Bacillus novalis sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli* sp. nov., *Bacillus bataviensis* sp. nov. and *Bacillus drementensis* sp. nov., from the Drentse A grasslands

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A group of 42 isolates were isolated from the soil of several disused hay fields, in the Drentse A agricultural research area (The Netherlands), that were taken out of production at different times. The group represents hitherto-uncultured *Bacillus* lineages that have previously been found, by a non-cultural method, to be predominant in soil. The strains were subjected to a polyphasic taxonomic study, including (GTG)₅-PCR, 16S rDNA sequence analysis, DNA–DNA hybridizations, DNA base-ratio determination, fatty acid analysis and morphological and biochemical characterization. By comparing the groupings obtained by (GTG)₅-PCR and 16S rDNA sequence analysis, six clusters of similar strains could be recognized. A DNA–DNA relatedness study showed that these clusters represented five novel genospecies. Further analysis supported the proposal of five novel species in the genus *Bacillus*, namely *Bacillus novalis* sp. nov. (type strain IDA3307^T=R-15439^T=LMG 21837^T=DSM 15603^T), *Bacillus vireti* sp. nov. (type strain IDA3632^T=R-15447^T=LMG 21834^T=DSM 15602^T), *Bacillus soli* sp. nov. (type strain IDA0086^T=R-16300^T=LMG 21838^T=DSM 15604^T), *Bacillus bataviensis* sp. nov. (type strain IDA1115^T=R-16315^T=LMG 21833^T=DSM 15601^T) and *Bacillus drementensis* sp. nov. (type strain IDA1967^T=R-16337^T=LMG 21831^T=DSM 15600^T).

INTRODUCTION

The Drentse A agricultural research area, along the Anlooër Diepje brook near Anloo (The Netherlands), comprises different plots, some of which are still used for agriculture and some of which have been taken out of production at different times from the late 1960s onwards. The bacterial communities in different soil samples taken from these sites were previously studied by a culture-independent approach based on temperature-gradient gel electrophoresis (Felske &

Akkermans, 1998). It was demonstrated that the spatial distribution of bacterial 16S rRNA from ribosomes directly extracted from soil was homogeneous among samples that represented a stretch of 1.5 km of the grassland. This suggested the presence of predominant bacteria occurring throughout the entire research area. Additional research by Felske *et al.* (1998) revealed that hitherto-uncultured *Bacillus* species were the most active bacteria, with approximately half of the sequenced 16S rRNA being attributable to this genus. Felske *et al.* (1999) made a first attempt to culture these dominant lineages of *Bacillus*. They compared the band positions of 659 isolates in temperature-gradient gel electrophoresis with those of the dominant uncultured bacteria. Initially, approximately 8% of the isolates had band positions identical to those of one of the uncultured ribotypes. However, sequence analysis (~500 bp) of these matching isolates indicated that their 16S rDNA sequences were clearly different from sequences representing the fingerprint bands. Although the apparently

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Abbreviations: rep-PCR, repetitive sequence-based PCR; *S*_G, Gower similarity coefficient.

The EMBL accession numbers for the 16S rRNA gene sequences of *Bacillus novalis* LMG 21837^T, *Bacillus vireti* LMG 21834^T, *Bacillus soli* LMG 21838^T, *Bacillus bataviensis* LMG 21833^T and *Bacillus drementensis* LMG 21831^T are respectively AJ542512, AJ542509, AJ542513, AJ542508 and AJ542506.

predominant taxa could not be retrieved from this culture collection, a remarkable variety of bacilli, including novel species, were isolated. Thousands of strains were isolated from the soils; here, we report on 42 isolates that represent novel *Bacillus* species. They were all most closely related to *Bacillus niacini*, albeit with relatively low sequence similarities (97.5–99.0%). After polyphasic characterization, this group of isolates could be recognized as members of five novel species within the genus *Bacillus*, which we propose as *Bacillus novalis* sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli* sp. nov., *Bacillus bataviensis* sp. nov. and *Bacillus drentensis* sp. nov.

METHODS

Strains and media. The isolates originate from the Drentse A agricultural research area (06°41'E, 53°02'N) in The Netherlands, a 1.5 km stretch of grassland alongside the Anlooër Diepje brook. Soil sampling, enrichment and cultivation were performed as described by Felske *et al.* (1999). All isolates originated from a general sample that was prepared by mixing together pooled samples from six plots representing different years during which fertilizer treatment for agricultural hay production had last occurred. Two dilution series were made of the sample; one series was plated directly on agar media and the other was plated after heating for 15 min at 80 °C to select for endospores. The isolate designations and culture conditions applied are shown in Table 1. Isolates were further maintained on nutrient-agar slopes and maintained in Microbank tubes (Pro-Lab Diagnostics) at –80 °C. For phenotypic-characterization studies, including microscopy, cultures were maintained on slopes of trypticase soy agar (TSA) containing 5 mg MnSO₄ 1^{–1} (to enhance sporulation); for strains IDA3120, IDA3351 and IDA3632^T, spore formation was determined on *Bacillus fumarioli* agar adjusted to pH 7.0 (Logan *et al.*, 2000).

DNA preparation. Total genomic DNA was purified for 16S rDNA sequencing and repetitive sequence-based PCR (rep-PCR) by using a slight modification of the method of Pitcher *et al.* (1989), as described by Heyndrickx *et al.* (1996). For determination of the G+C content and DNA–DNA hybridization, approximately 1 g biomass was harvested from agar plates. DNA was purified by a combination of the protocols of Marmur (1961) and Pitcher *et al.* (1989), as described by Logan *et al.* (2000).

16S rDNA sequencing and phylogenetic analysis. Sequence analysis was performed as described previously by Heyrman & Swings (2001). For partial sequencing, two primers were used (reverse 358–339 and reverse 536–519; Heyrman & Swings, 2001) to obtain the first 400–500 bp of the 16S rRNA gene, which, according to Goto *et al.* (2000), includes the hypervariable region for the genus *Bacillus*. A phylogenetic tree was constructed using BioNumerics 2.0 software (Applied Maths) by applying the neighbour-joining method of Saitou & Nei (1987) on a multiple-alignment similarity matrix. The stability of relationships was assessed by means of a bootstrap analysis of 1000 datasets.

Rep-PCR genomic fingerprinting. PCR was performed with the (GTG)₅ primer (Versalovic *et al.*, 1994) using the PCR conditions described previously by Rademaker & de Bruijn (1997). For each strain, 6 µl PCR product mixed with 2 µl loading buffer (Rademaker & de Bruijn, 1997) was electrophoresed in a 1.5% (w/v) agarose gel and TAE buffer (1.21 g Tris base 1^{–1}, 0.2 ml 0.5 M EDTA 1^{–1}, pH 8) for 15 h at a constant 55 V and 4 °C. The first lane and every sixth lane were loaded with 6 µl molecular ruler [45.5% (v/v) 100 bp ruler (Bio-Rad), 36.5% (v/v) 500 bp ruler (Bio-Rad) and

18% (v/v) loading buffer]. After staining with ethidium bromide (0.5 µg ml^{–1}), the patterns were digitized and Pearson's correlation of the resulting band patterns was calculated using BioNumerics 2.0.

G+C content and DNA–DNA hybridization. The G+C content of the DNA was determined by HPLC (Mesbah *et al.*, 1989) using the further specifications given by Logan *et al.* (2000). DNA–DNA hybridization was performed using a modification of the microplate method of Ezaki *et al.* (1989), as described by Willems *et al.* (2001). A hybridization temperature of 40 °C (calculated with correction for the presence of 50% formamide) was used.

Chemotaxonomic characterization. GC analysis of fatty acid methyl esters was performed starting from strains grown on TSA for 24 h at 28 °C. A quantitative analysis of cellular fatty acid compositions was further performed by using a GLC procedure as described previously (Mergaert *et al.*, 1993). Computer analysis of the resulting profiles was performed as described by Heyrman *et al.* (1999).

Phenotypic characterization. The strains were characterized phenotypically by using the methods of Logan & Berkeley (1984); other characteristics were determined, and the data analysed numerically, as described by Logan *et al.* (2000). Vegetative cells and sporangia were observed by phase-contrast microscopy for the presence of motile cells, chains of cells, curved rods, rods with tapered ends, vacuoles, spores, swollen sporangia, parasporal crystals, parasporal bodies, spores of ellipsoidal, cylindrical or spherical shape and spores positioned terminally, subterminally or centrally/paracentrally; the strains were also examined for casein and starch hydrolysis by using the methods of Gordon *et al.* (1973). Maximum and minimum growth temperatures were determined by incubating 10 ml TSA cultures in water baths set to 30, 40 and 50 °C; pH ranges for growth were determined using 10 ml TSA cultures adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0. Both series were examined for turbidity at 24 h intervals. Anaerobic growth was tested for by incubating cultures on TSA plates in a GasPak jar (BBL), with aerobically incubated plates as controls.

RESULTS AND DISCUSSION

Genomic fingerprinting

Partial 16S rDNA sequencing, including the hypervariable region for *Bacillus* (Goto *et al.*, 2000), and (GTG)₅ genomic fingerprinting were performed on the soil isolates. The latter technique revealed important genomic variability among the isolates, which might reflect the heterogeneity of the soil habitat. Comparison of the two techniques allowed grouping of consistent clusters of genomically closely related isolates (Fig. 1), from which representative strains were selected for further DNA–DNA relatedness experiments. In the partial 16S rDNA sequence clustering, four groups of isolates could be delineated that showed a high degree of internal sequence similarity (>99.8%). These clusters are denoted A, B, C and D in Fig. 1(a); adjacent outliers of these clusters are denoted by lower-case letters. The clustering of the (GTG)₅ fingerprints (Fig. 1b) is generally in good agreement with that obtained by sequencing. With the exception of IDA1746, all isolates belonging to the major 16S groups, A–D, also group together in the (GTG)₅ clustering. As might be expected, the rep-PCR patterns are much more heterogeneous and thus clusters A, B, C and D were cut off at low Pearson's correlation percentages,

Table 1. Strains used in this study, isolation conditions and overview of genomic methods used for characterization

Strain designations: IDA, isolate Drentse A, original strain designations (Felske *et al.*, 1999); R-, Research Collection, Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium; LMG, BCCM/LMG Bacteria Collection Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium.

Strain	Isolation conditions		(GTG) ₅	16S rDNA		DNA-DNA
	Medium*	Temperature†		Partial	Full	
IDA0061 (=R-16295)	PYAc	RT	+	+		
IDA0077 (=R-16296)	PYAc	RT	+	+		
IDA0078 (=R-16297=LMG 21836)	PYAc	RT	+	+	+	+
IDA0084 (=R-16298=LMG 21832)	PYAc	RT	+	+	+	+
IDA0086 ^T (=R-16300 ^T =LMG 21838 ^T)	PYAc	RT	+	+	+	+
IDA0103 (=R-16301)	PYAc	RT	+	+		
IDA0106 (=R-16302=LMG 21835)	PYAc	RT	+	+	+	
IDA0206 (=R-16307)	PYAc	RT	+	+		+
IDA0223 (=R-16308)	PYAc	RT	+	+		
IDA0325 (=R-16309)	PYB	RT	+	+		
IDA0361 (=R-16310)	PYB	RT	+	+		
IDA1046 (=R-16313)	PYAc	RT	+	+	+	
IDA1113 (=R-15414=LMG 21830)	PYAc	RT	+	+	+	+
IDA1115 ^T (=R-16315 ^T =LMG 21833 ^T)	PYAc	RT	+	+	+	+
IDA1626 (=R-16321)	PYB	37 °C	+	+		+
IDA1679 (=R-15415)	PYB	RT	+	+		
IDA1692 (=R-16324)	PYB	RT	+	+		
IDA1746 (=R-16325)	PYAc	37 °C	+	+		
IDA1796 (=R-15416)	PYAc	RT	+	+		
IDA1819 (=R-16328)	PYAc, pH 4.5	37 °C	+	+		
IDA1928 (=R-16336)	PYAc	RT	+	+		
IDA1967 ^T (=R-16337 ^T =LMG 21831 ^T)	PYB	RT	+	+	+	+
IDA1990 (=R-16338)	PYB	RT	+	+		
IDA2066 (=R-16339=LMG 21840)	PAc	37 °C	+	+	+	+
IDA2072 (=R-16340)	PAc	37 °C	+	+		
IDA2156 (=R-16342)	PAc	RT	+	+		
IDA2213 (=R-15418)	PYB	RT	+	+	+	
IDA2265 (=R-16345)	PAc	37 °C	+	+		+
IDA2269 (=R-17019)	PAc	37 °C	+	+		
IDA2290 (=R-16347)	PAc	RT	+	+		
IDA2473 (=R-15422=LMG 21839)	PAc	RT	+	+	+	
IDA2891 (=R-15427)	DSM1 (1/10)	4 °C	+	+		
IDA3120 (=R-15428)	PYB	37 °C	+	+		+
IDA3307 ^T (=R-15439 ^T =LMG 21837 ^T)	PYB/TP	RT	+	+	+	+
IDA3351 (=R-15441)	PYB	37 °C	+	+		+
IDA3432 (=R-15445)	PYB/RT	37 °C	+	+		
IDA3493 (=R-15446)	PYAc	RT	+	+		+
IDA3632 ^T (=R-15447 ^T =LMG 21834 ^T)	PYB	RT	+	+	+	+
IDA3829 (=R-15450)	PYB	RT	+	+		+
IDA3918 (=R-15453)	PYB/TP	RT		+		
IDA3943 (=R-15454)	PYB/TP	RT	+	+		
IDA4470 (=R-16986)	PYB	RT	+	+		

*Incubation media: P, mineral medium (Pichinoty *et al.*, 1977); Y, added yeast extract (2 g l⁻¹); B, added sodium benzoate (2 g l⁻¹); Ac, added sodium acetate (2 g l⁻¹); TP, added K₂TeO₃ (1.6 mg l⁻¹) and phenethyl alcohol (0.25 %, v/v).

†RT, Room temperature (~20 °C).

Fig. 1. Grouping based on partial 16S rDNA sequences (a) and normalized (GTG)₅-PCR patterns (b) of the Drentse A grassland isolates. Clusters A, B, C and D were delineated at 99.8% partial sequence similarity. Outliers in the immediate vicinity of these clusters are indicated by lower-case letters. Clusters X and Y comprise isolates that are more heterogeneous in partial rDNA sequence, although their intracluster rep-PCR patterning is similar. IDA0106 was used to test the reproducibility of (GTG)₅ analysis (PCR and gel electrophoresis). The very weak (GTG)₅ pattern from IDA3918 was excluded from the clustering.

respectively 67, 24, 36 and 41%. Furthermore, two clusters (X and Y; Fig. 1) could be delineated at a Pearson's correlation level above 50% in the rep-PCR analysis, showing internal partial sequence similarities definitely below 99.8%.

A more complete 16S rDNA sequence of representative strains of the different groups was analysed. By using a FASTA search (Pearson & Lipman, 1988), *B. niacini* (AB021194) was found to possess the most closely related sequence of the *Bacillus* species with validly published names in the database. Representatives of groups A, B, C, D, X and Y showed respective sequence similarities to this entry of 97.8, 97.9, 98.4, 99.0, 98.0 and 98.2%. Furthermore, the representatives of groups C and D showed sequence similarities to *B. fumarioli* (AJ210056) of 97.1 and 97.4%. 16S rDNA sequence similarities to all other species with validly published names in the EMBL database were below 97%. In a neighbour-joining tree (Saitou & Nei, 1987), the sequences form a distinct lineage, with *B. niacini* and *B. fumarioli* as the closest relatives (Fig. 2).

Representative strains of clusters A–D, X and Y together with their closest outliers were analysed in DNA–DNA reassociation experiments (Table 2). Generally recommended and accepted criteria for delineating bacterial species state that strains with a DNA–DNA relatedness below 70%, as measured by hybridization, or with 16S rDNA sequence dissimilarity above 3% are considered as belonging to separate species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). Yet, bacterial strains with a difference in 16S rDNA sequence of less than 3% cannot be allocated to the same species without support from DNA–DNA relatedness studies. The DNA–DNA relatedness of the representative strains with respect to *B. niacini* LMG 16677^T was low (<30%), while the internal group values were high (>80%; Table 2). Strains IDA3493, IDA2265 and IDA1626, which respectively group in rep-PCR analysis outside clusters A, B and C, could also clearly be attributed to the respective clusters on the basis of DNA–DNA relatedness values. Clusters C, D and Y are genotypically distinct from all other groups, with DNA relatedness values below 35%;

Fig. 2. Phylogenetic positions based on neighbour-joining of the 16S rDNA sequences of representative Drentse A grassland isolates, among related *Bacillus* species. *Paenibacillus polymyxa* was used as an outgroup. Bootstrap values (expressed as percentages of 1000 replications) greater than 60% are shown at branch points.

groups C, D and Y will be further denoted as *Bacillus bataviensis* sp. nov., *Bacillus drementensis* sp. nov. and *Bacillus soli* sp. nov., respectively. The DNA relatedness values between representatives of clusters A and B and the closely related strains IDA3493 and IDA2265 were between 68 and 73%. These values are an indication that the isolates belong to the same species, something which was supported by further phenotypic analysis, and thus the group will be further denoted as *Bacillus novalis* sp. nov. Overall DNA hybridization values of these strains with respect to representatives of cluster X are in the range 55–63%. Although these values are just below the threshold for species delineation, the two groups were found to be phenotypically

distinct from each other (see below), which supports separate species status for the two groups. Group X strains are proposed as *Bacillus vireti* sp. nov.

Fatty acid analysis

Overall, the fatty acid profiles of the grassland isolates are quite similar. However, on the basis of quantitative (percentage) distribution of the dominant fatty acids, two groups of isolates can be recognized: one group contains *B. novalis* (groups A and B), *B. vireti* (X) and *B. soli* (Y) isolates, while another group contains *B. bataviensis* (C) and *B. drementensis* (D) strains. This second group differs from the

Table 2. DNA–DNA relatedness among representatives of the Drentse A grassland isolates and *B. niacini* LMG 16677^T

Strain	Cluster	DNA–DNA relatedness (%) to:																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. IDA0078	A	100																
2. IDA3307 ^T	A	100	100															
3. IDA3493	a	79	–	100														
4. IDA3829	B	69	–	68	100													
5. IDA2265	b	73	–	73	70	100												
6. IDA3632 ^T	X	55	59	–	59	–	100											
7. IDA3351	X	58	61	–	–	–	99	100										
8. IDA3120	X	63	–	–	56	–	–	101	100									
9. IDA0206	Y	–	–	–	–	–	–	–	–	100								
10. IDA2066	Y	30	–	–	33	–	33	–	30	98	100							
11. IDA0086 ^T	Y	31	–	–	33	–	32	–	31	–	99	100						
12. IDA0084	C	–	–	–	–	–	–	–	–	–	–	–	100					
13. IDA1115 ^T	C	–	–	–	–	–	–	–	–	29	24	–	83	100				
14. IDA1626	c	–	–	–	–	–	–	–	–	32	30	–	–	94	100			
15. IDA1967 ^T	D	21	21	–	–	–	20	18	–	–	–	–	22	20	–	100		
16. IDA1113	D	–	–	–	–	–	–	–	–	–	–	–	21	21	–	95	100	
17. <i>B. niacini</i> LMG 16677 ^T	–	15	–	15	15	14	–	–	–	22	26	–	–	23	20	22	24	100

Table 3. Comparison of mean fatty acid profiles of the five novel taxa as measured by GC of fatty acid methyl esters

Data are mean percentages of total fatty acids $\pm$ SD. Only fatty acids accounting for at least 1.0% of the total fatty acid content are listed. The summed feature comprises iso-C_{17:1} I and/or anteiso-C_{17:1} B.

Fatty acid	<i>B. novalis</i> (13 isolates)	<i>B. vireti</i> (3 isolates)	<i>B. soli</i> (5 isolates)	<i>B. bataviensis</i> (11 isolates)	<i>B. drentensis</i> (10 isolates)
iso-C _{14:0}	5.3 $\pm$ 1.9	3.1 $\pm$ 1.2	3.3 $\pm$ 0.5	6.9 $\pm$ 1.1	8.7 $\pm$ 2.5
C _{14:0}	3.0 $\pm$ 1.3	1.6 $\pm$ 0.2	< 1.0	1.5 $\pm$ 0.6	1.4 $\pm$ 1.3
iso-C _{15:0}	43.9 $\pm$ 5.4	47.1 $\pm$ 3.5	42.9 $\pm$ 2.3	36.9 $\pm$ 4.3	32.2 $\pm$ 4.2
anteiso-C _{15:0}	31.0 $\pm$ 2.6	33.6 $\pm$ 2.0	33.5 $\pm$ 1.9	20.5 $\pm$ 3.0	21.8 $\pm$ 5.6
C _{16:1} ω 7c alcohol	1.7 $\pm$ 0.8	< 1.0	2.4 $\pm$ 0.6	2.3 $\pm$ 0.8	3.1 $\pm$ 0.8
iso-C _{16:0}	2.7 $\pm$ 1.3	2.5 $\pm$ 1.8	1.4 $\pm$ 0.6	2.4 $\pm$ 0.7	2.1 $\pm$ 1.0
C _{16:1} ω 11c	3.2 $\pm$ 1.1	1.5 $\pm$ 0.1	3.2 $\pm$ 0.8	11.3 $\pm$ 3.7	13.2 $\pm$ 5.5
C _{16:0}	4.8 $\pm$ 2.2	3.8 $\pm$ 1.0	1.6 $\pm$ 1.1	7.7 $\pm$ 3.2	3.4 $\pm$ 1.6
iso-C _{17:1} ω 10c	< 1.0	< 1.0	4.1 $\pm$ 1.1	1.7 $\pm$ 0.5	4.5 $\pm$ 1.4
Summed feature	< 1.0	< 1.0	1.8 $\pm$ 0.5	< 1.0	< 1.0
iso-C _{17:0}	< 1.0	2.0 $\pm$ 0.8	2.6 $\pm$ 0.7	1.4 $\pm$ 0.8	2.6 $\pm$ 0.6
anteiso-C _{17:0}	1.9 $\pm$ 0.7	2.7 $\pm$ 0.6	1.5 $\pm$ 0.4	1.1 $\pm$ 0.4	1.1 $\pm$ 0.3
C _{18:1} ω 9c	< 1.0	< 1.0	< 1.0	1.3 $\pm$ 0.8	1.8 $\pm$ 1.1
C _{18:0}	< 1.0	< 1.0	< 1.0	2.7 $\pm$ 1.4	1.3 $\pm$ 0.9

first by having smaller amounts of the dominant fatty acids iso-C_{15:0} and anteiso-C_{15:0} and by having moderate amounts of C_{16:1} ω 11c (Table 3).

Cultural and physiological characteristics

In the numerical analysis of API (bioMérieux) tests and other phenotypic characteristics (data not shown), strains representing clusters A and B (both attributed to *B. novalis*) were not separable, which was in accordance with the hybridization data. *B. novalis* strains showed an S_G (Gower similarity coefficient) of 90% and their nearest neighbours in the analysis were strains of *Bacillus oleronius* and isolates of *Bacillus shackletonii* from Candlemas Island (Logan *et al.*, 2004), at 82.5% S_G. Thus, *B. novalis* forms a group that is phenotypically distinct from other *Bacillus* species and from the other Drentse A isolates. For the *B. vireti* isolates, inconsistent results were obtained. Although IDA3632^T and IDA3351 showed quite high similarity (92.5% S_G) and joined with isolates of *Bacillus cereus* at only 85% S_G, strain IDA3120 showed high similarity (92.5% S_G) with *B. soli*. Such heterogeneous phenotypic patterns were unexpected, since (GTG)₅-PCR and DNA-DNA hybridization showed that the three isolates were genotypically very homogeneous, but, as IDA3120 generally showed weak reactions, it is probable that the standard test medium used did not provide suitable growth conditions for all of the strains, resulting in a less stable phenotypic pattern. All isolates of *B. soli*, on the other hand, showed very high similarity to each other (95% S_G), but were only separated from the type strain of *Bacillus horikoshii* by a difference of 5% in similarity, and from the type strain of *Bacillus firmus* by a 7.5% similarity difference. Therefore, although this group is phenotypically distinct and homogeneous, the characteristics used in this study may not always allow its reliable

separation from phenotypically similar species. Isolates belonging to *B. bataviensis* clustered quite loosely and then merged with *B. novalis* and the *B. oleronius* and *B. shackletonii* clusters at 80% S_G. *B. bataviensis*, therefore, shows greater heterogeneity than *B. novalis*, being less well separated from the other *Bacillus* species. *B. drentensis* isolates merged at 87.5% S_G, but were separated from representatives of *Bacillus lentus* (including the type strain) by a difference of only 5% in similarity. Therefore, although *B. drentensis* appears to form a coherent group in this analysis, it is not easily distinguishable from *B. lentus* by these phenotypic tests.

The phenotypic profiles used to distinguish the five groups of Drentse A isolates from each other and from phenotypically similar *Bacillus* species are shown in Table 4. Many tests give weak or variable reactions, so discrimination depends upon patterns of features, rather than on the presence or absence of features characteristic of an individual taxon.

Description of *Bacillus novalis* sp. nov.

Bacillus novalis (no.va'lis. L. gen. n. *novalis* of fallow land).

Cells are Gram-positive, facultatively anaerobic, motile, slightly curved, round-ended rods (0.6–1.2 μ m in diameter), occurring singly and in pairs and occasionally in short chains or filaments. Endospores are mainly ellipsoidal and lie in subterminal, and occasionally paracentral, positions in slightly swollen sporangia (Fig. 3a). When grown on TSA, colonies are raised, with slightly irregular margins and smooth or eggshell-textured surfaces, sometimes with an iridescent centre when viewed by low-powered microscopy; the consistency is butyrous. Colonies

Table 4. Differential characteristics of Drentse A grassland isolates and phenotypically related species

Taxa: 1, *B. novalis* sp. nov.; 2, *B. bataviensis* sp. nov.; 3, *B. drementensis* sp. nov.; 4, *B. vireti* sp. nov.; 5, *B. soli* sp. nov.; 6, *B. niacini* (data from Nagel & Andreessen, 1991); 7, *B. oleronius*; 8, *B. firmus*; 9, *B. lentus*. Data were from this study unless indicated otherwise. Symbols: +, >85% positive; +/w, positive or weakly positive; (+), 75–84% positive or weakly positive; –, <15% positive or weakly positive; (–), 16–25% positive or weakly positive; v, results vary between strains; w, always weak. ND, Not determined. All grassland isolates investigated in this study gave positive results for hydrolysis of aesculin and for acid production from *N*-acetyl-D-glucosamine, D-fructose, D-glucose and maltose. All strains gave negative results for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulfide production, urease, tryptophan deaminase, indole production and acid production from D-arabinose, L-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, 2-keto-D-gluconate, methyl D-xyloside, L-sorbose, D-tagatose, xylitol and L-xylose.

Characteristic	1	2	3	4	5	6	7	8	9
Gram stain	+	+/v	+/v	–	+/v	v	v	+	ND
Cell ends*	R	(T)	T	R/(T)	R	R	ND	ND	ND
Spore shape†	E	E(C)	E/C	E	E	E	E	E‡	E‡
Spore position§	St(Pc)	Ce/Pc/(St)	Pc(St)	Ce/Pc/(St)	Pc	Ce/St	St	ND	ND
Sporangia swollen	w	w	+	w	w	–/w	ND	–‡	–‡
Growth at 50 °C	+	+	+	–	–	–	–	–‡	–‡
Anaerobic growth	+	+	+	+	+	ND	–	–‡	–‡
Hydrolysis of:									
Casein	+	–	–	+	+	–	–	+	v‡
Gelatin	v	+	–	+	+	+	v	v	–
Storage inclusions	–	–	+	–	–	ND	–	–	–
ONPG	–	+	+	v	–	ND	–	–	+
Urease	–	–	–	–	–	(–)	–	–	+
Nitrate reduction to N ₂	+	+	v	+	+	(+)	+	+/w	+
Acid production from:									
Amygdalin	v	v	v	–	–	+			
D-Cellobiose	v	+	–	–	–	v	+/w	–	+/w
L-Fucose	–	v	–	w	–	–	–	–	–
β-Gentiobiose	v	+	–	–	–	v	–	–	+/w
Glycerol	v	w	–	–	–	–	w	w	–
Glycogen	–	–	–	+	+	(+)	–	v	–
Lactose	–	+	+	–	–	(+)	–	–	+/w
D-Mannitol	v	+	–	+	–	(–)	+/w	(+)	+/w
D-Melibiose	–	v	+	–	–	+	–	–	+/w
D-Melezitose	–	+	v	–	–	v	–	–	+/w
Methyl α-D-glucoside	–	v	v	w	–	v	–	–	v/w
Raffinose	–	+	v	–	–	(+)	–	–	+/w
Ribose	v	w	v	w	w	v	+/w	(–)	+/w
Salicin	–	w	+	–	–	v	–	–	+/w
Starch	–	v	v	+	+	ND	v	(+)	+/w
Sucrose	–	v	v	+	v	+	–	(+)	+/w
D-Turanose	–	+	v	–	–	(–)	–	–	v/w
G + C content (mol%)¶	40·0–40·5	39·6–40·1	39·3–39·4	39·8–40·3	40·1–40·4	37·0–39·0	35‡	36·1–47·4‡	36·5–47·3‡

*R, Rounded; T, tapered; (T), slightly tapered.

†C, Circular; E, ellipsoidal.

‡Data from Kuhnigk *et al.* (1995) for *B. oleronius* and from Claus & Berkeley (1986) for *B. firmus* and *B. lentus*.

§Ce, Central; St, subterminal; Pc, paracentral.

||Type strain DSM 2923^T was the only strain that gave a different result.

¶Determined for *B. novalis* IDA0078, IDA2265, IDA3307^T, IDA3493 and IDA3829, *B. bataviensis* IDA0084, IDA1115^T and IDA1626, *B. drementensis* IDA1113 and IDA1967^T, *B. vireti* IDA3120, IDA3351 and IDA3632^T and *B. soli* IDA0086^T, IDA0206 and IDA2066.

are cream-coloured and produce a light-brown pigment that diffuses into the agar. Optimal growth occurs at 30–40 °C; the maximum growth temperature lies between 50 and

55 °C. The minimum pH for growth lies between 4·0 and 5·0, the optimum pH is 7·0–9·0 and the maximum pH lies between 9·5 and 10·0. Casein is hydrolysed. In the API 20E

Fig. 3. Photomicrographs of sporangia and vegetative cells of (a) *B. novalis* sp. nov. LMG 21837^T, (b) *B. vireti* sp. nov. LMG 21834^T, (c) *B. soli* sp. nov. LMG 21838^T, (d) *B. bataviensis* sp. nov. LMG 21833^T and (e) *B. drementensis* sp. nov. LMG 21831^T. Bars, 3 µm.

strip, the Voges–Proskauer reaction is negative, gelatin is hydrolysed by most strains and nitrate reduction is positive (sometimes weakly); reactions for ONPG hydrolysis, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulfide production, urease, tryptophan deaminase and indole production are negative. Aesculin hydrolysis is positive in the API 50CH gallery. Acid without gas is produced (weakly by some strains) from the following carbohydrates in the API 50CH gallery, using CHB suspension medium (bioMérieux): *N*-acetyl-D-glucosamine, D-fructose, galactose (always weak), D-glucose, maltose, D-mannose and D-trehalose. The following reactions are variable between strains, and, when positive, are usually weak: amygdalin, arbutin, D-cellobiose, β -gentiobiose, gluconate, glycerol, 5-keto-D-gluconate, D-lyxose, D-mannitol, ribose, sorbitol, D-xylose. Acid is not produced from the following carbohydrates: D-arabinose, L-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, glycogen, inulin, 2-keto-D-gluconate, lactose, D-melezitose, D-melibiose, *meso*-inositol, methyl α -D-glucoside, methyl α -D-mannoside, methyl D-xyloside, raffinose, rhamnose, salicin, L-sorbose, starch, sucrose, D-tagatose, D-turanose, xylitol, D-xylose and L-xylose. The major cellular fatty acids are iso-C_{15:0} and anteiso-C_{15:0}, respectively present at levels of about 44 and 31 % of the total fatty acid content. The following fatty acids are present to at least 1 %: iso-C_{14:0}, C_{14:0}, C_{16:1} ω 7c

alcohol, iso-C_{16:0}, C_{16:1} ω 11c, C_{16:0} and anteiso-C_{17:0}. For the strains tested (Table 4), the G + C content is 40.0–40.5 mol%. Isolated from soil (Drentse A agricultural research area, The Netherlands).

In the variable reactions listed above, the type strain, LMG 21837^T (= R-15439^T = IDA3307^T = DSM 15603^T), was positive for amygdalin (weak), D-cellobiose (weak), β -gentiobiose (weak), D-mannitol (weak), sorbitol and D-xylose. The G + C content for the type strain is 40.5 mol%.

Description of *Bacillus vireti* sp. nov.

Bacillus vireti (vi.re'ti. L. gen. n. *vireti* of the field).

Cells are Gram-negative, facultatively anaerobic, motile, slightly curved, round-ended rods (0.6–0.9 µm in diameter) occurring singly and in pairs (Fig. 3b). Cells do not produce endospores on TSA supplemented with MnSO₄, but sporulate on *B. fumarioli* agar at pH 7 after 48 h. Endospores are ellipsoidal, lie in central, paracentral and sometimes subterminal positions and may swell the sporangia slightly; the ends of the sporangia may be slightly tapered. After 3 days growth on TSA, colonies are up to 4 mm in diameter, circular, raised, with entire edges and dark-cream in colour. The surface has an eggshell-like texture and the biomass is of loose consistency. The optimum temperature for growth is 30 °C and the

maximum growth temperature is 40–45 °C. The minimum pH for growth is 4.0–5.0 and the optimum and maximum pH values for growth are in the range 7.0–7.5. Casein is hydrolysed. In the API 20E strip, gelatin is hydrolysed and nitrate reduction is positive; ONPG hydrolysis is variable; reactions for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulfide production, urease, tryptophan deaminase, indole production and the Voges–Proskauer test are negative. Hydrolysis of aesculin is positive in the API 50CH gallery. Acid without gas is produced from the following carbohydrates in the API 50CH gallery, using the CHB suspension medium: *N*-acetyl-D-glucosamine, D-fructose, L-fucose (weak), galactose (weak), D-glucose, glycogen, maltose, D-mannitol, D-mannose, methyl α -D-glucoside (weak), ribose (weak), starch, sucrose and D-trehalose. The following reactions are variable between strains and, when positive, are usually weak: gluconate, *meso*-inositol, methyl α -D-mannoside, rhamnose. Acid is not produced from the following carbohydrates: adonitol, amygdalin, D-arabinose, L-arabinose, D-arabitol, L-arabitol, arbutin, D-cellobiose, dulcitol, erythritol, D-fucose, β -gentiobiose, glycerol, inulin, 2-keto-D-gluconate, 5-keto-D-gluconate, lactose, D-lyxose, D-melezitose, D-melibiose, methyl D-xyloside, raffinose, salicin, sorbitol, L-sorbose, D-tagatose, D-turanose, xylitol and D-xylose and L-xylose. The major cellular fatty acids are iso-C_{15:0} and anteiso-C_{15:0}, respectively present at about 47 and 34 %. The following fatty acids are present to at least 1 %: iso-C_{14:0}, C_{14:0}, iso-C_{16:0}, C_{16:1} ω 11c, C_{16:0}, iso-C_{17:0} and anteiso-C_{17:0}. For the strains tested (Table 4), the G + C content is 39.8–40.3 mol%. Isolated from soil (Drentse A agricultural research area, The Netherlands).

In the variable reactions listed above, the type strain, LMG 21834^T (=R-15447^T=IDA3632^T=DSM 15602^T), was weak for gluconate and methyl α -D-mannoside and negative for *meso*-inositol and rhamnose. The G + C content of the type strain is ~40.2 mol%.

Description of *Bacillus soli* sp. nov.

Bacillus soli (so'li. L. gen. n. *soli* of soil).

Cells are Gram-positive or Gram-variable, facultatively anaerobic, motile, round-ended rods (0.6–1.2 μ m in diameter), sometimes curved, occurring as single cells, in pairs and in chains. Endospores are ellipsoidal, lie paracentrally and may swell the sporangia (Fig. 3c). On TSA, colonies are butyrous, cream-coloured, low and slightly umbonate, with entire margins and glossy or eggshell-textured surfaces. The optimum growth temperature is 30 °C and the maximum growth temperature is between 40 and 45 °C. The minimum pH for growth lies between 4.0 and 5.0, the optimum is 7.0–8.0 and the maximum lies between 9.0 and 9.5. Casein is hydrolysed. In the API 20E strip, gelatin is hydrolysed and nitrate reduction is positive; reactions for ONPG hydrolysis, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulfide production,

urease, tryptophan deaminase, indole production and the Voges–Proskauer test are negative. Hydrolysis of aesculin is positive in the API 50CH gallery. Acid without gas is produced from the following carbohydrates in the API 50CH gallery, using CHB suspension medium: *N*-acetyl-D-glucosamine, D-fructose, D-glucose, glycogen, maltose (weak), D-mannose, ribose (weak), starch and D-trehalose (weak). The following reactions are variable between strains and, when positive, are usually weak: galactose and sucrose. Acid is not produced from the following carbohydrates: adonitol, amygdalin, D-arabinose, L-arabinose, D-arabitol, L-arabitol, arbutin, D-cellobiose, dulcitol, erythritol, D-fucose, L-fucose, β -gentiobiose, glycerol, inulin, 2-keto-D-gluconate, 5-keto-D-gluconate, lactose, D-lyxose, D-mannitol, D-melezitose, D-melibiose, methyl α -D-glucoside, methyl D-xyloside, raffinose, salicin, sorbitol, L-sorbose, D-tagatose, D-turanose, xylitol, D-xylose and L-xylose. The major cellular fatty acids are iso-C_{15:0} and anteiso-C_{15:0}, respectively present at about 43 and 34 %. The following fatty acids are present to at least 1 %: iso-C_{14:0}, C_{16:1} ω 11c, C_{16:0}, iso-C_{17:0} and anteiso-C_{17:0}. For the strains tested (Table 4), the G + C content is 40.1–40.4 mol%. Isolated from soil (Drentse A agricultural research area, The Netherlands).

In the variable reactions listed above, the type strain, LMG 21838^T (=R-16300^T=IDA0086^T=DSM 15604^T), was positive for galactose (weak) and negative for sucrose. The G + C content of the type strain is 40.1 mol%.

Description of *Bacillus bataviensis* sp. nov.

Bacillus bataviensis (ba.ta.vi.en'sis. L. masc. adj. *bataviensis* pertaining to *Batavia*, the name Julius Caesar gave to The Netherlands).

Gram-positive or variable (at 24 h), facultatively anaerobic, motile, slightly tapered rods (0.7–1.2 μ m in diameter) occurring singly, in pairs and in short chains. Endospores are mainly ellipsoidal but may be spherical, and lie centrally, paracentrally and occasionally subterminally, in slightly swollen sporangia (Fig. 3d). Colonies grown on TSA are butyrous, cream-coloured and produce a light-brown pigment that diffuses into the agar; they are slightly raised and umbonate, have regular margins and have smooth or rough, eggshell-textured surfaces. The optimum temperature for growth is 30 °C and the maximum growth temperature lies between 50 and 55 °C. The minimum pH for growth lies between 4.0 and 6.0, the optimum pH is 7.0–8.0 and the maximum pH lies between 9.5 and 10.0. Casein is not hydrolysed. In the API 20E strip, ONPG hydrolysis is positive, gelatin is hydrolysed by most strains and nitrate reduction is positive; the Voges–Proskauer reaction is negative and reactions for arginine dihydrolase (one strain positive), lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulfide production, urease, tryptophan deaminase and indole production are negative. Hydrolysis of aesculin is positive in the API 50CH gallery. Acid without gas is produced from the

following carbohydrates in the API 50CH gallery, using CHB suspension medium: *N*-acetyl-D-glucosamine, D-cellobiose, D-fructose, galactose, β -gentiobiose, D-glucose, glycerol (weak), lactose, maltose, D-mannitol, D-mannose, D-melezitose, raffinose, ribose (weak), salicin (weak), D-trehalose and D-turanose. The following reactions are variable between strains and, when positive, are usually weak: amygdalin, arbutin, L-fucose, inulin, D-melibiose, methyl α -D-glucoside, methyl α -D-mannoside, starch and sucrose. Acid is not produced from the following carbohydrates: adonitol, D-arabinose, L-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, gluconate, glycogen, 2-keto-D-gluconate, 5-keto-D-gluconate, D-lyxose, *meso*-inositol, methyl D-xyloside, rhamnose, sorbitol, L-sorbose, D-tagatose, xylitol, D-xylose and L-xylose. The major cellular fatty acids are iso-C_{15:0} and anteiso-C_{15:0}, respectively present at about 37 and 21 %, while C_{16:1} ω 11c accounts for about 11 % of the total fatty acids. The following fatty acids are present to at least 1 %: iso-C_{14:0}, C_{14:0}, C_{16:1} ω 7c alcohol, iso-C_{16:0}, C_{16:0}, iso-C_{17:1} ω 10c, iso-C_{17:0}, anteiso-C_{17:0}, C_{18:1} ω 9c and C_{18:0}. For the strains tested (Table 4), the G + C content is 39.6–40.1 mol%. Isolated from soil (Drentse A agricultural research area, The Netherlands).

In the variable reactions, the type strain, LMG 21833^T (= R-16315^T = IDA1115^T = DSM 15601^T), was positive but weak for arbutin, L-fucose, inulin, D-melibiose, methyl α -D-glucoside, methyl α -D-mannoside and sucrose and negative for amygdalin and starch. The G + C content for the type strain is 40.1 mol%.

Description of *Bacillus drentensis* sp. nov.

Bacillus drentensis (dren.ten'sis. N.L. masc. adj. *drentensis* of Drente, a province in The Netherlands).

Cells are Gram-positive or Gram-variable, facultatively anaerobic, motile, tapered rods (0.6–1.2 μ m in diameter) occurring singly and in pairs. Cells show pleomorphism (narrow and broad cells, the latter showing swellings) and produce intracellular storage inclusions on TSA. Endospores are spherical or ellipsoidal and lie in paracentral or occasionally subterminal positions in swollen sporangia (Fig. 3e). Colonies are slightly convex with regular margins when small and sometimes wrinkled with irregular margins and prominent centres when larger. Colonies are cream-coloured and produce a brownish soluble pigment; the consistency is butyrous, with an eggshell-like surface texture. The optimum temperature for growth is 30 °C and the maximum growth temperature lies between 50 and 55 °C. The minimum pH for growth lies between 5.5 and 6.0, the optimum pH is 7.0–8.0 and the maximum pH lies between 9.5 and 10.0. Casein is not hydrolysed. In the API 20E strip, ONPG hydrolysis is positive, the Voges–Proskauer reaction is variable (most strains negative, positive strains weak) and the nitrate reduction is variable; reactions for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen

sulfide production, urease, tryptophan deaminase, indole production and gelatin hydrolysis are negative. Hydrolysis of aesculin is positive in the API 50CH gallery. Acid without gas is produced from the following carbohydrates in the API 50CH gallery, using CHB suspension medium: *N*-acetyl-D-glucosamine, D-fructose, D-glucose (some strains, including the type strain, weak), lactose, maltose, D-melibiose and salicin (some strains, including the type strain, weak). The following reactions are variable between strains and, when positive, are usually weak: amygdalin, arbutin, galactose, gluconate, inulin, D-mannose, D-melezitose, methyl α -D-glucoside, raffinose, ribose, starch, sucrose, D-trehalose, D-turanose and D-xylose. Acid is not produced from the following carbohydrates: adonitol, D-arabinose, L-arabinose, D-arabitol, L-arabitol, D-cellobiose, dulcitol, erythritol, D-fucose, L-fucose, β -gentiobiose, glycerol, glycogen, 2-keto-D-gluconate, 5-keto-D-gluconate, D-lyxose, D-mannitol, *meso*-inositol, methyl α -D-mannoside, methyl D-xyloside, rhamnose, sorbitol, L-sorbose, D-tagatose, xylitol and L-xylose. The major cellular fatty acids are iso-C_{15:0} and anteiso-C_{15:0}, respectively present at about 32 and 22 %, while C_{16:1} ω 11c accounts for about 13 % of the total fatty acids. The following fatty acids are present to at least 1 %: iso-C_{14:0}, C_{14:0}, C_{16:1} ω 7c alcohol, iso-C_{16:0}, C_{16:0}, iso-C_{17:1} ω 10c, iso-C_{17:0}, anteiso-C_{17:0}, C_{18:1} ω 9c and C_{18:0}. For the strains tested (Table 4), the G + C content is 39.3–39.4 mol%. Isolated from soil (Drentse A agricultural research area, The Netherlands).

In the variable reactions, the type strain, LMG 21831^T (= R-16337^T = IDA1967^T = DSM 15600^T), was positive for inulin, D-mannose, D-melezitose, raffinose (weak), ribose (weak), starch (weak), sucrose and D-turanose (weak) and negative for amygdalin, arbutin, galactose, gluconate, methyl α -D-glucoside, D-trehalose and D-xylose. The G + C content for the type strain is 39.4 mol%.

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