Bacillus arenosi sp. nov., *Bacillus arvi* sp. nov. and *Bacillus humi* sp. nov., isolated from soil

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A group of nine Gram-positive endospore-forming bacteria was isolated from soil of the Drentse A agricultural research area in the Netherlands. Using (GTG)₅-PCR genomic fingerprinting and fatty acid analysis, the nine isolates were divided into three consistent groups. On the basis of 16S rRNA gene sequence similarity of representative strains, the nine isolates were shown to belong to the genus *Bacillus*. The first group of four isolates was most closely related to *Bacillus carboniphilus* (95.5%) and *Bacillus sporothermodurans* (95.5%). The second and third groups of three and two isolates, respectively, showed highest sequence similarity to *Bacillus neidei* (97.0 and 97.1%, respectively) and *Bacillus pycnus* (both 96.7%). A DNA–DNA relatedness study confirmed the consistency of the three groups delineated by (GTG)₅-PCR and fatty acid analysis. A small number of phenotypic characters allowed differentiation of the three groups of isolates. The three groups therefore represent novel species, for which the names *Bacillus humi*, *Bacillus arenosi* and *Bacillus arvi* are proposed, with LMG 22167^T (=DSM 16318^T), LMG 22166^T (=DSM 16319^T) and LMG 22165^T (=DSM 16317^T) as the respective type strains.

The genus *Bacillus* was first described by Cohn in 1872 and since then the number of *Bacillus* species has fluctuated widely among the eight editions of Bergey's Manual (Berkeley, 2002), ranging from a peak of 146 species in 1938 to a trough of 22 in 1974 (but with a further 26 appearing as *species incertae sedis*). The introduction of molecular methods, especially the use of 16S rRNA gene sequencing, has had a major impact on *Bacillus* taxonomy and has resulted in splitting of the genus. Ash *et al.* (1991), for example, studied the 16S rRNA gene sequences of 51 *Bacillus* species and revealed five phylogenetically distinct clusters of species and three ungrouped species. Three out of the five clusters and two out of the three ungrouped species have since been transferred to other genera

(Wisotzkey *et al.*, 1992; Ash *et al.*, 1993; Shida *et al.*, 1996; Nazina *et al.*, 2001) and currently 59 species originally described as *Bacillus* have been transferred to related genera and *Bacillus sensu stricto* contains 99 species. Despite the reduction in the number of species in the genus *Bacillus*, the phylogenetic and physiological heterogeneity of what can now be considered as *Bacillus sensu stricto* is still far too large and the need for further splitting is supported by its widely ranging G + C content, from 31 to 66 mol%.

The Drentse A agricultural research area, along the Anlooër Diepje brook near Anloo (the Netherlands), was the focus of a previous culture-independent study based on temperature-gradient gel electrophoresis (Felske & Akkermans, 1998). This study revealed the presence of bacterial groups that predominated throughout the entire research area. Subsequent research (Felske *et al.*, 1998) showed that hitherto-uncultured *Bacillus* species were the most active bacteria in these soils. Therefore, a large culturing campaign was undertaken using a multiplex PCR to screen specifically for novel *Bacillus*-related lineages in the soil (Felske *et al.*, 2003). Positive isolates were analysed by their partial 16S rRNA gene sequences to verify their taxonomic positions. Among the isolates, nine strains were present that, according

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of LMG 22165^T, LMG 22166^T, LMG 22167^T and LMG 22168 are AJ627211, AJ627212, AJ627210 and AJ627209, respectively.

The sporangial morphologies of *B. arenosi* and *B. humi* are provided as supplementary material in IJSEM Online.

Fig. 1. Grouping based on normalized (GTG)₅-PCR patterns (a) and cellular fatty acid profiles (b) of the grassland isolates. Groups obtained by partial 16S rRNA gene sequencing as explained in the text are shown in (a) (16S rRNA groups 1 and 2). Novel species names are indicated in (b).

to partial 16S rRNA gene sequencing, could represent at least two novel species; their strain numbers are given in Fig. 1. Sampling, enrichment, cultivation, high-throughput processing and screening with multiplex PCR were performed as described previously by Felske *et al.* (2003). Strains were subcultured further on nutrient agar (NA).

Purification of total genomic DNA for 16S rRNA gene sequencing and repetitive extragenic palindromic (rep)-PCR fingerprinting was performed as described by Heyndrickx *et al.* (1996). Sequence analysis was performed as described previously by Heyrman & Swings (2001). Phylogenetic trees were constructed using the BioNumerics software version 3.5 (Applied Maths) by applying the neighbour-joining and maximum-parsimony methods on a multiple-alignment similarity matrix. The stabilities of relationships were assessed by a bootstrap analysis of 1000 datasets. rep-PCR genomic fingerprinting was performed with the (GTG)₅ (microsatellite-specific) primer (Versalovic *et al.*, 1994) applying PCR conditions described by Rademaker & de Bruijn (1997). Electrophoresis and pattern analysis were performed as described by Heyrman *et al.* (2003). Analysis of the fatty acid content of the cell walls was performed as described previously (Heyrman *et al.*, 1999), starting from cells grown on tryptic soy agar (TSA) for 48 h.

On the basis of partial 16S rRNA gene sequencing targeting the first ~500 bp of the hypervariable region (Goto *et al.*, 2000), the nine strains could be divided into two groups. The partial 16S rRNA gene sequences (~480 bp) of the strains attributed to the first group (LMG 22167^T, LMG 22168, R-17036 and R-19269) were highly similar, showing only three positions that contained a double reading in some of the strains and not in the others. In addition, the sequence of R-17036 had an insertion of an extra base not

found in the other strains. Members of the second group of strains were also highly similar in their partial 16S rRNA gene sequences: strains LMG 22165^T and R-16994 showed identical sequences and this was also the case for LMG 22166^T, R-17172 and R-17173. LMG 22165^T and LMG 22166^T showed only one base pair difference in the first 480 bp of the 16S rRNA gene. The partial sequence similarity between the two groups of strains was only 84–86%.

To assess the variability among the grassland isolates further, they were investigated by (GTG)₅-PCR genomic fingerprinting (Fig. 1a) and fatty acid analysis (Fig. 1b). As expected, the two groups as revealed by 16S rRNA gene sequencing were also easily distinguishable by these two methods. Strains attributed to 16S rRNA group 1 showed quite variable (GTG)₅ patterns and formed a coherent fatty acid group. In 16S rRNA group 2, strains with identical partial sequences also showed identical (GTG)₅ patterns and the one base pair difference between these two subgroups was reflected by a different pattern. In fatty acid analysis, the difference between the two subgroups was more pronounced: the mean percentages of the main fatty acid iso-C_{15:0} were approximately 46 and 23% for the subgroups containing LMG 22166^T and LMG 22165^T, respectively (Table 1). This led to the hypothesis that, despite their high partial 16S rRNA gene sequence similarity, the two subgroups might represent two separate novel species. In a MIDI database search of the profiles generated for the grassland strains, the similarity with all existing *Bacillus* species included in the TSBA 4.0 database was below 50%.

Based on the data obtained by (GTG)₅-PCR and fatty acid analysis, representatives were selected for determination of their nearly complete 16S rRNA gene sequences (~1500 bp), their DNA–DNA relatedness and their G+C content

Table 1. Comparison of mean fatty acid profiles of the three novel taxa and *B. neidei* and *B. pycnus* 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. Data for *B. neidei* and *B. pycnus* are from Nakamura *et al.* (2002).

Fatty acid	<i>B. arvi</i> (n=2)	<i>B. arenosi</i> (n=3)	<i>B. humi</i> (n=4)	<i>B. neidei</i>	<i>B. pycnus</i>
iso-C _{14:0}	12.8 $\pm$ 0.3	5.2 $\pm$ 1.7	14.9 $\pm$ 2.1	5.0	—
C _{14:0}	1.9 $\pm$ 0.1	1.8 $\pm$ 0.4	< 1.0	—	—
iso-C _{15:0}	23.4 $\pm$ 2.1	46.2 $\pm$ 3.4	33.4 $\pm$ 2.7	23.6	70.3
anteiso-C _{15:0}	16.2 $\pm$ 1.1	9.8 $\pm$ 1.0	38.7 $\pm$ 4.7	17.8	8.1
C _{15:0}	2.2 $\pm$ 0.5	3.7 $\pm$ 0.9	< 1.0	1.7	—
C _{16:1} ω 7c alcohol	10.7 $\pm$ 1.2	4.3 $\pm$ 1.2	4.1 $\pm$ 0.7	4.7	6.0
iso-C _{16:0}	15.1 $\pm$ 1.2	5.4 $\pm$ 2.1	1.8 $\pm$ 1.1	8.1	3.1
C _{16:1} ω 11c	6.1 $\pm$ 0.4	7.3 $\pm$ 0.8	1.8 $\pm$ 0.4	14.5	1.4
C _{16:0}	1.3 $\pm$ 0.0	1.5 $\pm$ 0.4	< 1.0	5.4	—
iso-C _{17:1} ω 10c	1.3 $\pm$ 0.1	4.9 $\pm$ 0.1	< 1.0	—	—
Summed feature	2.4 $\pm$ 0.1	2.7 $\pm$ 0.2	1.0 $\pm$ 0.2	—	—
iso-C _{17:0}	1.9 $\pm$ 0.2	3.4 $\pm$ 0.5	< 1.0	—	—
anteiso-C _{17:0}	5.0 $\pm$ 0.4	3.1 $\pm$ 0.1	1.4 $\pm$ 0.6	—	—

(mol%). The selected strains were the two most divergent strains of 16S rRNA group 1 (LMG 22167^T and LMG 22168) and one strain from each subgroup of 16S rRNA group 2 (LMG 22165^T and LMG 22166^T). The nearly complete 16S rRNA gene sequences of LMG 22167^T and LMG 22168 only differed at one position (in the hypervariable region), which showed a T for the former and a double reading (C or T) for the latter. The sequences of LMG 22165^T and LMG 22166^T, each representing a subgroup of 16S rRNA group 2, showed mismatches at three positions. For LMG 22167^T and LMG 22168 (16S rRNA group 1), a FASTA search (Pearson & Lipman, 1988) gave *Bacillus carboniphilus* and *Bacillus endophyticus* as the closest related established species (both $\sim$ 95.5% sequence similarity). The sequences of LMG 22165^T and LMG 22166^T (16S rRNA group 2) showed highest similarities to the type strains of *Bacillus neidei* (97.1 and 97.0%, respectively), *Bacillus pycnus* (both 96.7%) and *Kurthia sibirica* (both 96.7%). The close relatedness with *Kurthia* is not unexpected since this genus, and also the genus *Caryophanon*, clearly group within *Bacillus* in a dendrogram based on 16S rRNA gene sequences (Fig. 2). The strains of 16S rRNA group 2 phylogenetically clustered together with *B. neidei* and formed endospores (although slow and scarce on rich media; see below), thereby supporting their attribution to the genus *Bacillus*. It is clear that the current taxonomic situation at the generic level can be criticized. From our data, a separate genus status for *Kurthia*, based on the absence of endospores (Keddie & Shaw, 1986), does not coincide with the phylogenetic position of at least some of the taxa. However, whether species of *Kurthia* and *Caryophanon* should be transferred to the genus *Bacillus*, or vice versa, remains an open question. Farrow *et al.* (1994) performed a 16S rRNA gene-based phylogenetic study on bacilli containing cell walls based on lysine (group 2 bacilli of Ash *et al.*, 1991) and

several non-spore-forming genera including *Kurthia*, and concluded that phylogenetically the most consistent policy would be to exclude the Ash *et al.* group 2 bacilli (*Bacillus sphaericus*, *Bacillus fusiformis*, *Bacillus insolitus*, etc.) from the genus *Bacillus*. However, the Ash *et al.* group 2 bacilli are embedded in a larger cluster of *Bacillus sensu stricto* species and renaming of the group 2 bacilli would also result in a phylogenetically unsatisfactory taxonomic situation. Because additional data are needed to clarify the taxonomic

Fig. 2. Phylogenetic positions based on neighbour-joining of the 16S rRNA gene sequences of representative grassland isolates among related *Bacillus* species and members of the genera *Kurthia* and *Caryophanon*. Bootstrap values (expressed as percentages of 1000 replications) greater than 60% are shown at branch points.

situation, the soil isolates studied here belonging to 16S rRNA group 2 are best attributed to the genus *Bacillus*.

For determination of the G+C content and DNA–DNA hybridization, approximately 1 g biomass was harvested from agar plates and DNA was purified as described by Logan *et al.* (2000). DNA–DNA hybridization was performed using a modification of the microplate method described by Ezaki *et al.* (1989), as described by Willems *et al.* (2001). A hybridization temperature of 37 °C was used. The G+C content of DNA was determined by HPLC (Mesbah *et al.*, 1989), using further specifications given by Logan *et al.* (2000). Generally recommended and accepted criteria for delineating bacterial species state that strains showing 3 % or more 16S rRNA gene sequence dissimilarity are considered to be representatives of separate species (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). Since the 16S rRNA gene sequence similarities of the grassland strains to all established species in the GenBank/EMBL/DDBJ databases were around or just below this level, DNA–DNA hybridization experiments were only performed between representatives of the grassland isolates. The DNA–DNA relatedness value for LMG 22167^T and LMG 22168, representative of 16S rRNA group 1, was 87.0 %, indicating that 16S rRNA group 1 represents a single novel *Bacillus* genomospecies, which will subsequently be referred to as *Bacillus humi*. The DNA–DNA relatedness between the representatives of the two subgroups of 16S rRNA group 2 was 42.0 %. Therefore the strains of the two subgroups can be allocated to two novel genomospecies. Strains LMG 22165^T and R-16994 are hereafter referred to as *Bacillus arvi*, and strains LMG 22166^T, R-17172 and R-17173 as *Bacillus arenosi*.

The G+C content of *B. humi* (determined for LMG 22167^T and LMG 22168) was 37.5 mol%. LMG 22165^T (*B. arvi*) and LMG 22166^T (*B. arenosi*) both had a G+C content of 35.0 mol%. For *B. arvi* and *B. arenosi*, the G+C content was in the range of the related *Bacillus* species listed in Fig. 2 (34–41 %; Claus & Berkeley, 1986; Rheims *et al.*, 1999; Abd El-Rahmanj *et al.*, 2002; Nakamura *et al.*, 2002) and of the genus *Kurthia* (36–38 %; Keddie & Shaw, 1986; Belikova *et al.*, 1986), but lower than the range given for *Caryophanon* (41–46 %; Trentini, 1986).

The strains were phenotypically characterized by the methods of Logan & Berkeley (1984); other characters were determined and the data numerically analysed, as described by Logan *et al.* (2000). For observations on sporangia, cells were grown on NA supplemented with 5 mg MnSO₄ l⁻¹ (NA MnSO₄) at 30 °C. Vegetative cells and sporangia were observed by phase-contrast microscopy. Strains were also examined for catalase and oxidase production and casein and starch hydrolysis using the methods of Gordon *et al.* (1973). Maximum and minimum growth temperatures were determined by incubating 10 ml tryptic soy broth (TSB) cultures in a water bath; pH ranges for growth were determined using 10 ml TSB cultures adjusted to pH 5.0, 7.0 and 9.0; both series were examined

for turbidity at 24 h intervals. Growth under anaerobic conditions was tested in GasPak (BBL) jars. Strains were also tested for assimilation of substrates as sole carbon sources, using the API Biotype 100 kit as described by Heyndrickx *et al.* (1997).

In the phenotypic characterization tests, none of the strains was particularly reactive, and in the Biotype 100 substrate assimilation tests, none of the strains gave readable positive results. Consequently, although the three new species could be recognized and differentiated on the basis of routine tests, as the descriptions below make clear, the separation of the three species and their distinction from phenotypically similar organisms relied on relatively few features (Table 2). Spore formation of *B. arvi* and *B. arenosi* on NA MnSO₄ was very slow and scarce: for some strains no spore formation could be recorded after 10 days of growth at 30 °C on this medium. Therefore, spore formation was tested further on a poor medium, half-strength *Bacillus fumarioli* agar (½ BFA; Logan *et al.*, 2000) including 5 mg MnSO₄. On ½ BFA spores were formed in both species after approximately 3 days incubation at 30 °C, and after 5 days vegetative cells could no longer be observed. The sporangial morphologies of *B. arenosi* and *B. arvi* are quite distinct from that of *B. humi* (Table 2; supplementary Figs A and B available in IJSEM Online). *B. arvi*, which was similar in fatty acid composition to *B. neidei* (Table 2), could be differentiated from the latter species by a positive urease reaction, slow and scarce endospore formation on NA, casein hydrolysis (weak) and anaerobic growth (weak).

Description of *Bacillus arenosi* sp. nov.

Bacillus arenosi (ar.en.o'si. L. gen. n. *arenosi* of a sandy place).

Cells are straight or slightly curved, round-ended, motile rods (0.8–1.0 × 3.0–8.0 µm) and occur singly and in pairs. Gram-stain reaction is variable amongst cells. Endospore formation is slow and scarce on NA MnSO₄ after 10 days incubation at 30 °C. On ½ BFA, endospores are formed abundantly after approximately 3 days incubation at 30 °C. Endospores are spherical and appear in terminal positions, swelling the sporangia slightly. After 24 h on NA at 30 °C, colonies are cream-coloured, semi-translucent and slightly raised, with irregular margins that may spread along the inoculation streak. They have glossy surfaces and their diameters are 1–2 mm. Strict aerobes. They grow at 20 and 30 °C but are not able to grow at 45 °C. Strains grow at pH 9 as profusely as at pH 7, but are unable to grow at pH 5. Casein is not hydrolysed but biomass on casein agar produces a pale-pink, diffusible pigment. Starch is not hydrolysed. Strains tolerate 5 but not 7 % (w/v) NaCl. Catalase-positive and oxidase-negative. In the API 20E strip, gelatin is weakly hydrolysed and nitrate is reduced, but reactions for *o*-nitrophenyl-β-D-galactoside (ONPG), arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulfide production, urease, indole production and Voges–Proskauer are

Table 2. Characters that differentiate between *B. arenosi*, *B. arvi*, *B. humi* and phenotypically related species

+, >85 % Positive; v, variable (26–74 % positive); –, 0–15 % positive; w, weak positive reaction; ND, no data.

Character	Grassland isolates			Related species*	
	<i>B. arvi</i> (n=2)	<i>B. arenosi</i> (n=3)	<i>B. humi</i> (n=4)	<i>B. sporothermodurans</i>	<i>B. oleronius</i>
Dominant spore shape	Spherical	Spherical	Ellipsoidal	Ellipsoidal	Ellipsoidal
Spore position	Terminal	Terminal	(Sub)Terminal	Terminal	Subterminal
Swelling of sporangia	Slight	Slight	+	–	Slight
Casein hydrolysis	w	–	No growth	–	–
Aesculin hydrolysis	–	–	+	+	w
Hydroxyquinoline- β -D-glucuronide hydrolysis	–	–	+	ND	ND
Pigment on casein agar	–	Pink	–	v	–
Anaerobic growth	w	–	w	+	–
Tolerance to 7 % NaCl	–	–	+	v	+
ONPG	w	–	–	–	–
Urease	+	–	–	–	–
Acid production from:					
D-Fructose	+	–	–	+	+
N-Acetylglucosamine	w	–	–	v	w

**B. neidei*, *B. pycnus* and *B. sphaericus* are strict aerobes, form spherical spores in terminal, swollen sporangia, and do not produce urease. Casein hydrolysis is negative for *B. neidei* and *B. pycnus* and variable for *B. sphaericus*. The *B. sporothermodurans* type strain is totally unreactive in API 20E/50CH tests and forms spherical to oval spores.

negative. In the API 50CHB gallery, acid is not produced from any of the carbohydrate substrates. In the Biotype 100 kit, no substrate is used as a single carbon source. The major cellular fatty acid is iso-C_{15:0}, present at approximately 46 %. The following fatty acids are present to at least 5 %: iso-C_{14:0}, anteiso-C_{15:0}, iso-C_{16:0} and C_{16:1} ω 11c.

The G + C content is 35.0 mol% for the type strain, LMG 22166^T (=DSM 16319^T). Isolated from soil (Drentse A agricultural research area, the Netherlands).

Description of *Bacillus arvi* sp. nov.

Bacillus arvi (ar'vi. L. gen. n. *arvi* of a field).

Cells are straight, round-ended, Gram-variable, motile rods (0.8–1.0 $\times$ 3.0–8.0 μ m), occurring singly and in pairs. Endospore formation is slow and scarce on NA MnSO₄ after 10 days incubation at 30 °C. On ½ BFA, endospores are formed abundantly after approximately 3 days incubation at 30 °C. Endospores are spherical and appear in terminal positions, swelling the sporangia slightly. After 24 h on NA at 30 °C, colonies are butyrous, cream-coloured, semi-translucent and slightly raised, with irregular margins that may spread along the inoculation streak. They have granular textures and eggshell to glossy textured surfaces. Colony diameter is 1–3 mm. Anaerobic growth occurs but is weak. Growth occurs at 20 and 30 °C but not at 45 °C. Strains grow at pH 9 as profusely as at pH 7, but are unable to grow at pH 5. Casein is weakly hydrolysed. Starch is not hydrolysed. Strains tolerate 5 % (w/v) NaCl but not 7 %.

Catalase-positive and oxidase-negative. In the API 20E strip, the ONPG reaction is weakly positive and urease is produced. Arginine dihydrolase-, lysine decarboxylase- and ornithine decarboxylase-negative. Citrate is not utilized. Hydrogen sulfide and indole are not produced. The Voges–Proskauer reaction is negative and gelatin is not hydrolysed. Nitrate is not reduced to nitrite. In the API 50CHB, aesculin is not hydrolysed and acid without gas is produced from D-fructose and weakly from N-acetylglucosamine. No acid is produced from the other substrates. No substrate was used as the sole carbon source in the API Biotype 100 kit. The major cellular fatty acid is iso-C_{15:0}, present at approximately 23 %. The following fatty acids are present to at least 5 %: iso-C_{14:0}, anteiso-C_{15:0}, C_{16:1} ω 7c alcohol, iso-C_{16:0}, C_{16:1} ω 11c and anteiso-C_{17:0}.

The G + C content is 35.0 mol% for the type strain, LMG 22165^T (=DSM 16317^T). Isolated from soil (Drentse A agricultural research area, the Netherlands).

Description of *Bacillus humi* sp. nov.

Bacillus humi (hu'mi. L. gen. n. *humi* of earth, soil).

Cells are thin, slightly curved, round-ended, Gram-positive, highly motile rods (0.7–0.9 $\times$ 4.0–7.0 μ m), occurring singly and in pairs. They produce ellipsoidal, sometimes spherical endospores that appear in subterminal or terminal positions and may swell the sporangia. After 24 h on NA at 30 °C, colonies are small (pin-head size, with diameters of approx. 1 mm), convex, whitish and glossy, and the

texture is watery. Growth occurs in anaerobic conditions but is weak. Optimum growth occurs at approximately 30 °C; growth occurs at 20 but not at 45 °C. Strains grow at pH 9 less profusely than at pH 7 and no growth occurs at pH 5. Strains are unable to grow on skimmed-milk agar. Starch is not hydrolysed. Strains grow profusely in the presence of 7 % (w/v) NaCl. Oxidase- and catalase-positive. In the API 20E strip, nitrate is reduced, but reactions for ONPG, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulfide production, urease, indole production, Voges-Proskauer and gelatin hydrolysis are negative. In the API 50CHB, aesculin is hydrolysed and acid without gas is weakly and variably produced from arbutin, lactose and salicin. Acid production is negative for other carbohydrates. In the Biotype100 kit, aesculin and hydroxyquinoline- β -glucuronide are hydrolysed and the following substrates are used weakly as sole carbon sources: D-glucosamine, D-glucuronate and 2-keto-D-gluconate. The major cellular fatty acids are anteiso-C_{15:0} and iso-C_{15:0}, present at approximately 39 and 33 %, respectively. The fatty acid iso-C_{14:0} is present to at least 5 %.

The G+C content is 37.5 mol% for the type strain, LMG 22167^T (=DSM 16318^T). Isolated from soil (Drentse A agricultural research area, the Netherlands).

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