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Development and Application of a Selective PCR-Denaturing Gradient Gel Electrophoresis Approach To Detect a Recently Cultivated *Bacillus* Group Predominant in Soil

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The worldwide presence of a hitherto-nondescribed group of predominant soil microorganisms related to *Bacillus benzoevorans* was analyzed after development of two sets of selective primers targeting 16S rRNA genes in combination with denaturing gradient gel electrophoresis (DGGE). The high abundance and cultivability of at least some of these microorganisms makes them an appropriate subject for studies on their biogeographical dissemination and diversity. Since cultivability can vary significantly with the physiological state and even between closely related strains, we developed a culture-independent 16S rRNA gene-targeted DGGE fingerprinting protocol for the detection of these bacteria from soil samples. The composition of the *B. benzoevorans* relatives in the soil samples from The Netherlands, Bulgaria, Russia, Pakistan, and Portugal showed remarkable differences between the different countries. Differences in the DGGE profiles of these communities in archived soil samples from the Dutch Wieringermeer polder were observed over time during which a shift from anaerobic to aerobic and from saline to freshwater conditions occurred. To complement the molecular methods, we additionally cultivated *B. benzoevorans*-related strains from all of the soil samples. The highest number of *B. benzoevorans* relatives was found in the soils from the northern part of The Netherlands. The present study contributes to our knowledge of the diversity and abundance of this interesting group of microbes in soils throughout the world.

The soil ecosystem is known to provide ecological niches for an extremely high number of microorganisms (31). Investigations in Drentse A grassland soils in The Netherlands have demonstrated the abundance of 16S rRNA gene sequences that originated from a group related to *Bacillus benzoevorans* (6). Analysis of a clone library based on direct recovery of 16S rRNA genes from this Dutch soil indicated that a substantial proportion (32%) of the bacterial sequences belonged to this novel phylogenetic lineage, which includes *B. benzoevorans*, *B. niacini*, *B. pseudomegaterium*, *B. jeotgali* (34), five recently isolated species comprising *B. novalis*, *B. vireti*, *B. soli*, *B. bataviae*, and *B. drentensis* (14), the uncultured Drentse A bacterium DA001, and their relatives (10). Their homogeneous distribution and activity over kilometer distances has been demonstrated previously in the Dutch Drentse A grasslands (7). Hence, it is feasible to broaden the focus for a comparison of more distant landscapes. After its discovery in Dutch soils, more uncultured members of this lineage were reported to be present as the predominant group of *Bacillus* spp. in soil samples from other countries, including the United States (Wisconsin) (2), Amazonia (3), The Netherlands (8), and the United Kingdom (19).

Since the *B. benzoevorans*-related bacteria are so abundant, they provide an attractive object for evolutionary studies and the opportunity to examine the microdiversity of one of the most dominant microbial groups in the grassland soil biosphere. Moreover, only a fraction of the bacteria from this cluster has been cultivated thus far (11a, 15). Although new approaches continue to be developed, it is well recognized that the larger part of the soil community remains inaccessible to detailed studies due to inadequate media and methods for cultivation (1, 2, 35).

The purpose of the present study was to develop tools for the fast and sensitive cultivation-independent detection of *B. benzoevorans*-related bacteria from soil samples. Molecular fingerprinting methods targeting 16S rRNA genes and rRNA, such as denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis can be used for the rapid evaluation of composition and activity of complex microbial assemblages at moderately high temporal and spatial resolution (21). The design and validation of 16S rRNA gene-targeted primer sets specific for the cluster of *B. benzoevorans* relatives for use in combination with DGGE are presented here. These primers allow for the monitoring of the distribution and succession of these bacteria over time and space. This molecular approach was complemented with cultivation of isolates from several European locations that revealed the presence and sometimes remarkable abundance of these bacteria.

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MATERIALS AND METHODS

Bacterial strains, growth conditions, and soil samples. Reference strains used for validation of the newly developed primers for detection of the predominant *B. benzoevorans* relatives are listed in Table 1 with their respective sources and

TABLE 1. Reference strains and pure cultures of *B. benzoevorans* relatives isolated and characterized in this study, growth conditions, and sources used for validation of the new *B. benzoevorans* cluster-specific primer sets (REX460f-REX1466r and REX576f-REX1446r)

Species or isolates[a]	Medium[b]	Collection or GenBank accession no.	Primer set[c]	
			REX460f-REX1466r	REX576f-REX1446r
Reference species				
Bacillus benzoevorans relatives				
Bacillus benzoevorans	1	LMG15526	+	+
Bacillus pseudomegaterium	1	LMG18519	+	+
Bacillus niacini	1	LMG16677^T	+	+
Bacillus jeotgali	Marine broth	JCM10885	+	+
Non-*Bacillus benzoevorans* relatives				
Paenibacillus polymixa	51	LMG6319^T	−	NP
Bacillus methanolicus	CM0129	NCIMB13113	−	NP
Bacillus cereus	1	LMG6923^T	−	NP
Bacillus simplex	1	LMG11160^T	+	−
Bacillus megaterium	1	LMG7127^T	+	−
Bacillus mycoides	1	LMG7128^T	−	NP
Bacillus subtilis	1	LMG7135^T	−	NP
Escherichia coli	1	LMG18221	−	NP
Staphylococcus hominis	1	LMG13348^T	−	NP
Streptomyces murinus	78	LMG10475^T	−	NP
Sporosarcina globispora	1 or 51	LMG6928^T	+	−
New isolates[d]				
Bacillus benzoevorans relatives				
Bacillus benzoevorans IDA4715 (97)	Sodium benzoate	AY289495	+	+
Bacillus benzoevorans IDA4919 (98)	Sodium benzoate	AY289496	+	+
Bacillus bataviensis IDA4789 (95)	Sodium benzoate	AY289499	+	+
Bacillus bataviensis IDA4730 (99)	Sodium benzoate	AY289497	+	+
Bacillus bataviensis IDA4740 (97)	Sodium benzoate	AY289498	+	+
Uncultured bacterium DA001 IDA4921 (97)	Sodium benzoate	AY289500	+	+
Non-*Bacillus benzoevorans* relatives				
Paenibacillus sp. strain IDA4508 (96)	Sodium acetate	AY289501	−	NP
Paenibacillus sp. strain IDA5358 (99)	Sodium acetate	AY289507	−	NP
Bacillus mycoides IDA4575 (99)	Sodium acetate	AY289502	−	NP
Bacillus megaterium IDA4770 (99)	Sodium benzoate	AY289503	+	−
Bacillus simplex IDA4917 (98)	Sodium benzoate	AY289504	+	−
Bacillus macroides IDA5330 (99)	Sodium benzoate	AY289506	+	−
Bacillus megaterium IDA5367 (99)	Sodium acetate	AY289508	+	−
Streptomyces mirabilis IDA5203 (99)	Sodium acetate	AY289505	−	NP

[a] Reference strains were from various strain collections as follows: LMG, Laboratory of Microbiology, University of Ghent, Ghent, Belgium; NCIMB, National Collections of Industrial, Food, and Marine Bacteria, NCIMB, Ltd., Aberdeen, United Kingdom; JCM, Japan Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research), Tokyo, Japan. New isolates were from Wageningen University.

[b] Media: 1, see reference 18; medium 51, medium 1 amended with glucose at 1 g/liter; CM0129, tryptone soya broth (Oxoid, Haarlem, The Netherlands); medium 78, BCCM (Bacterial Collection, LMG, University of Ghent).

[c] +, PCR products generated with respective primer pair; −, no PCR products generated with the respective primer pair. After a negative PCR result with REX460f-REX1466r, the subsequent REX576f-REX1466r nested PCR was not performed (NP).

[d] The cultivation temperature of all reference species and new isolates was 30°C, except for *B. methanolicus* NCIMB13113 (45°C) and *S. globispora* LMG6928^T (15°C). IDA, isolate from Drentse A. The percent similarity to the closest cultured relative is given in parentheses.

growth media. The primers were further tested and validated by using pure culture isolates that were isolated and identified by 16S rRNA gene sequence analysis (Table 1).

The soil samples in the present study can be divided in two main groups: (i) fresh, i.e., stored at 4°C for 6 to 12 months, and (ii) air dried, i.e., collected during different years and from different locations. The locations and soil characteristics of the fresh soils from The Netherlands were as follows: (i) Drentse A, where *B. benzoevorans* relatives were discovered, an agricultural research area (06°41′E, 53°03′N) with high bacterial diversity (10) representing a 1.5-km stretch of grasslands along the Anlooër Diepje river (11), 0- to 25-cm sampling depth; (ii) Hoeksma and Sikkema farms, Friesland, The Netherlands, 0- to 25-cm sampling depth; (iii) Wageningen University experimental field, sandy soil, 0- to 25-cm sampling depth; (iv) the shore of the river Rhine, Wageningen, The Netherlands, wet clay soil, 0- to 10-cm sampling depth; and (v) the root nodule surface of *Alnus glutinosa* and the surrounding bulk soil, 0- to 10-cm sampling depth (33). Additional fresh samples were obtained from various locations, including a river site, a grassland, a pine tree forest, a fruit tree garden, and a vegetable garden within Provinzende de Cima, Portugal, and from a vegetable garden in Sofia, Bulgaria. Air-dried samples originated from soil under *Coriaria nepalensis* plants from the Murree area of the Himalayan Mountains in Pakistan (collected in 1993) (20) and from the Russian tundra (collected in 2001). In addition, air-dried soil samples were used that had been collected from the top 0- to 25-cm layer of nonfertilized areas of an agricultural field in the Wieringermeer polder, The Netherlands. These samples were taken from an extensive archive for technical information and soil samples (TAGA; Alterra B.V., Wageningen, The Netherlands). The collection contains the results of field experiments performed between 1879 and 1998 in The Netherlands (5). Land reclamation by drainage of this former sea bottom was started in 1930 and completed in 1940, followed by transformation into agricultural lands (32). To describe the changes in the total bacterial and *B. benzoevorans*-related community after land reclamation, samples obtained in the years 1942, 1950, 1951, 1966, 1973, and 1975 were analyzed.

Two nonsoil samples were also analyzed: sludge from an anaerobic wastewater treatment system in Eerbeek, The Netherlands (K. Roest, H. G. H. J. Heilig,

TABLE 2. Sequences of PCR primers used in this study

Primer	Sequence	Source or reference
27f	5′-GTT TGA TCC TGG CTC AG-3′	17
1492r	5′-CGG CTA CCT TGT TAC GAC-3′	17
REX460f	5′-GAG TAA CTG C(T/C)(C/G) GTA CC-3′	This study
REX1466r	5′-CCA ATC ATC TGT CCC ACC TTA-3′	This study
REX576f	5′-AAG CGC GCG CAG GCG GTC CT-3′	This study
REX1446r	5′-CCT TAG GCG GCT GGC TCC TTA-3′	This study
GC-0968f	5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA CCT TAC-3′	23
1401r	5′-GCG TGT GTA CAA GAC CC-3′	23
SP6	5′-ATT TAG GTG ACA CTA TAG-3′	Promega
T7	5′-AAT ACG ACT CAC TAT AGG-3′	Promega
519r	5′-G(A/T)A TTA CCG CGG C(G/T)G CTG-3′	17
BACREXf	5′-GATCCTGGCTCAGGAYSAACGCTGGCGGC-3′	9
LGb1513r	5′-GGTGATCCAGCCGCACCTTCCGA-3′	9
ACB1409r	5′-CARCCSRCTTTCGTGAT-3′	9
VER877r	5′-GCGACCGTACTYCCCAGGCGGC-3′	9
Bmeg180r	5′-CATCTCCCATGAAGGAGAAGA-3′	9
PAE835f	5′-GGGGTTTCGATACCCTTGGTGC-3′	9
REX1426f	5′-CACCCGAAGTCGGTGGGGTAACCGYA-3′	9

H. Smidt, W. M. de Vos, A. J. M. Stams, and A. D. L. Akkermans, unpublished data) and piglet feces (16).

Strain isolation procedure. Two types of mineral media were used for strain isolation. Briefly, they contained the following (per liter of distilled water): sodium acetate or sodium benzoate, 2 g; yeast extract, 2 g; P-medium, 50 ml; and oligo-element solution (25), 200 μl. Agar (BBL; Becton Dickinson, Le Pont de Claix, France) was added at 20 g per liter for solidification where needed. P-medium comprised (per liter of distilled water): $Na_2HPO_4 \cdot 0.2H_2O$, 37.75 g; KH_2PO_4, 19.6 g; $MgSO_4 \cdot H_2O$, 0.36 g; and NH_4Cl, 2 g. The pH was adjusted to 7.0, and media were autoclaved for 20 min at 121°C. Cells were isolated from 0.1 g of soil via vertical shaking for 60 min at room temperature in 950 μl of 1× phosphate-buffered saline buffer (27). Cell suspensions were diluted to 10^{-5}, and 100 μl was plated on two different media. The plates were incubated for 5 to 7 days at 20°C. A total of 96 colonies were randomly chosen from each medium and from each sample for further investigation.

Platewash. After the colonies were picked, the plates were washed with 1 ml of 1× phosphate-buffered saline buffer.

DNA isolation, primer design, and PCR conditions. DNA was isolated from pure cultures (1 ml) and directly from soils (1 g) by using the Fast DNASPIN kit (for soil) (Q BIOgene, Cambridge, United Kingdom) according to the manufacturer's instructions. Preliminary experiments showed that using the kit-based extraction gave the best DNA isolation results (data not shown). DNA from *B. novalis*, *B. vireti*, *B. soli*, *B. bataviae*, and *B. drentensis* was kindly provided by Jeroen Heyrman.

All primers used in the present study are listed in Table 2. For primer design, 16S rRNA gene sequences of phylogenetically closely related species were retrieved from GenBank at www.ncbi.nlm.nih.gov and aligned by using CLUSTAL W (30). Two sets of primers (REX primers) were developed for specific detection of *B. benzoevorans*-related strains. The first primer set, REX460f-REX1466r, excludes *Paenibacillus* spp., *B. methanolicus*, *B. infernus*, *Streptococcus mirabilis*, and *B. macroides*. The second primer set, REX576f-REX1446r, excludes *B. mycoides*, *B. cereus*, *B. megaterium*, *B. simplex*, *B. macroides*, *B. subtilis*, *B. methanolicus*, *Paenibacillus* spp., *S. mirabilis*, *B. infernus*, and *B. globisporus* (Table 3).

The specificity of the primers was checked in silico by submitting the sequence to the Check Probe program of the Ribosomal Database Project II (4, 13) and to a BLAST search (www.ncbi.nlm.nih.gov/BLAST/). The decision on the specificity of the primers was made based on the number, type, and position of mismatches with nontarget sequences.

Primers were purchased from MWG Biotech AG (Ebersberg, Germany) and were used in a nested approach of two successive specific PCRs. PCR products that were obtained after amplification with primer pair REX460f-REX1466r were used as a template for PCR with primer pair REX576f-REX1446r at a concentration of 1.0 to 2.6 ng of DNA per 50-μl reaction. Subsequently, REX576f-REX1446r amplicons were used as a template for DGGE-PCR with the primers GC-0968f and 1401r (23).

PCR was performed with *Taq* polymerase (Life Technologies, Gaithersburg, Md.). DNA was amplified in a Whatman Biometra Thermocycler (Göttingen, Germany) under the following conditions: REX460f-REX1466r, 94°C for 1 min, 35 cycles of 94°C for 10 sec, 56°C for 20 s, and 68°C for 1 min, and finally 68°C for 7 min; and REX576f-REX1446r 94°C for 5 min, 25 cycles of 94°C for 1 min, 71°C for 20 s, and 72°C for 1 min, and finally 72°C for 3 min. PCR with the primer set 0968GCf-1401r was performed as previously described (23). Multiplex PCR was used in the present study to differentiate between *B. benzoevorans*-related and non-*B. benzoevorans*-related bacterial species. Seven primers (BACREXf, LGb1513r, ACB1409r, VER877r, Bmeg180r, PE835f, and REX1426f) were used to amplify different organisms. The sequences of the primers are listed in Table 2. The multiplex PCR was performed in 96-well microtiter plates according to a previously published procedure (9). PCR products from multiplex positive isolates were sequenced with primer 519r (Table 2), yielding ca. 500 bp of sequence information.

PCR conditions were optimized for specificity using genomic DNA extracted from reference strains, as well as from pure cultures isolates obtained at Wageningen University (Table 1). The primers were further tested with cloned 16S rRNA genes isolated from soil samples that had been previously demonstrated to contain high numbers of *B. benzoevorans*-related species (6).

Analysis of PCR products by DGGE and sequencing. The PCR products obtained after three nested PCRs with the primer sets REX460f-REX1466r, REX576f-REX1446r, and GC-0968f–1401r were separated by DGGE (21, 22). 16S rRNA gene-targeted PCR-DGGE fingerprinting was used to analyze the profiles of the *B. benzoevorans* relatives from different soil samples and also to identify different clones from clone libraries. A gradient of 38 to 50% of the denaturing chemicals (urea and formamide) was used, and gel electrophoresis was performed according to the method of Heilig et al. (13). The gels were stained with $AgNO_3$ according to the method of Sanguinetti et al. (28), scanned at 400DRI, and analyzed by using the software Molecular Analyst 1.12 (Bio-Rad). The similarity between the DGGE profiles was determined by calculating similarity indices of the densitometric curves of the profiles compared by using Pearson product-moment correlation (12, 36). The UPGMA (unweighted pair-group method with arithmetic averages) algorithm was used as implemented in the analysis software for the construction of dendrograms.

PCR amplicons after performing nested PCR with primer sets REX460f-REX1466r and REX576f-REX1446r were purified by using a Qiaquick PCR purification kit according to the manufacturer's instructions and cloned into *E. coli* JM109 by using the Promega pGEM-T vector system (Promega, Madison, Wis.) as previously described (13). The size of the inserts was confirmed by PCR with specific vector primers T7 and Sp6 (Table 2). Clones were screened by restriction fragment length polymorphism analysis with the restriction enzymes MspI and CfoI. Plasmids containing unique inserts were further purified, and inserts were sequenced. Sequencing reactions were performed with the Sequenase (T7) sequencing kit (Amersham Life Sciences, Slough, United Kingdom) as previously described (13). Sequences were automatically analyzed on a LI-COR DNA sequencer 4000L (Lincoln, Nebr.) and corrected manually.

TABLE 3. Alignment of primer sets REX460f-REX1466r and REX576f-REX1446r with the respective 16S rRNA gene target and nontargeted sequences from different *Bacillus* spp.

Species[a]	Sequence alignment[b] for:				
	REX460f	REX1466r	REX576f	REX1446r	
	5′-GAGTAACTGCYSGTACC-3′	5′-CCAATCATCTGTCCCACCTTA-3′	5′-AAGCGCGCGCAGGCGGTCCT-3′	5′-CCTTAGGCGGCTGGCTCCTTA-3′	
*Bacillus benzoevorans**	GAGTAACTGCTCGTACC	3′-GGTTAGTAGATAGGGTGGAAT-5′	AAGCGCGCGCAGGCGGTCCT	3′-GGAATCCGCCGACCGAGGATT-5′	
*Bacillus pseudomegaterium**	GAGTAACTGGGCGTACC	----------AGGGTGGAAT	AAGCGCGCGCAGGCGGTCCT	GGAATCCGCCGACCGAGGAAT	
*Bacillus niacini**	GAGTAACTGCCGGTACC	GGTTAGTAGACAGGGTGGAAT	AAGCGCGCGCAGGCGGTCCT	GGAATCCGCCGACCGAGGAAT	
*Bacillus jeotgali**	GAGTAACTGCCGGTACC	GGTTAGTAGACAGGGTGGAAT	AAGCGCGCGCAGGCGGTCCT	GGAATCCGCCGACCGAGGTTT	
Uncultured bacterium DA032*	GAGTAACTGCTGGTGCC	GGTTAGTAGACAGGGTGGAAT	AAGCGCGCGCAGGCGGTCCT	GGAATCCGCCGACCGTGGTAT	
Bacillus mycoides	GTTGAATAAGCTGGCAC	GGTTAGTAGACAGGGTGGAAT	AAGCGCGCGCAGGTGGTTTC	GGAATCCGCCGACCGAGGTAT	
Bacillus cereus AH-527	GTTGAATAAGCTGGCAC	GGTTAGTAGACAGGGTGGAAT	AAGCGCGCGCAGGTGGTTTC	GGAATCCGCCGACCGAGGTAT	
Bacillus megaterium	GAGTAACTGSTTGTACC	GGTTAGTAGACAGGGTGGAAT	AAGCGCGCGCAGGCGGTTTC	GGAATCCGCCGATCGAGGAAT	
Bacillus macroides	GAGTAACTGCTGGTACC	----------GGAAG	AAGCGCGCGCAGGTGGTTCC	GGAAGCCGCCGACCGAGGTAC	
Bacillus subtilis	TCGAATAGGGCGGTACC	----------	AAGGGCTCGCAGGCGGTTTC	----------	
Bacillus methanolicus	TCGAATAGGGCGGTACT	GGTTAGTAGACAGGGTGGAAT	AAGCGCGCGCAGGCGGTTCC	GGAATCCGCCGACCGAGGAAT	
Paenibacillus spp.	GAGTAACTGCTCCATAG	----------	AAGCGCGCGCAGGCGGGCTAA	----------AACGCCCAAT	

[a] *Bacillus* species belonging to the cluster of *B. benzoevorans* relatives are indicated with an asterisk.
[b] Mismatching bases are in boldface and underlined. Incomplete sequence information is indicated by a hyphen.

Nucleotide sequence accession numbers. The GenBank accession numbers of the 16S rRNA gene sequences of isolates obtained here are given in Table 1.

Statistical analysis. The Student *t* test was used for statistical analysis of comparison between similarity indices from the DGGE profiles.

RESULTS

Cultivation and identification of *B. benzoevorans* relatives and strain isolation. Samples from Drentse A, Hoeksma and Sikkema farms, the Wageningen University experimental field, *A. glutinosa*-associated rhizosphere nodules and bulk soil, soil from Bulgaria, five different Portuguese soils, Russian tundra soil, and six Wieringermeer soils were used for cultivation and isolation of potential *B. benzoevorans* relatives. All soils yielded ca. 10^4 to 10^5 CFU per g of soil on either of the two mineral media, except for the Wieringermeer samples, from which only ca. 10^3 CFU g^{-1} could be retrieved.

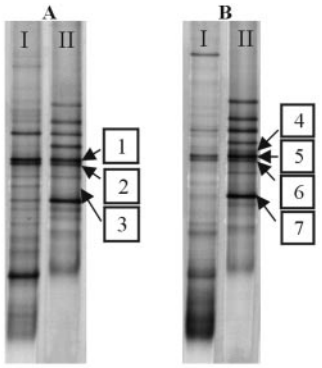
Multiplex PCR was used to tentatively identify *B. benzoevorans* relatives. A total of 192 CFU from each Dutch soil sample were screened, except for the Wieringermeer samples, in which the possible maximum of CFU was picked, and 48 CFU from the Portuguese, Bulgarian, and Russian soils were analyzed. In general, ca. 20% of all screened colonies retrieved from Dutch soil samples (except the Wieringermeer samples) tested multiplex positive, in contrast to the isolates from Portuguese (2.5%), Bulgarian (no positives), and *A. glutinosa*-associated (no positives) samples. *B. benzoevorans* relatives could not be detected in the Russian tundra, Bulgarian vegetable garden, Portuguese river site, grassland, and vegetable garden soil samples or in the Wageningen *A. glutinosa* rhizosphere soil. Single-colony isolates that were identified as potentially *B. benzoevorans*-related by multiplex PCR were subjected to 16S rRNA gene sequence analysis for further identification.

The highest number of *B. benzoevorans* relatives was found in the soils from the northern part of The Netherlands. About 9% of all 384 screened isolates obtained from the Drentse A and Friesland samples were identified as *B. benzoevorans* relatives, whereas the remaining multiplex-positive isolates belonged to other *Bacillus* spp. The fraction of *B. benzoevorans*-related isolates that could be retrieved from Wageningen and Portuguese soil samples was significantly lower (3.5% of the screened 96 CFU and ca. 1.7% of the screened 240 CFU, respectively). The length of the sequences was ~0.5 kb, and a threshold of 97% sequence similarity was used to consider strains as *B. benzoevorans* related (data not shown).

The isolates used for validation of the new *B. benzoevorans* cluster-specific primers are shown in Table 1. They were chosen to represent a range of *B. benzoevorans*-related and -unrelated microorganisms. Since the 16S rRNA gene sequence similarity of several isolates was <97%, some potentially novel *B. benzoevorans* relatives were cultivated.

Design and validation of new group specific primers for detection of *B. benzoevorans* relatives. Primer sets REX460f-REX1466r and REX576f-REX1446r for the specific detection of *B. benzoevorans* relatives were designed based on a multiple alignment of the complete 16S rRNA gene sequences of *B. benzoevorans* relatives, as well as non-*B. benzoevorans*-related species (Table 3).

The optimal conditions for PCR amplification were experi-



No	Closest relative	Length (bp)	Identity (%)	GenBank accession number
1	*Bacillus benzoevorans*	891	97	AY289513
2	*Bacillus benzoevorans*	916	98	AY289515
3	Uncultured bacterium DA001	884	97	AY289514
4	*Bacillus benzoevorans*	889	97	AY289511
5	Uncultured bacterium DA001	916	98	AY289509
6	Uncultured bacterium DA001	891	98	AY289510
7	Uncultured bacterium DA036	962	99	AY289512

FIG. 1. DGGE profiles of the total bacterial and *B. benzoevorans*-related communities in Drentse A (A) and Friesian (B) soil samples. Identified bands are indicated with numbers and explained in the table below. Lane I, total bacterial fingerprint (universal DGGE primer set GC-0968f–1401r); lane II, *B. benzoevorans*-related fingerprint (specific primer sets REX460f-REX1466r and REX576f-REX1446r and universal DGGE primer set GC-0968f–1401r).

mentally determined by using genomic DNA from 29 bacterial strains as the template (Table 1). The optimum for REX460f-REX1466r was annealing at 56°C and 35 cycles, and for REX576f-REX1446f it was annealing at 71°C and 25 cycles. In agreement with the multiple alignment, the primers discriminated against different *Bacillus* species, which do not belong to the cluster of *B. benzoevorans* relatives (Table 3). These primer sets, when applied in a nested approach, specifically amplified 16S rRNA gene fragments of *Bacillus benzoevorans*, *B. niacini*, *B. pseudomegaterium*, *B. jeotgali*, and uncultured bacterium DA001 as expected (Table 1).

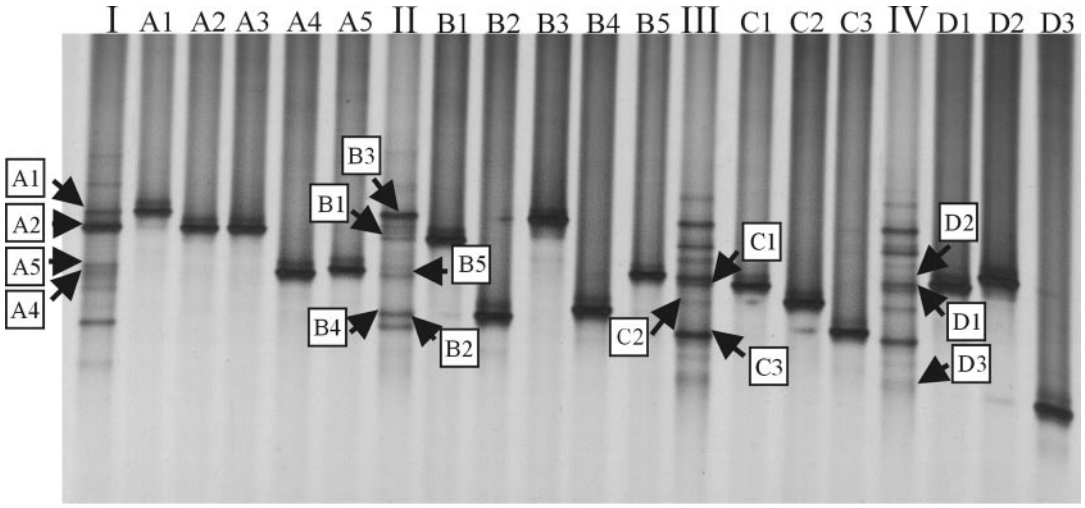
The diversity of *B. benzoevorans* relatives judged by DGGE was compared between DNA isolated directly from the soil and after the plate was washed (data not shown). The DGGE fingerprints showed different profiles.

Specific DGGE patterns of *B. benzoevorans* relatives from the Dutch soil samples. The applicability of the REX primers in combination with DGGE for monitoring of the *B. benzoevorans*-related community was initially tested with DNA isolated from soil samples from Drentse A and Friesian farms in The Netherlands (Fig. 1, lane II), since these grassland fields contain high numbers of *B. benzoevorans*-related isolates. PCR products generated by the two-step nested specific REX PCR were used as the template for PCR with universal primers, i.e., GC-0968f–1401r, and products were separated by DGGE. The resulting profiles of *B. benzoevorans* relatives were compared to the total bacterial community profiles of the two Dutch soil samples from Drentse A and Friesland (lane I). The results revealed that the predominant bands of the upper half of the bacterial DGGE patterns corresponded to bands in the DGGE fingerprint of *B. benzoevorans* relatives.

The DGGE profiles of *B. benzoevorans*-related microorganisms were compared in five soil samples (Fig. 1 and 2), which demonstrated differences in the diversity of *B. benzoevorans* relatives in the different soils. In order to confirm the specificity of the newly developed nested REX PCR approach, cloning and sequence analysis were performed for PCR products that were obtained from several soil samples, including Drentse A and Friesland (Fig. 1), the Wageningen University experimental field, the river Rhine, and Himalaya (Fig. 2). These analyses showed that all of the unique clones, selected after restriction fragment length polymorphism analysis, belonged to the cluster *B. benzoevorans* relatives. The DGGE bands of 20 of 22 studied clones (Fig. 1 and 2, lanes A1 to A5, B1 to B5, C1 to C3, and D1 to D3) from all tested samples corresponded to the predominant bands in the fingerprint of the *B. benzoevorans* relatives. The majority of sequences retrieved from the clone libraries were most closely related with clones from uncultured bacteria, also related to *B. benzoevorans*, detected in Drentse A grassland soils in a previous study (10).

Comparison of the diversity of *B. benzoevorans* relatives in different places. A remarkable richness of *B. benzoevorans* relatives in different places was observed based on DGGE profiles (Fig. 3). No *B. benzoevorans* relatives could be detected in the nonsoil samples, i.e., sludge from an anaerobic wastewater treatment reactor and piglet intestine, nor in the Bulgarian and Russian tundra soil samples (data not shown). In contrast, the other 13 locations showed some similar but also different DGGE profiles. To determine whether the *B. benzoevorans*-related communities from different soil samples were significantly different or similar, similarity indices of the DGGE profiles were calculated. Both Himalayan rhizosphere soil samples showed almost identical *B. benzoevorans*-related communities (similarity index of 95). Remarkably, they showed significant similarity (similarity indices from 82.5 to 88.8) with the *B. benzoevorans* cluster-specific DGGE fingerprints of Dutch soil samples. *B. benzoevorans*-related communities in the soils from Portugal and also in the samples from *A. glutinosa* nodules and surrounding soil were less diverse than the Dutch and Himalayan soils (Fig. 3). The *B. benzoevorans* cluster-specific DGGE banding patterns observed for the different Portuguese samples were significantly less similar and were also distinct from those obtained from the Dutch and Himalayan soils, based on the different positions of the bands in the profiles (Fig. 3b). The similarity indices between the Portuguese and the Himalayan rhizosphere samples varied from 22.2 to 69.7. The DGGE profiles of the samples from the river site and vegetable garden in Portugal showed only two dominant bands. In contrast to other Dutch soil samples, low-complexity *B. benzoevorans*-related communities were found on *A. glutinosa* nodules and the surrounding soil, where only one predominant species and possibly some species present in lower numbers could be detected. A Student *t* test revealed that there was a significant difference between the two separately clustering groups shown on the dendrogram (Fig. 3), since the lowest $P_{\text{two-tail}}$ observed was 0.199776×10^{-7} (df = 18).

Heterogeneity of the 16S rRNA genes of *B. benzoevorans* relatives. The heterogeneity of the 16S rRNA genes was studied to determine whether each single band from the DGGE profiles of *B. benzoevorans*-related members belongs to a different strain. The DGGE fingerprints of 12 pure cultures were compared (Fig. 4). Most of the *B. benzoevorans*-related isolates



No	Closest relative	Length (bp)	Identity (%)	GenBank accession number
A1	Uncultured bacterium DA001	901	97	AY289516
A2	*Bacillus benzoevorans*	901	97	AY289517
A4	Uncultured bacterium DA001	902	97	AY289518
A5	Uncultured bacterium DA001	901	99	AY289519
B1	*Bacillus benzoevorans*	901	98	AY289520
B2	*Bacillus benzoevorans*	906	98	AY289521
B3	*Bacillus benzoevorans*	907	97	AY289522
B4	*Bacillus benzoevorans*	901	97	AY289523
B5	Uncultured bacterium DA036	901	99	AY289524
C1	*Bacillus bataviensis*	911	97	AY289525
C2	*Bacillus benzoevorans*	902	97	AY289526
C3	*Bacillus benzoevorans*	901	96	AY289527
D1	*Bacillus benzoevorans*	901	98	AY289528
D2	*Bacillus benzoevorans*	891	98	AY289529
D3	*Bacillus benzoevorans*	901	97	AY289530

FIG. 2. DGGE analysis of the *B. benzoevorans*-related communities in four different soil samples. Identified bands are indicated with numbers and explained in the table below. Lane I, Wageningen University experimental field; lane II, river Rhine shore; lane III, Himalaya, bulk soil; lane IV, rhizosphere soil sample from *C. nepalensis*, Himalaya; A1 to A5, clones from DNA extracted from sample I; B1 to B5, clones from DNA extracted from sample II; C1 to C3, clones from DNA extracted from sample III; D1 to D3, clones from DNA extracted from sample IV.

showed a single dominant band, often with one to two fainter bands, except *B. benzoevorans* IDA4919, which had four bands. This indicates that at least some of the species have multiple, heterogeneous 16S rRNA gene alleles. In addition, the positions of the predominant bands obtained for some of the isolates were similar. These findings suggest that the diversity of the *B. benzoevorans* relatives can be either slightly over- or underestimated, depending on the populations present in an environmental sample.

Development and succession of *B. benzoevorans*-related communities in Wieringermeer polder soil samples. Changes in the *B. benzoevorans*-related communities over a period of 33 years were investigated in soil samples from an experimental field in the Wieringermeer polder (The Netherlands) with samples obtained in 1942, 1950, 1951, 1966, 1973, and 1975. The soil environment changed from anaerobic to aerobic during the reclamation process (32). *B. benzoevorans*-specific PCR-DGGE was applied to determine whether *B. benzoevorans*-related communities were present in these soils and if changes occurred over the years (Fig. 5). In the 1942 soil sample, only two major bands were detected, whereas the number of bands had increased by 1950 to at least four strong bands, indicating an increase in the diversity during these years. No major changes in the diversity of *B. benzoevorans*-related communities in the period between 1950 and 1975 were detected.

DISCUSSION

Both culture-dependent and independent strategies were developed here to monitor *B. benzoevorans*-related soil bacteria that are surprisingly predominant in some soils around the world. Specific primers for the group were developed and validated that allowed their rapid detection in environmental samples. The validity of the primers was demonstrated by determining the distribution of the group in different geographical locations and also their diversity over time in various soil samples.

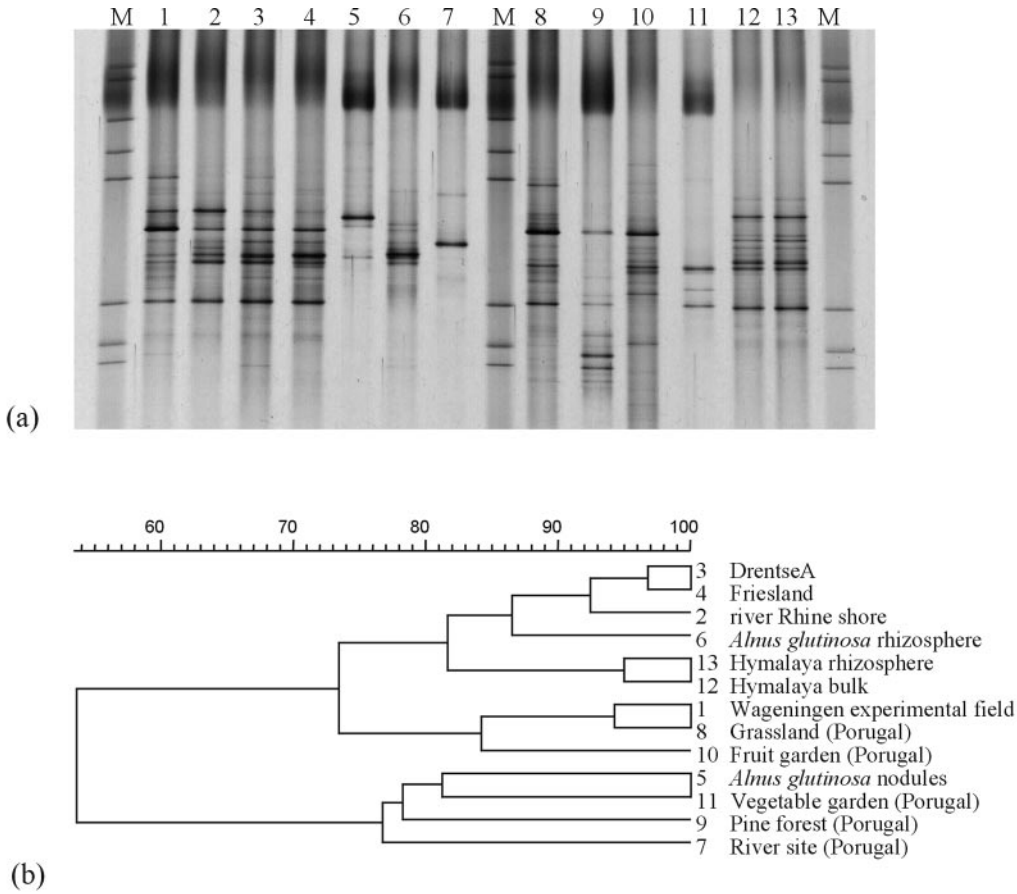
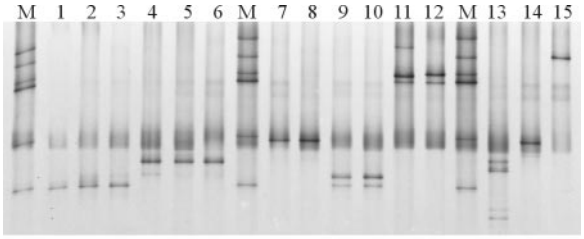


FIG. 3. DGGE profile of the *B. benzoevorans* related community in soil samples from different places (a) and corresponding dendrogram (UPGMA clustering) (b). Lanes: M, marker; 1, Wageningen University experimental field (The Netherlands); 2, river Rhine shore (The Netherlands); 3, Drentse A (The Netherlands); 4, Friesland (The Netherlands); 5, *A. glutinosa* nodules (The Netherlands); 6, *A. glutinosa* rhizosphere (The Netherlands); 7, river site (Portugal); 8, grassland (Portugal); 9, pine forest (Portugal); 10, fruit garden (Portugal); 11, vegetable garden (Portugal); 12, Himalaya, bulk soil; 13, Himalaya, rhizosphere soil from *C. nepalensis*.

A large number of *B. benzoevorans* isolates could be retrieved from a variety of soil samples by using mineral media supplemented with sodium acetate or sodium benzoate as substrates. It was possible to obtain and keep the bacteria viable on the mineral media developed in the present study, which will allow for further morphological and physiological characterization of isolates. The majority of *B. benzoevorans* relatives were isolated from the sodium benzoate medium, whereas sodium acetate amended media proved less suitable for the isolation of *B. benzoevorans* relatives. The species belonging to this cluster probably have better metabolic potential to utilize benzoate than acetate as a substrate (24). The isolation of a substantial collection of cultured representatives of this novel lineage of *Bacillus* now offers a solid basis to gain more insight into the physiology of these microorganisms. In this context it is noteworthy that recently obtained pure culture isolates (e.g., IDA1527 and IDA3504) (Fig. 4) of the *B. benzoevorans* group share an identical 16S rRNA gene sequence (similarities of 99.5 and 99.9%, respectively) with clones of the highly abundant uncultured bacterium DA001 (Jeroen Heyrman, unpublished data).

Nevertheless, the cultivation procedure utilized here was rather time-consuming, and the yield of cultured stains was still low. Fewer than 7% of all isolates obtained in the present study were *B. benzoevorans* relatives. Moreover, it is likely that not all members of this lineage can be cultivated with similar efficiency, since it is widely accepted that most of the soil microorganisms (estimated to be 99.5 to 99.9%) have not yet been isolated and cultivated on laboratory media (1, 2, 26, 31). To compensate for this well-recognized inability to comprehensively address microbial diversity solely by cultivation, we developed a fast and sensitive method for qualitative analysis of the *B. benzoevorans* relatives in the soil based on a group-specific PCR and DGGE. Since the present study focused on a fast and easy detection of *B. benzoevorans*-related bacteria in different soil samples, these two primer sets were constructed to consecutively exclude non-target *Bacillus* species. Because different *Bacillus* species showed 16S rRNA sequence similarities that were too high, it was not possible to develop only one primer pair that would be sufficiently specific to amplify only target sequences from *B. benzoevorans* relatives. Therefore, a two-step nested-PCR approach was necessary for molecular detection of the *B. benzoevorans* relatives. Indeed, the DGGE profiles showed that most of the predominant *B. benzoevorans* related microorganisms detected by these primers could not be cultivated.



No	Closest relative	GenBank accession number
1	*Bacillus novalis* IDA 0078	AJ542511
2	*Bacillus novalis* IDA 0106	AJ542510
3	*Bacillus vireti* IDA 3632	AJ542509
4	*Bacillus soli* IDA 0086	AJ542513
5	*Bacillus soli* IDA 2066	AJ542515
6	*Bacillus soli* IDA 2473	AJ542514
7	*Bacillus bataviensis* IDA 0084	AJ542507
8	*Bacillus bataviensis* IDA 1115	AJ542508
9	*Bacillus drentensis* IDA 1113	AJ542505
10	*Bacillus drentensis* IDA 1967	AJ542506
11	Uncultured bacterium DA001 IDA 1527 [a)]	AJ544783
12	Uncultured bacterium DA001 IDA 3504 [a)]	AJ544784
13	*Bacillus benzoevorans* IDA4919	AY289496
14	*Bacillus bataviensis* IDA4730	AY289497
15	Uncultured bacterium DA001 IDA4921 [a)]	AY289500

FIG. 4. DGGE fingerprint and list of 15 pure cultures of *B. benzoevorans*-related isolates, showing heterogeneity of their rRNA. M, marker. IDA, isolates from Drentse A. Cultured strains that show a 16S rRNA gene sequence identical to that of the uncultured bacterium DA001 are indicated by a superscript "a."

The products of REX576f-REX1446r PCR were subsequently used as a template for DGGE-PCR. We did not use a GC clamp for either of these primers because the products would have a size of ca. 900 bp, which is not optimal for separation by DGGE (22). We also observed that the high annealing temperature, required for specificity (71°C), negatively influenced the amplification efficiency of the GC clamp primer.

The specificity of the REX primers for *B. benzoevorans* relatives was demonstrated by cloning and sequencing PCR products obtained with DNA isolated directly from different soil samples. All of the sequenced clones belonged to the *B. benzoevorans*-related cluster. In most cases, the highest sequence similarity was found with the Drentse A clones corresponding to uncultured relatives of *B. benzoevorans*, which were retrieved from a Dutch Drentse A soil 16S rRNA gene clone library (10, 11).

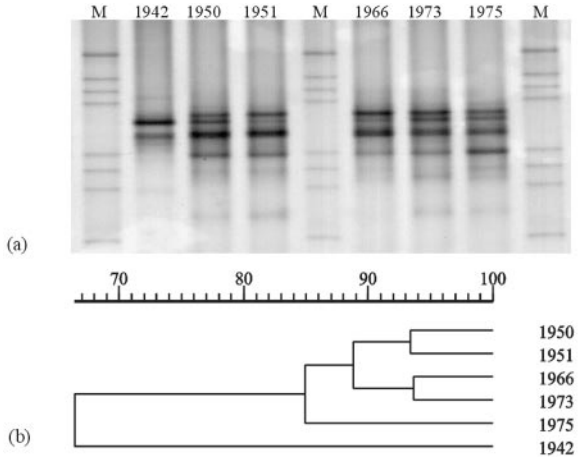


FIG. 5. DGGE profile of the *B. benzoevorans*-related community in soil samples from different years (Wieringermeer polder) (a) and the corresponding dendrogram (UPGMA clustering) (b). M, marker; 1942, 1950, 1951, 1966, 1973, or 1975 indicate the year of sampling.

The REX primers provide a convenient and fast method not only for detection of *B. benzoevorans* relative populations in the soil samples but also for monitoring of this part of the bacterial community over time and its distribution in different places. We studied the presence of these microorganisms in 15 soil samples and two nonsoil samples (anaerobic sludge and gut content). In neither of the two nonsoil environments were *B. benzoevorans* relatives detected, which was an expected result since these ecosystems are not among the preferred habitats for characterized *Bacillus* species belonging to this cluster. Specifically, the anaerobic conditions in the two environments are probably a limiting factor for the aerobic *B. benzoevorans* relatives, although the genus *Bacillus* includes some facultative anaerobic members. In the soil samples, *B. benzoevorans* relatives were present in 19 of the 21 studied soil samples from different countries. Although *B. benzoevorans* relatives could be found worldwide (8, 10), they were not detectable in all of the samples from the different countries and thus do not comprise the predominant soil bacteria everywhere. In the Russian and Bulgarian samples they are present, if at all, in numbers that are below the detection threshold of the PCR-DGGE method. The highest predominance of the *Bacillus* species from this cluster was demonstrated in the Dutch and Himalayan soil samples. In all of the Portuguese habitats *B. benzoevorans* relatives were present but not as the predominant population. When soil samples from similar environments but from different countries were compared (e.g., river sites in The Netherlands and Portugal), it was found that the *B. benzoevorans*-related community was also different. Significant differences in the *B. benzoevorans*-related communities were found not only between samples from the different countries but also between soils collected from different habitats in the same country.

In the period from 1942 to 1950, significant differences in the DGGE profiles of the *B. benzoevorans*-related community in soil samples from the Wieringermeer polder were observed. These changes in the diversity of the *B. benzoevorans* relatives can be explained by the fact that in 1945 the Wieringermeer polder was flooded during the war (29). It was subsequently reclaimed and, at that time, agricultural activities were also developed that can be another reason for the observed increase in the bacterial diversity of the reclaimed land. From 1950 onward, the richness did not alter, but the intensity of the DGGE bands slightly varied, suggesting changes in relative abundance of the populations within this bacterial community.

The strategy that combines the *B. benzoevorans* relatives-specific PCR with DGGE is widely applicable for monitoring of the temporal and spatial diversity of *B. benzoevorans*-related bacilli and their abundance in the bacterial community. Their abundance suggests that these broadly distributed and, in certain places, predominant microorganisms have an important role in the soil ecosystems, one that will be examined in further studies.

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