

## Specific Detection and Real-Time PCR Quantification of Potentially Mycophagous Bacteria Belonging to the Genus *Collimonas* in Different Soil Ecosystems<sup>∇†‡</sup>

Sachie Höppener-Ogawa,\* Johan H. J. Leveau, Wiecher Smant,  
Johannes A. van Veen, and Wietse de Boer

Netherlands Institute of Ecology (NIOO-KNAW), Centre for Terrestrial Ecology, Boterhoeksestraat 48, 6666 GA Heteren, The Netherlands

Received 19 February 2007/Accepted 24 April 2007

The bacterial genus *Collimonas* has the remarkable characteristic that it grows at the expense of living fungal hyphae under laboratory conditions. Here, we report the first field inventory of the occurrence and abundance of *Collimonas* in soils ( $n = 45$ ) with naturally different fungal densities, which was performed in order to test the null hypothesis that there is a relationship between the presence of *Collimonas* and fungal biomass. Estimates of fungal densities were based on ergosterol measurements. Each soil was also characterized in terms of its physical and chemical properties and vegetation and management types. Culturable *Collimonas* was identified in plate-spread soil samples by its ability to clear colloidal chitin, in combination with a *Collimonas*-specific restriction fragment length polymorphism analysis of 16S rRNA PCR amplified from individual colonies. Using this approach, we found culturable collimonads only in (semi)natural grasslands. A real-time PCR assay for the specific quantification of *Collimonas* 16S rRNA in total soil DNA was developed. *Collimonas* was detectable in 80% of the soil samples, with densities up to  $10^5$  cells  $g^{-1}$  (dry weight) soil. The numbers of *Collimonas* cells per gram of soil were consistently lowest in fungus-poor arable soils and, surprisingly, also in fungus-rich organic layers of forest soils. When all soils were included, no significant correlation was observed between the number of *Collimonas* cells and ergosterol-based soil fungal biomass. Based on this result, we rejected our null hypothesis, and possible explanations for this were addressed.

All described strains of the genus *Collimonas* are soil bacteria that have the capacity to grow at the expense of intact, living fungal hyphae (9). This property, termed mycophagy (7, 17, 27), has not been well examined for soil bacteria (7). In contrast, fungal mycophagy, which is better known as mycoparasitism, has been studied extensively (5, 6). This is especially the case for the mycoparasitic fungi (e.g., *Trichoderma* spp.) that are applied as biocontrol agents for plant-pathogenic soil fungi (19, 31). Although the mechanisms of mycophagous growth of collimonads have yet to be elucidated, it is known that these bacteria share some properties with mycoparasitic fungi, such as the production of chitinases (9–11, 27), which are thought to be involved in the destabilization of the fungal cell wall (14, 18, 48). However, de Boer et al. reported that for collimonads chitinase activity alone could not explain mycophagous growth (9), and other factors (for example, other lytic enzymes and antibiotics) should be involved (9, 13, 45).

Until now, collimonads have been quantified only for the acidic dune grassland soils from which they were originally isolated (11). In these soils, the numbers of collimonads ranged from  $10^3$  to  $10^5$  CFU per g (dry weight) soil. Enumeration was

based on plate counts of chitin-degrading colonies on agar plates containing colloidal chitin. On such plates, *Collimonas* strains can be recognized as halo-producing bacteria due to clearing of chitin, with concomitant production of translucent biomass. However, identification of collimonads on the basis of colony morphology cannot be conclusive without a more specific identification method. For this purpose, we developed and describe here a *Collimonas*-specific restriction fragment length polymorphism (RFLP) assay based on restriction analysis of PCR-amplified 16S rRNA.

Enumeration on chitin agar plates provides an indication of the abundance of collimonads. However, collimonads are relatively slow-growing bacteria, and, therefore, they can remain undetected when fast-growing chitinolytic bacteria are present as well. Additionally, this method does not detect potentially nonculturable collimonads. As an alternative to plate enumeration, we developed and applied a culture-independent real-time quantitative PCR (qPCR) assay (3, 21) for quantification of collimonads in soil. Real-time PCR has been used successfully to detect and quantify bacterial cells in various environmental samples (1, 3, 20, 25, 26), including soils (16, 21, 30, 34, 38, 42). In the current study, the presence of soil collimonads was examined for a wide range of soils (40 sites) using both the plate count-RFLP method and the real-time PCR assay. In order to identify the possible factors that determine collimonad soil population sizes, the presence or absence of culturable collimonads and the real-time PCR-based total numbers of collimonads were compared to fungal density based on ergosterol measurements, as well as other soil properties, vegetation composition, and management practices.

\* Corresponding author. Mailing address: Netherlands Institute of Ecology (NIOO-KNAW), Centre for Terrestrial Ecology, Boterhoeksestraat 48, 6666 GA Heteren, The Netherlands. Phone: 31-26-4791409. Fax: 31-26-4723227. E-mail: s.ogawa@nioo.knaw.nl.

† Supplemental material for this article may be found at <http://aem.asm.org/>.

‡ Publication no. 4047 NIOO-KNAW of The Netherlands Institute of Ecology.

<sup>∇</sup> Published ahead of print on 4 May 2007.

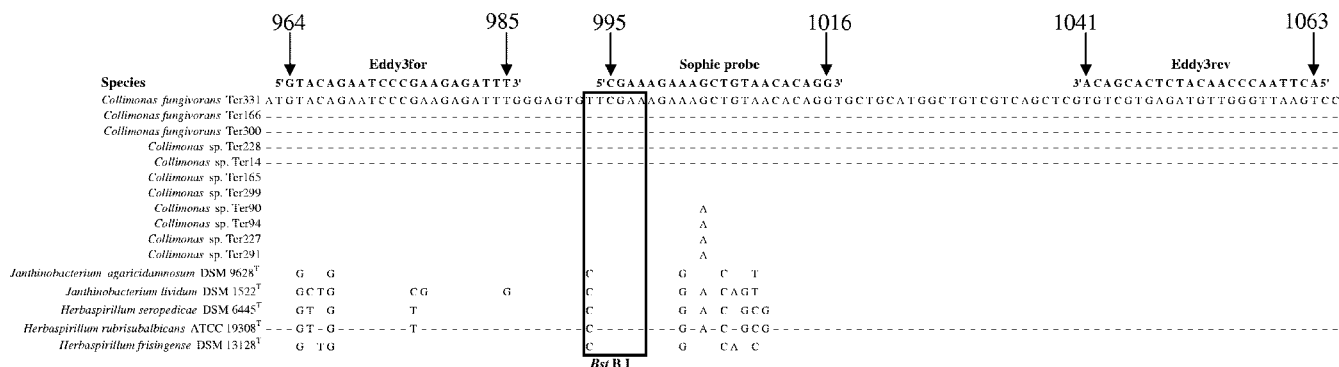


FIG. 1. Alignment of partial 16S rRNA sequences from previously described collimonads and closely related species. The numbers correspond to the numbers in the 16S rRNA sequence of *C. fungivorans* Ter331 (accession no. AJ310395). Dashes indicate nucleotides identical to those in the Ter331 sequence. The locations of primers Eddy3for and Eddy3rev, as well as the dual-labeled probe Sophie, are indicated. The boxed nucleotides indicate the BstBI restriction site unique to collimonads. The accession numbers for the 16S rRNA sequences of strains Ter166, Ter300, Ter228, Ter14, Ter165, Ter299, Ter90, Ter94, Ter227, Ter291, DSM 9628<sup>T</sup>, DSM 1522<sup>T</sup>, DSM 6445<sup>T</sup>, ATCC 19308<sup>T</sup>, and DSM 13128<sup>T</sup> are AY281140, AY281145, AY281148, AY281135, AY281139, AY281144, AY281136, AY281138, AJ496445, AY281143, Y08845, Y08846, Y10146, AB021424, and AJ238358, respectively.

## MATERIALS AND METHODS

**Soils, sampling procedure, and soil analyses.** A total of 40 sites in The Netherlands were selected on the basis of differences in vegetation (grassland, forest, agricultural crops, heathland, and shrubs) and management practices (agricultural sites, abandoned arable sites, and nature reserves), as well as physical and chemical soil characteristics (particle size, pH, moisture content, organic matter content, total phosphorus content, carbon content, nitrogen content, C/N ratio, and chloride content) (see Table S1 in the supplemental material). At each sampling site, soil was collected at at least 30 spots that were selected randomly in a 50- by 50-m plot using a corer with a 3.5-cm diameter, and they were pooled to obtain a composite sample. For most sites, only the upper 10-cm layer was sampled, but at five forest sites with a well-developed organic horizon, separate samples were taken from the organic layer and the upper 10 cm of the mineral soil. Hence, the total number of samples was 45. The composite samples were sieved (mesh size, <4 mm) and stored at 4°C for no more than 1 week until the analyses were started. Physical and chemical characteristics of soil were analyzed as described elsewhere (43, 44).

Estimates of soil fungal biomass were based on measurements of the soil ergosterol content (mg per kg soil). Ergosterol is the major sterol in the membrane of most fungi and is not common outside the fungal kingdom (46, 47). Soil ergosterol was extracted using an alkaline extraction procedure and was analyzed by high-performance liquid chromatography as described elsewhere (12).

Total DNA from soil samples was extracted using a Power Soil DNA isolation kit (MOBIO Laboratories, Solana Beach, CA) according to the manufacturer's instructions except that two 30-s bead-beating treatments with a Mixer Mill MM301 (Retsch, Haan, Germany) were used instead of vortex mixing. In addition, DNA was eluted in a final volume of 50  $\mu$ l instead of 100  $\mu$ l. The DNA extract was diluted 10-fold before it was used as a template for real-time PCR quantification.

**Detection and identification of culturable collimonads by RFLP analysis of 16S rRNA.** Soil suspensions were prepared as described elsewhere (10). Fifty-microliter portions of 10-fold dilutions were plated on chitin-yeast extract agar with the composition described previously (11) but with an extra 0.1 g per liter of the fungal inhibitor delvocid (DSM, Delft, The Netherlands) (11). The plates were sealed with Parafilm, incubated at 20°C for 2 weeks, and inspected regularly for halo-forming (chitinolytic) bacterial colonies. Among the chitinolytic bacterial colonies, we screened for *Collimonas*-like isolates based on the morphology described by de Boer et al. (11). In addition, other chitinolytic colony types were sampled. In total, 205 chitinolytic isolates were identified. All isolates were streaked on 0.1 $\times$  tryptone soy broth (TSB) (Oxoid) agar containing chitin (11). This TSB-chitin agar was used to see if the chitinolytic ability of the strains was repressed by the presence of TSB, as such catabolic repression of chitinase production has been reported for all *Collimonas* strains described so far (11). Plates were screened for chitinolytic activity after 2 weeks of incubation at 20°C.

Total DNA of the 205 chitinolytic isolates was extracted from 3-ml King's broth cultures (King's B) incubated at 27°C on a rotary shaker (200 rpm) for 48 h, using the procedure used for extraction of DNA from soil samples.

Nearly complete fragments of 16S rRNA genes were amplified from isolated genomic DNA using the universal bacterial primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GRTACCTGTGTACGACTT-3') (15). Bacterial DNA (1  $\mu$ l of 10 $\times$  diluted genomic DNA) was added to a 25- $\mu$ l (final volume) mixture containing 0.6  $\mu$ M of each primer, 200  $\mu$ M of each deoxynucleoside triphosphate, 2.5  $\mu$ l of 10 $\times$  buffer (Promega, Leiden, The Netherlands), and 0.056 U *Taq* polymerase (Promega). The PCR was performed using a touchdown program in which the annealing temperature initially decreased from 65 to 55°C by 2°C per cycle, followed by 12 cycles at 55°C for 1 min. The denaturing step was 30 s at 92°C, and the extension step was 2 min at 68°C.

The restriction enzyme BstBI (New England Biolabs, MA) was used to digest the PCR amplicons. Based on the current information from the Ribosomal Database Project (Ribosomal Database Project II; <http://rdp.cme.msu.edu/>), we determined that collimonads are the only members of the family *Oxalobacteraceae* which share a BstBI restriction site in the 16S rRNA gene at the position corresponding to nucleotides 993 to 998 of the 16S rRNA gene of *Collimonas fungivorans* Ter331 (accession number AJ310395). Restriction fragments were examined on 1.8% agarose gels in 0.5 $\times$  TSB buffer (11), using O'GeneRuler 1-kb DNA Ladder Plus (Fermentas, St. Leon-Rot, Germany).

The specificity of the RFLP assay for collimonads was tested using five representative strains belonging to genera closely related to *Collimonas*, namely, three *Herbaspirillum* type strains (*H. seropedicace* DSM 6445<sup>T</sup>, *H. rubrisubalbicans* ATCC 19308<sup>T</sup>, and *H. frisingense* DSM 13128<sup>T</sup>) and two *Janthinobacterium* type strains (*J. agaricidamnorum* DSM 9268<sup>T</sup> and *J. lividum* DSM 1522<sup>T</sup>) (11). *C. fungivorans* Ter331 was used as a positive control (11).

PCR products that were identified by RFLP analysis as products from *Collimonas*, as well as 38 PCR products that were identified as not originating from *Collimonas*, were sequenced (Greenomics, Wageningen, The Netherlands) using the universal primer U115R (5'-TCCCAGCAACGAGCGCAACC-3') (4) as the sequencing primer. DNA sequences up to 650 bp long were compared with sequences available in the GenBank (<http://www.ncbi.nlm.nih.gov/>) and Ribosomal Database Project II (<http://rdp.cme.msu.edu/>) databases. The sequences were aligned and compared using Clustal-W in the Lasergene DNA and protein analysis software (DNASTAR, Madison, WI).

**Development, validation, and application of a *Collimonas*-specific real-time qPCR assay.** In a dual-labeled probe assay, the primers used for real-time PCR quantification of collimonads were Eddy3for and Eddy3rev. The forward primer Eddy3for (5'-GTACAGAATCCCGAAGAGATTTGG-3') was based on a previously reported fluorescent in situ hybridization probe specific for *Collimonas* species (11). In combination with the nonspecific reverse primer Eddy3rev (5'-ACTTAACCCAACATCTCAGACA-3'), it yields a 100-bp amplicon.

To obtain assay specificity, we designed the *Collimonas*-specific probe Sophie (5'-FAM-CGAAAGA+AA+GC+TG+TA+ACACAGG-BHQ-3') (Fig. 1), which contains 6-carboxyfluorescein (FAM) as the fluorophore, butylhydroquinone (BHQ) as a quencher, and several locked nucleic acids (a plus sign indicates a locked nucleic acid base). A locked nucleic acid is a modified nucleic acid with increased binding affinity for cDNA sequences (22). The primers and probe were

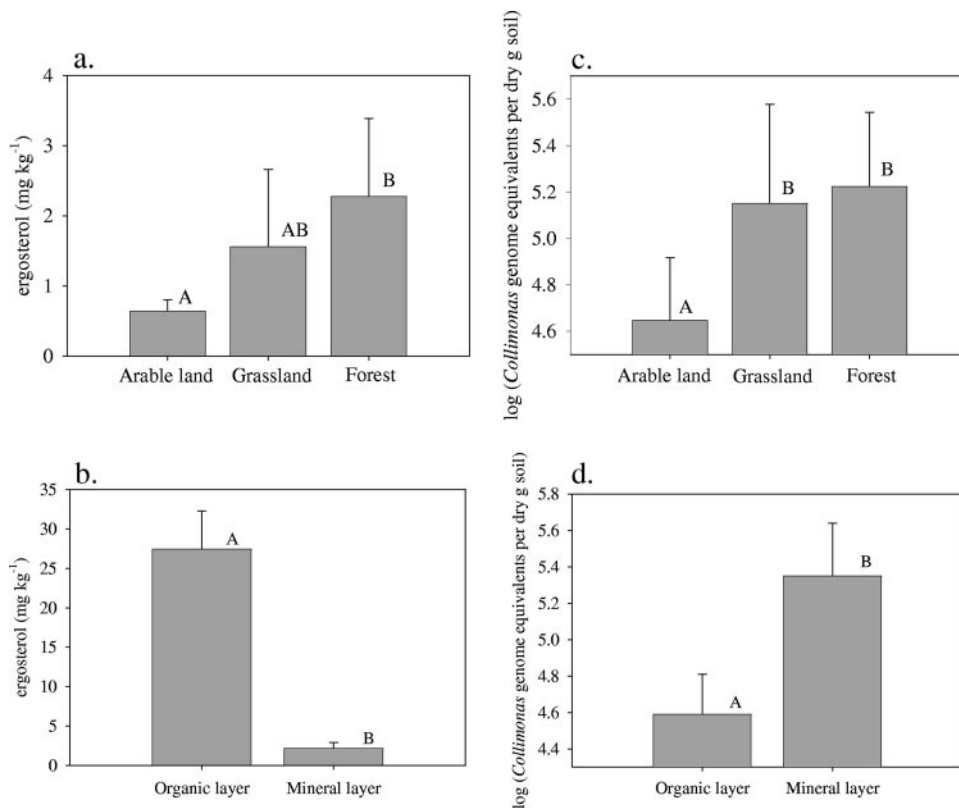


FIG. 2. (a and b) Average ergosterol contents ( $\text{mg kg}^{-1}$  [dry weight] soil) in the upper layer (0 to 10 cm) of the mineral soils at arable, forest, and (semi)natural grassland sites (a) and in the organic and mineral layers at five forest sites (b). (c and d) Average log-transformed numbers of *Collimonas* genome equivalents per gram (dry weight) of soil in the upper layer (0 to 10 cm) of the mineral soils at arable, forest, and (semi)natural grassland sites (c) and in organic and mineral layers at five forest sites (d). Different letters indicate significant differences at the 0.05% level (as determined by a modified Tukey's honestly significant difference test).

synthesized by Biologio BV (Nijmegen, The Netherlands) and Sigma-Proligo (Paris, France), respectively.

Real-time PCR was carried out using a Rotor-Gene 3000 instrument (Corbett Research, Sydney, Australia). Primer and probe concentrations were optimized according to the manufacturer's guide.

The ABsolute qPCR mixture (ABgene) was used at a final concentration of  $1\times$  for the real-time reaction. Primers Eddy3for and Eddy3rev were added to final concentrations of 100 and 70 nM, respectively. The final concentration of the Sophie probe was 50 nM. The volume of the reaction mixture was adjusted to 20  $\mu\text{l}$  using nuclease-free water. The reaction mixture contained 400 ng of bovine serum albumin per  $\mu\text{l}$  to minimize soil PCR inhibition (16, 24). After addition of 5  $\mu\text{l}$  DNA extracts from soil or control DNA (see below), amplifications were performed using the following conditions: 15 min at 95°C, followed by 45 cycles of 15 s for 95°C and 45 s for 66°C. Each DNA extract was tested in duplicate. In all cases, negative controls containing nuclease-free water instead of DNA extract were included.

For quantification of collimonads, a standard curve was generated using serial dilutions of genomic DNA isolated from *C. fungivorans* Ter331. Standard curves were generated by plotting threshold cycles ( $C_t$ ) versus genome equivalents of strain Ter331. The undiluted genomic DNA extract contained 40  $\mu\text{g/ml}$  genomic DNA, as measured spectrophotometrically. The size of the genomic DNA of *C. fungivorans* Ter331 has been estimated to be 5.1 Mbp (unpublished data). Considering that 1 kb of double-stranded DNA is equivalent to  $6.5 \times 10^5$  Da (1 Da =  $1.65 \times 10^{-24}$  g), one genome of *C. fungivorans* Ter331 weighs  $5.5 \times 10^{-15}$  g. Thus, undiluted DNA extract contained  $7.3 \times 10^6$  genomic DNA equivalents per 1  $\mu\text{l}$ . We considered one genome equivalent to one cell.

$C_t$  values indicate the minimum number of PCR cycles needed to obtain fluorescence signals that are significantly greater than the background for the exponential phase of PCR amplification. The Rotor-Gene 6 software (Corbett Research, Sydney, Australia) was used to establish  $C_t$  values for each sample.

**Statistics.** The chi-square test was used to compare the patterns of distribution of culturable collimonads in arable, forest, and (semi)natural grassland soils. For this test, sites cropped with maize and fertilized grassland were categorized as arable land, nonfertilized grassland and formerly arable lands that were abandoned for >10 years were categorized as (semi)natural grasslands, and recently abandoned arable sites (abandoned for <10 years) were not included in the analyses of land management since they represent a transient stage from arable lands to natural areas.

Data for real-time PCR-based (log-transformed) numbers of *Collimonas* cells and soil ergosterol contents were analyzed by one-way analysis of variance with different land management practices (arable, forest, and [semi]natural grassland) and different layers (mineral and organic) of forest soil as treatments. Differences between groups were tested for significance with a modified Tukey's honestly significant difference test at a  $P$  value of <0.05. Possible relationships between real-time PCR-based numbers of *Collimonas* cells and physical and chemical characteristics of soil were examined using correlation analyses. Principal-component analysis was performed to ordinate the soils on the basis of soil characteristics. Scaling was focused on intersample distances. Species scores (soil characteristics) were divided by the standard deviation and used for centering. The analysis was performed using CANOCO 4.5 (41). All other statistical analyses were performed using STATISTICA (Statsoft Inc., Tulsa, OK).

## RESULTS

**Soil characteristics and soil fungal biomass.** The 45 soil samples used in this study covered a wide range of physical and chemical properties, as well as different vegetation characteristics and land management practices (see Table S1 in the

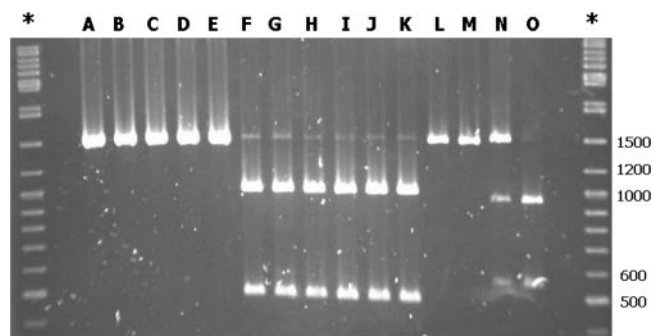


FIG. 3. RFLP analysis of BstBI-digested 16S rRNA genes amplified almost to completion from *J. agaricidammosum* DSM 9628<sup>T</sup> (lane A), *J. lividum* DSM 1522<sup>T</sup> (lane B), *H. rubrisubalbicans* ATCC 19308<sup>T</sup> (lane C), *H. seropedicae* DSM 6445<sup>T</sup> (lane D), *H. frisingense* DSM 13128<sup>T</sup> (lane E), *C. fungivorans* Ter331 (lane F), *Collimonas* sp. from soil sample 19 (lanes G and H), *Collimonas* sp. from soil sample 26 (lane I), *Collimonas* sp. from soil sample 15 (lane J), *Collimonas* sp. from soil sample 26 (lane K), isolate 142 from soil sample 16 (= *Flavobacterium* sp.) (lane L), isolate 134 from soil sample 27 (= *Burkholderia* sp.) (lane M), isolate 186 from soil sample 36 (= *Rhodanobacter* sp.) (lane N), and isolate 88 from soil sample 9 (= *Pedobacter* sp.) (lane O). The lanes indicated by an asterisk contained a 1-kb marker. The strains in lanes A to E were obtained from DSMZ (Braunschweig, Germany). The strains in lanes G to O were chitinolytic isolates from our inventory study. Identification of the strains in lanes G to O (see above) was based on sequence analysis of the 16S rRNA gene fragment amplified by primers pA and 1492r (15).

supplemental material). The concentration of ergosterol, which we used as an indicator of fungal biomass, in mineral soil ranged from 0.1 to 4.0 mg kg<sup>-1</sup> (dry weight) soil in grasslands, from 0.6 to 7.3 mg kg<sup>-1</sup> (dry weight) soil in forests, and from 0.5 to 2.6 mg kg<sup>-1</sup> (dry weight) soil in arable lands. The average amount of ergosterol in mineral soils was highest at the forest sites and lowest at the arable sites (Fig. 2a). The organic layers of forest soils were particularly rich in ergosterol (19.7 to 34.0 mg kg<sup>-1</sup> [dry weight] forest organic soil), indicating a high fungal biomass (Fig. 2b).

**Identification of culturable *Collimonas* by RFLP and 16S rRNA sequence analysis.** For four forest soils (sites 1, 2, 13, and 37), we were not able to detect the possible presence of culturable *Collimonas* strains due to the rapid expansion of fast-growing gliding bacteria over the whole agar plate. From the remaining 41 soil samples, a total of 205 chitinolytic isolates were obtained. Sixty-nine of these isolates showed colony morphologies similar to those of the described *Collimonas* strains (11). 16S rRNA of all 205 isolates was amplified by PCR and used for RFLP analysis using BstBI. Twenty-six of the amplified 16S rRNA fragments showed the same banding pattern (i.e., one 1-kb fragment and one 0.5-kb fragment) as the reference strain, *C. fungivorans* Ter331 (Fig. 3). The other isolates showed either a single undigested PCR product or two bands with sizes that were clearly different from the sizes of *Collimonas* bands (Fig. 3).

The 26 strains that were identified as collimonads by RFLP analysis, as well as 38 randomly selected other chitinolytic isolates, including a subset of strains with colony morphology resembling *Collimonas* colony morphology, were subjected to partial (0.4 to 0.6 kb) sequence analysis of the 16S rRNA gene. The results revealed that the isolates identified as *Collimonas*

sp. by RFLP analysis also had sequence similarities of >98% with published *Collimonas* sequences, and thus they were classified as confirmed collimonads. The 38 RFLP-negative strains were clearly not *Collimonas* species, since their 16S rRNA genes showed the highest sequence similarities to species belonging to the families *Burkholderiaceae* and *Xanthomonadaceae*. This confirms the accuracy of our RFLP assay for establishing *Collimonas* identity.

The 26 confirmed collimonads originated from nine different sampling sites, sites 4, 5, 6, 15, 18, 19, 22, 26, and 27. These sites were eight (semi)natural grassland sites and one heathland soil (Table 1). All 26 *Collimonas* isolates had a colony morphology that resembled the colony morphologies described for collimonads (11). However, two isolates obtained from grassland site 26 produced a purple pigment, which was previously described for species belonging to the related genus *Janthinobacterium* (11, 28, 33). The purple color was not clear on the original counting plates. However, when colonies were restreaked on both chitin-yeast extract agar and TSB-chitin plates for purity, the purple color became apparent. Production of purple pigments by collimonads has been reported previously (32).

Isolates were also studied for the ability to produce haloes on chitin agar containing TSB since it was reported that the chitinolytic ability of collimonads was completely repressed by TSB (11). This appeared to be the case for only one-half (13 strains) of the new isolates. These results show that colony morphology, colony color, and catabolic repression of chitinase production are not reliable characteristics for the identification of *Collimonas* sp., as was suggested previously (11).

**Development and validation of a *Collimonas*-specific dual-labeled probe assay.** Although the aforementioned combination of plate counts and RFLP-based identification gave us an indication of the distribution of collimonads in different soil types, there were still many limitations, including the presence of nonculturable collimonads and the apparent absence of collimonads due to the presence of fast-growing chitinolytic gliding bacteria. Therefore, we developed a 16S rRNA gene-based real-time PCR assay. A *Collimonas*-specific primer/probe set was designed by alignment of 16S rRNA gene sequences of 12 representative collimonads (11) and the 22 most closely related bacterial species belonging to the family *Oxalobacteraceae* (accession numbers AY167838, Y08845, Y08846, AF174648, AY247410, Y10146, AB021424, AJ238358, AB074524, U49757, PSJ001384, AF543312, AB008506, AB024305, AY133107,

TABLE 1. Numbers of arable sites, (semi)natural grasslands, and forests in which culturable collimonads were present or absent<sup>a</sup>

| Site        | Total no. of soil samples <sup>b</sup> | No. of sites with <i>Collimonas</i> present | No. of sites with <i>Collimonas</i> absent |
|-------------|--|---|--|
| Arable land | 8                                      | 0   | 8  |
| Grassland   | 18                                     | 8   | 10   |
| Forest      | 9                                      | 0   | 9  |
| Total       | 35                                     | 8   | 27   |

<sup>a</sup> The distribution is significant at the  $P < 0.05$  level (as determined by a chi-square test).

<sup>b</sup> The organic layers of forest soil, heathland, and formerly arable land were excluded from the analysis.

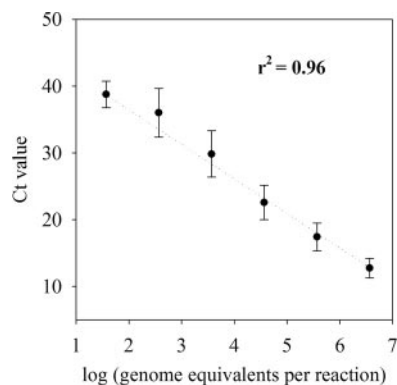


FIG. 4. Standard curve for the *Collimonas*-specific dual-labeled probe PCR assays. This curve was created using known amounts of DNA ranging from  $3.7 \times 10^1$  to  $3.7 \times 10^6$  genome equivalents of *C. fungivorans* Ter331 genomic DNA per reaction mixture. The values shown are representative results derived from multiple independent assays ( $n = 5$ ).

AF529336, AF154097, AY177773, AF529095, AF358019, AY214204, and AF407411).

The probe, which was designed based on a 22-bp stretch shared only by collimonads, had a single mismatch (G or A at position 10) (Fig. 1). The single mismatch of the probe did not influence the  $C_t$  value (data not shown), indicating that the probe was specific for real-time PCR analysis of collimonads.

The specificity of the dual-labeled probe assays was tested empirically using genomic DNA of representative species of genera closely related to *Collimonas* (i.e., DNA of *H. seropedicae* DSM 6445<sup>T</sup>, *H. rubrisubalbicans* ATCC 19308<sup>T</sup>, *H. frisingense* DSM 13128<sup>T</sup>, *J. agaricidamnosum* DSM 9268<sup>T</sup>, and *J. lividum* DSM 1522<sup>T</sup>). The dual-labeled probe assay did not give a signal during 40 cycles of the reaction (results not shown).

**Direct detection and quantification of *Collimonas* in soils using the dual-labeled probe PCR assay.** Figure 4 shows a composite standard curve that was derived using  $C_t$  values from five independent runs of PCR assays. The standard curve was obtained for the range from  $3.7 \times 10^1$  to  $3.7 \times 10^6$  genome equivalents per reaction mixture with an  $R^2$  value of 0.96 and a coefficient of variation of  $10.3\% \pm 2.58\%$ . The amplification efficiency, calculated using the methods described by Pfaffl (37), was  $1.54 \pm 0.07$ . The lower limit of quantification was  $3.7 \times 10^1$  genome equivalents per reaction mixture, which corresponds to a mean  $C_t$  value of 38, whereas the negative controls did not give a signal during the 40 cycles of the reaction. The lower limit,  $3.7 \times 10^1$  genome equivalents per reaction mixture, equals  $3.7 \times 10^1$  genome equivalents per 5  $\mu$ l of  $10\times$ -diluted DNA extract or  $3.7 \times 10^3$  genome equivalents per 50  $\mu$ l of undiluted DNA extract. The 50  $\mu$ l was extracted from 0.25 g (dry weight) soil, which implies that the quantification limit of our real-time PCR assay was  $1.5 \times 10^4$  *Collimonas* genome equivalents (cells)  $g^{-1}$  (dry weight) soil. The standard curve that was constructed was used to estimate the number of genome equivalents or cells of *Collimonas* in all soil samples.

Real-time PCR showed that 36 soil samples contained collimonads at levels above the quantification limit, up to  $10^5$  cells  $g^{-1}$  (dry weight) soil. The soils that did not show a detectable

level of collimonads were four arable soils, three forest soils, and two natural grassland soils. For all soils, the extent of inhibition of real-time PCR was examined. This was done by adding  $10^3$  or  $10^4$  genome equivalents of *C. fungivorans* Ter331 to soil DNA dilutions. The degree of PCR inhibition was calculated by measuring the recovery of added DNA. The recovery of genome equivalents of *C. fungivorans* Ter331 added to soil DNA was complete for most soils ( $n = 37$ ) and greater than 90% for the rest of the soils ( $n = 8$ ), indicating that there was no significant underestimation of native collimonad numbers in these soils due to PCR inhibition. The average numbers of collimonads per gram of soil were significantly different ( $P < 0.05$ ) for different land management practices (Fig. 2c). The numbers of collimonads were higher in grassland and forest soils than in arable land soils. The same trend was found for ergosterol-based fungal biomass (Fig. 2a). In forest soils, the number of collimonads per gram of soil was significantly higher ( $P < 0.05$ ) in the mineral layer than in the organic layer (Fig. 2d). This is in contrast to the fungal biomass, which was much greater in the organic layer than in the mineral layer (Fig. 2b). Correlation analysis did not show a significant relationship between the number of collimonads and any soil characteristic, including the ergosterol content. Furthermore, principal-component analysis did not point to factors contributing effectively to the presence or absence of *Collimonas* (data not shown).

## DISCUSSION

The *Collimonas*-specific RFLP assay and real-time PCR protocol described in this study were successfully used for specific detection of collimonads among culturable chitin-degrading soil bacteria and in total soil DNA, respectively. The RFLP analysis of 16S rRNA fragments proved to be a reliable method for genus-specific identification of culturable collimonads, whereas a combination of the primers and probe developed allowed highly specific real-time PCR quantification of *Collimonas* 16S rRNA genes in soil.

Real-time PCR has the advantage that it can be used to quantify both culturable and nonculturable bacteria in the soil microbial community (21, 30, 42). The discrepancy between real-time PCR detection and the results of the isolation experiments (Table 1) could be explained by the inability of some *Collimonas* strains to grow on the enumeration plates (chitin-yeast extract agar). Another explanation could be that fast-growing antagonistic or gliding bacteria prevented or masked the (slow) development of *Collimonas* colonies.

In theory, the quantification limit for plate enumeration is  $2.0 \times 10^2$  *Collimonas* cells  $g^{-1}$  (dry weight) soil. However, in practice, the detection limit is higher due to suppression of colony development by fast-growing antagonistic and gliding chitinolytic bacteria. The quantification limit for our real-time PCR assay was  $1.5 \times 10^4$  *Collimonas* cells  $g^{-1}$  (dry weight) soil. Our qPCR results indicate that the size of the population of collimonads was about  $10^5$   $g^{-1}$  (dry weight) soil in 60% of soils that we tested. In the other soils containing *Collimonas* (20%), the numbers were close to the quantification limit. The presence of  $10^5$  cells  $g^{-1}$  soil corresponds to at most 0.01% of the total soil bacteria (based on 4',6'-diamidino-2-phenylindole [DAPI] counts) (result not shown). In the nine soils where they

were detected, culturable collimonads accounted for 0.1% of the total culturable chitinolytic bacteria (data not shown). Hence, it seems that the abundance of collimonads is generally low. This does not indicate a strong competitive ability in soil, even though it has been shown that collimonads are metabolically versatile (11). *Collimonas* was originally isolated in a search for chitinolytic bacteria. However, it is known that collimonads are weak chitin degraders compared to fungi and actinomycetes (8). Hence, their growth on chitin sources, mainly dead fungal hyphae and exoskeletons of arthropods, in soil is probably insignificant.

As another approach to get an indication of the abundance of collimonads, we used BLAST to screen for the presence of *Collimonas* sequences in published gene clone libraries of bacteria. Collimonads were in 16S rRNA gene clone libraries of soils in only three studies, supporting our finding that these organisms do not occur in high numbers in soil. In one of these studies, collimonads were found in the lichen-dominated surface of forest soils, and the authors suggested that they may play a role in the decay of the lichen biomass, which consists largely of fungi (32). Another study showed that collimonads were found in a forest soil-based microbial biofilm exposed to 2,4-dichlorophenoxyacetic acid (2). Opelt and Berg found collimonads in the bacterial community associated with bryophytes living in the Baltic Sea coast area (36).

Real-time PCR results indicated that collimonads were widely distributed, but their numbers were significantly higher in (semi)natural ecosystems than in arable soils. In addition, culturable *Collimonas* cells were detected only in natural grasslands. The relatively low numbers of collimonads in arable soils and production grasslands are in line with mycophagous growth, as these sites are fungus poor due to agricultural management practices (44). The low numbers are also compatible with a parasitic or predatory growth strategy (12). For example, *Bdellovibrio* species, which are bacterial predators of other bacteria, also occur in low numbers ( $10^2$  to  $10^4$  CFU per  $g^{-1}$ ) in soils (23).

Despite the apparent preference for the more fungus-rich natural ecosystems, the numbers of collimonads were not pairwise correlated with ergosterol-based soil fungal biomass. The lack of a statistically significant correlation between the fungal biomass and the numbers of collimonads could imply that mycophagy is not an essential growth strategy for collimonads under natural conditions. Alternatively, enumeration of collimonads by qPCR may also include as-yet-unknown strains that are not mycophagous. Furthermore, preferential feeding of *Collimonas* strains on specific taxonomic or functional fungal groups may interfere with a correlation between ergosterol-based soil fungal biomass and numbers of collimonads. Indeed, under lab conditions preferential growth of *Collimonas* strains was demonstrated. The proliferation of collimonads on the zygomycete *Mucor hiemalis* was much greater than that on the ascomycetes *Chaetomium globosum* and *Fusarium culmorum* (10).

Surprisingly, within the forest soil profile, collimonads were present in relatively low numbers in the fungus-rich forest organic layers. If mycophagy is an important in situ growth strategy for collimonads, this must indicate that the environmental conditions or the type of fungal species in the organic layer of forest soils is not favorable for collimonads. Recently,

Lindahl et al. (29) reported spatial separation in the vertical distribution of different functional groups of fungi in the organic layer of boreal forest soils. It was observed that saprotrophic fungi were primarily confined to the top organic layer (litter), whereas ectomycorrhizal fungi dominated the lower layers (29, 39). Hence, it may be that the higher numbers of collimonads in the mineral layer are due to a preference for hyphae of ectomycorrhizal fungi.

A functional group of fungi with a short turnover time for hyphae is the arbuscular mycorrhizal (AM) fungi (40). If these fungi are an important source of food for collimonads, then this should not be apparent from ergosterol measurements as AM fungi do not contain ergosterol (35).

Obviously, other approaches are needed to indicate whether mycophagy is important for collimonads under natural growth conditions. One possible strategy is to introduce fungal hyphae as bait in soil samples that naturally contain collimonads. Application of the real-time PCR assay that we developed here would allow us to follow changes in *Collimonas* numbers and assess whether the mycophagous property is essential for in situ growth. Future studies also need to be aimed at increasing our knowledge of the types of fungal species that are preferably targeted by collimonads. This should enable reassessment of a possible link between the abundance of collimonads and the presence of fungi by focusing more specifically on the presence of fungal species that are actually susceptible to collimonads. Depending on their specificity for different fungi, collimonads could have different functions in soil environments and could range from being deleterious (e.g., due to disturbance of symbiosis between plants and AM fungi) to being beneficial to plants by targeting plant-pathogenic fungi.

#### ACKNOWLEDGMENTS

We thank Bio-Rad (Veenendaal, The Netherlands) for helping to design the Eddy primers. Sigma-Proligo (Paris, France) is acknowledged for its help in designing the Sophie probe.

#### REFERENCES

1. Abu Al-Soud, W., and P. Radstrom. 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. *J. Clin. Microbiol.* **38**:4463–4470.
2. Aspray, T. J., S. K. Hansen, and R. G. Burns. 2005. A soil-based microbial biofilm exposed to 2,4-D: bacterial community development and establishment of conjugative plasmid pJP4. *FEMS Microbiol. Ecol.* **54**:317–327.
3. Bach, H. J., J. Tomanova, M. Schloter, and J. C. Munch. 2002. Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification. *J. Microbiol. Methods* **49**:235–245.
4. Baker, G. C., J. J. Smith, and D. A. Cowan. 2003. Review and re-analysis of domain-specific 16S primers. *J. Microbiol. Methods* **55**:541–555.
5. Baker, R. 1987. Mycoparasitism—ecology and physiology. *Can. J. Plant Pathol.* **9**:370–379.
6. Brimmer, T. A., and G. J. Boland. 2003. A review of the non-target effects of fungi used to biologically control plant diseases. *Agric. Ecosyst. Environ. Appl. Soil Ecol.* **100**:3–16.
7. de Boer, W., L. B. Folman, R. C. Summerbell, and L. Boddy. 2005. Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS Microbiol. Rev.* **29**:795–811.
8. de Boer, W., S. Gerards, P. J. A. Klein Gunnewiek, and R. Modderman. 1999. Response of the chitinolytic microbial community to chitin amendments of dune soils. *Biol. Fertil. Soils* **29**:170–177.
9. de Boer, W., P. J. A. Klein Gunnewiek, G. A. Kowalchuk, and J. A. van Veen. 2001. Growth of chitinolytic dune soil beta-subclass *Proteobacteria* in response to invading fungal hyphae. *Appl. Environ. Microbiol.* **67**:3358–3362.
10. de Boer, W., P. J. A. Klein Gunnewiek, P. Lafeber, J. D. Janse, B. E. Spit, and J. W. Woldendorp. 1998. Anti-fungal properties of chitinolytic dune soil bacteria. *Soil Biol. Biochem.* **30**:193–203.
11. de Boer, W., J. H. J. Leveau, G. A. Kowalchuk, P. J. A. Klein Gunnewiek,

- E. C. A. Abeln, M. J. Figge, K. Sjölema, J. D. Janse, and J. A. van Veen. 2004. *Collimonas fungivorans* gen. nov., sp. nov., a chitinolytic soil bacterium with the ability to grow on living fungal hyphae. *Int. J. Syst. Evol. Microbiol.* **54**:857–864.
12. De Ridder-Duine, A. S., W. Smant, A. van der Wal, J. A. van Veen, and W. de Boer. 2006. Evaluation of a simple, non-alkaline extraction protocol to quantify soil ergosterol. *Pedobiologia* **50**:293–300.
  13. Dijksterhuis, J., M. Sanders, L. G. M. Gorris, and E. J. Smid. 1999. Antibiosis plays a role in the context of direct interaction during antagonism of *Paenibacillus polymyxa* towards *Fusarium oxysporum*. *J. Appl. Microbiol.* **86**:13–21.
  14. Donzelli, B. G. G., and G. E. Harman. 2001. Interaction of ammonium, glucose, and chitin regulates the expression of cell wall-degrading enzymes in *Trichoderma atroviride* strain P1. *Appl. Environ. Microbiol.* **67**:5643–5647.
  15. Edwards, U., T. Rogall, H. Blocker, M. Emde, and E. C. Bottger. 1989. Isolation and direct complete nucleotide determination of entire genes—characterization of a gene coding for 16S-ribosomal RNA. *Nucleic Acids Res.* **17**:7843–7853.
  16. Fierer, N., J. A. Jackson, R. Vilgalys, and R. B. Jackson. 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.* **71**:4117–4120.
  17. Fritsche, K., J. H. J. Leveau, S. Gerard, S. Ogawa, W. de Boer, and J. A. van Veen. 2006. *Collimonas fungivorans* and bacterial mycophagy. *IOBC/WPRS Bull.* **29**:27–30.
  18. Haran, S., H. Schickler, and I. Chet. 1996. Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. *Microbiology* **142**:2321–2331.
  19. Harman, G. E. 2006. Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* **96**:190–194.
  20. Henry, S., D. Bru, B. Stres, S. Hallet, and L. Philippot. 2006. Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG*, *nirK*, and *nosZ* genes in soils. *Appl. Environ. Microbiol.* **72**:5181–5189.
  21. Hermansson, A., and P. E. Lindgren. 2001. Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. *Appl. Environ. Microbiol.* **67**:972–976.
  22. Johnson, M. P., L. M. Haupt, and L. R. Griffiths. 2004. Locked nucleic acid (LNA) single nucleotide polymorphism (SNP) genotype analysis and validation using real-time PCR. *Nucleic Acids Res.* **32**:e55.
  23. Jurkevitch, E., D. Minz, B. Ramati, and G. Barel. 2000. Prey range characterization, ribotyping, and diversity of soil and rhizosphere *Bdellovibrio* spp. isolated on phytopathogenic bacteria. *Appl. Environ. Microbiol.* **66**:2365–2371.
  24. Kreder, C. A. 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl. Environ. Microbiol.* **62**:1102–1106.
  25. Lebuhn, M., M. Effenberger, G. Garces, A. Gronauer, and P. A. Wilderer. 2004. Evaluating real-time PCR for the quantification of distinct pathogens and indicator organisms in environmental samples. *Water Sci. Technol.* **50**:263–270.
  26. Lebuhn, M., M. Effenberger, A. Gronauer, P. A. Wilderer, and S. Wuerz. 2003. Using quantitative real-time PCR to determine the hygienic status of cattle manure. *Water Sci. Technol.* **48**:97–103.
  27. Leveau, J. H. J., S. Gerards, K. Fritsche, G. Zondag, and J. A. van Veen. 2006. Genomic flank-sequencing of plasposon insertion sites for rapid identification of functional genes. *J. Microbiol. Methods* **66**:276–285.
  28. Lincoln, S. P., T. R. Fermor, and B. J. Tindall. 1999. *Janthinobacterium agaricidamnosum* sp. nov., a soft rot pathogen of *Agaricus bisporus*. *Int. J. Syst. Bacteriol.* **49**:1577–1589.
  29. Lindahl, B. D., K. Ihrmark, J. Boberg, S. E. Trumbore, P. Höglberg, J. Stenlid, and R. D. Finlay. 2007. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. *New Phytol.* **173**:611–620.
  30. Lloyd-Jones, G., A. D. Laurie, and A. C. Tizzard. 2005. Quantification of the *Pseudomonas* population in New Zealand soils by fluorogenic PCR assay and culturing techniques. *J. Microbiol. Methods* **60**:217–224.
  31. Lorito, M., S. L. Woo, I. G. Fernandez, G. Colucci, G. E. Harman, J. A. Pintor-Toro, E. Filippone, S. Muccifora, C. B. Lawrence, A. Zoina, S. Tuzun, and F. Scala. 1998. Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. *Proc. Natl. Acad. Sci. USA* **95**:7860–7865.
  32. Mannisto, M. K., and M. M. Haggblom. 2006. Characterization of psychrotolerant heterotrophic bacteria from Finnish Lapland. *Syst. Appl. Microbiol.* **29**:229–243.
  33. Matz, C., P. Deines, J. Boenigk, H. Arndt, L. Eberl, S. Kjelleberg, and K. Jurgens. 2004. Impact of violacein-producing bacteria on survival and feeding of bacterivorous nanoflagellates. *Appl. Environ. Microbiol.* **70**:1593–1599.
  34. Okano, Y., K. R. Hristova, C. M. Leutenegger, L. E. Jackson, R. F. Denison, B. Gebreyesus, D. Lebauer, and K. M. Scow. 2004. Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. *Appl. Environ. Microbiol.* **70**:1008–1016.
  35. Olsson, P. A., L. Larsson, B. Bago, H. Wallander, and I. M. van Aarle. 2003. Ergosterol and fatty acids for biomass estimation of mycorrhizal fungi. *New Phytol.* **159**:7–10.
  36. Opelt, K., and G. Berg. 2004. Diversity and antagonistic potential of bacteria associated with bryophytes from nutrient-poor habitats of the Baltic Sea coast. *Appl. Environ. Microbiol.* **70**:6569–6579.
  37. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**:e45.
  38. Rodrigues, J. L. M., M. R. Aiello, J. W. Urbance, T. V. Tsoi, and J. M. Tiedje. 2002. Use of both 16S rRNA and engineered functional genes with real-time PCR to quantify an engineered, PCB-degrading *Rhodococcus* in soil. *J. Microbiol. Methods* **51**:181–189.
  39. Rosling, A., R. Landeweert, B. D. Lindahl, K. H. Larsson, T. W. Kuyper, A. F. S. Taylor, and R. D. Finlay. 2003. Vertical distribution of ectomycorrhizal fungal taxa in a podzol soil profile. *New Phytol.* **159**:775–783.
  40. Staddon, P. L., C. B. Ramsey, N. Ostle, P. Ineson, and A. H. Fitter. 2003. Rapid turnover of hyphae of mycorrhizal fungi determined by AMS microanalysis of C-14. *Science* **300**:1138–1140.
  41. Ter Braak, C. J. F., and P. Šmilauer. 2002. CANOCO reference manual and CanoDraw for Windows user's guide: software for canonical community ordination (version 4.5). Microcomputer Power, Ithaca, NY.
  42. Tomlinson, J. A., N. Boonham, K. J. D. Hughes, R. L. Griffen, and I. Barker. 2005. On-site DNA extraction and real-time PCR for detection of *Phytophthora ramorum* in the field. *Appl. Environ. Microbiol.* **71**:6702–6710.
  43. Troelstra, S. R., L. A. P. Lotz, R. Wagenaar, and L. Sluimer. 1990. Temporal and spatial variability in soil nutrient status of a former beach plain. *Plant Soil* **127**:1–12.
  44. Van der Wal, A., J. A. van Veen, W. Smant, H. T. S. Boschker, J. Bloem, P. Kardol, W. H. van der Putten, and W. de Boer. 2006. Fungal biomass development in a chronosequence of land abandonment. *Soil Biol. Biochem.* **38**:51–60.
  45. Vazquez-Garciduenas, S., C. A. Leal-Morales, and A. Herrera-Estrella. 1998. Analysis of the beta-1,2-glucanolytic system of the biocontrol agent *Trichoderma harzianum*. *Appl. Environ. Microbiol.* **64**:1442–1446.
  46. Weete, J. D. 1989. Structure and function of sterols in fungi. *Adv. Lipid Res.* **23**:115–167.
  47. Weete, J. D., A. Elmoughith, and J. M. Touzesoulet. 1989. Inhibition of growth, lipid, and sterol biosynthesis by monensin in fungi. *Exp. Mycol.* **13**:85–94.
  48. Zeilinger, S., C. Galhaup, K. Payer, S. L. Woo, R. L. Mach, C. Fekete, M. Lorito, and C. P. Kubicek. 1999. Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. *Fungal Genet. Biol.* **26**:131–140.