Bacillus invictae sp. nov., isolated from a health product

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A Gram-positive, rod-shaped, endospore-forming Bacillus isolate, Bi.FFUP1T, recovered in Portugal from a health product was subjected to a polyphasic study and compared with the type strains of Bacillus pumilus, Bacillus safensis, Bacillus altitudinis and Bacillus xiamenensis, the phenotypically and genotypically most closely related species. Acid production from cellobiose, D-glucose and D-mannose and absence of acid production from D-arabinose, erythritol, inositol, maltose, mannitol, raffinose, rhamnose, sorbitol, starch and L-tryptophan discriminated this new isolate from the type strains of the most closely related species. Additionally, a significant different protein and carbohydrate signature was evidenced by spectroscopic techniques, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and Fourier transform IR spectroscopy with attenuated total reflectance. Using a chemometric approach, the score plot generated by principal component analysis clearly delineated the isolate as a separate cluster. The quinone system for strain Bi.FFUP1T comprised predominantly menaquinone MK-7 and major polar lipids were diphosphatidylglycerol, an unidentified phospholipid and an unidentified glycolipid. Strain Bi.FFUP1T showed ≥99% 16S rRNA gene sequence similarity to B. safensis FO-036bT, B. pumilus (7061T and SAFR-032), B. altitudinis 41KF2bT and B. xiamenensis HYC-10T.

Abbreviations: DDH, DNA–DNA hybridization; FTIR-ATR, Fourier transform IR spectroscopy with attenuated total reflectance; gyrB, β-subunit of DNA gyrase; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight MS; ML, maximum-likelihood; NJ, neighbour-joining; PCA, principal component analysis; rpoB, β-subunit of RNA polymerase; FAME, fatty acid methyl ester; MK, menaquinone; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, gyrB and rpoB gene sequences of strain are JX183147, JX183192 and JX183163, respectively.

Two supplementary figures and four supplementary tables are available with the online version of this paper.
The genus *Bacillus* includes organisms of great medical, biotechnological and economic importance (Logan & De Vos, 2009), with resistance to heat, radiation, chemical oxidizing agents and desiccation, due to spore production, an important condition for their adaptation to different ecological niches (Stackebrandt & Swiderski, 2008). This resistance to acute stress factors makes them difficult to eliminate from clean room environments and consequently they are a frequent cause of significant contamination of many industrial products, including pharmaceuticals. Recently, the National Aeronautics and Space Administration has reported *Bacillus safensis* and *Bacillus pumilus* (Satomi *et al.*, 2006), as major contaminants in spacecraft, associated with clean room assembly facility surfaces. Taxonomically, species of the *B. pumilus* group belong to the *Bacillus subtilis* clade (Bhandari *et al.*, 2013) and encompass *B. pumilus*, the first species described, in addition to *B. safensis*, *Bacillus altitudinis*, *Bacillus stratosphericus*, *Bacillus aerophilus* and the more recently discovered species *Bacillus xiamenensis* (Satomi *et al.*, 2006; Liu *et al.*, 2013; Lai *et al.*, 2014).

During a study assessing the diversity of *Bacillus* species isolates recovered from terrestrial sources, we observed the presence of strain Bi-FFUP1\(^T\) as a contaminant of a health product, which initially showed high similarities to members of the *B. pumilus* group. Bi-FFUP1\(^T\) was isolated according to the general procedure described by the European Directorate for the Quality of Medicines (2014). Thus, in the present study, physiological and biochemical characteristics, which included carbohydrate and amino acid metabolic profiles, whole-cell fatty acid methyl esters, respiratory quinones, polar lipids, protein and carbohydrate content, together with phylogenetic features derived from 16S rRNA, rpoB and gyrB gene sequences and DNA–DNA hybridization (DDH) studies, were determined and compared with those of the type strains of *B. safensis*, *B. pumilus*, *B. altitudinis* and *B. xiamenensis*, to assess the taxonomic position of this strain, which is of relevance in food and pharmaceutical quality control and for infection control purposes. Unfortunately, the type strains of *B. stratosphericus* and *B. aerophilus*, isolated from the same sample as *B. altitudinis* 41KF2b\(^T\), are no longer available in the depositor’s collections and for this reason were not included in our polyphasic analysis.

Strain Bi-FFUP1\(^T\) was recovered from a health product in Portugal. It was maintained on trypticase soy agar (TSA; Sigma-Aldrich) for short-term storage and in trypticase soy broth (TSB; Sigma-Aldrich) supplemented with 20 % (v/v) glycerol at −80 °C for long-term storage. Liquid culture was grown in TSB at 37 °C for 24 h. In addition, the type strains of phenotypically and genotypically most closely related species, *B. pumilus* ATCC 7061\(^T\) (DSM 27\(^T\)), *B. safensis* FO-036b\(^T\) (DSM 19292\(^T\)), *B. altitudinis* 41KF2b\(^T\) (DSM 21631\(^T\)) and *B. xiamenensis* HYC-10\(^T\) (LMG 27143\(^T\)), were obtained from the respective culture collections and cultivated at 37 °C in TSA and were used as reference strains for comparative analysis during the study.

To characterize strain Bi-FFUP1\(^T\) phenotypically, standard phenotypic tests were performed. Gram staining was determined using the bioMérieux Gram Stain kit according to the manufacturer’s instructions. Oxidase activity was tested by oxidation of 1 % (w/v) tetramethyl-p-phenylenediamine (Merck) and catalase activity was evaluated by the production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution. Growth at different NaCl concentrations [0, 5.0, 7.5, 10.0, 11.0, 12.0, 13.0, 14.0 and 15.0 % (w/v)] and different temperatures (5, 10, 15, 20, 25, 30, 37, 50, 60, 65 and 70 °C) was examined by growing strain Bi-FFUP1\(^T\) in TSB as the basal medium. To determine the pH range for growth, basal medium was adjusted with HCl or NaOH to reach values of 4.0–11.0, at intervals of 1.0 pH unit. To confirm anaerobic growth, cells were inoculated into TSB tubes with paraffin film on top under optimal growth conditions. Carbohydrate and amino acid metabolic profiles were obtained with a commercial BBL Crystal Gram Positive ID kit (Becton Dickinson) and the API 50CH system (bioMérieux), which were interpreted according to the manufacturers’ instructions.

For quantitative analysis of whole-cell fatty acid methyl ester profiles, the novel isolate was cultivated on TSA at 30 °C for 24 h, which preparation and analysis were conducted according to the instructions of the Sherlock Microbial Identification System (MIDI). Moreover, quinones and polar lipids were harvested from biomass of strain Bi-FFUP1\(^T\) grown in peptone yeast extract broth (Sigma-Aldrich), which were extracted and analysed according to the integrated procedure described by Tindall (1990a, b) and Altenburger *et al.* (1996). HPLC analysis was carried out using the HPLC apparatus described by Stolz *et al.* (2007). Polar lipids were separated by two-dimensional silica gel TLC and then identified according to a previously described method (Tindall *et al.*, 2007).

Differences in strain Bi-FFUP1\(^T\) gyrB and rpoB sequences in comparison with the most closely related species and DNA–DNA hybridization experiments with Bi-FFUP1\(^T\) and *B. pumilus* ATCC 7061\(^T\), *B. safensis* FO-036b\(^T\), *B. altitudinis* 41KF2b\(^T\) and *B. xiamenensis* HYC-10\(^T\) gave relatedness values of 39.6 % (reciprocal 38.0 %), 49.9 % (reciprocal 42.9 %), 61.9 % (reciprocal 52.2 %) and 61.7 % (reciprocal 49.2 %), respectively, supported the delineation of strain Bi-FFUP1\(^T\) as a representative of a novel species of the genus *Bacillus*, for which the name *Bacillus invictae* sp. nov. is proposed, with strain Bi-FFUP1\(^T\) (=DSM 26896\(^T\)=CCUG 64113\(^T\)) as the type strain.
The snapshot of different protein compositions detected by matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF/MS) was acquired by a MALDI-TOF/TOF mass spectrometer (4800 Plus MALDI-TOF/TOF Analyser; AB SCIEX) operating in linear positive mode. Each spectrum was the accumulated sum of at least 2000 laser shots for \( m/z \) 2500–12 000 due to the good reproducibility of the spectral profile in that mass range. All spectra were externally calibrated using a commercial mixture of angiotensin I, adrenocorticotropic hormone and insulin (AB SCIEX). Strain Bi.FFUP1\(^T\) was grown under aerobic conditions on Luria–Bertani agar (Merck) for 24 h at 37 °C. Cells were harvested by transferring the equivalent of three full blue plastic loops (\( \#30 \) ml) from each agar plate into 20 ml of sterile water and bacterial material was resuspended by vortexing. Sample inactivation was carried out by applying the modified trifluoroacetic acid (Sigma-Aldrich) inactivation protocol (Lasch et al., 2008). Subsequent supernatant filtration (0.22 μm) was carried out.

For MALDI-TOF/MS experiments, 2 μl of the filtered microbial dilution was mixed with 2 μl of a solution of α-cyano-4-hydroxycinnamic acid (12 mg ml\(^{-1}\); Sigma-Aldrich), prepared in 100 % acetonitrile (Sigma-Aldrich) and 0.3 % trifluoroacetic acid. Then, 1 μl of the mixture was spotted onto a stainless steel MALDI sample plate (Opti-TOF 384-well insert; AB SCIEX) and allowed to dry at room temperature. For each isolate, two biological replicates (obtained from two different agar plates) were carried out and the mean spectra were considered for the analysis. Mass spectra were analysed with the Data Explorer software (version 3.7, build 126; AB SCIEX). Subsequently, \( m/z \) values were extracted from the raw experimental mass spectra that included all the mass peaks with a relative signal to noise (S/N) ratio intensity above 2.

### Table 1. Bacillus pumilus group species used in this study, their sources and corresponding GenBank accession numbers

<table>
<thead>
<tr>
<th>Species</th>
<th>Isolate</th>
<th>Year/origin/location</th>
<th>GenBank accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. invictae sp. nov.</strong></td>
<td>Bi.FFUP1(^T)*</td>
<td>2005/Health products/Portugal</td>
<td>JX183147, JX183163, JX183192</td>
</tr>
<tr>
<td><strong>B. pumilus</strong></td>
<td>ATCC 7061(^T)</td>
<td>1999/Clean room air particulate/USA</td>
<td>GQ911554.1, EU138862.1, AY167869.1</td>
</tr>
<tr>
<td><strong>B. safensis</strong></td>
<td>FO-036b(^T)</td>
<td>1999/Clean room air particulate/USA</td>
<td>AF234854, KC895451, AY167867</td>
</tr>
<tr>
<td><strong>B. xiamenensis</strong></td>
<td>HYC-10(^T)</td>
<td>2014/Mullet Mugil cephalus/China</td>
<td>JX680066, JX679987, JX680142</td>
</tr>
<tr>
<td><strong>B. altitudinis</strong></td>
<td>41KF2b(^T)</td>
<td>2006/High-elevation air sample/India</td>
<td>AJ831842, JX680064, JX680219</td>
</tr>
</tbody>
</table>

*Obtained from the Quality Control Department (INFARMED), Lisbon, Portugal.

### Table 2. Differential metabolic profiles of strain Bi.FFUP1\(^T\) and type strains of closely related Bacillus pumilus group species

| Strains: | 1, Bi.FFUP1\(^T\); 2, B. pumilus ATCC 7061\(^T\); 3, B. safensis FO-036b\(^T\); 4, B. xiamenensis HYC-10\(^T\); 5, B. altitudinis 41KF2b\(^T\). |
|---|---|---|---|---|
| Carbohydrate acid production profile | 1 | 2 | 3 | 4 | 5 |
| Cellobiose | + | – | + | + | + |
| D-Arabinose | – | + | + | – | + |
| Erythritol | – | + | + | – | – |
| D-Glucose | + | – | + | – | – |
| Inositol | – | + | – | – | – |
| Maltose | – | + | – | + | – |
| Mannitol | – | + | + | + | + |
| Mannose | + | – | + | + | + |
| Raffinose | – | + | – | + | – |
| Rhamnose | – | – | – | + | + |
| Sorbitol | – | – | – | + | + |
| Starch | – | – | – | + | + |
| Amino acid utilization | – | – | – | + | – |
| L-Arginine | – | – | – | + | – |

**Fig. 1.** Total polar lipid profile of strain Bi.FFUP1\(^T\) after two-dimensional TLC and detection with 5 % ethanolic molybdatephosphoric acid. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; GL1–3, unidentified glycolipids; PL1, unidentified phospholipid; AL1, unidentified aminolipid; L1–5 unidentified polar lipids not containing a phosphate, an amino or sugar residues.
(a) Mass spectra of five different bacterial strains:

- **B. pumilus ATCC 7061T**
- **B. safensis FO36-bT**
- **B. xiamenensis HYC-10T**
- **B. altitudinis 41KF2bT**
- **Bi_n FFUP1T**

Intensity (a.u.)

**Scores on PC1 (84.50%)**, **Scores on PC2 (11.25%)**, **Scores on PC3 (2.96%)**

(b) Score plots for the bacterial strains:

- **B. pumilus ATCC 7061T**
- **B. safensis FO36-bT**
- **B. altitudinis 41KF2bT**
- **B. xiamenensis HYC-10T**
- **Bi_n FFUP1T**
In addition, whole-cell content of the bacterial isolates studied was analysed by Fourier transform IR spectroscopy with attenuated total reflectance (FTIR-ATR). Spectra were acquired using a FTIR System spectrophotometer (PerkinElmer Spectrum BX) in the ATR mode with a PIKE Technologies Gladi ATR accessory from 4000 to 600 cm\(^{-1}\) and a resolution of 4 cm\(^{-1}\) and 32 scan co-additions. Strain Bi\(_{-}\)FFUP\(_{1}\)\(^T\) was grown for 37 °C for 17 h on Mueller–Hinton agar (bioMérieux) and colonies were directly applied in the ATR crystal and dried in a thin film. For each strain, six spectra were acquired, corresponding to three biological replicates (obtained from two different agar plates) and two instrumental replicates (obtained from the same agar plates) and the mean spectra were considered in the analysis.

Chemometric analyses of MALDI-TOF/MS and FTIR-ATR spectra were performed in Matlab version 6.5 release 13 (MathWorks) and the PLS Toolbox version 3.5 for Matlab (Eigenvector Research). FTIR-ATR spectra were processed with standard normal variate (Næs et al., 2002) followed by the application of a Savitzky–Golay filter (nine smoothing points, second-order polynomial and second derivative) (Savitzky & Golay, 1964) and mean-centred. MALDI-TOF/MS spectra were mean-centred. Spectra were analysed by principal component analysis (PCA) (Jolliffe, 1986) considering the whole spectra for MALDI-TOF/MS and the carbohydrates region (1200–900 cm\(^{-1}\)) for FTIR-ATR, which was chosen due to the higher variability among isolates.

Comparative phylogenetic analyses were also performed with strain Bi\(_{-}\)FFUP\(_{1}\)\(^T\) and closely related organisms assigned

**Fig. 2.** (a) Mass spectra comparison of strain Bi\(_{-}\)FFUP\(_{1}\)\(^T\) and closely related B. safensis FO-36b\(^T\), B. pumilus ATCC 7061\(^T\), B. altitudinis 41KF2b\(^T\) and B. xiamenensis HYC-10\(^T\) generated by MALDI-TOF/MS. Species-specific peaks are indicated by an asterisk. A. u. means arbitrary units. (b) Score plot corresponding to the first three components of the PCA model.

**Fig. 3.** (a) FTIR-ATR spectra of strain Bi\(_{-}\)FFUP\(_{1}\)\(^T\) and closely related B. safensis FO-36b\(^T\), B. pumilus ATCC 7061\(^T\), B. altitudinis 41KF2b\(^T\) and B. xiamenensis HYC-10\(^T\) processed with standard normal variate and Savitzky–Golay filter (9 points filter size, 2nd degree polynomial, 2nd derivative) in the region 1200–900 cm\(^{-1}\). (b) Score plot corresponding to the first three components of the PCA model.
Fig. 4. NJ tree based on rpoB gene sequences, showing the relationship of strain Bi-FFUP1\(^T\) and species related to the *B. pumilus* group. The type strains of *B. stratosphericus* and *B. aerophilus*, isolated from the same sample as *B. altitudinis* 41KF2b\(^T\), are no longer available in the depositor's collection, and therefore were not included in this analyses. Genetic distances were constructed using the Jukes–Cantor model (Jukes & Cantor, 1969). Numbers at branch points indicate bootstrap percentages from both ML (before the slash '/') and NJ (after the slash '/') analyses. As the NJ tree was very similar to the ML tree, only the first is shown. *B. subtilis* subsp. *subtilis* 168\(^T\), *B. subtilis* subsp. *spizizenii* DSM 15029\(^T\), *B. mojavensis* DSM 9205\(^T\), *B. vallismortis* DSM 11031\(^T\), *B. amyloliquefaciens* DSM7\(^T\), *B. atrophaeus* DSM 7264\(^T\), *B. licheniformis* ATCC...
to the *B. subtilis* clade (Bhandari et al., 2013). DNA was extracted using an InstaGene Matrix (Bio-Rad) and was used directly for PCR amplification. Sequences of the 16S rRNA (Héritier et al., 2003), β-subunit of DNA gyrase (gyrB) (Yamamoto & Harayama, 1995) and β-subunit of RNA polymerase (rpoB) genes were amplified using the primers pair and PCR conditions outlined in Table S1 (available in the online Supplementary Material), performed using a GRS PCR & Gel Band Purification kit (Grisp). The entire rpoB gene sequence and its hypervariable region, located between positions 2300 and 3300 bp (Adékambi et al., 2008), were used for the phylogenetic assignments. As similar phylogenetic patterns were obtained for both rpoB gene sequences (data not shown), the tree obtained with the hypervariable region was presented. For phylogenetic analyses, the 16S rRNA, gyrB and rpoB genes sequences of strain Bi.FFUP1 T, deposited in GenBank and assigned with NCBI accession numbers (Table 1), were compared with homologous nucleotide sequence data from *B. pumilus* group members, namely *B. pumilus* ATCC 7061 T and SAFR-032, *B. safensis* FO-036bT, *B. altitudinis* 41KF2bT and *B. xiamenensis* HYC-10 T. Moreover, other *B. subtilis* clade members, i.e. *B. subtilis* subsp. subtilis 168 T, *B. subtilis* subsp. spizizenii DSM 15029 T, *Bacillus mojavensis* DSM 9205 T, *Bacillus vallismortis* DSM 11031 T, *Bacillus amyloyticus* DSM 7 T, *Bacillus atrophaeus* DSM 7264 T, *Bacillus licheniformis* ATCC 14580 T and *Bacillus cereus* ATCC 14579 T, were used as outgroups. Nucleotide sequences were aligned and similarity scores for each gene comparison were generated using MEGA version 5.2.2 (http://www.megasoftware.net/) (Tamura et al., 2011). Phylogenetic trees were then reconstructed using the neighbour-joining (NJ) and maximum-likelihood (ML) (Felsenstein, 1981; Saitou & Nei, 1987) methods. In addition, genetic distances were estimated using Kimura’s two-parameter model (Kimura, 1980), where all substitutions were included in pairwise distance calculations. The reliability of internal branches was assessed from a bootstrap analysis based on 1000 resamplings (Felsenstein, 1985).

According to Adékambi et al. (2009), the total genomic G + C content can be inferred from the G + C content of universally conserved genes, with a strong correlation (coefficient of determination $r^2=0.97$) between the G + C content of the *rpoB* gene and that of the correspondent genome. Thus, the genomic G + C content (GCg) of strain Bi.FFUP1 T was estimated based on the *rpoB* gene G + C content (GCr) using the following formula: GCg=1.2065 × GCr−11.495 (Adékambi et al., 2009).

For DDH studies, cells were disrupted using a Constant Systems TS 0.75 kW machine (IUL Instruments) and the DNA in the crude lysate was purified by chromatography on hydroxypatite, as described by Cashion et al. (1977). DNA—DNA hybridizations between strain Bi.FFUP1 T and the type strains of *B. pumilus*, *B. safensis*, *B. altitudinis* and *B. xiamenensis* were carried out according to the hydroxyapatite/microtitre plate method described by Ziemke et al. (1998).

The novel isolate stained Gram-positive. The rod-shaped cells (0.5–0.7 μm in diameter and 1.0–1.5 μm in length) were motile and contained terminally/subterminally located oval spores, similar to cells of *B. safensis*, *B. pumilus*, *B. altitudinis* and *B. xiamenensis*. Growth occurred at 20, 25, 30, 37 and 50 °C but not at 5, 10, 15, 60, 65 or 70 °C, and in the range of 0–14% (w/v) NaCl (when grown in TSA) and at pH 5.0–10.0. Phenotypic details derived from metabolic tests are detailed in the species description and in Table S2. Acid production from cellobiose, D-glucose and D-mannose and absence of acid production from D-arabinose, erythritol, inositol, maltose, manniitol, raffinose, rhamnose, sorbitol, starch and L-tryptophan allowed the discrimination of the novel isolate from related species (Table 2). The profile of major fatty acids of the novel isolate consisted of iso-C15:0 (41.3 %), anteiso-C15:0 (35.3 %), iso-C17:0 (4.9 %) and anteiso-C17:0 (6.6 %), similar to those of closely related species (Table S3); minor fatty acids were iso-C14:0 (1.0 %), iso-C16:0 (3.0 %) and anteiso-C16:0 (2.0 %).

The quinone system consisted predominantly of menaquinone MK-7 (99.4 %), which was consistent with members of the genus *Bacillus* (Lai et al., 2014), and also traces of MK-6 (0.3 %) and MK-8 (0.2 %). In the polar lipid profile (Fig. 1), diphosphatidylglycerol (DGP), an unidentified phospholipid (PL1) and an unidentified glycolipid (GL2) were detected. Furthermore, moderate amounts of phosphatidylglycerol (PG), another unidentified glycolipid (GL1) and three lipids (L1–3) not containing a sugar, phosphate or amino residues were detected. In addition, some other minor lipids were observed, including a third unidentified glycolipid (GL3), an aminolipid (AL1) and two lipids (L4, L5) not containing a sugar, phosphate or amino residues. However, GL2 showed a chromatographic motility similar to a glycolipid detected in *B. subtilis* and considered to be β-gentibiosydilaidyglycerol (Kämpfer et al., 2006).

Spectral profiles comparison of *B. pumilus* ATCC 7061 T, *B. safensis* FO-36b T, *B. altitudinis* 41KF2b T, *B. xiamenensis* HYC-10 T and strain Bi.FFUP1 T showed the presence of several common mass-to-charge ratio ion peaks (m/z) in all *B. pumilus* group members tested (Fig. 2a, marked with an asterisk). Species-specific m/z values (Table S4) were observed for strain Bi.FFUP1 T, namely the presence of m/z signal of 6820 and the absence of m/z 3400 which were recognized as characteristic. A PCA of the mass spectra of all isolates (*B. pumilus* ATCC 7061 T, *B. safensis* FO-036b T, *B. altitudinis* 41KF2b T, *B. xiamenensis* HYC-10 T and Bi.FFUP1 T) showed that they were clearly positioned in distinct quadrant of the score map, highlighting their dissimilarity (Fig. 2b). Moreover *B.
safensis FO-036b<sup>T</sup> was discriminated from the remaining species in the first principal component (PC1), which accounts for 84.5% of the spectral variability, whereas PC2 and PC3 contributed to the discrimination between strain Bi<sub>FFUP1</sub><sup>T</sup>, B. altitudinis 41KF2b<sup>T</sup>, B. pumilus ATCC 7061<sup>T</sup> and B. xiamenensis HYC-10<sup>T</sup>.

![NJ tree based on gyrB gene sequences, showing the relationship of strain Bi<sub>FFUP1</sub><sup>T</sup> and species related to the B. pumilus group. The type strains of B. stratosphericus and B. aerophilus, isolated from the same sample as B. altitudinis 41KF2b<sup>T</sup>, are no longer available in the depositor's collection, and therefore are not included in this analysis. Genetic distances were constructed using the Jukes–Cantor model (Jukes & Cantor, 1969). Numbers at branch points indicate bootstrap percentages from both ML (before the slash '/') and NJ (after the slash '/') analyses. As the NJ tree was very similar to the ML tree, only the first is shown. B. subtilis subsp. subtilis 168<sup>T</sup>, B. subtilis subsp. spizizenii DSM 15029<sup>T</sup>, B. mojavensis DSM 9205<sup>T</sup>, B. vallismortis DSM 11031<sup>T</sup>, B. amyloliquefaciens DSM 7<sup>T</sup>, B. atrophaeus DSM 7264<sup>T</sup>, B. licheniformis ATCC 14580<sup>T</sup> and B. cereus ATCC 14579<sup>T</sup> were used as outgroups. Accession numbers are given in parentheses. Bar, genetic distance of 0.05.

**Fig. 5.** NJ tree based on gyrB gene sequences, showing the relationship of strain Bi<sub>FFUP1</sub><sup>T</sup> and species related to the B. pumilus group. The type strains of B. stratosphericus and B. aerophilus, isolated from the same sample as B. altitudinis 41KF2b<sup>T</sup>, are no longer available in the depositor's collection, and therefore are not included in this analysis. Genetic distances were constructed using the Jukes–Cantor model (Jukes & Cantor, 1969). Numbers at branch points indicate bootstrap percentages from both ML (before the slash '/') and NJ (after the slash '/') analyses. As the NJ tree was very similar to the ML tree, only the first is shown. B. subtilis subsp. subtilis 168<sup>T</sup>, B. subtilis subsp. spizizenii DSM 15029<sup>T</sup>, B. mojavensis DSM 9205<sup>T</sup>, B. vallismortis DSM 11031<sup>T</sup>, B. amyloliquefaciens DSM 7<sup>T</sup>, B. atrophaeus DSM 7264<sup>T</sup>, B. licheniformis ATCC 14580<sup>T</sup> and B. cereus ATCC 14579<sup>T</sup> were used as outgroups. Accession numbers are given in parentheses. Bar, genetic distance of 0.05.
IR spectra of the whole bacterial cell content have demonstrated great potential for bacterial identification and/or discrimination at different taxonomic levels (Kuhm et al., 2009; Sousa et al., 2014a, b). Whereas other methods require the identification of specific markers to allow bacterial differentiation, IR spectroscopy provides global information about bacterial composition concerning lipids, phospholipids, carbohydrates, proteins and DNA/RNA (Naumann et al., 1991). IR spectra of B. pumilus ATCC 7061T, B. safensis FO-036bT, B. altitudinis 41KF2bT, B. xiamenensis HYC-10T and Bi_FFUP1T presented a high degree of spectral similarity, corroborating the relatedness of the species. The main differences were found in the carbohydrate region (1200–900 cm–1) (Fig. 3a), which revealed a different sugar composition among the five species. Furthermore and similarly to the MALDI-TOF/MS analysis, a PCA (Fig. 3b) of FTIR-ATR spectra located the isolates in distinct quadrants, confirming their dissimilarity. PC1 allowed the discrimination of B. pumilus ATCC 7061T, B. altitudinis 41KF2bT and B. xiamenensis HYC-10T from Bi_FFUP1T and B. safensis FO-036bT and accounted for 58.11% of the spectral variability. In addition, PC2 and PC3, encompassing 40.78% of the spectral variability, accounted for the discrimination between B. pumilus ATCC 7061T and B. altitudinis 41KF2bT and B. xiamenensis HYC-10T.

Strain Bi_FFUP1T shared 97.1, 96.1, 98.4 and 97.9% rpob gene sequence similarity (bootstrap values >72%) with B. pumilus ATCC 7061T and SAFR-032, B. safensis FO-036bT, B. altitudinis 41KF2bT and B. xiamenensis HYC-10T, respectively, and 93.0, 90.8, 98.5 and 92.2% gyrB gene sequence similarity (bootstrap values >94%), respectively (Figs 4 and 5). Thus, rpob and gyrB gene sequence-based phylogenetic analysis proved to be more discriminative than 16S rRNA gene sequences (Fig. S1), which clearly delineated strain Bi_FFUP1T in a separate branch from the type strains of related species enclosed in the B. pumilus group.

DDH analyses were carried out to evaluate the genomic DNA–DNA relatedness between strain Bi_FFUP1T and the most closely related reference strains, B. pumilus ATCC 7061T, B. safensis FO-036bT, B. altitudinis 41KF2bT and B. xiamenensis HYC-10T. Values of 39.6% (reciprocal 38.0%), 49.9% (reciprocal 42.9%), 61.9% (reciprocal 52.2%) and 61.7% (reciprocal 49.2%) were obtained, respectively, supporting the species status of the novel isolate.

Due to the unavailability of B. stratosphericus and B. aerophilus type strains it was not possible, by other methodologies, to clarify their apparent similarity depicted by 16S rRNA gene analysis. Nevertheless, data from the polyphasic studies described above clearly support that strain Bi_FFUP1T from a health product represents a novel species of the genus Bacillus, for which the name Bacillus invictae sp. nov. is proposed.

Description of Bacillus invictae sp. nov.

Bacillus invictae (in.vic`tae. L. masc. adj. invictae unconquered, invincible, referring to the city of Oporto, ‘a cidade invicta’).

Vegetative cells are rod-shaped and 0.5–0.7 μm in diameter and 1.0–1.5 μm in length (Fig. S2). Gram-stain-positive, motile and catalase- and oxidase-positive. Does not grow anaerobically. Growth occurs at 20–50°C (optimum, 30–37°C) but not at 5, 10, 15, 20, 60, 65 or 70°C. Growth occurs in the range 0–14% (w/v) NaCl and at pH 5.0–10.0 (optimum, 6.0–8.0) in TSB. Colonies are round, smooth, yellowish, have irregular margins and are approximately 2.8±0.2 mm on TSA plates incubated at 37°C for 24 h. Nitrate is not reduced to nitrite and N₂ production is negative. Gelatin, aesculin and citrate are utilized and ONPG is hydrolysed. Utilizes L-proline, L-leucine and L-phenylalanine, but not L-valine, L-tryptophan, L-arginine, L-ornithine or L-lysine. Urea hydrolysis is negative. β-Haemolytic on 5% sheep blood agar. Acid is produced from glycerol, D-ribose, D-glucose, D-fructose, mannose, arbutin, aesculin, salicin, cellobiose, sucrose, trehalose, gentiobiose, D-tagatose and L-arabinose, but not from L-xyllose, adonitol, L-sorbosone, rhamnose, dulcitol, inositol, mannotol, D-sorbitol, methyl α-D-glucoside, maltose, melibiose, inulin, raffinose, starch, glycerogen, xylitol, turanose, D-lyxose, D-fucose, D-arabitol, D-arabitol, erythritol, methyl β-D-mannoside, melezitose, glucosone, 2-ketogluconate, 5-ketogluconate, D-arabinose or lactose. Major fatty acids are iso-C₁₅:₀ and anteiso-C₁₅:₀ iso-C₁₇:₀ anteiso-C₁₇:₀ iso-C₁₄:₀ iso-C₁₆:₀ and anteiso-C₁₆:₀ are detected in minor amounts. The predominant quinone is menaquinone MK-7. The polar lipid profile consists of the major lipids diphosphatidylglycerol, an unidentified phospholipid, phosphatidylglycerol, two unidentified glycolipids and three lipids not containing a sugar, phosphate or amino residue. The DNA G+C content is 41 mol%. MALDI-TOF/MS reveals m/z of 2620, 2760, 3690, 3820, 4300, 4340, 4420, 5230, 5270, 5945, 6090, 6410, 6700, 6790, 6820 and 7410, and characteristically the presence of m/z 6820 and absence of m/z 3400.

The type strain, Bi_FFUP1T (= DSM 26896T=CCUG 64113T), was isolated from a contaminated health product in Portugal.

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