Bacillus farraginis sp. nov., *Bacillus fortis* sp. nov. and *Bacillus fordii* sp. nov., isolated at dairy farms

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Forty-eight bacterial strains were isolated at dairy farms from raw milk, the milking apparatus, green fodder or feed concentrate after a heat treatment of 30 min at 100 °C. In this way, spore-forming bacteria with a very high intrinsic heat resistance were selected for. The aerobic spore-forming isolates were subjected to a polyphasic taxonomical study, including repetitive element sequence-based PCR typing, whole-cell protein profiling, 16S rDNA sequence analysis, DNA–DNA hybridizations, DNA base composition, fatty acid analysis, and morphological and biochemical characteristics. A comparison of the REP- and (GTG)₅-PCR and whole-cell protein SDS-PAGE profiles resulted in three clusters of similar strains. Analysis of the 16S rDNA sequences and DNA–DNA relatedness data showed that these clusters represented three novel species. The highest 16S rDNA similarity to a recognized species found for the three groups was around 94 % with *Bacillus lentus* and *Bacillus sporothermodurans*. Further phenotypic characterization supported the proposal of three novel species in the genus *Bacillus*, *Bacillus farraginis*, *Bacillus fortis* and *Bacillus fordii*. The respective type strains are R-6540^T (=LMG 22081^T=DSM 16013^T), R-6514^T (=LMG 22079^T=DSM 16012^T) and R-7190^T (=LMG 22080^T=DSM 16014^T); their G + C DNA base contents are 43.7, 44.3 and 41.9 mol%, respectively. Although in variable amounts, a predominance of the branched fatty acids iso-C_{15:0} and anteiso-C_{15:0} was observed in all three novel species.

INTRODUCTION

Milk is a highly nutritious substrate that can support the growth of a wide variety of both Gram-negative and Gram-positive bacteria (Ternström *et al.*, 1993; Griffiths & Philips, 1990). Aerobic spore-forming bacteria of the genus *Bacillus* and related genera are commonly present in raw milk (Waes, 1976; Crielly *et al.*, 1994; McGuiggan *et al.*, 2002). Owing to their natural resistance properties, spores

can survive during food processing. After germination and outgrowth to high levels, they may cause spoilage and even outbreaks of food-borne illness. They have been responsible for adversely affecting the quality of pasteurized milk and milk products (Guirguis *et al.*, 1983; Meer *et al.*, 1991) and ultra heat-treated (UHT) products (Hammer *et al.*, 1995). Highly heat resistant spores of *Bacillus sporothermodurans* were detected in UHT products in

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Abbreviations: FAME, fatty acid methyl ester; rep-PCR, repetitive element sequence-based PCR; UHT, ultra heat treated.

The GenBank accession numbers for the 16S rRNA gene sequences of *B. farraginis* R-8039, R-6538, R-6540^T and R-6915, *B. fortis* R-6514^T and *B. fordii* R-7190^T are AY443034–AY443039, respectively.

Details of DNA–DNA hybridization results and photomicrographs of sporangia and vegetative cells of the type strains of *B. farraginis*, *B. fortis* and *B. fordii* are available as supplementary material in IJSEM Online.

different countries both inside and outside Europe (Guillaume-Gentil *et al.*, 2002).

The presence of *B. sporothermodurans* spores in feed concentrate, silage, soy and pulp was demonstrated for individual dairy farms (de Silva *et al.*, 1998; Vaerewijck *et al.*, 2001; Scheldeman *et al.*, 2002). In order to prevent contamination of milk and dairy products with aerobic spore-forming bacteria, it is important to ensure that contamination of raw milk is minimized. To achieve this, the nature and origin of spores and in particular of spores with a potential high heat resistance in raw milk must be better understood. In a survey of dairy farms, several strains were isolated from raw milk, the milking apparatus and animal feeds after 30 min heat treatment at 100 °C, as a selection procedure for spore-forming bacteria with potential high heat resistance (Scheldeman *et al.*, 2002).

In this study, we elucidated the taxonomic positions of 48 potentially highly heat-resistant isolates from different sources at dairy farms. In addition to molecular typing, we used extensive phenotypic characterization as well as 16S rRNA gene sequence determinations and whole-genome hybridization studies to group and to identify these strains.

METHODS

Strains and culture conditions. Details of the strains included in this study are listed in Table 1. All strains were isolated after 30 min heat treatment at 100 °C of samples taken at dairy farms (Scheldeman *et al.*, 2002). All isolates were grown on brain heart infusion (BHI) (Oxoid) supplemented with bacteriological agar no. 1 (Oxoid) and filter-sterilized vitamin B₁₂ (1 mg l⁻¹) (Sigma).

DNA preparation. Whole-cell DNA for PCR amplification was extracted from pure cultures by the method described by Pitcher *et al.* (1989). For DNA–DNA reassociation experiments, high-quality total genomic DNA was purified using a modification of the method described by Gevers *et al.* (2001), as outlined by De Clerck *et al.* (2004).

rep-PCR genomic fingerprinting. The conditions for genomic fingerprinting by means of repetitive element sequence-based PCR (rep-PCR), directed against the REP (repetitive extragenic palindromic) element or the (GTG)₅ repetitive sequence, were previously described by Herman *et al.* (1998). The (GTG)₅ primer (Versalovic *et al.*, 1994) was used under the following PCR conditions. Amplification reactions were performed in a final volume of 25 µl containing PCR buffer (10 mM Tris/HCl, pH 8.3, and 50 mM KCl) (Applied Biosystems), 1.5 mM MgCl₂, 0.2 mM of each dNTP (Pharmacia), 0.3 µg primer (Isogen), 1 U Goldstar DNA polymerase (Eurogentec) and 25 ng DNA template. The PCR program (Versalovic *et al.*, 1994) was run on a DNA thermal cycler (Perkin Elmer 9600). To avoid inter-PCR differences, all samples were included in one single PCR run.

The rep-PCR amplicons, obtained with the REP- and (GTG)₅ primers, were separated on a 1.5% LSI LE agarose gel (Life Science International) (20 × 25 cm) for 4 h at a constant voltage of 4 V cm⁻¹ in 1 × TBE (100 mM Tris/HCl, 100 mM boric acid, 2 mM EDTA, pH 8.0). The rep-PCR profiles were visualized after staining with ethidium bromide, followed by digital image capturing using a GelDoc 2000 camera (Bio-Rad). A mixture of molecular mass markers VIII, IX and X (Roche) was used as reference for intra- and

inter-gel comparison. Numerical analysis of the resulting fingerprints was performed using the GELCOMPAR II version 2.0 software package (Applied Maths). The similarity among digitized profiles was calculated using Pearson's correlation coefficient and an average linkage (UPGMA) dendrogram was derived.

Whole-cell protein profiling. Cells used for whole-cell protein profiling through SDS-PAGE analysis were obtained after 24 h of growth on NAG (nutrient agar supplemented with 1% glucose, pH 7.4). SDS protein extracts were prepared and electrophoresed according to Pot *et al.* (1994). Data collection and interpretation were conducted as described by Vauterin & Vauterin (1992).

16S rDNA sequencing and phylogenetic analysis. The 16S rRNA genes of strains R-6514^T, R-6521, R-6538, R-6540^T, R-6915 and R-7190^T were amplified by PCR using conserved primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3'). The PCR products were purified and subsequently sequenced using an ABI 310 Sequencer (Applied Biosystems) as previously described (Scheldeman *et al.*, 2002). A combination of the primers given by Coenye *et al.* (1999) was used to obtain a continuous stretch of the 16S rRNA gene sequence. Sequence assembly was implemented using the GENEBASE software (Applied Maths). The BIONUMERICS 3.0 software package (Applied Maths) was used for construction of a phylogenetic tree based on the neighbour-joining method.

DNA–DNA reassociation experiments. DNA–DNA hybridizations were performed at 37 °C with photobiotin-labelled probes in microplate wells according to the method of Ezaki *et al.* (1989), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements as described in detail by Willems *et al.* (2001).

DNA G+C content. The DNA base composition was determined by HPLC using further specifications given by Logan *et al.* (2000).

Fatty acid analysis. The culture conditions for fatty acid methyl ester (FAME) analysis were as described by Scheldeman *et al.* (2002). The methods used for fatty acid extraction, methyl ester preparation and separation by gas chromatography were as described by Vancanneyt *et al.* (1996).

Physiological and morphological characterization. Phenotypic tests were performed as described by Logan & Berkeley (1984) and Heyman *et al.* (2004); other characters were determined and the data analysed numerically using the general similarity coefficient of Gower (S_G) as described by Logan *et al.* (2000). For observations on sporangia, cells were grown on tryptic soy agar (TSA) supplemented with 5 mg MnSO₄ l⁻¹ at 30 °C. Tests for assimilation of substrates as the sole carbon source were performed following the methods described by Heyndrickx *et al.* (1997).

RESULTS AND DISCUSSION

Grouping based on whole-cell protein and genomic profiling

To assess the diversity in the collection of 48 potentially highly heat-resistant spore-forming strains from dairy farms, two genome-based molecular fingerprinting methods were applied together with whole-cell protein profiles (SDS-PAGE). The result of a numerical analysis based on whole-cell protein profiles is summarized in Table 1. Three clusters could be delineated with an internal similarity of at least 90%. The 32 strains grouping together in cluster A (and accordingly denominated in Table 1) mainly

Table 1. Details of the origin of *Bacillus* strains used in this study

Culture collection abbreviations: R, Research collection of the BCCM/LMG Bacteria Culture Collection, Gent, Belgium; MB, Culture Collection, Molecular Bacteriology Laboratory of the Department of Animal Product Quality, Melle, Belgium.

Strain	Isolation source*	Sample*	Temperature† (°C)	Farm‡	Cluster			G + C content (mol%)	
					SDS- PAGE	(GTG) ₅	REP		
Group I. <i>Bacillus farraginis</i> sp. nov.									
R-6538 (=MB 1884=LMG 22143)	MI: cluster	MI3	55	1	A	B	A	43.7	
R-6539 (=MB 1886)	MI: cluster	MI3	55	1	A	B	A		
R-6540 ^T (=MB 1885 ^T =LMG 22081 ^T =DSM 16013 ^T)	MI: cluster	MI3	55	1	A	B	A		
R-6692	Feed concentrate	KV8	55	4	A	B	A		
R-6714	Feed concentrate	KV4	55	3	A	B	A	43.8	
R-6730	Feed concentrate	KV13	55	7	A	B	A		
R-6732	Feed concentrate	KV13	55	7	A	B	A		
R-6735 (=MB 1853)	Green fodder	SI5	55	3	A	B	A		
R-6776	Grass	SI8	37	5	A	B	B		
R-6915 (=MB 1891=LMG 22144)	Feed concentrate	KV13	37	7	A	B	A		
R-6924	Feed concentrate	KV13	55	4	A	B	A		
R-6925	Feed concentrate	KV13	55	7	A	B	A		
R-6929	Feed concentrate	KV13	37	7	A	B	A		
R-6932	Grass silage	SI12	37	7	A	B	A		
R-6952	MI: connection point	MI35	37	8	Single	B	A		
R-7148 (=MB 1854)	Lucerne	SI18	55	11	A	B	A		
R-7343 (=MB 1860)	Feed concentrate	KV19	55	12	A	B	A		
R-7346 (=MB 1892)	Feed concentrate	KV19	37	12	A	B	A		
R-7351	Feed concentrate	KV20	37	12	A	B	A		
R-7436	Feed concentrate	KV22	55	13	A	B	B		
R-7439	Feed concentrate	KV22	55	13	A	B	A		
R-7448	Feed concentrate	KV21	55	13	A	B	A		
R-7503	Hay	SI27	55	14	A	B	B		
R-7640	Feed concentrate	KV27	55	15	A	B	A		
R-7643	Feed concentrate	KV27	55	15	A	B	A		
R-7648	Feed concentrate	KV27	37	15	A	B	A		
R-7789 (=MB 1856)	Feed concentrate	KV30	55	16	A	B	A		
R-7796	Feed concentrate	KV30	37	16	A	B	B		
R-7813	Feed concentrate	KV29	37	16	A	B	A		
R-7819	Feed concentrate	KV31	55	17	A	B	B		
R-8039 (=LMG 22145)	Feed concentrate	KV32	55	17	A	B	A		43.8
R-8125	Feed concentrate	KV32	37	17	A	B	A		
Group II. <i>Bacillus fortis</i> sp. nov.									
R-6514 ^T (=LMG 22079 ^T =DSM 16012 ^T)	MI: cluster	MI3	55	1	B	C	C	44.3	
R-6684	Soy	KV5	55	4	B	D	C		
R-6696	Feed concentrate	KV8	55	4	B	C	D		
R-7163	Raw milk	RM13	37	12	B	C	C		
R-7426	Feed concentrate	KV21	37	13	B	Single	C		
R-7795 (=MB 1876=LMG 22141)	Feed concentrate	KV30	37	16	B	C	Single		
R-8029	Feed concentrate	KV31	37	17	B	C	C		
Group III. <i>Bacillus fordii</i> sp. nov.									
R-6437	MI: cluster	MI3	37	1	C	A	E	44.4	
R-6446	MI: cluster	MI3	37	1	C	A	E		
R-6459 (=LMG 22142)	MI: filter cloth	MI6	37	1	C	A	E		
R-6674 (=MB 1874)	MI: filter cloth	MI20	37	4	C	A	E		

Table 1. cont.

Strain	Isolation source*	Sample*	Temperature† (°C)	Farm‡	Cluster			G + C content (mol%)
					SDS-PAGE	(GTG) ₅	REP	
R-7190 ^T (=MB 1878 ^T =LMG 22080 ^T =DSM 16014 ^T)	Raw milk	RM12	37	11	C	A	E	41.9
R-7810	Feed concentrate	KV29	37	16	C	A	D	
R-8028	Feed concentrate	KV31	37	17	C	A	E	
Unassigned strains								
<i>Bacillus</i> sp. R-6521 (=MB 1851)	MI: cluster	MI3	55	1	Single	D	Single	40.8
<i>Bacillus</i> sp. R-6685	Soy	KV5	55	4	A	Single	Single	

*MI, Milking apparatus; KV, cattle feed; SI, green fodder; RM, raw milk.

†Temperature of isolation, temperature used for incubation following heat treatment for 30 min at 100 °C.

‡Samples were taken from 17 dairy farms at geographically diverse locations in Belgium (Scheldeman *et al.*, 2002).

originated from feed concentrate for dairy cattle. Clusters B and C each included seven isolates and showed 83 % mutual similarity. Cluster B comprised four feed concentrate isolates and one isolate from raw milk, from the milking apparatus (cluster) and from soy. The seven strains from cluster C were isolated from the milking equipment [namely, the filter cloth (two strains) and the cluster (two)], from feed concentrate (two) and from raw milk (one). Only two strains occupied distinct positions in the dendrogram, although their profiles closely resembled those of cluster A. R-6521 still showed 87 % similarity to cluster A, whereas R-6952 only showed 79 % similarity to this cluster, probably due to one single additional dense band in the lower molecular mass region (data not shown).

Molecular typing by means of (GTG)₅- and REP-PCR revealed respectively four and five groups of profiles among the strains studied (Table 1). The seven strains included in (GTG)₅ cluster A corresponded to those included in SDS-PAGE cluster C. The 32 strains in (GTG)₅ cluster B largely matched those in SDS-PAGE cluster A, with the exception of R-6952, which took a single position in the SDS-PAGE clustering (see above). Although included in SDS-PAGE cluster A, strain R-6685 held a distinct position in the (GTG)₅ clustering. The five strains included in (GTG)₅ cluster C were also included in SDS-PAGE cluster B. The two remaining strains of SDS-PAGE cluster B, R-6684 and R-7426, were respectively included in (GTG)₅ cluster D or distinctly located in the clustering. Strain R-6521, with a separate position in the SDS-PAGE clustering, grouped together with R-6684 in (GTG)₅ cluster D. Finally, R-6685 did not cluster with any of the (GTG)₅ groups. Numerical analysis of the REP-PCR data revealed five clusters and three separate strains. The 27 and five strains of, respectively, REP clusters A and B corresponded to the 32 strains in (GTG)₅ cluster B, and hence largely to those in SDS-PAGE cluster A. Although REP clusters A and B showed a mutual similarity of only 32 %, some common bands in the profiles of these two groups

could be observed (data not shown). REP cluster C consisted of five strains, which also grouped together in SDS-PAGE cluster B. The two remaining strains in SDS-PAGE cluster B, R-6696 and R-7795, were respectively included in REP cluster D or distinctly located in the dendrogram. Finally, the seven strains included in SDS-PAGE cluster C and (GTG)₅ cluster A were grouped together in REP cluster E, except for R-7810, which was grouped in REP cluster D. Overall, there was a good agreement between the three typing methods used, resulting in three groups of strains for which at least two of the three typing techniques supported the overall pattern of the group. Group I comprised 32 isolates corresponding to those in (GTG)₅ cluster B, group II of seven isolates comprised all those included in SDS-PAGE cluster B and group III included all isolates of SDS-PAGE cluster C (Table 1). Ultimately, two strains could not be considered to belong to these delineated groups on these criteria and were therefore considered as single strains.

16S rDNA-based phylogeny and DNA–DNA relatedness

To investigate the taxonomic position of the three groups of strains, 16S rDNA sequence analysis and genomic DNA–DNA reassociation studies were performed. For the latter study, one representative of each group was included. In addition, the internal relatedness of the large group I was evaluated by including two strains (R-6540^T and R-8039). A reassociation value of 87 % indicated that R-6540^T and R-8039 (group I) indeed belong to the same species. The DNA–DNA reassociation values between the representatives of groups I, II and III proved to be low (<32 %; for more details see supplementary table in IJSEM Online). As a result, the three groups delineated on the basis of SDS-PAGE, REP- and (GTG)₅-PCR can be considered to represent three separate species. The DNA base compositions presented in Table 1 for strains R-6540^T, R-8039, R-6514^T and R-7190^T are in accordance with the DNA–DNA

reassociation values (supplementary table in IJSEM Online). Moreover, the G+C value for R-6521 further confirmed its exclusion from groups I and II.

16S rRNA gene sequences for four group I strains (R-6538, R-6540^T, R-6915, R-8039) and strains R-6514^T and R-7190^T, representatives of respectively groups II and III, were determined in the present study. The lowest pairwise similarity found within the 16S rDNA sequences of the four group I strains was 98.8 %, for strains R-6540^T and R-8039. All other values ranged between 99.0 and 99.8 % (data not shown). The 16S rDNA sequence similarity of R-6514^T (group II) with the strains from group I ranged from 96.8 % with R-6540^T to 97.5 % with R-6538. For group III strain R-7190^T a 16S rDNA sequence similarity with group I ranging from 95.8 % with R-6540^T to 97.1 % with R-6538 was observed. The 16S rDNA sequence similarity of R-6514^T and R-7190^T, representing groups II and III, was 97.9 %. These data indicate the close relationship among the three groups.

The highest similarity found by a FASTA search (Pearson & Lipman, 1988) for strain R-7190^T (group III) was 99.3 % with the 16S rDNA of *Bacillus* sp. 112442 JS2 (GenBank accession no. AF071857). The latter strain was isolated from silage after heating samples for 60 min at 100 °C (de Silva *et al.*, 1998), also suggesting the possible formation of very heat-resistant spores by this strain. For group I and II strains R-6540^T and R-6514^T, the FASTA search revealed 96.0 and 99.1 % sequence similarity, respectively, with the low G+C Gram-positive bacterium M52 (AB116130) isolated from compost. Both strains R-6540^T and R-6514^T furthermore showed 95.8 and 98.0 % sequence similarity, respectively, with the 16S rDNA of the previously mentioned *Bacillus* sp. 112442 JS2. The highest 16S rDNA sequence similarity with a recognized species found for strain R-6514^T was only 94.9 % with *Bacillus*

lentus NCIMB 8773^T (AB021189) and 94.5 % with *B. sporothermodurans* M215^T (U49078). The 16S rDNA sequence of strain R-6540^T showed only 94.5 % similarity with *B. lentus* NCIMB 8773^T (AB021189) and 94.5 % with *Bacillus galactosidilyticus* LMG 17892^T (AJ535638). For the 16S rDNA sequence of R-7190^T, only 94.3 % similarity with *B. lentus* NCIMB 8773^T, 94.3 % similarity with *Bacillus firmus* IAM 12464^T (D16268) and 94.1 % with *B. sporothermodurans* M215^T (U49078) was observed. It can therefore be concluded that groups I, II and III, represented by R-6540^T, R-6514^T and R-7190^T, constitute three novel species within the genus *Bacillus*. The phylogenetic positions of these three novel species are presented in Fig. 1, by means of a neighbour-joining tree with a selection of recognized *Bacillus* species. All groups (I, II and III) were clearly separated from the other *Bacillus* species included. The four strains comprising group I clearly appeared as a well-separated branch in the phylogenetic tree, whereas R-7190^T showed a high relatedness to *Bacillus* sp. 112442 JS2, as discussed above. Group II strain R-6514^T was located in the same deeper branching group.

Standard bacteriological characterization and fatty acid composition

All strains were unreactive in the API 20E and 50CHB tests. In the API Biotype100 tests all strains did react, and some characters that differentiated the three groups were observed (Table 2). Most of the group I strains showed a reactive and consistent profile in which amino acids and organic acids were utilized as sole carbon and energy sources, but very few carbohydrates were assimilated. Members of groups II and III also attacked amino acids and organic acids and very few carbohydrates, but their ranges of substrates utilized were narrower than for group I strains, their reactions were weaker and considerable between-strain variation was observed. As the three novel

Fig. 1. Neighbour-joining tree derived from 16S rDNA sequences, showing the phylogenetic positions of *Bacillus farraginis* sp. nov., *Bacillus fortis* sp. nov. and *Bacillus fordii* sp. nov. strains and the type strains of the most closely related *Bacillus* species. GenBank accession numbers are given and bootstrap values (expressed as percentages of 1000 replications) are shown at branch points when higher than 70 %. Unknown bases were not considered for the analysis.

Table 2. Phenotypic characteristics that distinguish *B. farraginis* sp. nov., *B. fortis* sp. nov. and *B. fordii* sp. nov. in the Biotype100 system

Unless otherwise indicated, characters refer to the use of different compounds as sole carbon sources in the Biotype100 system. +, Positive; –, negative; V, variable among strains; W, weak for all strains tested; *n*, number of strains analysed.

Character	Group I <i>B. farraginis</i> (n=29)	Group II <i>B. fortis</i> (n=6)	Group III <i>B. fordii</i> (n=7)
Production of pigment in casein	–	–	+
Growth at pH 5	–	–	+
D-Alanine	+	–	–
L-Alanine	+	–	V
4-Aminobutyrate	+	V	–
Fumarate	+	–	V
L-Glutamate	+	V	–
3-Hydroxybutyrate	+	–	V
L-Malate	+	V	–
Methyl α-galactopyranoside	–	–	W
Methyl β-galactopyranoside	V	–	W
3-Methyl D-glucopyranose	–	–	W
Methyl α-D-glucopyranoside	V	–	W
L-Proline	+	V	–
Succinate	+	–	V

species are unreactive in the API 20E and 50CHB kits, this distinguishes them from their closest phylogenetic neighbours (e.g. *B. lentus*), and so a more extended diagnostic table is of no informative or practical value.

All strains were found to be Gram-negative, aerobic, motile rods, which formed ellipsoidal spores positioned paracentrally to subterminally (groups I and III) or centrally to paracentrally (group II). Photomicrographs of cells and spores for the three species are available as supplementary material in IJSEM Online. In a cluster analysis (data not shown) based on similarities calculated from both positively and negatively matching Biotype100 data, all but two of the 29 strains forming group I merged at 82.5 % *S*_G and joined with clusters containing the remaining two group I strains and all of the group II and III strains at only 75 %. Such relatively low percentage similarities are typical of Biotype100 data with similarities calculated using *S*_G (Logan *et al.*, 2002). Strains forming group II merged at 90 % *S*_G, but this cluster contained one of the atypical group I strains (R-7351), and the other atypical group I strain (R-6952) joined this cluster at 87.5 % *S*_G. These atypical strains could be recognized as members of group I on the basis of molecular data, but their phenotypic profiles were narrower and weaker than the other group I strains. Strain R-6952, however, was also found to be atypical in its whole-cell protein profile. Strains belonging to group III showed even greater within-group variation and all but one of the strains (R-7810) formed two subclusters at 90 %; one of these subclusters merged with group II at 85 % and the other, along with the remaining ungrouped strain (R-7810), joined at only 82.5 %. The

atypical group III strain R-7810 also showed a different REP pattern to other members of this group.

The mean fatty acid profiles for the *Bacillus* strains examined are listed in Table 3. A predominance of iso-C_{15:0} could be observed for each novel species. Group I strains could be differentiated from group II and III by means of a larger amount of the branched fatty acids anteiso-C_{15:0} and anteiso-C_{17:0} on the one hand and a smaller amount of the saturated fatty acids C_{14:0} and C_{16:0} on the other hand. Discrimination between group II and groups I and III was possible based on a larger amount of the saturated fatty acids C_{15:0} and C_{17:0} for group II strains. Finally, group III strains could be distinguished from groups I and II on the basis of a larger amount of the unsaturated fatty acids C_{16:1ω11c} and C_{18:1ω9c} and the branched fatty acids iso-C_{17:0} and iso-C_{17:0ω10c}. These data are in accordance with the previous findings that groups I, II and III represent separate novel species within the genus *Bacillus*.

Conclusions

Combining all of the genotypic and phenotypic data presented here, it is clear that the 48 isolates form three homogeneous groups that are clearly different from all other related species within the genus *Bacillus*. Therefore, they represent three novel species of the genus *Bacillus*, for which we propose the names *Bacillus farraginis* sp. nov., *Bacillus fortis* sp. nov. and *Bacillus fordii* sp. nov. All strains of these novel species were isolated after heat treatment of 30 min at 100 °C, suggesting that these strains might be capable of forming very heat-resistant endospores. Further research is needed to reveal to what extent the spores of these

Table 3. Cellular fatty acid composition of the three novel *Bacillus* species isolated at dairy farms

Values are percentages of total fatty acids expressed as means $\pm$ SD for the number of strains analysed. ND, Not detected.

Fatty acid	Group I <i>B. farraginis</i> (n=17)	Group II <i>B. fortis</i> (n=7)	Group III <i>B. fordii</i> (n=7)
Saturated fatty acids:			
C _{14:0}	0.3 $\pm$ 0.4	1.4 $\pm$ 0.2	1.6 $\pm$ 0.5
C _{15:0}	1.7 $\pm$ 1.6	5.9 $\pm$ 2.0	1.9 $\pm$ 0.6
C _{16:0}	1.1 $\pm$ 0.6	3.5 $\pm$ 0.6	3.0 $\pm$ 0.8
C _{17:0}	ND	2.0 $\pm$ 0.5	0.9 $\pm$ 0.2
C _{18:0}	<0.2*	<0.2*	0.3 $\pm$ 0.3
Unsaturated fatty acids:			
C _{16:1} ω 7c alcohol	5.3 $\pm$ 2.0	3.8 $\pm$ 0.6	2.9 $\pm$ 0.5
C _{16:1} ω 11c	3.0 $\pm$ 0.7	4.2 $\pm$ 0.6	6.4 $\pm$ 0.8
C _{18:1} ω 7c	ND	ND	<0.2†
C _{18:1} ω 9c	ND	<0.1‡	1.1 $\pm$ 0.2
Branched fatty acids:			
iso-C _{13:0}	ND	ND	<0.2†
iso-C _{14:0}	3.3 $\pm$ 2.5	5.3 $\pm$ 1.4	2.5 $\pm$ 0.5
iso-C _{15:0}	36.4 $\pm$ 8.2	34.9 $\pm$ 2.8	42.0 $\pm$ 2.9
iso-C _{16:0}	6.4 $\pm$ 4.3	9.9 $\pm$ 2.0	4.3 $\pm$ 0.6
iso-C _{17:0}	3.4 $\pm$ 1.0	3.2 $\pm$ 0.6	5.9 $\pm$ 1.0
anteiso-C _{15:0}	26.7 $\pm$ 4.0	19.2 $\pm$ 1.9	18.0 $\pm$ 1.9
anteiso-C _{17:0}	11.0 $\pm$ 1.8	5.7 $\pm$ 1.2	6.7 $\pm$ 1.7
iso-C _{17:0} ω 10c	0.3 $\pm$ 0.6	0.3 $\pm$ 0.4	1.6 $\pm$ 0.4
anteiso-C _{17:1} ω 9c	ND	ND	<0.2†
Summed feature 3§	ND	ND	0.3 $\pm$ 0.7†
Summed feature 4§	1.2 $\pm$ 0.7	0.6 $\pm$ 0.5	0.4 $\pm$ 0.4

*C_{18:0} was detected only in strains R-6932 (*B. farraginis*) and R-7795 (*B. fortis*), at less than 1%.

†C_{18:1} ω 7c, iso-C_{13:0}, anteiso-C_{17:1} ω 9c and summed feature 3 were detected only in strain R-7810, at low levels (respectively 1.0, 0.4, 0.8 and 1.8%).

‡C_{18:1} ω 9c was detected only in strain R-6514, at less than 1%.

§Summed feature 3 represents C_{15:0} 2OH and/or C_{16:1} ω 7c, which could not be separated by GLC using the MIDI system. Summed feature 4 similarly represents iso-C_{17:1} I and/or anteiso-C_{17:1} B.

strains can be highly heat resistant and as a result adversely affect the quality of heat-treated dairy products.

Description of *Bacillus farraginis* sp. nov.

Bacillus farraginis (far.ra.gin'is. L. gen. fem. n. *farraginis* from mixed fodder for cattle, referring to feed concentrate for dairy cattle as the principal isolation source).

Cells are long, straight, round-ended, motile, strictly aerobic, Gram-negative rods, occurring singly, in pairs or filaments. Cell diameter is 0.5–0.8 μ m and cell length 1.2–4 μ m. Spores are ellipsoidal and occur paracentrally or subterminally in occasionally slightly swollen sporangia. Colonies grown for 3 days at 30 °C on nutrient agar are cream coloured or translucent, slightly raised, with irregular margins and granular, glossy surfaces. Colony diameter is no greater than 1 mm. Good growth occurs at 30 and 45 °C and weak growth occurs at 20 °C. Some strains are

capable of growth at pH 9 but none grows at pH 5. Growth is not inhibited by 7% (w/v) NaCl. Hydrolysis of starch and casein is not observed within 7 days of incubation at 30 °C, and growth in casein agar is poor or negative. Catalase and oxidase are positive. All strains are unreactive in the API 20E and API 50CHB test kits. In the Biotype100 kit using the Biotype 2 medium, nearly all strains (>83%) belonging to the species are able to use the following substrates as sole carbon sources: 4-aminobutyrate, 5-aminovalerate, D- and L-alanine, fumarate, L-glutamate, glutarate, L-histidine, 3-hydroxybutyrate, 2-oxoglutarate, D- and L-malate, DL-lactate, L-proline, putrescine, succinate, L-tryptophan and L-tyrosine. Many strains (>45%) are able to use L-aspartate, dulcitol, *m*-hydroxybenzoate, malonate, D-mannitol, D-ribose and D-sorbitol. Other substrates are used less frequently (17–44%): L-arabinose, D-galactose, gentisate, D-glucuronate, *p*-hydroxybenzoate, *myo*-inositol and α -L-rhamnose. The following substrates are seldom or

never used as sole carbon sources: *cis*- and *trans*-aconitate, adonitol, D- and L-arabitol, benzoate, betaine, caprate, caprylate, D-cellobiose, citrate, *m*-coumarate, erythritol, aesculin, ethanolamine, β -D-fructose, α -L-fucose, D-galacturonate, β -gentiobiose, D-gluconate, D-glucosamine, α -D-glucose, DL-glycerate, glycerol, histamine, hydroxyquinoline β -glucuronide, itaconate, 2- and 5-keto-D-gluconate, α -lactose, lactulose, D-lyxose, maltitol, maltose, maltotriose, D-mannose, α -D-melibiose, D-melezitose, methyl α -galactopyranoside, methyl β -galactopyranoside, 3-methyl D-glucopyranose, methyl α -D-glucopyranoside, methyl β -D-glucopyranoside, mucate, *N*-acetyl D-glucosamine, palatinose, phenylacetate, 3-phenylpropionate, propionate, protocatechuate, quinate, D-raffinose, D-saccharate, sucrose, L-serine, L-sorbose, D-tagatose, L-, *meso*- and D-tartrate, D-trehalose, D-turanose, tricarballoylate, trigonelline, tryptamine, D-xylitol and xylitol.

The type strain, R-6540^T (=MB 1885^T=LMG 22081^T=DSM 16013^T), utilizes the following substrates as sole carbon sources: 4-aminobutyrate, 5-aminovalerate, D- and L-alanine, L-aspartate, dulcitol, fumarate, D-glucosamine, L-glutamate, glutarate, histamine, L-histidine, *m*-hydroxybenzoate, 3-hydroxybutyrate, 2-oxoglutarate, D- and L-malate, malonate, DL-lactate, L-proline, putrescine, D-ribose, D-sorbitol, succinate, *meso*-tartrate, L-tryptophan and L-tyrosine. The major cellular fatty acids (>5% of total cellular fatty acids) are iso-C_{15:0}, anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0} and C_{16:1} ω 7c alcohol. The DNA G+C composition of the type strain is 43.7 mol%.

Description of *Bacillus fortis* sp. nov.

Bacillus fortis (for'tis. L. adj. *fortis* strong, referring to the fact that the strains were isolated after heat treatment for 30 min at 100 °C).

Cells are straight, round-ended, motile, strictly aerobic, Gram-negative rods, occurring singly or in pairs. Cell diameter is 0.6–0.8 μ m and length 1.0–3.5 μ m. Spores are oval and occur centrally or paracentrally in slightly swollen sporangia. Colonies grown on nutrient agar at 30 °C for 3 days are cream coloured, raised with entire margins and smooth, glossy surfaces. Their maximum diameter is 1 mm. Good growth occurs at 30 and 45 °C and weak growth occurs at 20 °C. Growth does not occur at pH 9 or 5. Growth is not inhibited by 7% (w/v) NaCl. Hydrolysis of starch and casein is not observed within 7 days of incubation at 30 °C, and growth on casein agar is poor or negative. Catalase and oxidase are positive. All strains are unreactive in the API 20E and API 50CHB test kits. In the Biotype100 kit using the Biotype 2 medium, strains use L-tryptophan and L-histidine as sole carbon sources. Most strains (>50%) use the following substrates as sole carbon sources: 4-aminobutyrate, 5-aminovalerate, aesculin, ethanolamine, glutarate, hydroxyquinoline β -glucuronide, 2-oxoglutarate, DL-lactate, malonate, phenylacetate, L-proline, putrescine, D-ribose and L-tyrosine. Some strains (<50%) use the following substrates: citrate, erythritol, D-gluconate,

D-glucosamine, α -D-glucose, L-glutamate, DL-glycerate, histamine, *m*-hydroxybenzoate, 2- and 5-keto-D-gluconate, L- and D-malate, α -D-melibiose, protocatechuate, L-sorbose and L-tartrate. Only the type strain uses D-glucuronate and no strain utilizes the following substrates as sole carbon sources: *trans*- and *cis*-aconitate, adonitol, D- and L-alanine, L-arabinose, D- and L-arabitol, L-aspartate, betaine, benzoate, caprate, caprylate, D-cellobiose, *m*-coumarate, dulcitol, β -D-fructose, α -L-fucose, fumarate, D-galactose, D-galacturonate, β -gentiobiose, gentisate, glycerol, *p*-hydroxybenzoate, 3-hydroxybutyrate, *myo*-inositol, itaconate, α -lactose, lactulose, D-lyxose, maltitol, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, methyl α -galactopyranoside, methyl β -galactopyranoside, 3-methyl D-glucopyranose, methyl α -D-glucopyranoside, methyl β -D-glucopyranoside, mucate, *N*-acetyl D-glucosamine, palatinose, 3-phenylpropionate, propionate, quinate, D-raffinose, α -L-rhamnose, D-saccharate, sucrose, L-serine, D-sorbitol, succinate, D-tagatose, D- and *meso*-tartrate, D-trehalose, tricarballoylate, trigonelline, tryptamine, D-turanose, xylitol and D-xylitol.

The type strain is R-6514^T (=LMG 22079^T=DSM 16012^T). Where results are variable, the type strain uses the following substrates: aesculin, D-gluconate, D-glucosamine, D-glucuronate, DL-glycerate, hydroxyquinoline- β -glucuronide, 2-oxoglutarate, 2- and 5-keto-D-gluconate, DL-lactate, malonate, phenylacetate, protocatechuate, L-tartrate and L-tyrosine. The major cellular fatty acids (>5% of total cellular fatty acids) are iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0}, C_{15:0} and anteiso-C_{17:0}. The DNA G+C content of the type strain is 44.3 mol%.

Description of *Bacillus fordii* sp. nov.

Bacillus fordii (for'di.i. N.L. gen. n. *fordii* named after W. W. Ford, an American microbiologist working on aerobic spore-forming bacteria at the beginning of the twentieth century).

Cells are long, straight, round-ended, motile, strictly aerobic, Gram-negative rods, occurring singly or in pairs. Cell diameter is 0.6–0.8 μ m and length 1.6–3.5 μ m. Spores are ellipsoidal and occur paracentrally or subterminally in, occasionally, slightly swollen sporangia. Colonies grown on nutrient agar at 30 °C for 3 days are cream coloured, raised, with entire margins and smooth glossy surfaces. Their maximum diameter is 2 mm. Good growth occurs at 30 and 45 °C and weak growth occurs at 20 °C. Growth occurs at pH 9 and some strains grow at pH 5. Growth is not inhibited by 7% (w/v) NaCl. Hydrolysis of starch and casein is not observed within 7 days of incubation at 30 °C. Growth on casein agar is coloured faint pink. Catalase and oxidase are positive. All strains are unreactive in the API 20E and API 50CHB test kits. In the Biotype100 kit using the Biotype 2 medium, all strains show very good production of biomass using malonate and L-tyrosine as sole carbon sources. Variable results with good production of biomass are obtained for L-histidine, 2-oxoglutarate,

glutarate, DL-lactate, 5-aminovalerate and L-tryptophan. All or most strains are capable of weak growth from the following carbon sources: aesculin, gentisate, protocatechuate, *meso*-tartrate, D-glucosamine, DL-glycerate, quinate, ethanolamine, D-glucuronate, *p*-hydroxybenzoate, hydroxyquinoline β -glucuronide and 2- and 5-keto-D-gluconate. Growth occurs seldom and weakly from the following substrates: adonitol, L-alanine, L-arabinose, L-arabitol, benzoate, fumarate, D-galacturonate, D-gluconate, α -D-glucose, histamine, 3-hydroxybutyrate, D-lyxose, D-malate, methyl α -galactopyranoside, methyl β -D-glucopyranoside, *N*-acetyl D-glucosamine, phenylacetate, propionate, putrescine, D-raffinose, D-ribose, D-saccharate, L-sorbose, succinate, D-tagatose, L-tartrate, tricarballoylate, trigonelline and D-xylose. The following substrates are not used as sole carbon source: *cis*- and *trans*-aconitate, D-alanine, 4-aminobutyrate, D-arabitol, L-aspartate, betaine, caprate, caprylate, D-cellobiose, citrate, *m*-coumarate, dulcitol, erythritol, β -D-fructose, α -L-fucose, D-galactose, β -gentiobiose, L-glutamate, glycerol, *m*-hydroxybenzoate, *myo*-inositol, itaconate, α -lactose, lactulose, L-malate, maltitol, maltose, maltotriose, D-mannitol, D-mannose, α -D-melibiose, D-melezitose, methyl β -galactopyranoside, 3-methyl D-glucopyranoside, methyl α -D-glucopyranoside, mucate, palatinose, 3-phenylpropionate, L-proline, α -L-rhamnose, sucrose, L-serine, D-sorbitol, D-tartrate, D-trehalose, tryptamine, D-turanose and xylitol.

The type strain is R-7190^T (=MB 1878^T=LMG 22080^T=DSM 16014^T). Where results are variable, the type strain uses the following substrates: aesculin, 5-aminovalerate, L-arabinose, benzoate, ethanolamine, D-galacturonate, gentisate, D-glucuronate, glutarate, DL-glycerate, histamine, *p*-hydroxybenzoate, 3-hydroxybutyrate, hydroxyquinoline- β -glucuronide, 2- and 5-keto-D-gluconate, 2-oxoglutarate, DL-lactate, D-lyxose, *N*-acetyl D-glucosamine, phenylacetate, protocatechuate, putrescine, quinate, D-raffinose, *meso*-tartrate, tricarballoylate, L-tryptophan and D-xylose. The major cellular fatty acids (> 5% of total cellular fatty acids) are iso-C_{15:0}, anteiso-C_{15:0}, anteiso-C_{17:0}, C_{16:1} ω 11c and iso-C_{17:0}. The DNA G+C content of the type strain is 41.9 mol%.

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