**Paenibacillus graminis** sp. nov. and Paenibacillus odorifer sp. nov., isolated from plant roots, soil and food

Odile Berge,1 Marie-Hélène Guinebretière,2 Wafa Achouak,1 Philippe Normand3 and Thierry Heulin1

Author for correspondence: Odile Berge. e-mail: oberge@cea.fr

Sixteen Gram-positive endospore-forming bacteria previously isolated from soil, plant rhizospheres, plant roots and pasteurized pureed vegetables were studied to determine their taxonomic positions. The isolates were formerly identified as *Bacillus circulans* based on their biochemical characters using API galleries. Two of these strains, RSA19T and TOD45T, were recently assigned to the genus *Paenibacillus* based on phylogenetic analysis of their 16S rRNA (rrs) gene sequence. In the present work, the sixteen isolates were assigned to two genomospecies using DNA–DNA hybridization, in agreement with rrs gene sequence analysis. These genomospecies can also be differentiated on the basis of their cultural and biochemical characters into two novel species, for which the names *Paenibacillus graminis* sp. nov. (type strain RSA19T = ATCC BAA-95T = LMG 19080T) and *Paenibacillus odorifer* sp. nov. (type strain TOD45T = ATCC BAA-93T = LMG 19079T) are proposed.

**Keywords:** *Paenibacillus graminis* sp. nov., *Paenibacillus odorifer* sp. nov., wheat roots, food

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**INTRODUCTION**

Members of the genus *Bacillus sensu lato* from terrestrial habitats are currently considered to be essentially saprophytic bacteria living mostly in bare soils. However, as early as 1908, nitrogen-fixing *Bacillus polymyxa* strains were found associated with plants (Von Bredemann, 1908). In 1963, a nitrogen-fixing *B. polymyxa* strain that was able to promote wheat, maize and tomato growth after inoculation was isolated from the wheat rhizosphere (Rovira, 1963). Since that date, a lot of root-associated *Bacillus* strains have been isolated from various environments and their taxonomic status has sometimes been unclear. Recently, revisions made in the genus *Bacillus* have led to its splitting into distinct genera (Ash et al., 1991). One of them is the new genus *Paenibacillus* (Ash et al., 1993), which currently contains 32 species including *Paenibacillus polymyxa* and *Paenibacillus azotofixans*, frequently found in association with plants. *P. polymyxa* has been isolated from various environments in the world, on different plant roots (Abdel Wahab, 1975; Heulin et al., 1994; Holl et al., 1988; Lethbridge et al., 1982; Lindberg & Granhall, 1984; Nelson et al., 1976; Rennie et al., 1982; Subba Rao & Dart, 1981; von der Weid et al., 2000). *P. azotofixans* has also been frequently isolated from rhizosphere soils and plant roots (Seldin et al., 1984, 1998). Unlike the ubiquitous *P. polymyxa*, *P. azotofixans* has only been found in Brazilian and Hawaiian soils (Rosado et al., 1998) and not in soils from temperate zones (L. Seldin, personal communication).

In the course of a study of nitrogen-fixing bacteria associated with the rhizosphere of cereals, *P. polymyxa* was frequently isolated from wheat cultivated in various French soils (Mavingui et al., 1992; Heulin et al., 1994). In some cases, *P. polymyxa* and other phenotypically related nitrogen-fixing spore-formers were isolated from wheat and maize rhizosphere (Berge et al., 1991). Two of these bacteria, RSA19T and TOD45T, formed a monophyletic cluster with *P. azotofixans* based on 16S rDNA gene sequences.
whereas phenotypic identification using the API 50CHB system erroneously classified these isolates as *Bacillus circulans* (Achouak et al., 1999b). Actually, *B. circulans* has often been described as a ‘complex’ rather than a species and long encompassed a variety of genotypically unrelated bacteria (Nakamura & Swezey, 1983a, b; Priest et al., 1988). Following extensive phylogenetic analysis, the *B. circulans* complex has since undergone major taxonomic revisions leading to new affiliations at the levels of genera and species (Nakamura, 1984, 1987a; Alexander & Priest, 1989), whereas phenotypic identification systems have not yet been updated.

A set of sixteen strains, isolated from plant rhizosphere or food samples and closely related to RSA19, respectively. Phenotypic and molecular methods, these strains can be assigned to two novel species, for which the names *Paenibacillus graminis* sp. nov. and *Paenibacillus odorifer* sp. nov. are proposed.

**METHODS**

**Bacterial strains and culture conditions.** All strains of *Paenibacillus* and *Bacillus* used in this study are listed with their origins in Table 1. Of these, three were previously isolated from the rhizosphere of maize growing in two different French soils (Berge et al., 1991). A total of thirteen new isolates from different origins and phenotypically closely related to those isolated from maize was collected. Five strains (TOD strains) were isolated from a French soil (Dieulouard) without pasteurization. Appropriate dilutions of macerated wheat roots were plated onto nitrogen-free solid RCV4C medium containing RCV mineral medium and 0.01% yeast extract, 0.5% glucose, malate, starch and mannitol according to a previously described method (Mavingui et al., 1990). Sporulating isolates able to fix nitrogen were then selected and preserved. Four strains were isolated from an Australian soil (GJK strains) together with *P. polymyxa*: one strain was isolated from wheat root macerates and three were from bulk soil. Appropriate dilutions were pasteurized to isolate spore-forming bacteria and inoculated on modified LG broth (Ledingham et al., 1945) containing RCV mineral medium, 5% (w/v) glucose, soluble starch and 1% (w/v) peptone. Inverted small glass tubes were placed in culture tubes to select for gas production during bacterial growth. Further purification was performed on RCV4C medium. The last four strains were isolated from pasteurized and chilled pureed leeks or courgettes, produced by an industrial French processing plant. Samples of leek and courgette purées were stored for 4 d at 4 °C before isolation of strains L42-09 and Z42-19, respectively (Carlin et al., 2000). Samples of courgette purée were stored for 21 d at 10 °C and 5 d at 20–25 °C to obtain strains P14-7 and P22-9, respectively.

Purification and screening for sporulation and nitrogen fixation were performed on RCV4C medium. Strain cultures were stored in 20% (v/v) glycerol at −80 °C. The strains were grown on modified LB broth containing 1% (w/v) bacto-tryptone, 0.5% yeast extract and 0.5% NaCl. Working stock cultures were grown on tryptic soy agar (TSA) (Difco) for 24–48 h at 28 °C.

**Metabolic and growth characteristics.** The isolates were examined for the following characters: growth at 5 and 10 °C, position and shape of spores and sporangia, reduction of nitrates, catalase activity and anaerobic growth, as previously described (Gordon et al., 1973; Brenner et al., 1982). The microtube system API 20E/50CHB (API; bioMérieux) was used according to Logan & Berkeley (1984). Oxidase activity was tested both with this system and with dimethyl-p-phenylenediamine discs. Bacterial carbohydrate metabolism was studied using the microtube system API 50CHB and analysed with the API-LAB database identification system. Production of exopolysaccharides was tested by culturing strains on plates containing RCV mineral medium supplemented with 0.01% yeast extract and 4% (w/v) sucrose or glucose.

**Acetylene reduction assay (ARA).** Nitrogenase activity was measured using the ARA on slant agar medium under air. Strains were grown on solid RCV mineral medium containing 0.5% glucose, malate, starch and mannitol (Mavingui et al., 1990). After 48 h at 30 °C, strains were incubated under 2% (v/v) acetylene/air for 5 d and then analysed for ethylene production by GC.

**Amplified rDNA restriction analysis (ARDRA).** The procedure described by Laguerre et al. (1994) was used for PCR amplification of the 16 rDNA (rrs) gene fragments (approx. 1500 bp) using primers F1 and rD1 (Weisburg et al., 1991), which correspond to positions 8–27 and 1524–1540, respectively, of the *Escherichia coli* rrs gene. PCRs were carried out with bacterial suspensions as template DNA. PCR products (10 μl) were digested with 6 U of each restriction enzyme and the fragments were resolved on agarose gels (1.8%, w/v). The amplified rrs gene of the type strains of twelve taxonomically related species (Table 1) were digested with thirteen restriction enzymes: *Alu*, *Bsi*ZI, *CfoI*, *DdeI*, *HaeIII*, *HindIII*, *MspI*, *NciI*, *NdeI*, *RsaI*, *ScrFI*, *TaqI*, *TaqII*, *Tru9I* (result not shown). Four of them (*DdeI*, *HindIII*, *Bsi*ZI and *ScrFI*) generated different patterns and were selected for further studies. The amplified rrs genes of the sixteen strains under investigation were digested with these enzymes and the combination of the different restriction patterns detected with each enzyme was used to assign strains to ARDRA groups.

**REP-PCR fingerprinting.** REP primers, REP1R-I and REP2-I, which target conserved sequences known as bacterial repetitive elements (Versalovic et al., 1991), were used for PCR by mixing cell suspensions with PCR reagents according to Frey et al. (1997). Stained gels were digitized as gel images using Enhanced Analysis System software (easy 3.16; Herolab). Strains were then assigned to REP genotypes by comparing their electrophoretic patterns.

**Determination of the rrs gene sequences.** Amplification of the rrs gene of strains was performed as described above. PCR products of strains TRO4 and the *P. azotoferans* type strain from two collections, LMG 14658T and ATCC 35681T, were purified using the QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed using the ABI PRISM Dye Terminator Ready Reaction kit as specified by the manufacturer (Perkin Elmer). Sequences were obtained with an automatic sequencer (ABI PRISM 377 DNA sequencer; Perkin Elmer) using S6, S10, S12 and S17 primers (Achouak et al., 1999a). The rrs gene sequence of strains TOD45T and RSA19T have been previously determined (accession numbers AJ223987 and AJ223990, respectively; Achouak et al., 1999b).
Two new *Paenibacillus* species

### Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>ARDRA*</th>
<th>REP genotype</th>
<th>Origin, place of isolation and reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group</td>
<td>Pattern</td>
<td></td>
</tr>
<tr>
<td><em>P. graminis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSA19 (= LMG 19080T)</td>
<td>1</td>
<td>AAAA a</td>
<td>Maize rhizosphere soil, Ramonville, France (Berge et al., 1991)</td>
</tr>
<tr>
<td>RSA20</td>
<td>1</td>
<td>AAAA a</td>
<td>Maize rhizosphere soil, Ramonville, France (Berge et al., 1991)</td>
</tr>
<tr>
<td>TOD61</td>
<td>1</td>
<td>AAAA b</td>
<td>Wheat roots, Dieulouard, France (this study)</td>
</tr>
<tr>
<td>TOD111</td>
<td>1</td>
<td>AAAA b</td>
<td>Wheat roots, Dieulouard, France (this study)</td>
</tr>
<tr>
<td>TOD221</td>
<td>1</td>
<td>AAAA b</td>
<td>Wheat roots, Dieulouard, France (this study)</td>
</tr>
<tr>
<td>TOD302</td>
<td>1</td>
<td>AAAA b</td>
<td>Wheat roots, Dieulouard, France (this study)</td>
</tr>
<tr>
<td>GJK201</td>
<td>1</td>
<td>AAAA c</td>
<td>Wheat roots, Kapunda, Australia (this study)</td>
</tr>
<tr>
<td>GJK9</td>
<td>1</td>
<td>AAAA c</td>
<td>Soil, Kapunda, Australia (this study)</td>
</tr>
<tr>
<td>GJK8</td>
<td>1</td>
<td>AAAA c</td>
<td>Soil, Kapunda, Australia (this study)</td>
</tr>
<tr>
<td>GJK5</td>
<td>1</td>
<td>AAAA c</td>
<td>Soil, Kapunda, Australia (this study)</td>
</tr>
<tr>
<td><em>P. odorifer</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOD45 (= LMG 19079T)</td>
<td>2</td>
<td>BBBB d</td>
<td>Wheat roots, Dieulouard, France (this study)</td>
</tr>
<tr>
<td>L42-09</td>
<td>2</td>
<td>BBBB e</td>
<td>Pasteurized pureed leeks, Avignon, France (this study)†</td>
</tr>
<tr>
<td>Z42-19</td>
<td>2</td>
<td>BBBB f</td>
<td>Pasteurized pureed courgettes, Avignon, France (this study)†</td>
</tr>
<tr>
<td>P14-7</td>
<td>2</td>
<td>BBBB g</td>
<td>Pasteurized pureed courgettes, Avignon, France (this study)†</td>
</tr>
<tr>
<td>P22-9</td>
<td>2</td>
<td>BBBB h</td>
<td>Pasteurized pureed courgettes, Avignon, France (this study)†</td>
</tr>
<tr>
<td><em>Paenibacillus</em> sp.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRO4 (= LMG 19081)</td>
<td>3</td>
<td>CABC i</td>
<td>Maize rhizosphere soil, Troy, France (Berge et al., 1991)</td>
</tr>
<tr>
<td>Standard references</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. azotofixans</em> ATCC 35681T</td>
<td>4</td>
<td>ABBB j</td>
<td>Wheat roots, Parana state, Brazil (Seldin et al., 1984)</td>
</tr>
<tr>
<td><em>P. azotofixans</em> LMG 14658T</td>
<td>4</td>
<td>ABBB j</td>
<td>Wheat roots, Parana state, Brazil (Seldin et al., 1984)</td>
</tr>
<tr>
<td><em>P. maccarini</em> ATCC 8244T</td>
<td>5</td>
<td>ACCC k</td>
<td>Potato puree (Schardinger, 1905)</td>
</tr>
<tr>
<td><em>P. peoriae</em> LMG 14832T</td>
<td>6</td>
<td>EDBD l</td>
<td>Soil, Delaporte strain 11.B.9 (Nakamura, 1987b)</td>
</tr>
<tr>
<td><em>P. polymyxa</em> ATCC 842T</td>
<td>6</td>
<td>EDBD m</td>
<td>Kluyver strain (Smith et al., 1964)</td>
</tr>
<tr>
<td><em>P. laitii</em> LMG 11157T</td>
<td>7</td>
<td>DCBE ND</td>
<td>Intestinal tract of children, University of Washington (Batchelor, 1919)</td>
</tr>
<tr>
<td><em>P. glucanolyticus</em> LMG 12239T</td>
<td>7</td>
<td>DCBE ND</td>
<td>Soil, England, Priest strain 593T (Priest et al., 1988)</td>
</tr>
<tr>
<td><em>P. chibensis</em> LMG 14457T</td>
<td>8</td>
<td>ACBD ND</td>
<td>Streptomycin assay (Nakamura &amp; Swezey, 1983a)</td>
</tr>
<tr>
<td><em>P. pabuli</em> LMG 11158T</td>
<td>9</td>
<td>ECBC ND</td>
<td>Animal food (Schieblach, 1923)</td>
</tr>
<tr>
<td>‘<em>B. longisporus</em>’ LMG 13275T</td>
<td>10</td>
<td>ADBC ND</td>
<td>Coarse reddish sand, Sweden (Delaporte, 1972)</td>
</tr>
<tr>
<td><em>B. circulans</em> ATCC 4513T</td>
<td>11</td>
<td>FEED ND</td>
<td>Human dejecta, Ford strain 26 (Ford, 1916, Smith et al., 1964)</td>
</tr>
<tr>
<td><em>B. cereus</em> LMG 6923T</td>
<td>12</td>
<td>FEED ND</td>
<td>Ford strain 13 (Smith et al., 1964)</td>
</tr>
</tbody>
</table>

ND: Not determined.

* Four letters were arbitrarily assigned to represent specific fingerprint patterns obtained from ARDRA of *rrs* genes digested with restriction endonucleases, *Dde*I, *Hin*fI, *Bsi*ZI and *Scr*FI respectively. Different combinations of letters were then used to define ARDRA groups.

† These industrial purees were made of vegetables; they contained no additives or preservatives.

**16S rRNA gene sequence alignment and phylogenetic analysis.** The *rrs* gene sequences were aligned with **clustal x** software (Thompson et al., 1997). Indel-containing regions were excluded from the analysis. Matrix pairwise comparisons were corrected for multiple base substitutions by the two-parameter method of Kimura (1980). Phylogenetic trees were constructed by the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods. A bootstrap confidence analysis was performed on 1000 replicates to determine the reliability of the distance tree topologies obtained (Felsenstein, 1985). A graphic rep-
presentation of the resulting tree was obtained using NJPLOT software (Perrière & Gouy, 1996). The strain designations and sequence accession numbers of their rrs genes used in the phylogenetic analysis are as follows: Bacillus subtilis NCDO 1769T, X60646; Paenibacillus algignolyticus DSM 5050T, D78465; Paenibacillus alvei ATCC 6344T, X57304; Paeni-
bacillus amylyolyticus NRRL NRS-290T, D85396; Paeni-
bacillus aptarius NRRL NRS-1438T, U49247; P. azotofixans ATCC 35681T, AJ251192; P. azotofixans LMG 14658T,
AJ251195; Paenibacillus campinensis 324T, AF021924; Paenibacillus chibensis NRRL B-142T, D85395; Paenibacillus
dendritiforms T168T, Y16128; Paenibacillus glucanolyticus DSM 5162T, D78470; Paenibacillus gorgonae ATCC 29948T,
X60617; Paenibacillus illinoiensis NRRL NRS-1356T,
D85397; Paenibacillus kobensis IFO 15729T, D78471; Paeni-
bacillus larvae subsp. larvae ATCC 9545T, X60619; P. larvae subsp. pulvificans NCDO 1141T, X60636; Paenibacillus
lautus NRRL NRS 666T, D78473; Paenibacillus lentimorbus
ATCC 14707T, AF071861; Paenibacillus macerans NCDO
1764T, X60624; Paenibacillus macquiriensis NCTC 10419T,
X60625; Paenibacillus pabuli NCIMB 12781T, X60630;
Paenibacillus peoriae IFO 15541T, D78476; P. polymyxa
IAM 13419T, D16276; Paenibacillus popilliae ATCC 14706T,
AF071859; Paenibacillus thiaminolyticus JCM 8360T,
D78475; and Paenibacillus validus DSM 3037T, D78320.

DNA–DNA hybridization. Extraction and purification of
DNA was performed using previously described methods
(Brenner et al., 1982). Total DNAs from TOD45T, RSA19T,
TRO4 and P. azotofixans ATCC 35681T were labelled in
vitro with tritium-labelled nucleotide using the Megaprime
DNA labelling system (Amersham). DNA–DNA hybridiza-
tion experiments were performed at the optimal temperature
for DNA reassociation (65 °C) using the S1 nuclease-
trichloroacetic acid method (Grimont et al., 1980).

DNA base composition. The G + C contents of the DNA
from strains TOD45T, RSA19T and TRO4 were determined
by the thermal denaturation temperature (Marmur & Doty,
1962) and calculated using the equation of Owen & Lapage
(1976). E. coli strain K-12 CIP 54-117 (DNA G + C content
of 50-6 mol %) was used as a control.

RESULTS AND DISCUSSION

Previously, Achouak et al. (1999b) have shown by rrs
gene analysis that two misnamed B. circulans strains, RSA19T
and TOD45T, belonged to the genus Paeni-
bacillus and were phylogenetically closely related to P.
azotofixans. This result confirmed that several mis-
classified B. circulans strains belonged to different
species of the genus Paenibacillus (Nakamura, 1984; Ash et al., 1993; Heyndrickx et al., 1996; Shida et al., 1997a, b). The API 50CHB system recommended for
the identification of Bacillus and relatives thus has to be
up-dated for this group. Nevertheless, this system was
used as a first step for the phenotypic screening of
strains closely related to RSA19T or TOD45T and
likely to belong to the same species. Eighty-one isolates
identified as ‘Bacillus circulans 2’ from clinical (API
collection), food, rhizosphere, soil and root environ-
ments were selected for ARDRA analysis as a second
screening step.

ARDRA and REP typing of strains

ARDRA is a rapid and powerful technique able to
discriminate between species (Laguerre et al., 1994) and
it gives reliable genotypic classification of large
numbers of strains (Achouak et al., 2000). Four
restriction enzymes were selected from the thirteen
tested (data not shown) generating different patterns
among almost all the Paenibacillus species, except for
the closely related species such as P. peoriae and P.
polymyxa, which both belonged to ARDRA group 6, and
P. lautus and P. glucanolyticus, both assigned to
ARDRA group 7 (Table 1).

In the set of 81 strains studied in this work only
thirteen, and none from a clinical source, showed
ARDRA patterns similar to those of strains RSA19T
or TOD45T (Table 1). Strain TRO4, which exhibited a
single original pattern (Table 1), was selected because it
was isolated from maize rhizosphere by the same
procedure as strain RSA19T, except that the soil was
different (Berge et al., 1991).

REP-PCR fingerprinting of all sixteen strains
(RSA19T, TOD45T, TRO4 and the thirteen selected
strains) was carried out to determine their diversity.
Complex patterns of 10–20 DNA fragments were
recorded after gel electrophoresis of REP-PCR prod-
ucts. The fingerprints were highly reproducible; REP-
PCR profiles obtained in two independent experiments
were always identical. Fingerprints were compared
and the 16 strains were assigned to nine REP geno-
types, which were different from those recorded with
the type strains of P. azotofixans, P. polymyxa, P.
macerans and P. peoriae (Table 1).

REP-PCR fingerprinting is a simple and rapid tech-
nique that discriminates between strains at the intra-
specific level (Guemouri-Athmani et al., 2000). It was
assumed that all the strains sharing the same REP
genotype were clonal and one representative strain per
REP genotype was selected for further tests such as
DNA–DNA hybridization and nitrogenase activity to
determine their taxonomic status.

Phylogenetic study of the rrs gene

The phylogenetic position of the representative strains
of ARDRA group 1 (RSA19T) and ARDRA group 2
(TOD45T) based on their rrs gene sequences have been
published previously (Achouak et al., 1999b). In this
work, the almost complete rrs gene sequence (1440 bp)
of the single strain of ARDRA group 3, strain TRO4,
was determined. After phylogenetic study, it was
shown that this strain also belonged to the genus Paenibacillus, forming a monophyletic branch with
strains RSA19T, TOD45T and P. azotofixans (Fig. 1).
The similarities of the TRO4 rrs sequence with those of
strains RSA19T, TOD45T and the type strain of P.
azotofixans were 98-9, 97-8 and 96-4 %, respectively.
This monophyletic branch contains mostly nitrogen-
fixing strains from plant rhizosphere, P. azotofixans
Two new *Paenibacillus* species

![Neighbour-joining tree of strain TRO4 and related *Paenibacillus* species rrs genes. Bootstrap results above 50% are given above the nodes and groupings also found by maximum-likelihood (L) and parsimony (P) analyses, respectively, are indicated below the nodes. Bar, 0.01 substitutions per site. The *B. subtilis* sequence was used as an outgroup. An expanded phylogenetic tree is available as supplementary data in IJSEM Online (http://ijs.sgmjournals.org).](image)

**Fig. 1.**

**Table 2.** DNA–DNA reassociation between the strains studied

<table>
<thead>
<tr>
<th>Source of unlabelled DNA</th>
<th>ARDRA group*</th>
<th>Relative binding ratio (%) of labelled DNA from strain:</th>
<th>Hybridization group†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RSA19&lt;sup&gt;T&lt;/sup&gt;</td>
<td>TOD45&lt;sup&gt;T&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P. graminis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSA19&lt;sup&gt;T&lt;/sup&gt; (= LMG 19080&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>1</td>
<td>100</td>
<td>36</td>
</tr>
<tr>
<td>TOD302</td>
<td>1</td>
<td>98</td>
<td>7</td>
</tr>
<tr>
<td>GJK9</td>
<td>1</td>
<td>71</td>
<td>21</td>
</tr>
<tr>
<td><em>P. odorifer</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOD45&lt;sup&gt;T&lt;/sup&gt; (= LMG 19079&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>2</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>L42-09</td>
<td>2</td>
<td>29</td>
<td>97</td>
</tr>
<tr>
<td>Z42-19</td>
<td>2</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td><em>Paenibacillus sp.</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRO4 (= LMG 19081)</td>
<td>3</td>
<td>23</td>
<td>14</td>
</tr>
</tbody>
</table>

**References**

- *P. azotofixans* ATCC 35681<sup>T</sup>
- *P. macerans* ATCC 8244<sup>T</sup>
- *P. peoriae* LMG 14832<sup>T</sup>
- *P. polymyxa* ATCC 842<sup>T</sup>
- *P. lautus* LMG 11157<sup>T</sup>
- *P. glucanolyticus* LMG 12239<sup>T</sup>
- *P. chibensis* LMG 14457<sup>T</sup>
- *B. circulans* ATCC 4513<sup>T</sup>
- *B. cereus* LMG 6923<sup>T</sup>

*See Table 1.*

† Letters were arbitrarily assigned to represent specific hybridization patterns obtained from DNA–DNA experiments.

from tropical soils and strains RSA19<sup>T</sup>, TOD45<sup>T</sup> and TRO4 from temperate soils.

In the course of this phylogenetic study, confusion arose concerning the position of the *P. azotofixans* type strain that needed to be addressed. The type strains of *P. azotofixans* delivered by two culture collections (ATCC 35681<sup>T</sup> and LMG 14658<sup>T</sup>) were carefully checked for both phenotypic and molecular characterization and their *rrs* gene sequences were determined independently. These sequences were identical to each other (accession numbers AJ251192 and AJ251195) and very close to the *P. azotofixans* type strain sequence deposited by J. A. E. Farrow under accession number X60608. Conversely, the *rrs* sequences were very different from that of the *P. azotofixans* type strain deposited by O. Shida under accession number D78318, which was close to *P. peoriae* *rrs* sequence (accession number D78476) (data not shown). It is therefore recommended that accession numbers AJ251192 or AJ251195 be used for future studies of *P. azotofixans*. The phylogenetic tree in Fig.
Control tube was non-inoculated. Values are means ± confidence interval (\( P = 0.05 \)).

<table>
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<th>Strain</th>
<th>High (( \mu \text{mol C}_3\text{H}_4 ))</th>
<th>Weak (( \mu \text{mol C}_3\text{H}_4 ))</th>
<th>None (( \mu \text{mol C}_3\text{H}_4 ))</th>
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<td><em>P. graminis</em></td>
<td></td>
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<tr>
<td>RSA19(^T) (= LMG 19080(^T))</td>
<td>4.2 ± 0.3</td>
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<td>TOD61</td>
<td>1.8 ± 0.9</td>
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<td>GJK201</td>
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<td><em>P. odorifer</em></td>
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<td>TOD45(^T) (= LMG 19079(^T))</td>
<td>0.12 ± 0.08</td>
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<td>L42-09</td>
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<tr>
<td>TRO4 (= LMG 19081)</td>
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<td>Control tube</td>
<td>0.005 ± 0.006</td>
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Table 3. Nitrogenase activity (ARA) of strains

Measured in \( \mu \text{mol C}_3\text{H}_4 \) was measured per tube after 5 d incubation under 2\% \( \text{C}_3\text{H}_2 \). Control tube was non-inoculated. Values are means ± confidence interval (\( P = 0.05 \)).

Phenotypic analysis

All the sixteen strains were phenotypically very homogeneous: they produced acid from 23 of the 49 carbohydrates tested with the API50 CHB system and did not acidify 17 of them. However, in agreement with genotypic classification, it was possible to split them into three phenotypic groups corresponding to the three ARDRA groups. The growth temperature of strains ranged from 5 to 40, 5 to 35 and 10 to 35 °C for strains of ARDRA group 1 (including strain RSA19\(^T\)), group 2 (including strain TRO4\(^T\)) and group 3 (strain TRO4), respectively. Colony morphology on agar media containing glucose or sucrose resulting from exopolysaccharide production was also studied: strains of ARDRA group 2 were unable to produce mucous colonies; and strains of ARDRA groups 1 and 3 produced mucous colonies, but to a lesser extent than strains of *P. polymyxa*, which are known for their levan production in the presence of sucrose. The APILAB identification system assigned the 16 strains to *Bacillus circulans* 2' with similarities of 99-9% for group 1, 60-80% for group 2 and less than 60% for group 3. The type strain of *B. circulans* (ATCC 4513\(^T\)) was assigned to the ' *B. circulans* 1' taxon by this phenotypic identification.

Phenotypic characteristics that differentiate ARDRA groups 1, 2 and 3 are summarized in Table 4. ARDRA group 2 could be clearly differentiated from ARDRA groups 1 and 3: strains of group 2 acidified ribose, but not melezitose; they were unable to produce polysaccharide either from glucose- or sucrose-rich media; and they grew at 5 °C, which is consistent with the isolation of most of these strains from food preserved at 4 °C. These strains were easily differentiated from all other *Paenibacillus* species by a very simple character:
they displayed an intense, easily recognizable, fruity volatile aroma after 24–48 h growth on liquid or agar media. A weak nitrogenase activity was measured for these strains (Table 3) and the G + C content of TOD45T (44 mol%) was rather low compared with the closest Paenibacillus species (Table 4). In this group, strains that were dominant in pasteurized vegetable purées incubated at cold temperature were found, probably present on the vegetable surface before processing. This species could be frequently found in soils and is particularly adapted to survive on plant surfaces.

As well as the difference in the level of nitrogenase activity (Table 3), only two characters could be used to differentiate between strains from ARDRA groups 1 and 3: production of gas from carbohydrates and acidification of mannitol (Table 4). This result is consistent with the high DNA similarity between these strains (23–33 %), high similarity between RSA19T (ARDRA group 1) and TRO4 (ARDRA group 3) rrs sequences (98.9 %) and their close G + C content (52.1 and 50.0 mol % respectively). However, it was possible to discriminate between them using ARDRA with only four enzymes (Table 1).

On the basis of phenotypic properties, rrs gene sequencing and DNA–DNA reassociation values, strains included in ARDRA groups 1 and 2 may be considered as two new Paenibacillus species. The names Paenibacillus graminis sp. nov. and Paenibacillus odorifer sp. nov. are proposed. Strain TRO4 clearly belongs to a third genomospecies. In the absence of other strains belonging to this genomospecies, the description of a third new species is reported. The different characteristics of these species and phylogenetically related species of Paenibacillus are shown in Table 4.

**Description of Paenibacillus graminis sp. nov.**

*Paenibacillus graminis* (gra’mi.nis. L. gen. neut. n. graminis of grass).

Motile vegetative cells are rods, 0.5–1.0 × 3.0–4.0 µm (as determined by phase-contrast microscopy), and occur singly or in short chains. Cells produce oval spores, located terminally in swelling sporangia, and are Gram-positive (aminopeptidase assay). Nutrient agar colonies are cream coloured, smooth with regular entire margins and measure 1.0–2.0 mm in diameter

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**Table 4. Phenotypic characteristics that differentiate* P. graminis*, *P. odorifer* and *Paenibacillus* sp. TRO4 from their closest relatives among the genus *Paenibacillus* **

Abbreviations: +, > 90 % strains positive; −, < 10 % strains positive; v, 11–89 % strains positive; NT, not tested. Species: 1, *P. graminis*; 2, *P. odorifer*; 3, *Paenibacillus* sp. TRO4; 4, *P. azotofixans* (Seldin et al., 1984; Seldin & Penido, 1986); 5, *P. maccarrensis* (Logan & Berkeley, 1984; Priest et al., 1988; Heyndrickx et al., 1996); 6, *P. peoriae* (Nakamura, 1987b; Montefusco et al., 1993; Heyndrickx et al., 1996); 7, *P. polymyxa* (Nakamura, 1987b; Logan & Berkeley, 1984); 8, *P. latus* (Nakamura & Swezey, 1983b; Heyndrickx et al., 1996); 9, *P. glucanolyticus* (Priest et al., 1988; Alexander & Priest, 1989); 10, *P. clausisludus* (Yoon et al., 1998); 11, *P. chibensis* (Shida et al., 1997b); 12, *P. macquariensis* (Heyndrickx et al., 1996; Priest et al., 1988); 13, *P. illinoisensis* (Shida et al., 1997b); 14, *P. amylyofuscus* (Heyndrickx et al., 1996; Shida et al., 1997b); 15, *P. pabuli* (Nakamura & Swezey, 1983b; Heyndrickx et al., 1996).

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5 °C | 10 °C | G + C content (mol %) |
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* Data obtained in this work with the type strain of the species (see Table 1).
† For *P. clausisludus*, carbohydrates were tested for their oxidation: +, oxidized; −, non-oxidized.
‡ From Fahmy et al. (1985)
after 3 d at 30 °C on TSA medium. Colonies are mucous on media containing 2% glucose or sucrose. Catalase is produced. Oxidase is not produced. Facultatively anaerobic. Maximum and minimum growth temperatures are 5–10 °C and 35–40 °C, respectively. With API systems, acid is produced from glycerol, t-arabinose, d-xylene, methyl β-d-xylloside, galactose, glucose, fructose, mannose, mannotol, methyl α-d-glucoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose, raffinose, starch, glycogen, gentiobiose and β-d-turanose. Acid is not produced from erythritol, d-arabinose, ribose, l-xylene, adonitol, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-d-mannoside, xylitol, d-lyxose, d-tagatose, l-fucose, d-arabitol, l-arabitol, 2-ketogluconate or 5-ketogluconate. Acid may or may not be produced from inulin, d-fucose and gluconate. Aesculin is hydrolysed. Nitrate is reduced to nitrite. Acetylene is reduced to ethylene by all strains and the nifH gene is present at least in the type strain. Isolated from maize rhizosphere, wheat roots and soil. G+C content of the type strain is 52·1 mol%. Type strain is RSA19T (= LMG 19080T = ATCC BAA-95T).

**Description of Paenibacillus odorifer sp. nov.**

*Paenibacillus odorifer* (o.do’ri.fer. L. n. odor smell; L. suff. n. -fer carrier; L. n. odorifer carrier of smell).

Motile vegetative cells are rods, 0·5–1·0 × 2·0–4·0 μm (as determined by phase-contrast microscopy) and occur singly or in short chains. Cells produce oval colonies, 1·5–3·0 mm in diameter after 3 d at 30 °C on TSA medium. Colonies are not mucous on media containing 2% glucose or sucrose. Catalase is produced, but not oxidase. Facultatively anaerobic. Maximum and minimum growth temperatures are 5 and 35 °C, respectively. With API systems, acid is produced from t-arabinose, ribose, d-xylene, methyl β-d-xylloside, galactose, glucose, fructose, methyl α-d-glucoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, raffinose, starch, glycogen, gentiobiose and β-d-turanose. Acid is not produced from erythritol, d-arabinose, l-xylene, adonitol, sorbose, rhamnose, dulcitol, inositol, mannotol, sorbitol, methyl α-d-mannoside, melezitose, xylitol, d-lyxose, d-tagatose, d-fucose, d-arabitol, l-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Acid may or may not be produced from glycerol, mannoside and l-fucose. Aesculin is hydrolysed. Nitrate is reduced to nitrite. Acetylene is weakly reduced to ethylene by all strains and the nifH gene is present at least in the type strain. A typical fruity volatile aroma is produced on nutrient media. Isolated from wheat roots and pasteurized and chilled leek and courgette purées. G+C content of the type strain is 44·0 mol%. Type strain is TOD45T (= LMG 19079T = ATCC BAA-93T).

**ACKNOWLEDGEMENTS**

This work was supported, in part, by an International Program for Scientifique Cooperation (PICS), CNRS: Micro-organisms associated with durability of soil structure and fertility: effect of crop management on their diversity. Some of it was presented at ‘Bacillus 2000, Applications and Systematics of *Bacillus* and Relatives’, Bruges, Belgium, 30–31 August, 2000. We thank Laurent Sutra for G+C content measurement, Jacques Balandreau for Australian soils from which *P. graminis* strains GJJK201, GJK9, GJK8 and GJK5 were isolated, Françoise Allard from bioMérieux (La Balme Les Grottes, France) for strains belonging to the API collection and Hans G. Trüper for help in giving the correct name to the new *Paenibacillus* species.

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