Brevibacillus brevis* NBRC 15304
22.	FJ347755 *Allobacillus halotolerans* B3A
23.	LN812018 *Pelagirhabdus alkalitolerans* S5
24.	FJ746578 *Streptohalobacillus salinus* H96B60
25.	Y319933 *Tenuibacillus multivorans* 28–1

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