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Genotypic and phenotypic differentiation of an antifungal biocontrol strain belonging to *Bacillus subtilis*

P. Marten[1], K. Smalla[2] and G. Berg[1]

[1]University of Rostock, Department of Biology, Microbiology, and [2]Federal Biological Research Center for Agriculture and Forestry, Institute for Plant Virology, Microbiology and Biosafety, Braunschweig, Germany

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P. MARTEN, K. SMALLA AND G. BERG. 2000. Physiological and molecular fingerprints of biotechnologically relevant rhizobacteria are necessary for registration, patenting, recognition and quality checking of the strains. To characterize the biological control agent, *Bacillus subtilis* B2g, the strain was compared with other plant-associated *B. subtilis* isolates. Phenotypic characterization included biochemical and nutritional properties, *in vitro* activity and analysis of potential antagonistic mechanisms towards several plant pathogenic fungi. According to the phenotypic characteristics, it was not possible to differentiate the biocontrol agent from the other strains, although the enzymatic fingerprint was unique. Genotypic diversity among the isolates was characterized by molecular fingerprinting methods using REP–PCR (repetitive extragenomic palindromic PCR), and macrorestriction of genomic DNA and electrophoretic separation of DNA fragments by pulsed-field gel electrophoresis (PFGE). A protocol for PFGE analysis using restriction enzyme *Sfi*I for *B. subtilis* was developed. PFGE typing of *B. subtilis* B2g resulted in a unique fingerprint. Therefore, it was possible to differentiate *B. subtilis* B2g, the biocontrol agent of Phytovit®, from other antifungal *B. subtilis* isolates.

INTRODUCTION

Environmentally friendly control of plant disease is a pressing need for agriculture in the 21st century (Emmert and Handelsman 1999). Biological control using antifungal rhizobacteria to suppress plant diseases offers a powerful alternative to the use of synthetic chemicals. Numerous studies have demonstrated the ability of several bacteria to suppress diseases caused by soil-borne and seed-borne plant pathogens (Weller 1988; O'Sullivan and O'Gara 1992; Whipps 1997). The spore-forming Gram-positive bacteria have received less attention than the fluorescent pseudomonads in the publications on biocontrol, and less is known about the mechanisms by which they suppress disease and their ecology (Handelsman and Stabb 1996; Wipat and Harwood 1999).

Bacillus subtilis is not a typical rhizobacterium; it also occurs in the soil surrounding the root (Miller *et al.* 1989). However, it has often been reported as an antifungal agent against plant pathogens (Huber *et al.* 1987; Hebbar *et al.* 1991; Milus and Rothrock 1993). *Bacillus subtilis* A13 was categorized as a plant growth-promoting rhizobacterium (PGPR) by Kloepper *et al.* (1989). In view of the antifungal effect, some biocontrol products based on *B. subtilis* are commercially available for the control of plant diseases (http://www.barc.usda.gov/psi/bpdl/bioprod.htm).

Study of the diversity of antifungal rhizobacteria using fingerprint techniques is important not only for understanding their ecological role in the rhizosphere, but also for the characterization of biological control agents for (i) registration and patenting biocontrol strains, (ii) recognizing the strains, (iii) quality checking during production and (iv) ecological characterization (Plimmer 1993; Lemanceau *et al.* 1995). Several classical and molecular techniques are available for analysing the biodiversity of strains (Tichy and Simon 1994; Rademaker and de Bruijn 1997; De Urzi *et al.* 1999). Although they have been successfully applied to *Bacillus* species, e.g. repetitive extragenomic palindromic PCR (REP–PCR) for investigations of the relationships between *B. subtilis* isolates (Versalovic *et al.* 1992), or macrorestriction of genomic DNA and electrophoretic

Correspondence to: G. Berg, University of Rostock, Department of Biology, Microbiology, Gertrudenstrasse 11a, D-18051 Rostock, Germany (e-mail: gabriele.berg@biologie.uni-rostock.de).

separation of large DNA fragments with pulsed-field gel electrophoresis (PFGE) for the soil micro-organism *B. anthracis* and the related environmental species *B. cereus* and *B. mycoides* (Priest 1993), no molecular fingerprinting protocol for macrorestriction of DNA and PFGE for *B. subtilis* was available.

In this study, an approved biological control agent belonging to the species *B. subtilis* was investigated. *Bacillus subtilis* B2g is able to suppress soil-borne pathogens (e.g. *Pythium ultimum*, *Rhizoctonia solani*) in the rhizosphere of plants and this strain has been developed as a commercial biological control agent (Marten *et al.* 1999). *Bacillus subtilis* B2g was characterized by various phenotypic and genotypic fingerprinting methods and compared with other plant-associated strains of *B. subtilis* to obtain a unique fingerprint for the biocontrol strain and demonstrate intraspecies diversity.

MATERIALS AND METHODS

Isolation of bacterial antagonists

Between 1991 and 1995, bacteria were isolated from the roots of oilseed rape (*Brassica napus* L. subsp. *oleifera* Metzg. Sinsk.) near Rostock in Mecklenburg-Western Pomerania (Table 1). The strains B2a, B2g, B4b, M9a and B909 were obtained from the strain collection of Prophyta GmbH (Malchow/Poel, Germany) and were also isolated from the rhizosphere of oilseed rape in 1993 (Table 1).

Plant roots with adhering soil particles were washed for 20 min in sterile 0·85% NaCl solution. The suspension was heated for 10 min at 80 °C and plated in serial dilutions on nutrient agar (Merck). After incubation for 7 days at 20 °C, selected isolates (12 h culture, 30 °C) were stored in nutrient broth with 15% glycerol at −80 °C.

Table 1 Origin, identification data and antagonistic properties of *Bacillus subtilis* strains included in this study

				Production of secondary metabolites			
Number	Number of strains	Isolation data location*	Date	Antibiosis†	Siderophore‡	Lytic exoenzymes β-1,3-glucanase§	Chitinase¶
B1	B2a	Biestow	7/93	++	+++	+++	–
B2	B2g	Biestow	7/93	++	+++	+	–
B3	B4b	Biestow	7/93	++	+++	+++	–
B4	M9a	Biestow	7/93	++	+++	+++	–
B5	B909	Biestow	7/93	++	+++	+++	–
B6	DSM8563	Rostock	8/91	+++	+	++	–
B7	DSM8565	Rostock	8/91	+++	++	++	–
B8	62	Biestow	10/94	–	–	+++	+
B9	75	Biestow	10/94	–	–	+++	+
B10	113	Biestow	10/94	–	–	+++	+
B11	118	Biestow	10/94	–	–	+++	+
B12	143	Biestow	10/94	–	–	++	+
B13	681	Biestow	3/95	++	+	++	–
B14	907	Biestow	3/95	++	+	++	–
B15	909	Biestow	3/95	++	+	++	+
B16	1473	Biestow	6/95	++	–	++	–
B17	1475	Biestow	6/95	++	+	++	+
B18	1556	Biestow	7/95	++	+	++	–

*Town and village in Mecklenburg-Western Pomerania, Germany.

†Antibiosis: *in vitro* Bioassay with 15 min at 121°C autoclaved sterile filtrate, ++ represents 20–50 % inhibition of growth of *R. solani*; +++ represents > 50 % inhibition of growth of *R. solani*.

‡Siderophores activity *in vitro*: + represents < 5 mm wide orange zone; ++ represents 5–10 mm wide orange zone; +++ represents > 10 mm wide orange zone.

§β-1,3-glucanase-activity in culture filtrate *in vitro*: + represents < 100 nkat mg^{-1} protein; ++ represents 100–200 nkat mg^{-1} protein; +++ represents > 200 nkat mg^{-1} protein.

¶Chitinase activity *in vitro* (plate assay): + represents hydrolysis; – represents no hydrolysis.

Bioassay for *in vitro* inhibition of fungal growth

Antifungal activity was determined according to the paired *in vitro* bioassay I of Berg and Ballin (1994).

Bacterial identification and metabolic fingerprint

For identification and metabolic fingerprinting, the standardized micromethod API 20 B (Bio Mérieux, Marcy-L'Etoile, France), employing the Analytical Profile Index strip which consists of a gallery of 20 biochemical tests separated into seven groups, was used for the Gram-positive bacteria according to the manufacturer's instructions. For investigation of growth at different temperatures, bacteria were cultivated on nutrient agar (Merck) and incubated at 37, 50 and 60 °C for 24 h. The ability to hydrolyse starch and gelatine was examined by plating bacteria on nutrient agar (Merck), containing 2% starch (Merck) or 1% gelatine (Merck), and incubating for 48 h at 30 °C. Starch hydrolysis was assayed after overlaying Petri dishes with Lugol's solution (Bio Mérieux). A positive reaction was shown by a colourless zone (starch = blue colour) around the bacterial colonies. For determination of gelatine hydrolysis, the plates were incubated with a saturated ammonium sulphate solution for 5 min. Hydrolysis was detected by clear zones around bacterial colonies. For examination of enzymatic activities, bacteria were tested using the standardized system API ZYM (Bio Meriéux). Enzyme activity was shown by coloration of the suspension in the wells.

Production of secondary metabolites

The relative inhibition of *Rhizoctonia solani* DSMZ 63010 (obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) by extracellular products of the bacterial strains was assayed according to bioassay II of Berg and Ballin (1994). Siderophore production was assayed according to the method of Schwyn and Neilands (1987). To detect cyanide production, the Aquaquant 14417-Testsystem (Merck) with culture broth of the isolates was used.

Production of cell wall-degrading enzymes

Colonies were screened for chitinases by plating on chitin–agar plates [CA: containing nutrient broth (Sifin) 1·62 g, NaCl 0·5 g, M9 salts 6 g, chitin 5 g (all from Sigma-Aldrich), Bacto-Agar (Difco) 15 g, distilled water 1 l]. Clearance halos indicating enzymatic degradation were measured after 5 days of incubation at 30 °C. β-1,3-glucanase activity was determined by measuring the production of reducing sugars from laminarin (Fluka, Buchs, Switzerland) according to Daugrois *et al.* (1990) and described by Berg (1996).

Production of plant growth-promoting hormones

Production of indole-3-acetic acid (IAA) was investigated using the method developed by Sawar and Kremer (1995). IAA content in 3 ml culture filtrate was spectrophotometrically determined after 30 min incubation with 2 ml Salkowski reagent at 530 nm.

DNA extraction and PCR assay using REP-primers

Bacterial cultures (1·5 ml, Luria Bertani Medium, 28 °C, 130 rev min^{-1}, overnight) were harvested by centrifugation. Genomic DNA was extracted according to Wilson (1987). The REP–PCR mixture [destilled water 28·7 μl; stoffel buffer 5 μl; 2 mmol l^{-1} dNTPs 5 μl; 25 mmol l^{-1} $MgCl_2$ 7·5 μl; DSMO 2·5 μl; primer rep1 and rep2 (Rademaker and de Bruijn 1997) each 0·5 μl; and stoffel fragment (polymerase) 0·5 μl overlaid with two drops paraffin oil and 1 μl template] was centrifuged (30 s, 13 000 rev min^{-1}) and the REP–PCR performed as previously described by Brim *et al.* (1999) and Rademaker and de Bruijn (1997). The amplified products were analysed on 2% agarose gels and stained with ethidium bromide. All strains were tested in three independent replicates.

Macrorestriction of genomic DNA and electrophoretic separation of large DNA fragments with PFGE

A 10 ml early log-phase culture was grown in Luria Bertani medium without yeast extract (shaking at 30 °C for 3 h) and centrifuged for 8 min at 13 000 rev min^{-1}. Pellets were washed twice in 1 ml SE buffer (0·5 mol l^{-1} EDTA; 5 mol l^{-1} NaCl, pH 7·4) and centrifuged at 13 000 rev min^{-1} for 5 min. The cell pellet was resuspended in 1 ml SE buffer (pH 4·5,) mixed, and temperated at 37 °C. The bacterial suspension was then mixed with 1 ml 1·5% Low-Melting-Agarose [peqLab Biotechnology, in TE buffer (10 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA; pH 7·5)], dispensed in a plug mould (Biometra biomedical Analytic GmbH, Göttingen, Germany) and allowed to solidify. For lysis, the resulting plugs were placed in lysis buffer (pH 4·5) containing 100 mmol l^{-1} NaCl, 50 mmol l^{-1} Tris–HCl (pH 7·6), 5 mmol l^{-1} EDTA (pH 9·0); 10 mg ml^{-1} lysozyme (Merck) and 5 U ml^{-1} lysostaphin (Sigma) were added. Following incubation (2 h, 37 °C), the plugs were transferred to a solution which contained 1% sodium lauroylsarcosin (Sigma), 0·5 mol l^{-1} EDTA (pH 9·0) and 4 mg ml^{-1} proteinase K (Merck). The mixture was incubated overnight at 56 °C under gentle shaking. The plugs were washed once for 1 h at room temperature in TE buffer,

once for 1 h at 37 °C in TE buffer containing 5 mmol l^{-1} phenylmethylsulphonylfluoride (Sigma), and twice in TE buffer for 1 h at 4 °C. A quarter slice of each plug was cut out and incubated overnight with 20 U of the restrictions endonuclease *Sfi*I (Pharmacia, Freiburg, Germany), using the buffer and the reaction conditions recommended by the manufacturer. The plugs were then loaded into the wells of 1% agarose gel [peqGold 'pulsed-field'-agarose (peqLab), in 0·5 × TBE buffer (44·5 mmol l^{-1} bor acid, 1 mmol l^{-1} EDTA, 44·5 mmol l^{-1} Tris)]. PFGE was done in a Rotaphor Type V (Biometra) for 18 h at 14 °C, with an electric field of 200 V; the pulse time was increased from 2 to 25 s. A *Saccharomyces cerevisiae* WAY 5–4 A-marker (Biometra) was used as the molecular weight marker. The experiments were repeated twice.

Statistical analysis

Data for phenotypic analysis were converted to binary code and inter-isolate relationships were measured by the Euclidian metric unweighted pair-group average (UPGMA) method using the STATISTICA programme (StatSoft, Hamburg, Germany). Molecular fingerprint patterns generated for each strain were compared and grouped using the GelCompare programme (Kortrijk, Belgium).

RESULTS

To differentiate the biocontrol strain B2g from other strains, 17 rhizobacterial *B. subtilis* isolates obtained from the rhizosphere of oilseed rape (*Brassica napus* L. subsp. *oleifera* Metzg. Sinsk.) in Mecklenburg-Western Pomerania were selected for investigation (Table 1).

Phenotypic characterization

Metabolic and enzymatic fingerprint. The isolates of *B. subtilis* (Table 1) were compared by their metabolic and enzymatic activities using the API systems API 20 B and API ZYM. By combining the results of these tests with microscopic observations (morphology, motility), a total of 46 tests was carried out. The metabolic fingerprint was very homogenous, i.e., all of the isolates were able to hydrolyse starch and gelatine, and grew at temperatures of 37 and 50 °C but not at 60 °C. The strains showed similar properties in utilization of carbon sources and biochemical reactions.

In the enzymatic activities, strain B2g differed from the other isolates in the activity of alkaline phosphatase and α-galactosidase. Therefore, the enzymatic fingerprint of the biocontrol strain B2g was unique and discrimination from the other isolates was possible.

Antifungal activity and mechanisms. The *Bacillus* isolates showed an *in vitro* antagonism towards important fungal pathogens such as *Verticillium dahliae* and *Rhizoctonia solani* investigated by dual culture assay. There was a strong suppression of fungal growth in general, and the isolates could not be differentiated by their antifungal activity.

The rhizobacteria were characterized by the production of antifungal secondary metabolites *in vitro* (Table 1). For most of the strains, antibiotic effects were shown by analysing the culture filtrate. Twelve isolates of the rhizosphere bacteria produced siderophores of different intensity. For seven strains, chitinolytic activity could be detected, whereas all isolates showed activity of β-1,3-glucanase. None of the *Bacillus* strains produced cyanide or the plant growth hormone indol-3-acetic-acid. In summary, the mode of action and quantity of the active metabolites produced were strain-specific.

Genotypic characterization

The 18 different strains of *B. subtilis* isolated from the rhizosphere were subjected to REP–PCR, and to macrorestriction of genomic DNA and electrophoretic separation of DNA fragments with PFGE. PCR amplification with the primer pair, rep1 and rep2, of nine of the 18 isolates (B2a, B2g, B4b, B909, 62, 75, 143, 907 and 1475) resulted in an amplification fragment (data not shown). Analysis of the amplified DNA produced REP–PCR patterns (Fig. 1). The REP fingerprints showed a high similarity of patterns between the *Bacillus* strains. Three groups of isolates with similar patterns, i.e., group I: 62, 75, 143; group II: 907, 1475; group III: B2a, B2g, B4b and B909, could be recognized. The biocontrol strain B2g could not be differentiated from the other isolates by REP–PCR. Therefore, all *Bacillus* strains were analysed by macrorestriction of genomic DNA with the restriction endonuclease *Sfi*I and electrophoretic separation of large DNA fragments with PFGE (Fig. 2). The method was more discriminating than REP–PCR and the profiles were more heterogeneous. The macrorestriction profiles were compared by numerical methods, and the resultant dendrogram (Fig. 3), based on the percentage similarity between isolates, shows the genetic diversity. Four major groups were defined at a 90% similarity level (Fig. 3).

The patterns of the biocontrol strain B2g could be distinguished from the *Bacillus* strains of group II (strains 907, 909, 1475 and 1556) at the 83% similarity level, and at the 68% similarity level from groups III (strains B2a, B4b, B909, 113, 681, 1473, DSM8563 and DSM8565) and IV (strains 62, 75, 118 and 143). The highest conformity of the strain B2g was detected when comparing the patterns of isolate M9a. The patterns were similar in size and intensity of the bands, but different in number of bands (B2g 13

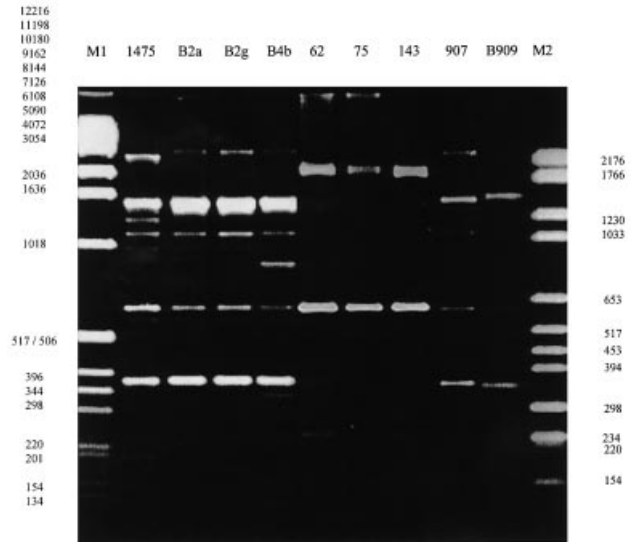

Fig. 1 REP fingerprints of the *Bacillus subtilis* strains. M1: molecular weight markers X, Roche Molecular Biochemicals; M2: molecular weight markers VI, Roche Molecular Biochemicals. The sizes (in basepairs) of the molecular size marker bands are indicated on the left and right of the figure

bands, M9a 16 bands). Seven DNA fragments had the same size (molecular weight), and four of these bands showed a similar intensity. At the 93% similarity level, B2g could be discriminated from the strain M9a. Consequently, the biocontrol strain B2g could be differentiated from the other rhizobacterial *Bacillus* strains only by its genomic DNA macrorestriction profile.

Comparison of physiological and molecular properties

To compare the isolates specifically, a further numerical analysis of their physiological characteristics and antagonistic mechanisms was performed. This analysis allowed a dendrogram to be drawn (results not shown), and strains showing a level of similarity of 70% were clustered. The origin, grouping of phenotypic (metabolic, enzymatic and antagonistic properties) and molecular patterns according to clusters of *Bacillus* isolates are shown in Table 2. The biocontrol strain B2g was grouped into the same cluster by metabolic and antagonistic fingerprint as the isolates B2a, B4b, M9a and B909, but the strains were different in their enzymatic activities. Isolates B2g and M9a were clustered in one group by their molecular fingerprint based on macrorestriction of DNA, but at the 93% similarity level, B2g could be distinguished from this strain.

DISCUSSION

Bacillus subtilis B2g was characterized by various phenotypic and genotypic fingerprinting methods and compared with other strains of *B. subtilis* to obtain unique fingerprints

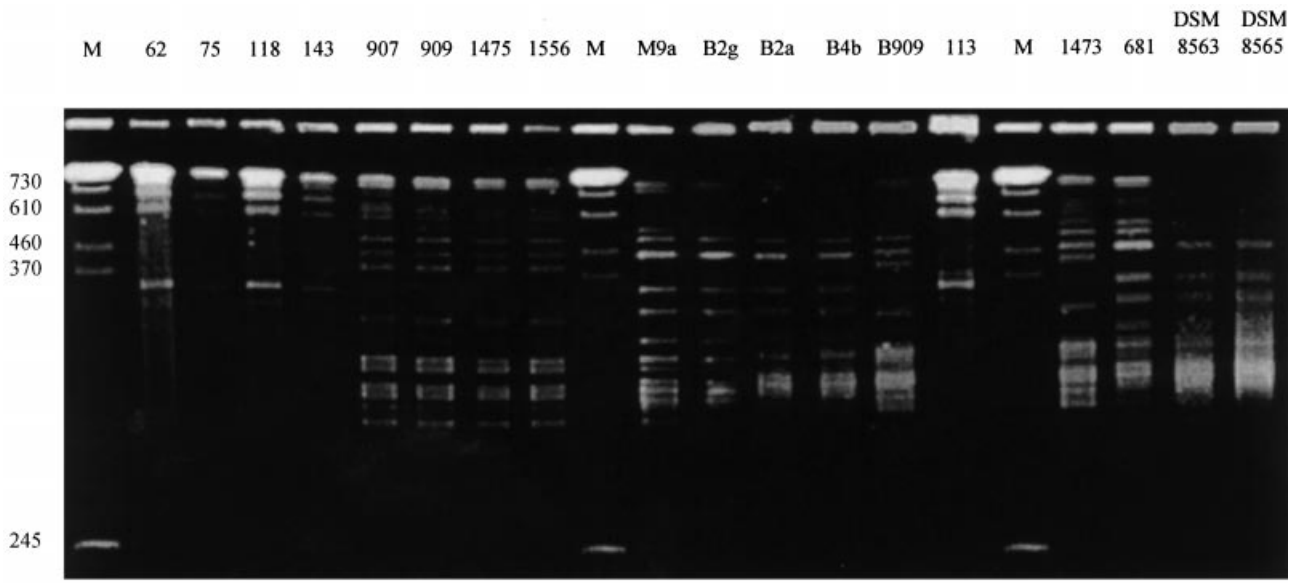

Fig. 2 Genomic DNA macrorestriction profiles of *Bacillus subtilis* strains produced by PFGE after *Sfi*I digestion. M: molecular weight marker *Saccharomyces cerevisiae* WAY 5–4 A (Biometra). The sizes (in kilobase) of the molecular size marker bands are indicated on the left of the figure

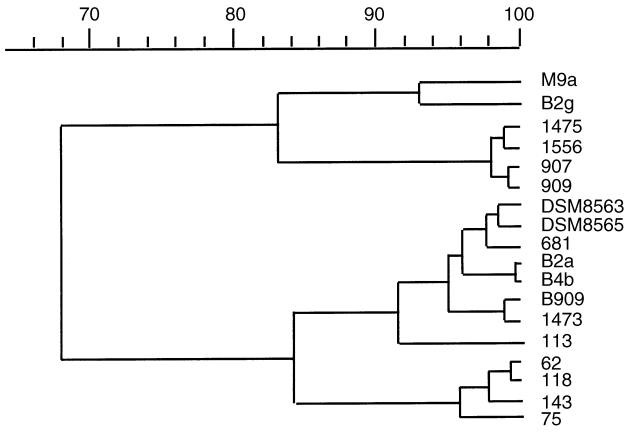

Fig. 3 UPGMA dendrogram showing relationships between *Bacillus subtilis* based on macrorestriction profiles produced by PFGE after *Sfi*I digestion

for the biocontrol strain and to show intra-species diversity.

On the whole, correlations were found in this study between groupings of isolates established by the phenotypic and genotypic characterization. According to its phenotype (metabolic and antifungal properties), the biocontrol strain B2g could not be differentiated from the other *B. subtilis* strains. The enzymatic fingerprint of B2g was unique. However, data from several studies suggest that phenotypic markers are more likely to change over time than are the results of molecular techniques (Carles-Nurit *et al.* 1992; Schlichting *et al.* 1993; van Belkum *et al.* 1993), so the enzymatic fingerprint would not be a certain criterion for differentiation of the biocontrol strain. Therefore, the bacterial isolates were genotypically characterized by molecular methods based on analysis of polymorphisms of DNA (REP–PCR and macrorestriction of genomic DNA).

Table 2 Grouping of *Bacillus subtilis* isolates according to cluster analysis based on physiological and molecular fingerprints

		Cluster group*					
			Physiology				
Number	Number of strains	Origin	Metabolic fingerprint†	Enzymatic fingerprint‡	Antagonistic mechanisms§	Molecular fingerprint¶ REP-PCR	PFGE
B1	B2a	VI	I	I	I	III	III
B2	B2g	VI	I	VI	I	III	I
B3	B4b	VI	I	II	I	III	III
B4	M9a	VI	I	I	I	ND	I
B5	B909	VI	I	II	I	III	III
B6	DSM8563	I	III	I	I	ND	III
B7	DSM8565	I	I	I	I	ND	III
B8	62	II	V	II	IV	I	IV
B9	75	II	I	III	IV	I	IV
B10	113	II	VII	III	IV	ND	III
B11	118	II	VI	III	IV	ND	IV
B12	143	II	IV	III	IV	I	IV
B13	681	III	V	I	I	ND	III
B14	907	III	I	IV	I	II	II
B15	909	III	II	I	II	ND	II
B16	1473	IV	III	V	III	ND	III
B17	1475	IV	I	I	II	II	II
B18	1556	V	I	I	I	ND	II

*Data of phenotypic and genotypic properties were clustered using UPGMA. Strains showing a level of similarity of 70% (physiological and antagonistic fingerprint) and 90% (molecular fingerprint based on macrorestriction with *Sfi*I) were clustered into the same group.
†Based on physiological properties obtained with API 20 B.
‡Based on enzymatic activities obtained with API ZYM.
§Based on antagonistic mechanisms *in vitro*.
¶Based on REP-PCR and macrorestriction of genomic DNA with restriction endonuclease *Sfi*I.
ND, not detected.

Although REP–PCR allowed grouping of strains, it was not possible to distinguish the biocontrol strain from the other isolates. The macrorestriction method was more highly discriminant than REP–PCR and allowed grouping of the isolates and differentiation of the biocontrol strain B2g from the other *B. subtilis* isolates. As far as is known, this is one of the first published reports of the use of PFGE for separating strains of this species. Macrorestriction of genomic DNA and PFGE are powerful tools for differentiation or typing of bacterial strains (Swaminathan and Matar 1993; Tenover *et al*. 1994; Arbeit 1995; Yao *et al*. 1995; Tynkkynen *et al*. 1999). Liu *et al*. (1997) described the PFGE technique for DNA macrorestriction as a highly discriminatory and reproducible method for epidemiological investigations of *B. cereus* infections. A disadvantage of this method is that it is relatively costly and time consuming.

On the other hand, marker systems could be used for the recognition of introduced biocontrol strains from diverse indigenous microbial populations (Errampalli *et al*. 1999). Antibiotic resistance markers are most frequently used (Prosser 1994), but genetic recombination to introduce foreign genes ('marker genes') into a root-colonizing bacterium has been suggested as an alternative to spontaneous antibiotic resistance (Hemming and Drahos 1984; Drahos *et al*. 1986; Kluepfel *et al*. 1991). Such marker genes, e.g. *lacZY*, *xylE*, *gfp* or *lux* genes, have been tested for detection in the environment (Prosser 1994; Errampalli *et al*. 1999). The *lacZY* system has been exploited as a marker system for fluorescent soil pseudomonads which lack the *lacZY* genes and cannot utilize lactose (Drahos *et al*. 1986; Hofte *et al*. 1990; Ryder *et al*. 1994). Introduction of bioluminescence (*lux*) genes from *Vibrio fischeri* and *V. harveji* into *B. subtilis* on a plasmid vector and by chromosomal integration was reported by Cook *et al*. (1993). No bioluminescent bacteria have been reported as natural constituents of rhizosphere bacterial communities based on spread plate or broth enrichment techniques (Beauchamp *et al*. 1991), suggesting that *lux* may be a practical marker gene for measuring root colonization (Kloepper and Beauchamp 1992). The introduction of recombinant micro-organisms to the field is still restricted in many countries because of a lack of knowledge of the potential hazards that might be associated with their large-scale use (van Elsas *et al*. 1998). Last, but not least, there are many reservations about the release of GEMs into the environment, and a low acceptance of application of GEMs in the environment by the potential users of biological control products. Nevertheless, in addition to fingerprinting techniques, the use of marker genes for easy detection is an excellent method of monitoring introduced micro-organisms in the environment in the field of research. Unique molecular fingerprints are also necessary for registration and patenting of the strain. On the basis of the present results and additional information on biocontrol efficacy, it was possible to register the biocontrol candidate *Bacillus subtilis* B2g at the Federal Biological Research Center for Agriculture and Forestry (BBA, Braunschweig, Germany). The biocontrol product Phytovit® is commercially available from Prophyta GmbH (Malchow/Poel, Germany).

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