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NOTES

Rapid Identification of Heterotrophic, Thermophilic, Spore-Forming Bacteria Isolated from Hot Composts

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The restriction enzyme profiles of 16S ribosomal DNAs (rDNAs) amplified by PCR from thermophilic heterotrophic bacterial strains isolated from composts were compared with those of reference strains. This allowed us to assign all but 1 of 16 strains to four different *Bacillus* species (namely, *Bacillus stearothermophilus*, *Bacillus pallidus*, *Bacillus thermoglucosidasius*, and "*Bacillus thermodenitrificans*"). This study showed that PCR restriction analysis of 16S rDNA contributes to rapid and reliable identification of newly isolated strains belonging to recognized species.

A few studies have reported the presence of thermophilic bacteria in hot compost (3, 4, 7, 19, 20). Strom (19, 20) isolated more than 750 heterotrophic spore-forming strains from compost; very few of these strains grew at temperatures above 60°C, and growth at 65°C was restricted to *Bacillus coagulans* (type A) and *Bacillus stearothermophilus*. Until recently, only strains related to *B. stearothermophilus* were identified from the hottest compost samples screened (65 to 69°C) (7, 19, 20). The great diversity of thermophilic bacteria related to the genus *Bacillus* has frequently been emphasized (16, 22), but it appears that only a few of the isolates have properly been identified to date. The morphology of sporulating cells, the shape of colonies, and growth abilities have proved to be insufficient for unequivocal identification of *Bacillus* strains (10, 11). The purpose of the present study was to identify heterotrophic, thermophilic, spore-forming strains isolated from hot composts by using a rapid molecular method based on the restriction profiles of 16S ribosomal DNA (rDNA) amplified by PCR.

Serial dilutions of compost sample suspensions were carried out in five different media. B and DN media consisted simply of nutrient broth (Merck, Darmstadt, Germany); DN medium was supplemented with 2 g of KNO_3 per liter. GA, P, and PN media were synthetic media composed of a basal mineral medium (1) supplemented with various growth substrates at a concentration of 2 g liter^{-1} [GA medium contained D-glucose and sodium acetate; P medium contained sodium pyruvate; PN medium contained sodium pyruvate and $(NH_4)_2SO_4$)]. The cultures were incubated under air at 65°C for 1 to 6 days, and pure colonies were isolated by repeated streaking on the same media solidified with agar. Colonies varying in appearance were picked deliberately to try to increase the number of different species isolated (the second and third letters of the compost strain designations in Fig. 1 refer to the isolation medium). Pure strains were then routinely cultivated at 60°C on B medium supplemented with 2 g yeast extract per liter and solidified with agar (NAY medium). The type and reference strains are listed in Table 1.

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Metabolic tests were carried out at 55°C with API 20 NE strips (BioMérieux, Marcy-l'Etoile, France) by using a few fresh colonies suspended in the basal mineral medium supplemented with 0.1 g of yeast extract per liter and 0.1 g of peptone per liter, unless indicated otherwise. Starch hydrolysis was tested in the basal mineral medium as described by Smibert and Krieg (17). Anaerobic growth (denitrification) was tested on NAY medium plates supplemented with 10 g of KNO_3 per liter. Cultures were incubated for 4 days at 60°C in desiccator jars; oxygen was eliminated by using an Anaerocult IS bag (Merck).

DNA extraction and purification were carried out as previously described (4), except that the cells were treated with lysozyme prior to guanidium thiocyanate DNA extraction (13). The 16S rDNA was selectively amplified by using oligonucleotide primers designed to anneal to bacterial 16S rRNA genes as previously described (4). The reaction conditions were as follows: 1 to 5 ng of template DNA, 0.8 U of Goldstar *Taq* DNA polymerase (Eurogentec, Seraing, Belgium), 5 μl of 10× Goldstar PCR buffer, 1.5 mM (final concentration) $MgCl_2$, 0.25 μM forward primer, 0.25 μM reverse primer, and each deoxynucleoside triphosphate at a concentration of 170 μM were combined in a total volume of 50 μl. Amplification was carried out in a model PTC-100 thermal cycler (MJ Research, Inc., Watertown, Mass.) with the following program: a preliminary denaturation step was carried out at 95°C for 1 min and was followed by 35 cycles consisting of 30 s at 94°C (denaturation), 30 s at 62°C (except for the three first touchdown cycles, which were successively at 68, 66, and 64°C), and 1 min at 72°C (extension). For restriction enzyme digestion, 50 ng of the PCR product was mixed with 2 U of *Hae*III or 1 U of *Hin*fI (New England Biolabs, Inc., Beverly, Mass.), *Taq*I, or *Rsa*I (Gibco BRL) and incubated for 4 h according to the manufacturer's instructions. PCR products and restriction digests were separated by electrophoresis as previously described (4).

Phenotypic characterization. The strains studied were isolated from 2- to 80-day-old composts as previously described (4); the sample temperatures ranged from 57 to 78°C. All of the strains were rods and formed oval endospores. Spore position varied from central to terminal. These strains were initially supposed to belong to the genus *Bacillus*, according to the description of Gordon et al. (8). Their maximum growth temperatures were between 65 and 72°C. Except for the *Bacillus pallidus* group, as pointed out previously (22), all of the strains

TABLE 1. Reference strains used in this study

Species	Strain[a]	EMBL 16S rDNA sequence accession no.
B. stearothermophilus	DSM 22^T (= ATCC 12980^T) (6)[b]	X60640 (2)
B. stearothermophilus	DSM 494 (12)	NA[c]
B. thermoglucosidasius	DSM 2542^T (= ATCC 43742^T) (21)	X60641 (2)
Bacillus sp.	DSM 6499 (18)	NA
"*B. thermodenitrificans*"	DSM 465 (= ATCC 29492) (9)	Z26928(14)
B. pallidus	DSM 3670^T (= ATCC 51176^T) (15)	Z26930(14)
Bacillus sp.	DSM 2349 (5)	Z26929(14)

[a] DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; ATCC, American Type Culture Collection, Rockville, Md.
[b] The numbers in parentheses are reference numbers.
[c] NA, not available.

could grow anaerobically with nitrate as a respiratory substrate. Glucose, mannose, and maltose were utilized as growth substrates by all but two strains, as reported previously for most thermophilic bacilli (22), and starch was hydrolyzed by most strains (data not shown).

PCR restriction analysis (PRA) of 16S rDNA. The restriction fragment length polymorphism profiles of 16S rDNAs (digested with *Hae*III) of the strains isolated from composts and of the reference strains listed in Table 1 formed five groups with distinctive patterns (Fig. 1). To avoid confusion with primer dimer bands, and because of the detection threshold, restriction fragments shorter than 90 bp were disregarded.

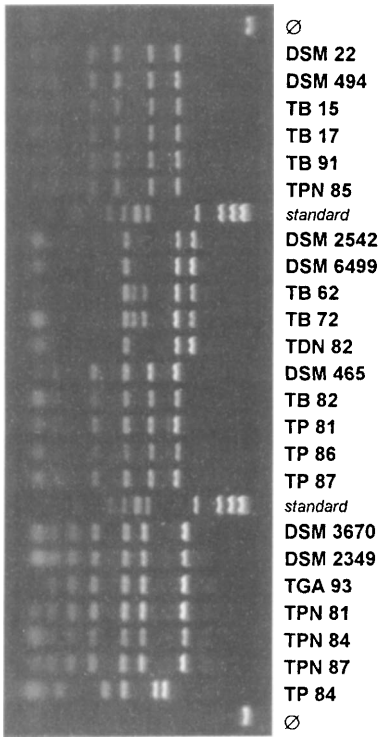

FIG. 1. PRA profiles of 16S rDNAs, selectively amplified and digested with *Hae*III, of *Bacillus* reference strains and of 16 strains isolated from hot composts. ∅, undigested amplification product; *standard*, ΦX174RF digested with *Hae*III. See Table 1 for reference strains.

These profiles permitted us to group all but 1 of the 16 strains with the seven reference strains belonging to four species (namely, *B. stearothermophilus*, *B. pallidus*, *Bacillus thermoglucosidasius* and "*Bacillus thermodenitrificans*"). The profiles obtained with restriction enzymes *Hin*fI and *Rsa*I showed no distinctive patterns for the strains tested (data not shown).

Theoretical restriction profiles calculated from 16S rDNA sequences available in the EMBL nucleotide sequence database were compared with the results obtained for our strains and for the reference strains. Within the first group, the profiles of reference strains DSM 22 and DSM 494 matched the theoretical profile calculated from the corresponding sequence of *B. stearothermophilus* (EMBL accession no. X60640). In the second group, the profile of reference strain DSM 2542 matched the theoretical profile of the sequence EMBL X60641. No 16S rDNA sequence could be found for strain DSM 6499. However, the restriction profiles did show that this strain was related to *B. thermoglucosidasius* DSM 2542, as described previously (18).

In the third group, the profile of the reference strain "*B. thermodenitrificans*" DSM 465 matched the theoretical profile of the corresponding sequence EMBL Z26928, except for the calculated 206-bp fragment that appeared to be 240 ± 5 bp long on the gel, and this was also true for the four related compost strains. This may have been due to the lack of recognition of a restriction site that caused a 25-bp fragment to remain attached to the 206-bp fragment. *Taq*I restriction profiles confirmed that this group was distinct from the three other groups (data not shown).

In the fourth group, the profiles of reference strains DSM 3670 and DSM 2349 matched the theoretical profiles of the corresponding sequences EMBL Z26930 and EMBL Z26929, respectively. Strain DSM 2349 could be linked to *B. pallidus* DSM 3670, as has been proposed in previous studies (14, 22).

The profile of strain TP-84 could not be related to any 16S rDNA sequence available for thermophilic bacilli.

PRA profiles are a powerful tool for identifying new strains related to heterotrophic thermophilic bacilli. The results obtained showed, however, that in some cases (e.g., the "*B. thermodenitrificans*" group [see above]) the digestion profiles of new strains should be compared with profiles obtained experimentally from reference strains, and the restriction profiles calculated from published sequences should not be relied on.

By using five different isolation media, we showed that there is a taxonomically and metabolically diverse population of heterotrophic thermophilic spore-forming bacteria in thermogenic composts. Few growth tests had diagnostic value for any single group of strains; only the strains related to *B. pallidus* had common features (particularly the absence of growth under denitrificating conditions) that clearly distinguished them from the other groups. Strain TP-84, despite its lack of reactivity with most of the substrates tested, like *Bacillus thermocloacae* strains (22), could not be related to this species by its PRA profile. Phenotypic tests can therefore not be relied on for taxonomic grouping of new *Bacillus* strains, while PRA of 16S rDNA appears to be a promising tool for rapid and reliable identification of newly isolated strains of recognized species.

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