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Certification of a reference material consisting of genomic DNA inserts of *Bacillus subtilis* DSM 5750 for PFGE IRMM-312

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IRMM-312

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Abstract

This report describes the production and certification of IRMM-312, a reference material of genomic DNA (gDNA) of *Bacillus subtilis* DSM 5750 in agarose inserts. This CRM (IRMM-312) is intended to be used for the taxonomic identification of authorised probiotic feed additives by Pulsed Field Gel Electrophoresis (PFGE). The homogeneity and stability of the batch was assessed by monitoring the PFGE pattern of a *Sfi*I restriction enzyme digest of gDNA of *Bacillus subtilis* DSM 5750 in agarose inserts. The batch was found to be homogeneous and the material is stable at 4 °C. The batch was characterised by five laboratories determining the PFGE pattern of a *Sfi*I restriction enzyme digest of IRMM-312. The pattern should be considered as a whole and restriction fragments in the size range from 15 kb to 97 kb are certified for their fragment length. DNA sequence analysis of the 3' end of the 16S rRNA gene and the entire 16S-23S internal transcribed spacer (ITS) coding region confirmed the identity of the strain as *Bacillus subtilis.* Each vial contains one agarose insert containing gDNA of *Bacillus subtilis* DSM 5750.

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Glossary

BLAST	Basic Local Alignment Search Tool
bp	base pair
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTCS	dye terminator cycle sequencing
EDTA	ethylenediaminetetraacetic acid
ES	0.5 mol/L EDTA pH 8.0, 1 % (m/v) sodium lauryl sarcosine
ESP	ES containing proteinase K at 1 mg/mL
gDNA	genomic DNA
ITS	internal transcribed spacer
kb	kilo base pairs
LMP	low melting point
LTS	long-term stability
N	number of vials
n	number of replicates
n.a.	not applicable
n.d.	not detected
no.	number
NB	nutrient broth
OD_{600}	optical density at a wavelength of 600 nm
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PMSF	phenylmethanesulfonyl fluoride
rDNA	ribosomal DNA, DNA sequences encoding ribosomal RNA
RE	restriction enzyme
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RSD	relative standard deviation
s	standard deviation
SDS	sodium dodecyl sulphate
SE	standard error
STS	short-term stability
TBE	89 mmol/L tris, 89 mmol/L boric acid, 2 mmol/L EDTA, pH 8.3
TE	10 mmol/L tris, 1 mmol/L EDTA, at specified pH
Tris	Tris(hydroxymethyl)aminomethane

1 Introduction

Probiotic micro-organisms authorised for use as feed additives are described in Commission Regulation (EC) No 2148/2004 [1]. Pulsed Field Gel Electrophoresis (PFGE) is a powerful molecular typing method which can be used to discrimate different strains of bacterial species [2]. Bacterial strains yielding the same PFGE patterns are considered to be the same strain [3]. This report describes the production and certification of a batch of 650 vials of a reference material composed of genomic DNA (gDNA) of *Bacillus subtilis* DSM 5750 in agarose (IRMM-312), in support of EU legislation. Council Directive 70/524/EEC concerning additives in feed regulates the use of probiotic bacilli and Directive 93/113/EC regulates the marketing of micro-organisms and their preparations in animal nutrition. According to both Directives, the use of specific genera of bacteria as feed additives are approved. One of the officially approved probiotics is *Bacillus subtilis* DSM 5750. The certification of IRMM-312 was performed according to IRMM Reference Materials Unit procedures applying a quality management system according to ISO Guide 34 [4 - 7]. Homogeneity and stability of the produced batch was analysed at IRMM. Short-term stability (STS) measurements were performed following incubation at 4 °C and 18 °C in the frame of the certification project. Long-term stability (LTS) measurements were performed following incubation at 4 °C. The batch was characterised by monitoring the PFGE pattern of a restriction enzyme (RE) digest of selected samples by IRMM and 4 different external laboratories. Identification of *Bacillus subtilis* DSM 5750 was confirmed by DNA sequencing of the nucleotide sequences of the 3' end of the 16S rDNA and the 16S–23S internal transcribed spacer (ITS) [9].

2 Participants

Bacillus subtilis strain DSM 5750 originates from the Chr. Hansen A/S culture collection (Hørsholm, DK).

The processing and certification of IRMM-312 was performed at IRMM.

Participants for the batch characterisation were:

- Chr. Hansen A/S, Hørsholm, DK.
- Animal Sciences Group, Lelystad, NL.
- Food and Consumer Product Safety Authority, Zutphen, NL.
- University of Helsinki, Helsinki, FI.
- European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE.

A summary of the different steps in the processing and certification of IRMM-312 is given in Table 1.

Table 1. Summary of different steps in the production of IRMM-312.

Project phases	**Time period**
Processing	Jul. - Aug. 2006
Certification	Sep. - Dec. 2006
Certification report and certificate	Mar. - Aug. 2007

3 Processing

IRMM-312 consists of single agarose inserts, prepared from cultured bacterial cells of *Bacillus subtilis* DSM 5750, mixed with melted agarose, allowed to solidify using 100 µL insert molds. Molecular biology grade (sterile, DNase and RNase free) consumables and biochemicals were used at all time.

The processing steps for the production of IRMM-312 were:

1. Start 10 mL overnight culture of *Bacillus subtilis* DSM 5750 in Nutrient Broth (NB) at 30 °C.
2. Inoculate the overnight culture into 100 mL NB medium, preheated to 30 °C in a 1 L Erlenmeyer flask. Grow culture to OD_{600} of 0.6 (optical path length 10 mm) at 30 °C. Add chloramphenicol to a final concentration of 180 µg/mL and incubate further for 1 h at 30°C with shaking at 200 rpm.
3. Chill the culture on ice for 10 min. Spin down the cells by centrifugation (5000 × *g*) at 4 °C for 30 min. Resuspend the pellet in 10 mL solution A (1 mol/L NaCl , 50 mmol/L Tris-HCl pH 7.6). Centrifuge the cells at 5000 × *g* at 4 °C for 30 min. Resuspend cells in 5 mL cold (4 °C) solution A.
4. Prepare a 1 % (m/v) solution of low melting point (LMP) agarose in 50 mmol/L Tris- HCl buffer (pH 7.6) and cool it to 42 °C.
5. Mix 500 µL 1 % (m/v) LMP agarose solution with 500 µL cell suspension by inverting the tube several times. Use 10 sets of 10 reusable plug molds (BioRad) and with a pipet fill each insert mold with 100 µL of the suspension. Put the insert molds on ice or at 4 °C for 20 min.
6. Fill 50 mL Falcon tubes with 25 mL of solution B (50 mmol/L Tris-HCl pH 7.6, 100 mmol/L EDTA, 100 mmol/L NaCl, 2 % (v/v) lysozyme) per tube. Push 50 agarose inserts into each tube and incubate in a shaking water bath overnight at 50 °C with shaking at 50 rpm.
7. Add Proteinase K (Qiagen) to a final concentration of 0.5 mg/mL.
8. Incubate 24 h in a shaking water bath (50 rpm) at 50 °C.
9. Remove solution carefully, and add 25 mL solution C to each tube (50 mmol/L Tris-HCl pH 7.6, 100 mmol/L EDTA, 100 mmol/L NaCl, 1 % SDS).
10. Incubate 24 h in a shaking water bath (50 rpm) at 50 °C.
11. Add RNase A (Qiagen) to a final concentration of 0,075 mg/mL, and incubate for another 24 h at 50 °C with shaking at 50 rpm.
12. Remove the solution, add 25 mL TE solution (10 mmol/L Tris-HCl pH 7.6, 1 mmol/L EDTA) containing 1 mmol/L phenylmethanesulfonyl fluoride (PMSF), and incubate 1 h at room temperature. Repeat once.
13. Incubate insert three times 30 min. in TE solution at room temperature.
14. Label 100 plastic 3 mL vials (Nunc).

15. Transfer one agarose insert to each labeled plastic vial containing 1 mL TE.
16. Store the vials at 4 °C until digestion with REs.
17. Perform homogeneity and stability study and batch characterisation.

Steps 1 to 16 were repeated in order to produce 650 samples. As a control, PFGE analysis was performed on 2 random samples as described in section 4 and revealed the presence of intact high molecular mass DNA in the plugs.

4 Procedures

4.1 Minimum sample volume.

One agarose insert is used per analysis.

4.2 Digestion of agarose inserts with a RE

Digestion of agarose inserts with a RE was performed as described in section 11.2. More information on the procedure can be found in reference [8]. Four different REs (*Not*I, *Spe*I, *Asc*I and *Sfi*I) were tested of which *Sfi*I appeared to be the most suitable.

4.3 Pulsed Field Gel Electrophoresis.

The measurements in this report were performed using the Bio-Rad CHEF-DR® III System (Bio-Rad Laboratories, BE) using the method described in section 11.2. Other PFGE systems can be used as well. More information on the procedure can be found in reference [8].

4.4 16S-RNA strain identification and DNA sequencing.

16S-RNA strain identification was performed as described in [9]. Briefly, gDNA was isolated using the Qiagen genomic tip 20/G kit. 16S rRNA and 16S-23S ITS were amplified by PCR using primers L516SF and L523SR. Amplified fragments were visualised on agarose gels, extracted from gel using the Qiagen gel extraction kit and cloned into pCR2.1 (Invitrogen). Plasmids were purified using the QIAGEN Plasmid Mini Kit and sequenced using the dye terminator cycle sequencing (DTCS) reaction, according to the DTCS chemistry protocol [10]. Primers for sequencing were the universal M13 primers annealing to the 5' terminus of *lac*Z. Samples were analysed on a CEQ™ 8000 genetic analysis system (Beckman Coulter, Inc., Fullerton, CA, US), using the following method: denaturation 120 s at 90 °C, injection 15 s at 2.0 kV, separation 85.0 min at 4.2 kV. As a control for the sequencing reaction the CEQ™ 8000 pUC18 plasmid was included on each sequencing plate. Validation of DNA sequencing using the CEQ™ 8000 system is described in detail in reference [11].

4.5 Sequencing of Pulse marker

Pulse marker is a mixture of λ DNA *Hin*d III fragments, λ DNA, and λ concatemers embedded in 1% LMP agarose. The concatamers are two or more identical linear molecular units, covalently linked in tandem by means of single-stranded extensions or cos sites. In order to check the completeness of the concatemers of λ, i.e. no parts are missing at the covalent bond, the nucleotide sequence at the linking region of two DNA molecules in the marker was determined. A 1 mm slice of Pulse marker D 2291 (Sigma) was used as template in a PCR reaction amplifying a 216 base pair (bp) region including the end of 1 λ molecule and the start of a second. Based on the sequence in GenBank (J02459) primers were chosen 76 bp upstream and 87 bp downstream from the cos sites respectively (Figure 1). PCR was performed under the following conditions: initial denaturation at 94 °C for 5 min was followed by 30

cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 20 s and 1 cycle of final elongation at 72 °C for 10 min. Amplified fragments were visualised on agarose gels, extracted from gel using the Qiagen gel extraction kit and cloned into pCR2.1 (Invitrogen). Plasmids were purified using the QIAprep spin Miniprep Kit and sequenced with M13 primers on an ABI Prism® 3130xl Genetic Analyser (Applied Biosystems, Lennik, BE) using the BigDye® Terminator v1.1 Cycle Sequencing kit.

```
48339 acgatacctg cgtcataatt gatttatttga cgtggtttga tggcctccac gcacgttgtg
      tgctatggac gcagtattaa ctataaact gcaccaaact accggaggtg cgtgcaacac

48399 atatgtagat gataatcatt atcactttac gggtcctttc cggtgatccg acaggttacg
      tatacatcta ctattagtaa tagtgaaatg cccaggaaag gccactaggc tgtccaatgc

1     gggcggcgac ctcgcgggtt ttcgctattt atgaaaattt tccggtttaa ggcgtttccg
      cccgccgctg gagcgcccaa aagcgataaa tactttaaa aggccaaatt ccgcaaaggc

61    ttcttcttcg tcataactta atgtttttat ttaaaatacc ctctgaaaag aaaggaaacg
      aagaagaagc agtattgaat tacaaaaata aattttatgg gagacttttc tttcctttgc

121   acaggtgctg aaagcgaggc tttttggcct ctgtcgtttc ctttctctgt ttttgtccgt
      tgtccacgac tttcgctccg aaaaaccgga gacagcaaag gaaagagaca aaaacaggca
```

Figure. 1: Nucleotide sequence of λ DNA (J02459) starting from base pair 48339 (end of first molecule) to base pair 171 (beginning of second molecule). Primers are indicated in bold upstream and downstream from the cos sites (highlight).

5 Homogeneity

The total number of samples produced was 650. The total number of samples to be used in the homogeneity study (*N*) was calculated as $\sqrt[3]{650} = 8.7$ [5]. In this study *N* is set to 8 and the number of replicates (*n*) is set to 1.

The homogeneity study was performed with by monitoring the PFGE pattern of a *Sfi*I digest of the samples in comparison with commercial size standards for PFGE (D 2291 and D 2416, Sigma). Identical banding patterns were found for all 8 samples. Imaging was done using GeneSnap software and fragment length for restriction bands in the size range 15 kb - 97 kb were assigned using GeneTools software, copyright© of Synoptics Ltd., Cambridge, UK. The results of the homogeneity measurements are summarised in Figure 2 and Table 2.

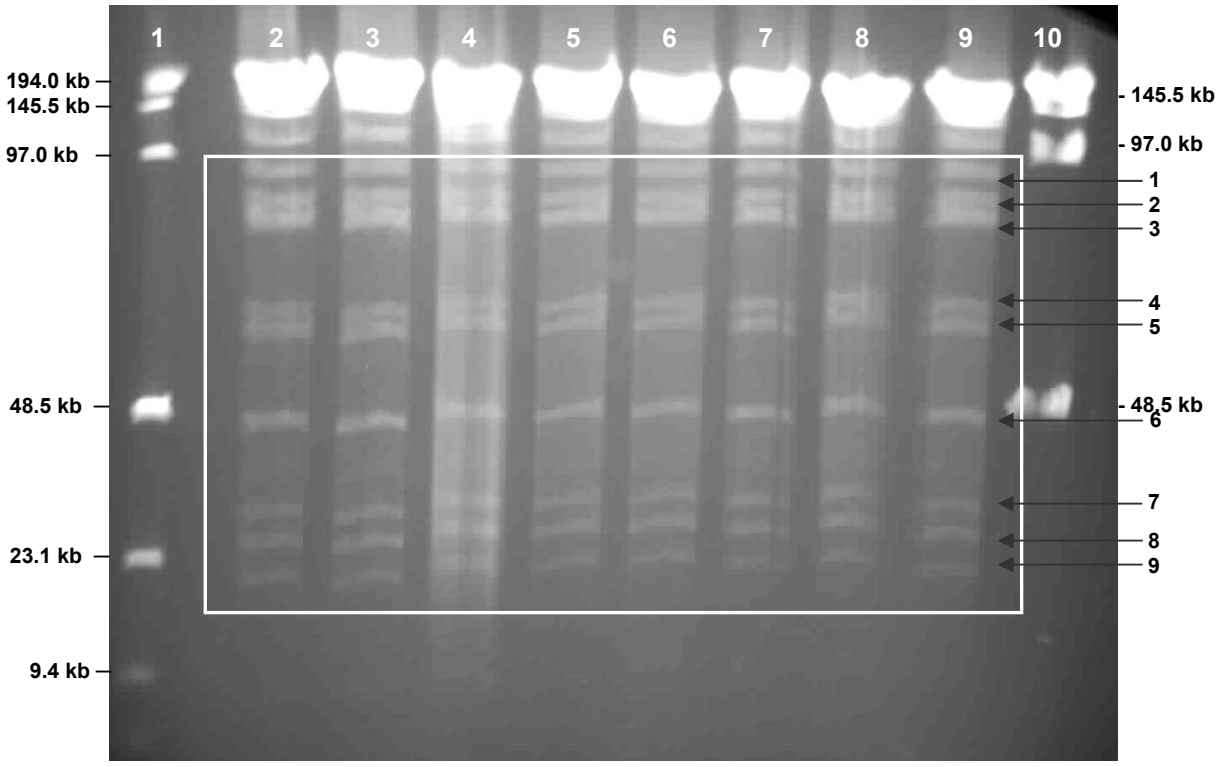

Figure 2. PFGE of *Sfi*I digest of gDNA of the samples used in the homogeneity measurements of IRMM-312. Run conditions: initial switch time (= final switch time) = 3 s, run time = 20 h, 6 V per cm, angle = 120°, buffer temperature 14 °C. Lane 1: PFGE standard 0.1 - 200 kb (D 2291, Sigma); Lane 2-9: corresponds to the series of sample numbers in Table 2; Lane 10: PFGE standard 50 - 1000 kb (D 2416, Sigma). Restriction bands between 15 kb and 90 kb are marked from 1 to 9.

Table 2. Homogeneity of the PFGE pattern of IRMM-312. Assignment of fragment length [kb] to restriction bands between 15 kb and 97 kb of a *Sfi*I digest of each sample.

Sample / Band no.	0033	0112	0175	0288	0354	0421	0524	0626	
1	92.5	94.7	94.7	93.5	93.7	95.0	95.5	94.7	Fragment sizes [kb]
2	85.4	86.6	86.6	86.9	86.3	87.7	86.8	88.2	
3	81.2	82.3	82.8	82.5	83.4	83.6	84.5	84.3	
4	64.2	63.0	64.5	64.5	65.6	64.9	66.3	65.7	
5	60.6	60.1	61.5	61.2	62.5	61.7	63.5	62.4	
6	46.0	45.2	48.0	48.3	48.8	48.3	49.7	48.5	
7	28.9	28.7	30.9	31.3	32.1	31.2	33.0	31.5	
8	25.0	25.0	26.9	27.3	27.9	27.0	28.7	27.2	
9	19.5	19.9	22.4	22.9	23.9	23.2	24.1	22.1	

No suspicious data were detected based on the number and the positioning of the bands. No bands were missing and no new bands appeared. Therefore the complete set of data was used. No major differences in the intensity of the restriction patterns of the different samples was visually observed. Therefore the batch was found to be homogenous.

6 Stability

6.1 Short-term stability

The proper conditions for transport of the material to the customer were determined in a STS study consisting of isochronous incubation of samples at 4 °C and 18 °C for 0, 2 and 4 weeks. For each time/temperature combination two samples were tested. The PFGE pattern of a *Sfi*I digest of selected samples was analysed using commercial size standards.

The data from the STS study are summarised in Figure 3 and Table 3. No suspicious data were detected. Therefore, the whole data set was used. At 4 °C and 18 °C the material is stable, with all restriction bands between 15 kb and 97 kb detected by PFGE analysis. The material must be shipped at 4 °C (use of cooling elements). At this temperature, degradation of the material is negligible.

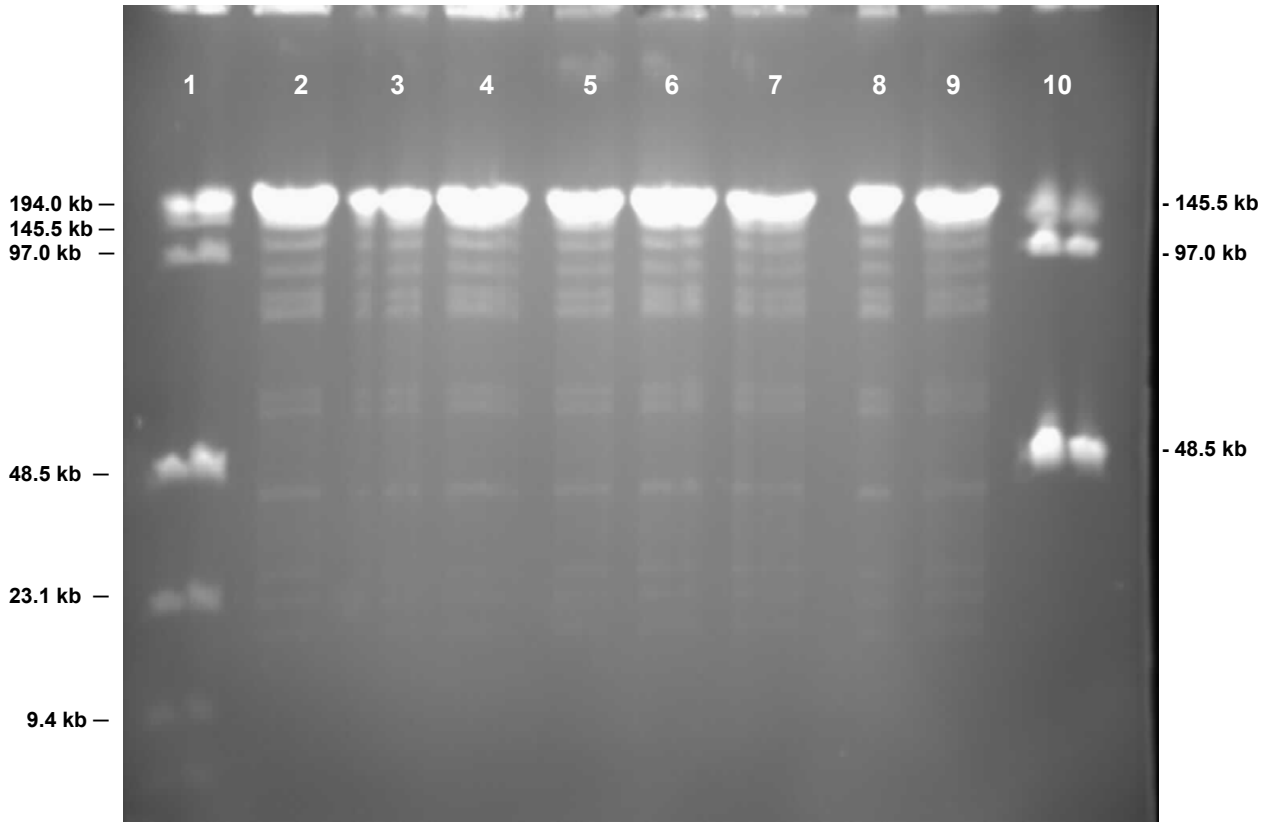

Figure 3. PFGE of *Sfi*I digests of the samples used for STS measurements of IRMM-312. Run conditions: initial switch time (= final switch time) = 3 s, run time = 20 h, 6 V per cm, angle = 120°, buffer temperature 14 °C. Lane 1: D 2291 (Sigma); Lane 2-9: corresponds to the series of sample numbers in Table 3; Lane 10: D 2416 (Sigma).

Table 3. STS of IRMM-312 stored at two different temperatures. At each time/temperature combination two different vials were tested. Assignment of fragment length (kb) of restriction bands between 15 kb and 97 kb of a *Sfi*I digest of each sample.

Temperature [°C]	n.a.		4				18				
Time [weeks]	0		2		4		2		4		
Sample / Band no.	0033	0112	0049	0364	0080	0398	0108	0440	0153	0453	
1	92.5	94.7	91.1	89.8	88.4	88.2	91.2	91.1	89.8	88.4	Fragment sizes [kb]
2	85.4	86.6	84.2	82.7	81.1	80.9	83.6	84.0	82.5	81.6	
3	81.2	82.3	80.4	78.4	77.0	77.2	79.5	79.2	79.4	77.5	
4	64.2	63.0	60.8	60.0	57.9	58.4	60.2	60.7	59.6	58.7	
5	60.6	60.1	57.2	56.6	54.6	55.3	57.2	57.3	56.8	55.8	
6	46.0	45.2	41.8	41.8	41.1	42.1	40.6	40.9	42.0	41.9	
7	28.9	28.7	27.4	28.5	30.7	31.6	25.8	26.3	29.8	31.0	
8	25.0	25.0	23.9	25.0	28.2	28.9	21.8	22.6	26.7	28.0	
9	19.5	19.9	19.2	20.6	25.5	25.9	17.2	17.9	22.6	24.5	

6.2 Long-term stability

The stability of the material during storage was determined from LTS studies. An isochronous study was carried out at 4 °C with time points of 0 and 6 months. Two samples (n = 1) per time point were tested. The PFGE pattern of a *Sfi*I digest of selected samples was analysed using commercial size standards. No spurious results were visible. Therefore, the whole data set was used. At 4 °C the material is stable, with all restriction bands between 15 kb and 97 kb detected by PFGE analysis. The data from the LTS study are summarised in Figure 4 and Table 4.

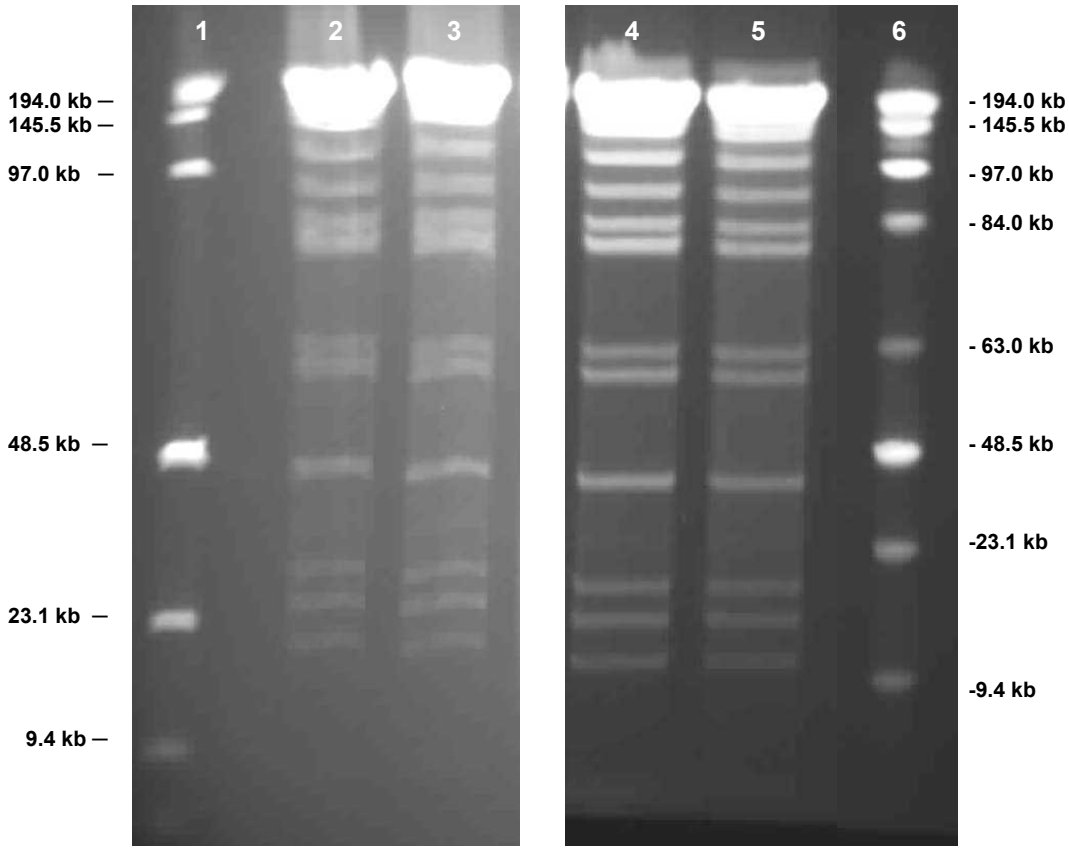

Figure 4. PFGE of *Sfi*I digest of gDNA of the samples used in the LTS measurements of IRMM-312. Run conditions: initial switch time (= final switch time) = 3 s, run time = 20 h, 6 V per cm, angle = 120°, buffer temperature 14 °C. Lane 1: D 2291 (Sigma); Lane 2 - 3: 0033 - 0112, 0 months; Lane 4 – 5: 0030 – 0359, 6 months 4 °C; Lane 6: N3551S (New England Biolabs) .

Table 4. LTS of IRMM-312 stored at 4 ºC. At each time/temperature combination two samples were tested. Assignment of fragment length (kb) to restriction bands between 15 kb and 97 kb of a *Sfi*I digest of each sample.

Temperature	n.a.		4 °C		
Time	0 weeks		6 months		
Sample / Band no.	0033	0112	0030	0359	
1	92.5	94.7	90.3	88.9	Fragment sizes [kb]
2	85.4	86.6	81.5	80.9	
3	81.2	82.3	78.3	77.5	
4	64.2	63.0	62.7	62.2	
5	60.6	60.1	59.1	58.8	
6	46.0	45.2	43.5	43.3	
7	28.9	28.7	26.5	26.5	
8	25.0	25.0	21.8	21.9	
9	19.5	19.9	16.8	17.0	

7 Batch characterisation

The batch of IRMM-312 was characterised by IRMM and four external laboratories. Each laboratory analysed two samples of IRMM-312 by PFGE following digestion with *Sfi*I according to the protocol in section 11.2 and reported the image of the PFGE gel. Fragment lengths of the characteristic restriction bands in the region between 15 kb and 97 kb were assigned using an appropriate software. The results are summarised in Table 5, Figure 5 and annex 1. Not all gels are of the same quality. However it is obvious that all labs obtained an identical banding pattern except for sample 608 that did not result in detectable restriction fragments.

Table 5. Batch characterisation of IRMM-312. Two samples were tested by each participant. Assignment of fragment length (kb) of restriction bands between 15 kb and 97 kb of a *Sfi*I digest. Mean of laboratory means, standard deviation (*s*) and relative standard deviation (RSD) are indicated. n.d.: not detectable

Laboratory code	A		B		C		
sample / Band no.	0116	0210	0550	0608	0435	0507	
1	88.8	89.0	88.4	n.d.	89.8	88.8	
2	81.0	81.6	80.8	n.d.	81.8	81.0	
3	77.7	77.8	77.2	n.d.	77.2	77.1	
4	62.0	62.7	62.9	n.d.	60.2	60.3	
5	59.6	59.7	60.1	n.d.	56.7	56.9	
6	43.5	43.8	45.6	n.d.	40.9	41.0	
7	27.4	27.8	29.3	n.d.	31.8	31.9	
8	22.0	22.3	24.1	n.d.	24.8	24.7	
9	17.6	18.0	18.3	n.d.	20.4	20.4	
Laboratory code	**D**		**E**				
sample / Band no.	**0241**	**0318**	**026**	**0326**	**mean**	***s***	**RSD [%]**
1	90.3	90.3	89.0	89.0	89.2	0.7	0.8
2	82.7	82.3	81.2	81.2	81.4	0.6	0.8
3	78.3	78.3	78.0	77.7	77.6	0.5	0.6
4	64.1	64.0	63.3	63.0	62.5	1.4	2.3
5	61.5	61.4	59.9	59.4	59.5	1.7	2.8
6	45.9	45.4	44.6	44.1	44.0	1.9	4.4
7	28.8	29.0	28.5	28.3	29.2	1.6	5.5
8	23.4	23.7	23.3	23.1	23.5	0.9	3.7
9	18.1	18.5	18.4	18.4	18.6	0.9	4.8

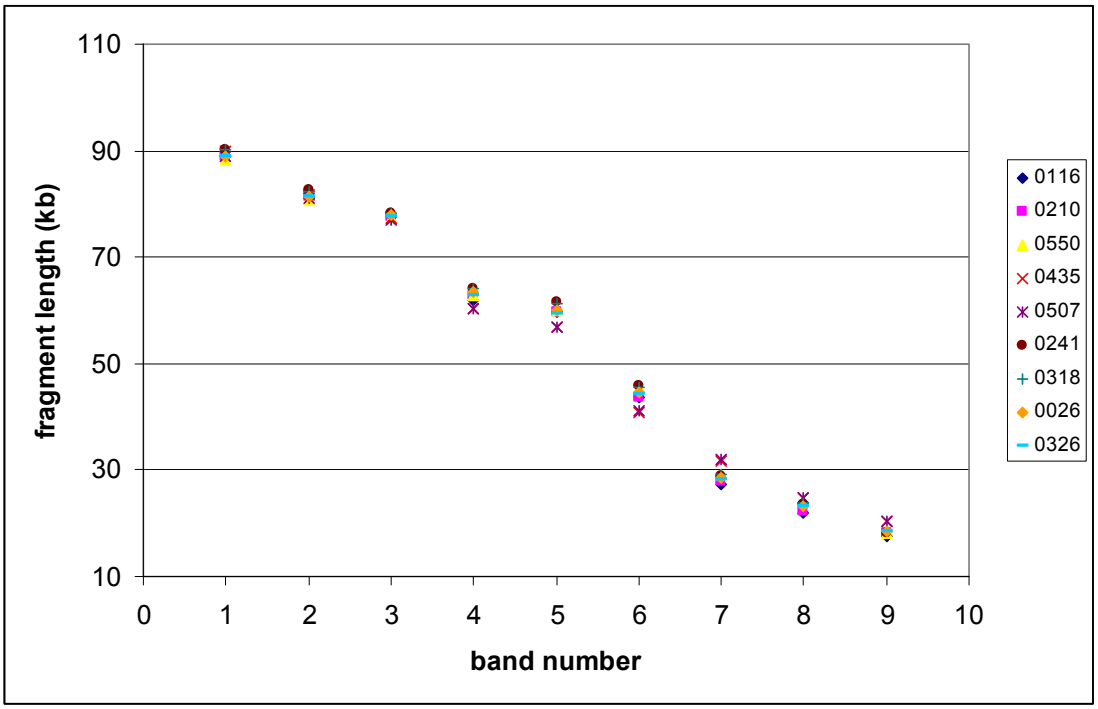

Figure 5. Results of batch characterisation. Graphical representation of fragment length of 9 restriction bands between 15 kb and 97 kb. Each symbol corresponds to a different sample analysed by one of the participating laboratories.

8 Additional characterisation

Identification of *Bacillus subtilis* was confirmed by DNA sequencing of the nucleotide sequences of the 3' end of the 16S rDNA and the 16S–23S ITS [9]. The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. Using nucleotide-nucleotide BLAST the obtained sequence was compared to other nucleotide sequences in the GenBank database and showed 99 % homology with the reported DNA sequence of the 16S rDNA and the 16S–23S ITS of different strains of *Bacillus subtilis* (Figure. 6). For DSM 5750 this sequence is not published in the GenBank. Therefore a match of 100% was not found.

```
>gi|32468687|emb|Z99104.2|BSUB0001  Bacillus subtilis complete
genome (section 1 of 21): from 1 to
213080
Length=213080

 Features in this part of subject sequence:
   rRNA-ribosomal RNA-16S
   rRNA-ribosomal RNA-23S

 Score =  860 bits (434),  Expect = 0.0
 Identities = 443/446 (99%), Gaps = 0/446 (0%)
 Strand=Plus/Minus

Query  58     GCATATCGGTGTTAGTCCCGTCCTTCATCGGCTCCTAGTGCCAAGGCATCCACCGTGCGC  117
              ||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
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Query  298    TAGAAAGGAGGTGATCCAGCCGCACCTTCCGATACGGCTACCTTGTTACGACTTCACCCC  357
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92086  TAGAAAGGAGGTGATCCAGCCGCACCTTCCGATACGGCTACCTTGTTACGACTTCACCCC  92027

Query  358    AATCATCTGTCCCACCTTCGGCGGCTGGCTCCTAAAAGGTTACCTCACCGACTTCGGGTG  417
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92026  AATCATCTGTCCCACCTTCGGCGGCTGGCTCCTAAAAGGTTACCTCACCGACTTCGGGTG  91967

Query  418    TTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCG  477
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  91966  TTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCG  91907

Query  478    GCATGCTGATCCGCGATTACTAGCGA  503
              |||||||||||||||||||||||||
Sbjct  91906  GCATGCTGATCCGCGATTACTAGCGA  91881
```

Figure 6. Comparison of the homology of the nucleotide sequences of the 3' end of the 16S rDNA and the 16S–23S ITS obtained from IRMM-312 (Query) with the reported DNA sequence of the complete genome of *Bacillus subtilis* in Genbank (accession no. Z99104), using nucleotide-nucleotide BLAST (blastn) on www.ncbi.nlm.nih.gov. Primer sites L516SF and L523SR are highlighted.

9 Metrological traceability

The obtained sequence of Pulse marker showed 100 % homology with the λ sequence (J02459). No part was missing, no part was added and no repetitions were observed (Figure 7). We conclude that the marker consists of full λ concatamers. Therefore the fragment length of the *Sfi*I digested DNA fragments is traceable to intact λ DNA (isolated from the bacteriophage λ cI857 ind1 Sam7).

A.

ATTTGACGTGGTTTGATGGCCTCCACGCACGTTGTGATATGTAGATGATAATCATTATCACTTTACGGGTCCTTTC
CGGTGATCCGACAGGTTACGGGGCGGCGACCTCGCGGGTTTTCGCTATTTATGAAAATTTTCCGGTTTAAGGCGTT
TCCGTTCTTCTTCGTCATAACTTAATGTTTTTATTTAAAATAC**CCTCTGAAAAGAAAGGAAACG**

B.

```
 Score =  231 bits (120),  Expect = 1e-57
 Identities = 120/120 (100%), Gaps = 0/120 (0%)
 Strand=Plus/Plus

Query  97   GGGCGGCGACCTCGCGGGTTTTCGCTATTTATGAAAATTTTCCGGTTTAAGGCGTTTCCG  156
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GGGCGGCGACCTCGCGGGTTTTCGCTATTTATGAAAATTTTCCGGTTTAAGGCGTTTCCG  60

Query  157  TTCTTCTTCGTCATAACTTAATGTTTTTATTTAAAATACCCTCTGAAAAGAAAGGAAACG  216
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TTCTTCTTCGTCATAACTTAATGTTTTTATTTAAAATACCCTCTGAAAAGAAAGGAAACG  120


Score =  185 bits (96),  Expect = 7e-44
 Identities = 96/96 (100%), Gaps = 0/96 (0%)
 Strand=Plus/Plus

Query  1      ATTTGACGTGGTTTGATGGCCTCCACGCACGTTGTGATATGTAGATGATAATCATTATCA  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  48407  ATTTGACGTGGTTTGATGGCCTCCACGCACGTTGTGATATGTAGATGATAATCATTATCA  48466

Query  61     CTTTACGGGTCCTTTCCGGTGATCCGACAGGTTACG  96
              ||||||||||||||||||||||||||||||||||||
Sbjct  48467  CTTTACGGGTCCTTTCCGGTGATCCGACAGGTTACG  48502
```

Figure 7 A. Nucleotide sequence of Pulse Marker D 2291 obtained with M13 primers on an ABI Prism® 3130xl Genetic Analyser. The highlighted part is the start of the second λ molecule.
B. Comparison of sequence identities between obtained sequence (A.) and λ sequence (J02459).

The identity of the IRMM-312 has been confirmed by dye terminator cycle sequencing of the nucleotide sequences of the 3' end of the 16S rDNA and the 16S–23S internal transcribed spacer (ITS) [9].

10 Certified value and uncertainty

The certified properties of IRMM-312 are the fragment lengths [kb] of 9 DNA fragments in a PFGE pattern as determined by each of five laboratories. The certified value for each band is the mean fragment length calculated from the means obtained by five different laboratories (Table 6). The PFGE pattern was obtained by *Sfi*I digestion of gDNA of *Bacillus subtilis* DSM 5750 in agarose inserts (Figure 8). The obtained band pattern of an unknown sample should always be compared to that of the reference material on the same agarose gel, considering all *Sfi*I fragments in size between 15 kb and 97 kb. Uncertainties for the certified values were taken as the half-width of the 95 % confidence interval of the mean.

Table 6. Unweighted mean of the means of five accepted sets of results, obtained by a *Sfi*I digest of genomic DNA of *Bacillus subtilis* DSM 5750.

IRMM-312: *Bacillus subtilis* DSM 5750	
Certified value (kb)	**Uncertainty (kb)**
89.2	0.9
81.4	0.8
77.7	0.6
62.5	1.8
59.5	2.1
44.0	2.4
29.2	2.0
23.6	1.3
18.6	1.3

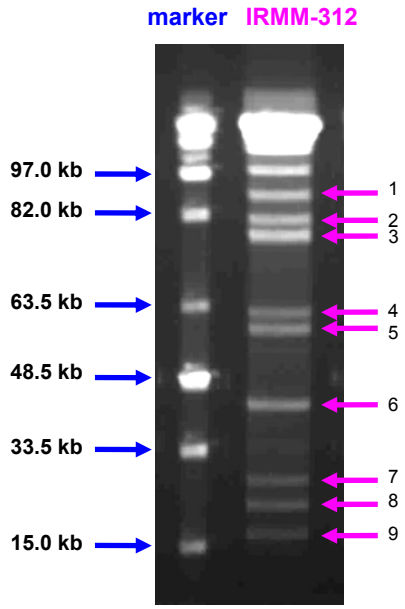

Figure 8. PFGE pattern of a *Sfi*I digest of gDNA of *Bacillus subtilis* DSM 5750 in agarose inserts when analysed according to a specified procedure (see section 11). Restriction bands between 15 kb and 97 kb are marked from 1 to 9.

11 Instructions for use

11.1 Dispatch

Dispatch to the customer shall be done at 4 °C (use of cooling elements). Upon receipt by the customer, the material should be stored at 4 °C until use.

11.2 Handling and intended use

This CRM is intended to be used for the taxonomic identification of the authorised probiotic feed additive *Bacillus subtilis* DSM 5750 by PFGE.

Molecular biology grade (sterile, Dnase-, Rnase-free) consumables and biochemicals shall be used in all procedures.

11.2.1 Digestion of agarose inserts with *Sfi*I restriction enzyme

- Transfer the agarose insert of IRMM-312 to a 1.5 mL tube containing 1 mL 10 mmol/L Tris-HCl buffer (pH 7.6) and incubate 30 min at room temperature.
- Remove the solution and add another 1 mL 10 mmol/L Tris-HCl buffer (pH 7.6), incubate again 30 min at room temperature.
- Remove solution without destroying the plug.
- Incubate insert two times 30 min at room temperature in 0.5 mL RE buffer for *Sfi*I (use RE and appropriate buffer from manufacturer of choice). Renew restriction buffer in between.
- Remove restriction buffer
- Add to each insert: 30 µL 10 × concentrated RE buffer for *Sfi*I, 30 U *Sfi*I and add H_2O to a final volume of 300 µL.
- Incubate at 50 °C for 15 -17 h.
- Remove solution, add 1 mL of ES solution (0.5 M EDTA pH 8.0, 1 % sodium lauryl sarcosine).
- Incubate overnight at 50 °C.
- Remove solution, add 250 µL ESP (ES containing proteinase K at 1 mg/mL).
- Incubate 2 h at 50 °C.
- Dilute Tris-Borate-EDTA (TBE) buffer (89 mmol/L Tris-borate, 2 mmol/L EDTA, pH 8.3), e.g. Sigma T7527, with distilled water in a volume ratio 1:3 (buffer:water).
- Remove solution, add 1 mL diluted TBE buffer and incubate 1 h at room temperature. Inserts can be kept in this solution for several days at 4 °C.
- Take a 1mm slice of agarose inserts of the PFGE standards D2291, D2416 (Sigma) and/or N3551S (New England Biolabs) and incubate in 1 mL diluted TBE for 1 h at room temperature.

11.2.2. PFGE

For other systems than Bio-Rad CHEF-DR® III System (Bio-Rad Laboratories, BE) use the instructions of the manufacturer, applying run conditions for electrophoresis as described below.

- Prepare a 1.2 % gel of PFGE certified agarose using diluted TBE.
- Melt the agarose suspension.
- Assemble the casting stand and pour in the agarose solution, remove any air bubbles and place comb near one of the ends. Allow the gel to solidify for at least 30 min.
- Fill the chamber with 2,5 L diluted TBE.
- Start buffer pump and remove air bubbles trapped in the pump. Set running temperature at 14 °C and start cooler. Circulate buffer at least 20 min.
- Melt 0.8 % LMP agarose prepared in diluted TBE, and cool it to 45-50 °C.
- Remove the comb from the gel.
- Load the samples carefully into the slots. Load the 1mm slice of size markers on both sides of the sample lanes.
- Remove excess buffer and seal the holes with 0.8 % LMP agarose in diluted TBE.
- Take gel and plate out of the casting stand. Remove any agarose which might stick underneath the plate.
- Place plate with gel in the frame in the electrophoresis chamber, and start run using the following conditions: initial switch time (= final switch time) = 3 s, run time = 20 h, 6 V per cm, angle = 120°, buffer temperature 14 °C.
- After electrophoresis stain gels 30 min in 250 mL 1 µg/mL Ethidium Bromide in diluted TBE.
- Destain in 250 mL H_2O for 2 h.
- Photograph the gel over UV-source and assign fragment lengths to size marker.

Standard Sigma D2291 contains the following size markers in the region of interest: 194, 145.5, 97, 48.5 (more concentrated band), 23.1 and 9.4 kb.

Standard Sigma D2416 contains the following size markers in the region of interest: 194, 145.5, 97, 48.5 kb.

MidRange PFG Marker I, NEB N3551S, contains the following size markers in the region of interest: 15.0, 33.5, 48.5, 63.5, 82.0 and 97.0 kb.

- Analyse data for sample tracks according to Gel Documentation System in place. Analyze pattern and fragment length of all bands between 15 kb and 97 kb.
- If necessary destain for additional 24 h in H_2O. The gel can be stored in diluted TBE at 4 °C, if necessary.

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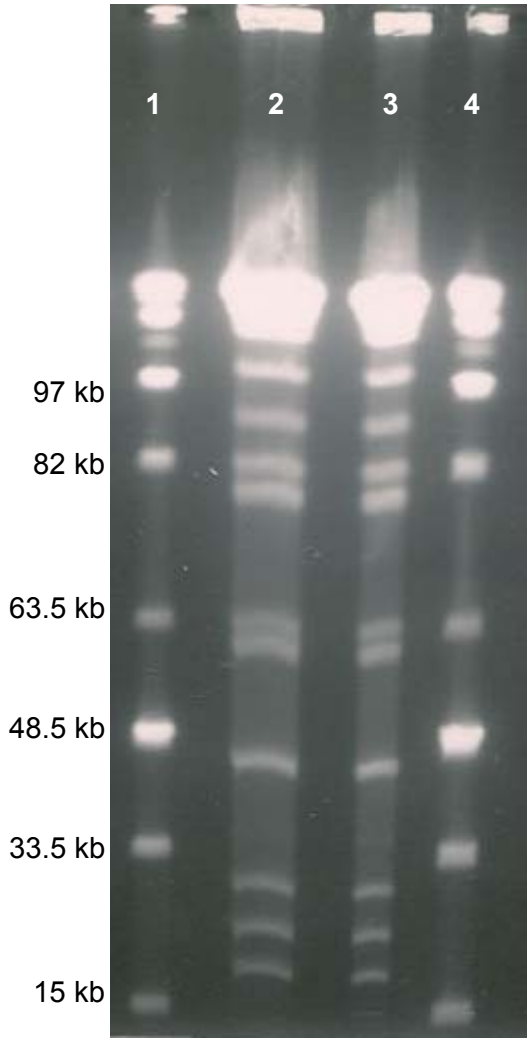
Annex 1: IRMM-312 batch characterisation – results received from five laboratories

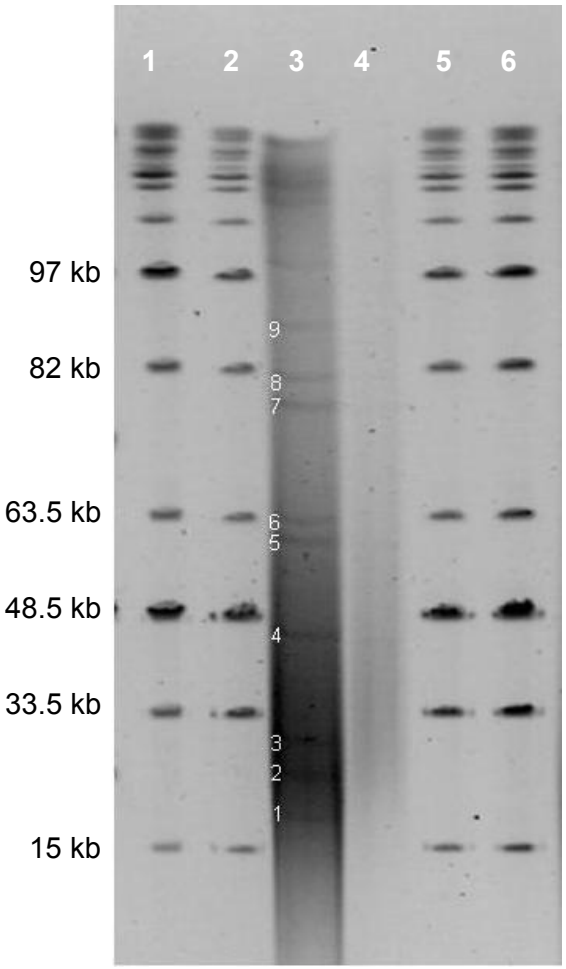
Laboratory A

1. MidRange PFG Marker I, NEB N3551S
2. IRMM – 312 # 0210
3. IRMM – 312 # 0116
4. MidRange PFG Marker I, NEB N3551S



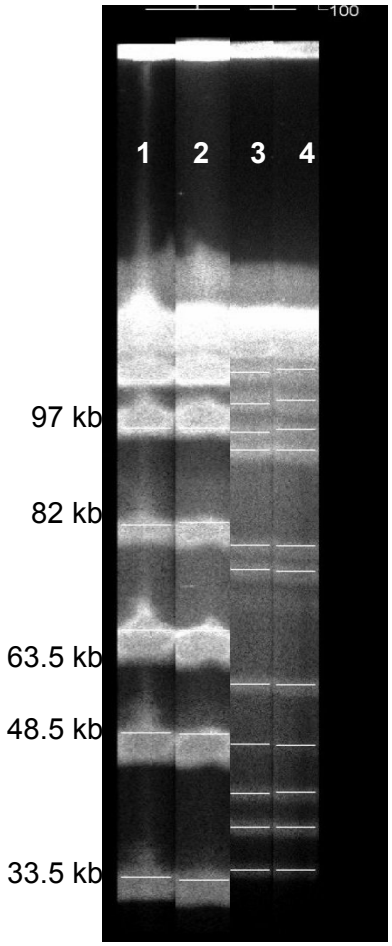
Laboratory B

1. MidRange PFG Marker I, NEB N3551S
2. MidRange PFG Marker I, NEB N3551S
3. IRMM – 312 # 0550
4. IRMM – 312 # 0608
5. MidRange PFG Marker I, NEB N3551S
6. MidRange PFG Marker I, NEB N3551S



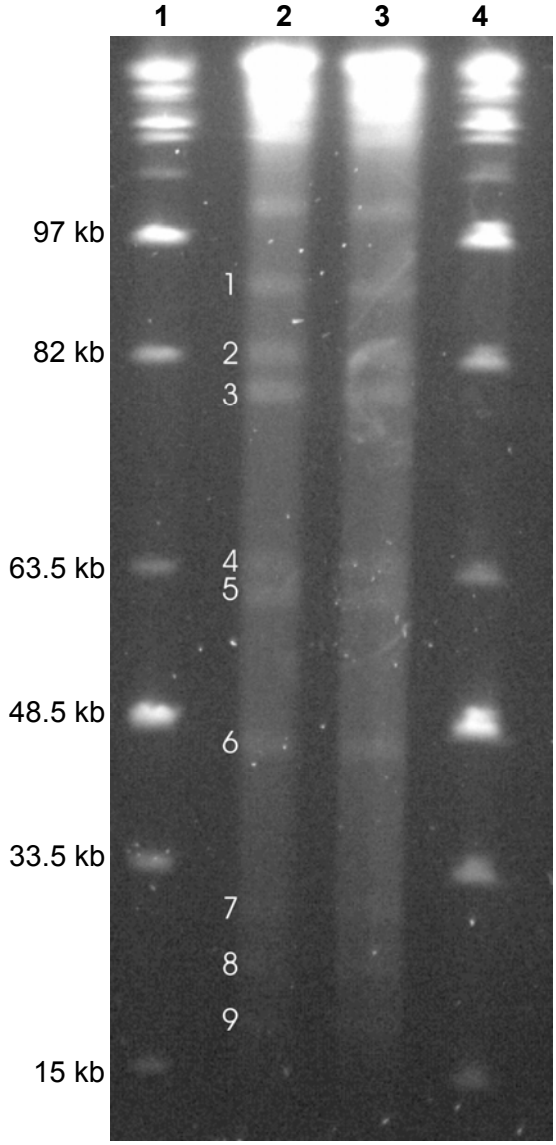
Laboratory C

1. MidRange PFG Marker I, NEB N3551S
2. MidRange PFG Marker I, NEB N3551S
3. IRMM – 312 # 0507
4. IRMM – 312 # 0435



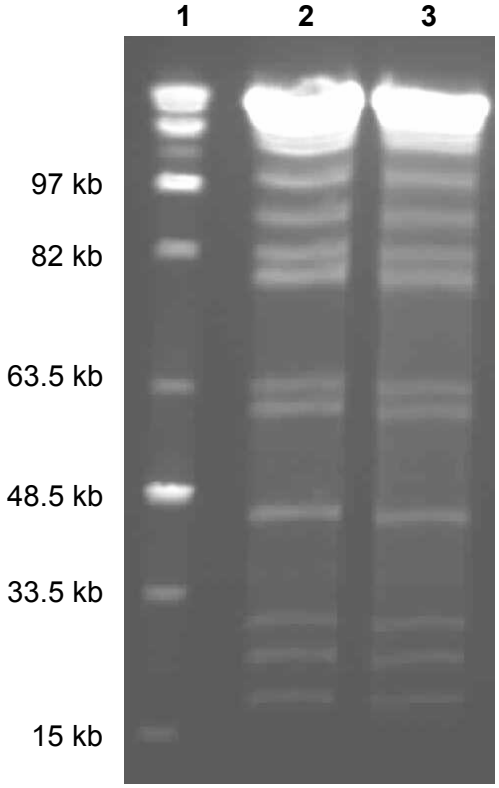
Laboratory D

1. MidRange PFG Marker I, NEB N3551S
2. IRMM - 312 # 0241
3. IRMM – 312 # 0318
4. MidRange PFG Marker I, NEB N3551S



Laboratory E

1. MidRange PFG Marker I, NEB N3551S
2. IRMM - 312 # 0
3. IRMM - 312 # 0



European Commission

EUR 23372 EN – Joint Research Centre – Institute for Reference Materials and Measurements
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Authors: L. De Baets, N. Meeus, P. van Iwaarden, W. Philipp, H. Schimmel
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Abstract

This report describes the production and certification of IRMM-312, a reference material of genomic DNA (gDNA) of *Bacillus subtilis* DSM 5750 in agarose inserts. This CRM (IRMM-312) is intended to be used for the taxonomic identification of authorised probiotic feed additives by Pulsed Field Gel Electrophoresis (PFGE). The homogeneity and stability of the batch was assessed by monitoring the PFGE pattern of a *Sfi*I restriction enzyme digest of gDNA of *Bacillus subtilis* DSM 5750 in agarose inserts. The batch was found to be homogeneous and the material is stable at 4 °C. The batch was characterised by five laboratories determining the PFGE pattern of a *Sfi*I restriction enzyme digest of IRMM-312. The pattern should be considered as a whole and restriction fragments in the size range from 15 kb to 97 kb are certified for their fragment length. DNA sequence analysis of the 3' end of the 16S rRNA gene and the entire 16S-23S internal transcribed spacer (ITS) coding region confirmed the identity of the strain as *Bacillus subtilis.* Each vial contains one agarose insert containing gDNA of *Bacillus subtilis* DSM 5750.

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