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Germination and persistence of Bacillus anthracis and Bacillus thuringiensis in soil microcosms.

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ORIGINAL PAPER

Comparative studies to assess bacterial communities on the clover phylloplane using MLST, DGGE and T-RFLP

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Abstract Denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) were used to characterise the changes that occurred in *Bacillus cereus* group strains present in the phylloplane of clover *Trifolium hybridum* over 4 months. These strains had previously been analysed by multiple locus sequence typing (MLST). DGGE displayed many equally intense bands which indicated many equally abundant ribotypes. The bacterial community composition was variable and the leaves sampled as little as a week apart were found to have some temporal variability, indicating that diverse phylloplane bacterial communities follow sequential patterns from time to time. The *B. cereus* group community clearly clustered into early, mid and late branches, possibly due to multiple successional sequences occurring during growing seasons. The functionally and phylogenetically diverse microbial communities appeared to exhibit predictable successional patterns over shorter time scales. DGGE analysis with the molecular marker *rpoB* gave better resolution than 16S rRNA amplicons. There were no strong similarities between the dendrograms produced by DGGE, MLST and T-RFLP and the clustering produced by the automated T-RFLP method was variable even between the three restriction enzymes used. The DGGE–MLST method emerged as a superior method to T-RFLP–MLST for rapid typing of bacterial communities.

Keywords *Bacillus thuringiensis* · Fingerprinting · PCR–DGGE · T-RFLP · MLST

Electronic supplementary material The online version of this article (doi:10.1007/s11274-013-1434-x) contains supplementary material, which is available to authorized users.

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Introduction

Endospore-forming bacteria play diverse and important roles in many habitats (Volker et al. 2011). Nevertheless, their ecology is relatively under-studied, probably because of their characteristic ability to form dormant, resistant, long-lived spores. *Bacillus cereus* sensu lato is a group of species that includes members with an extraordinary and highly varied impact on human beings (Jensen et al. 2003; Vilas-Bôas et al. 2007): *B. anthracis* is a pathogen of cattle and an important threat agent; *B. thuringiensis* is used as a biopesticide with an unequalled history of global exploitation and *B. cereus* can be a cause of food-borne infection (Jensen et al. 2003). The *B. cereus* group has been found to have more reticulate population structure with more numerous or less dominant clonal complexes, based on previous analysis of the *B. cereus* database, food-borne isolates or clinical isolates (Didelot and Falush 2007; Cardazzo et al. 2008; Hoffmaster et al. 2008) with exceptions of *B. anthracis* and *B. cereus* emetic strains (Vassileva et al. 2007). Despite their long history, the relationship between these organisms has yet to be completely resolved (Rasko et al. 2005) These markedly different phenotypes are the justification for maintaining these organisms as separate species although there are insufficient genomic differences to support this (Helgason et al. 2000). All three species can be isolated from the soil and phyllosphere and interact with soil invertebrates (Damgaard 2000; Jensen

et al. 2003; Raymond et al. 2010). In spite of their very different spectra of pathogenicity it has been proposed (Raymond et al. 2010) that epizootic outbreaks of *B. thuringiensis* on the phylloplane bear a striking resemblance to the ecology and population structure of *B. anthracis*.

More than 85 different species of microorganisms in about 37 genera have been reported on the phylloplane, all by traditional culture-based methods (Hirano and Upper 2000). The uses of conventional molecular approaches have shown the phyllosphere communities to be very complex in nature and have raised many fundamental questions pertaining to their spatial and temporal variability (Bizzarri and Bishop 2007; Coelho et al. 2007; Collier et al. 2005; Costa et al. 2006; Damgaard 2000). The occurrence of seasonal patterns in the bacterial community structure of the phyllosphere is still an important issue to be addressed (Legard et al. 1994; Collier et al. 2005; Redford and Fierer 2009).

In recent years bacterial taxonomy has been revolutionized by the application of molecular techniques and, in particular, analysis of 16S rRNA and functional gene-based phylogenies to study population dynamics (Da Mota et al. 2004; Felske et al. 2003) in different environments (Andersen et al. 2010; Coelho et al. 2007; Da Mota et al. 2004; Felske et al. 2003; Garbeva et al. 2003; Muyzer et al. 1993; Ryu et al. 2005; Gomes et al. 2005; Garbeva et al. 2008). Direct extraction of DNA (Da Mota et al. 2004) allows access to non-cultivable organisms but this approach may under-represent spores because they are resistant to lysis.

The 16S rRNA gene is now used as a framework for the modern classification of bacteria, including those in the genus *Bacillus* (Andersen et al. 2010; Da Mota et al. 2004). As a result of the conserved nature of 16S rRNA gene sequences there is limited variation for members of closely related taxa (Felske et al. 2003). Due to this constraint, DNA sequencing of certain housekeeping genes in multiple locus sequence typing (MLST) can provide more DNA sequence sub-typing than 16S rRNA sequencing for a number of bacterial species, thereby increasing taxonomic resolution (Bizzarri et al. 2008; Cardazzo et al. 2008; Helgason et al. 2004). Current molecular community profiling methods, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) analysis of amplified 16S or 18S rRNA genes represent powerful tools for studying bacterial and fungal diversities in complex environments (Muyzer et al. 1993; Ticknor et al. 2007). The use of 16S rRNA and functional gene-based phylogenies provides genus and species level delineation but the use of chromosome-wide differences increases taxonomic resolution and enables intraspecies discrimination (Meintanis et al. 2008). This study addressed the issue of how well techniques using the 16S rRNA and the *rpoB* genes reconstruct the bacterial phylogenies and whether the *rpoB* gene fulfils the criteria as a molecular marker in microbial ecology. To address this question, the analysis of isolates of *B. cereus* and *B. thuringiensis*, recovered from the phylloplane, by MLST, T-RFLP and DGGE was compared. All data were evaluated by cluster analysis.

Materials and methods

Sampling and sample characteristics

A collection of *Bacillus thuringiensis* strains (Table 1) collected from the phylloplane of clover *Trifolium hybridum* over one growing season was used for the initial analysis (Bizzarri et al. 2008). The MLST typings referred to here come from that publication. A different collection of *B. thuringiensis* (Table S2) and *B. cereus* (Table S3) strains was made from the phylloplane of *T. hybridum* growing in a glasshouse by the same method (Bizzarri

Table 1 Catalogue of the strains used in this study showing their: identity (ID) as assigned after their introduction in the http://www.mlst.net database; species, *B. thuringiensis* (*Bt*) and *B. cereus* (*Bc*); time of isolation and sequence type (ST)

ID	Strain	Species	Lineage	Isolation time	ST
5	169-S-5	Bt	*thuringiensis*	16th September	9
297	219-S-1	Bt	*thuringiensis*	21st September	189
298	1710-S-3	Bt	*thuringiensis*	17th October	190
299	1230-88	Bc	*cereus*	NA	191
300	164-S-1	Bt	*thuringiensis*	16th April	8
301	65-S-47	Bt	*thuringiensis*	6th May	192
302	2810-S-4	Bt	*thuringiensis*	28th October	193
303	286-S-1	Bt	*thuringiensis*	28th June	194
304	67-S-1	Bt	*thuringiensis*	6th July	195
305	1710-S-1	Bt	*thuringiensis*	17th October	196
306	158-S-3	Bt	*thuringiensis*	15th August	197
307	127-S-1	Bt	*thuringiensis*	12th July	198
308	65-S-2	Bt	*thuringiensis*	6th May	199
309	216-S-2	Bt	*thuringiensis*	21th June	200
310	2810-S-8	Bt	*thuringiensis*	28th October	201
311	1710-S-2	Bt	*thuringiensis*	17th October	194
312	186-S-1	Bc	*cereus*	18th June	202
313	158-S-2	Bt	*thuringiensis*	15th August	203
314	286-S-6	Bt	*sotto*	28th June	23
315	HD-1(4D-1)	Bt	*thuringiensis*	NA	10
316	248-S-1	Bt	*Kurstaki*	24th August	8
318	169-S-16	Bt	*Sotto*	16th September	23
319	127-S-3	Bt	*Sotto*	12th July	23

and Bishop 2007). All of the phylloplane strains were labelled with a number, indicating the date, month and year during which they had been isolated (Table S2). The origin of *B. thuringiensis* strain AND 508 has been described previously (Bizzarri et al. 2008). Strain HD-1 was obtained from the Bacillus Genetic Stock Center (BGSC) (Ohio, USA) and has the BGSC designation 4D-1. Leaves were weighed in order to ensure consistent leaf masses for each sampling.

PCR amplification and DGGE analysis of the 16S rRNA gene fragments and the *rpoB* gene

DNA was isolated from bacterial cultures grown overnight at room temperature on Nutrient Agar (Oxoid, UK) by the boiling cell-lysis method (Raffel et al. 1996). The primers were designed, by interrogating the database for regions matching a selection of *Bacilli*. A test for specificity of the designed primer was performed by comparison to sequences, available in the NCBI database, showing similarity to the majority of sequences from *Bacillus*. The primers for the 16S rRNA analysis (Integrated DNA Technologies, USA) used for the first PCR were BacF, (5′-GGGAAACCGGGGCTAATACCGGAT-3′, specific for *Bacillus* and related taxa, this study) and R1378 (5′-CGGTGTGTACAAGGCCCGGGAACG-3′, a universal bacterial 16S rDNA reverse primer) (Garbeva et al. 2003). The thermal cycling was as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C, 1 min; 65 °C, 90 s; 72 °C, 2 min and a final extension at 72 °C, 10 min. The PCR products (expected sizes, about 1,300 bp) were analysed by running 5–10 µl aliquots of the reaction mixtures in 1.2 % agarose gels.

For DGGE analysis, the product of the first PCR reaction was diluted 1:100 and used as a template for a second PCR using the universal bacterial primers (F984GC-R1378) targeting 16S rDNA at 968–1,401 bp (relative to the *Escherichia coli* rDNA sequence) (Gomes et al. 2005). The program used for the second PCR was as follows: initial denaturation at 94 °C for 5 min; 2 cycles of 94 °C, 1 min; 63 °C, 1 min; 72 °C, 2 min; followed by 10 times the same cycle with every second one a 2 °C lower annealing temperature (until 55 °C); 20 cycles of 94 °C, 1 min; 55 °C, 1 min and 72 °C, 2 min, followed by final extension at 72 °C, 10 min.

The set of primers for the *rpoB* gene analysis were *rpoB* 1698F and *rpoB* 2041R (Costa et al. 2006). A GC clamp was added to the forward primers. The amplification reaction was carried out as follows: a denaturing step of 94 °C for 3 min, followed by 10 cycles of denaturing for 1 min at 94 °C, annealing for 1.5 min at 40 °C and extension for 2 min at 72 °C followed by 25 cycles of denaturing for 1 min at 94 °C, annealing for 1.5 min at 50 °C and extension for 2 min at 72 °C, and a final extension at 72 °C for 10 min.

Denaturing gradient gel electrophoresis

DGGE was performed using 9 % acrylamide gels (ratio of acrylamide to bis acrylamide of 37:1) with a gradient of 45–65 % to enhance the band resolution and sharpness (Garbeva et al. 2003). One hundred percent corresponds to 7 M urea and 40 % (v/v) formamide (Promega, France). The gels were run at 60 °C at 100 V for 19 h in a PhorU_2 apparatus (Ingeny, Goes, Netherlands) in TAE buffer (40 mM Tris–HCl, 40 mM acetic acid, 1 mM EDTA, pH 8.0) and stained with ethidium bromide (Sigma, UK) for 10 min.

T-RFLP analysis of PCR amplified gene fragments

The bacterial primers 8-27f were labelled at the 5′-end with 6-carboxyfluorescein (6-FAM) and used to amplify a 919 bp fragment of the 16S rRNA gene (Legard et al. 1994). Each PCR mix (50 µl) contained PCR buffer, 1.75 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates, 25 pmol of each primer, 2.5 U of Red Hot Taq DNA polymerase (Abgene) to which 10 ng of template DNA were added. The thermal cycling conditions were: 3 min denaturation at 95 °C, followed by 25 cycles of 30 s at 94 °C, 40 s at 50 °C and 90 s at 72 °C, and a final extension for 8 min at 72 °C. The choice of the restriction enzymes, for use in the T-RFLP analysis of the PCR products, was made using the primers 8-27f and 907-926r following the 130 computer-based predictions of the expected T-RFs from a range of 16S rRNA gene sequences from representatives of *B. thuringiensis* and *B. cereus*. T-RFLP analysis was performed with digests of the PCR products with each of the restriction enzymes *Msp*I, *Rsa*I and *Hha*I using an ABI PRISM 3130xl Genetic Analyser (Molecular Biology Service, HRI, Warwick, United Kingdom) using the dye set DS-33 and Liz 500 as internal standard. The T-RFLP profiles were used for further cluster analysis.

MLST analysis of the clover leaf samples

Multiple locus sequence analysis was carried out as described previously (Bizzarri et al. 2008).

Similarity of the community profiles and cluster analysis

Analysis of the bacterial community DGGE was performed with the software package Diversity database SW/PC/Hercules, (CA, USA) according to the provider's

instructions. The gel patterns were converted to binary data (presence or absence of particular bands) and the similarity between samples visualized by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis. The ABI files with the T-RFLP data were analysed using the Peak Scanner software (Applied Bio Systems).

Significance test for comparing the complex microbial community fingerprints

The Pearson's similarity matrix was used for a permutation test to confirm the significance of effects which were indicated by the difference of means between the group similarities. Mantel statistics were carried out to distinguish among these three cases by assessing the extent of spatial autocorrelation among subjects, in this case, the three restriction enzymes.

Results

PCR and DGGE analysis: 16S rRNA and *rpoB* genes

The initial amplifications using the 16S rRNA and *rpoB* specific primers yielded PCR products of the correct sizes. The DGGE analysis of the PCR-amplified gene fragments displayed equally intense bands, indicating the presence of differences in the patterns. The *rpoB* profiles had fewer bands. The use of the *rpoB*–DGGE (Fig. 1) was more straightforward and superior, resolving much better than the 16S rRNA profiles (Fig. 2). This supports the previous finding (Costa et al. 2006) that single copy genes are better biomarkers for community analysis than heterogeneous, multiple copy genes. The analysis of the DGGE profiles by UPGMA clustering revealed that bacterial community profiles were separated by sample date and that there was some variability in community profile. The reproducibility of the DGGE method was determined using cluster analysis. PCR replicates run on the same gel were highly reproducible with DGGE, reflecting that the heterogeneity is not an artefact of the PCR step and that reproducibility is not related, in this or in any other case, to accuracy. This demonstrates the need to employ another gene, such as *rpoB*, as a suitable candidate for microbial studies. The *B. thuringiensis* and *B. cereus* samples were subjected to nested PCR using the *rpoB* primer set, resulting in a clear impact on the character of the DGGE profile. The dendrograms from the entire sample types showed that there were two major clusters that were formed: (a) the strains predominantly from the early sampling period i.e. samples isolated on 16th July and 23rd July, and (b) the strains from the late sampling period, an exception being the two samples collected on 20th August and 1st October which formed a separate cluster for the *B. thuringiensis* samples (Fig. 3a). For the *B. cereus* samples, however, the strains separated into the early and late sampling types as shown in Fig. 3b. In the dendrogram for the MLST-typed vegetative isolates (Table 1) analysed by DGGE, 4D-1 and Bc1230-88 did not cluster together and emerged as separate branches (Fig. 3c). The strains with identical sequence types (ST-8: 248-S-1 and 164-S-1) aligned closely with MLST (Bizzarri et al. 2008), whereas DGGE placed these identical STs as members of a different cluster, separating into two branches as shown in Fig. 3c. From the phylogenetic trees obtained with the MLST (Bizzarri et al. 2008) and DGGE both the techniques generated four different groups and evolved as two different branches. The members of cluster IV (1710-S-1, 1710-S-3 and 216-S-2) align very close to each other (Bizzarri et al. 2008), whereas in the dendrogram generated with DGGE (Fig. 3c) these three members aligned far from each other.

T-RFLP analysis

The resulting bacterial profiles of the clover leaf samples were characterised by the presence of distinct peaks, indicating a dominant bacterial group. For all of the isolates analysed with T-RFLP, assuming a resolution in size

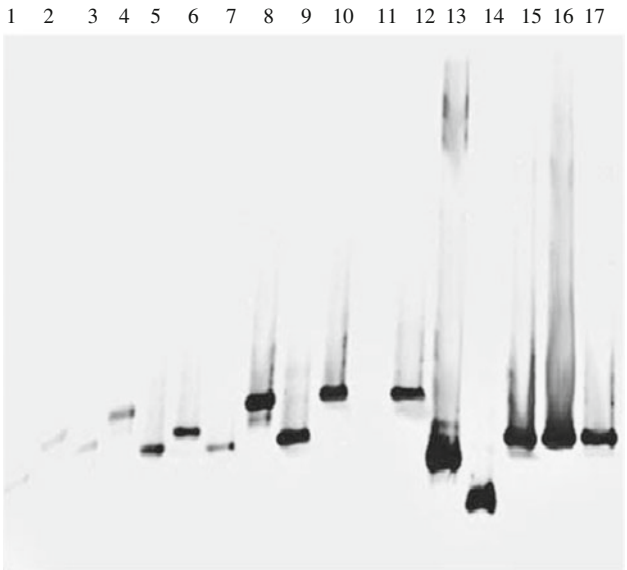


Fig. 1 DGGE gel image of the *B. thuringiensis* strains obtained from the clover leaf phylloplane used for the *rpoB* analysis on a 9 % acrylamide gel; 110 V, 66 mA, 7 W, on a 45–65 % gradient for a period of 20 h. The primers used were the *rpo*B gene primer set *rpo*1698F and *rpo*2041R. The strains used were *lane 1* 020707, *lane 2* 060707, *lane 3*090707, *lane 4* 160707, *lane 5* 230707, *lane 6* 130807, *lane 7* 200807, *lane 8* 270807, *lane 9* 010907, *lane 10* 100907, *lane 11* no loading, *lane 12* 160907, *lane 13* 200907, *lane 14* 011007, *lane 15* 091007, *lane 16* 151007, *lane 17* 221007. The strain names represent the sampling date with the month and year of collection as listed in the supplemental tables

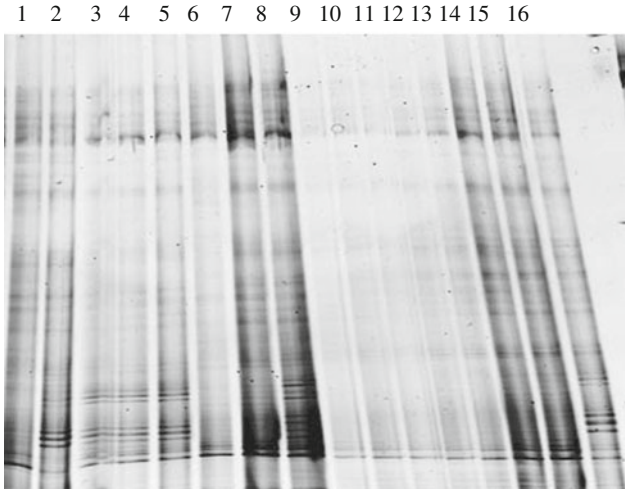


Fig. 2 DGGE gel image of the *B. thuringiensis* strains obtained from the clover leaf phylloplane used for the 16S rRNA analysis on a 9 % acrylamide gel; 110 V, 66 mA, 7 W, on a 45–65 % gradient for a period of 20 h. The primers used are the 16s rDNA primers F984GC and the R1494. The strains used were *lane 1* 020707, *lane 2* 060707, *lane 3* 090707, *lane 4* 160707, *lane 5* 230707, *lane 6* 130807, *lane 7* 200807, *lane 8* 270807, *lane 9* 010907, *lane 10* 100907, *lane 11* 160907, *lane 12* 200907, *lane 13* 011007, *lane 14* 091007, *lane 15* 151007, *lane 16* 221007. The strain names represent the sampling date with the month and year of collection as listed in the supplemental tables

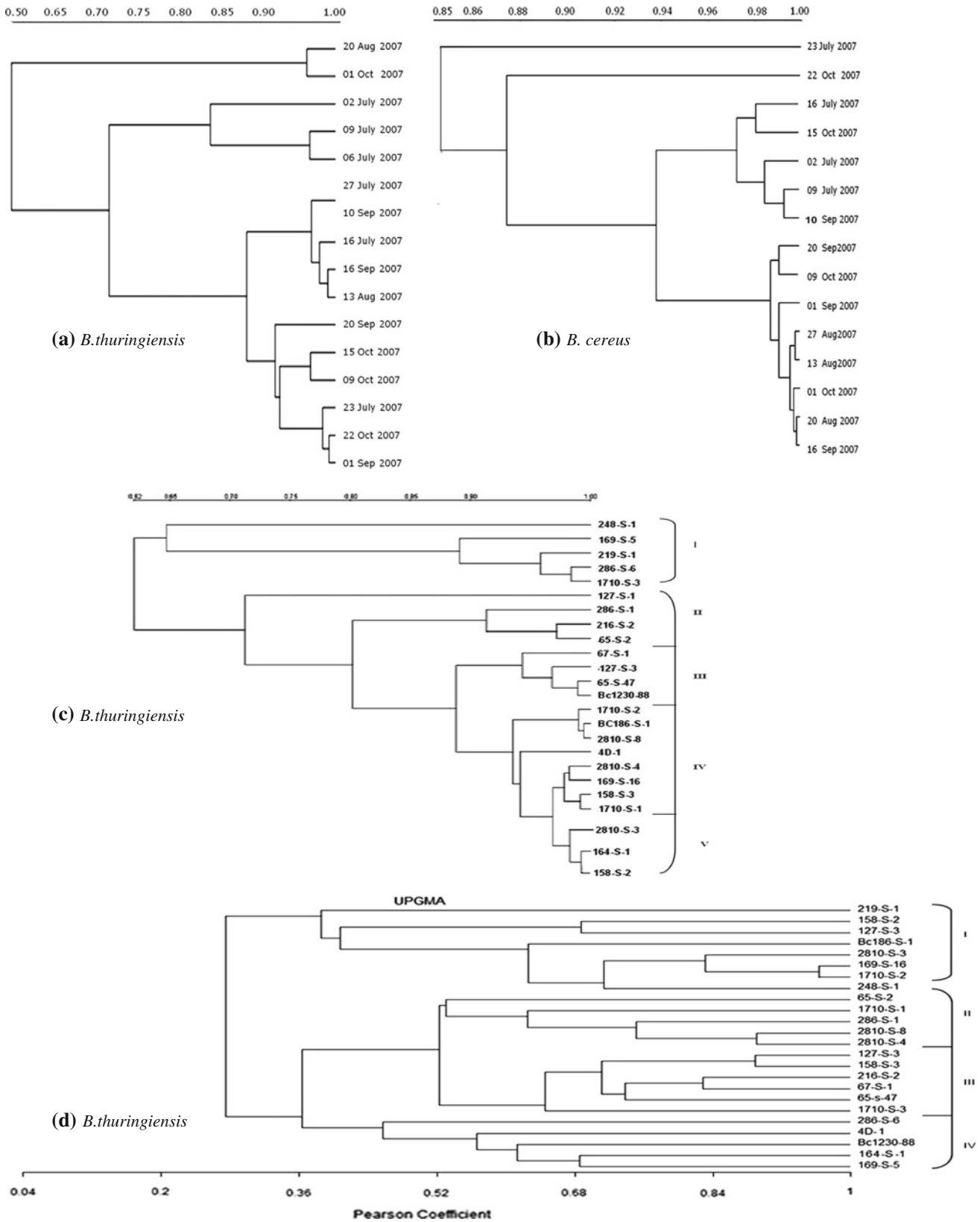
of ±1 bp, different restriction patterns were observed for all the samples. In the studies carried out using the DNA from the leaves colonized by *B. thuringiensis*, the greater number of phylogenetically informative T-RFs (terminal restriction fragments) were obtained from single enzyme digestion using the *Rsa*I enzyme as compared to *Msp*I and *Hha*I. A total of 42, 19 and 12 informative T-RFs were obtained from the data from separate enzyme digestions of the bacterial DNA samples with *Rsa*I, *Msp*I and *Hha*I, respectively. These data demonstrate that the use of single enzyme digests will typically be the best strategy for general profiling of bacterial communities. In the T-RFLP dendrogram using the MLST-typed strains (Table 1), standardized with use of restriction enzyme *Rsa*I (Fig. 3d), strain Bc1230-88 aligned with *B. thuringiensis* isolate HD-1(designated 4D-1). The relationship between the strains using MLST (Bizzarri et al. 2008) and T-RFLP showed close alignment of the *B. cereus* and *B. thuringiensis* strains. Using T-RFLP, vegetative isolates 1710-S-3 and 216-S-2 aligned closely in cluster III (Fig. 3d) whereas strain 1710-S-1 aligned separately in cluster II. The same results were obtained in clustering identical STs: ST-8 for example, where the members aligned separately in different clusters. For the same set of vegetative isolates (Table 1) there were no strong similarities produced by the three different methods: the clustering obtained by T-RFLP was variable even between the three restriction enzymes used (Fig. 3d).

A high reproducibility of profiles was found concerning both the presence and relative abundance of T-RFs: a few variations in the presence of the T-RFs among the samples were mostly restricted to small peaks. However, one peak can comprise numerous T-RFs, differing only in a few base pairs (Giovannoni et al. 1990). The resulting bacterial profiles isolated at different periods of time and digested with the use of three different enzymes were characterised by distinct peaks, indicating dominant bacterial groups over a period of time. The UPGMA dendrogram, generated using pairwise similarities for the T-RFLP fingerprints (Figs. 4, 5), showed levels of difference amongst them, indicating the choice of the restriction enzymes to be a deciding factor in the resolution of the microbial community structure. Clear differences in the community structure between the samples collected at different times could be observed. Also, for the T-RFLP profiles all of the statistical comparisons between the groups showed significant differences, as calculated by the Mantel statistics (data not shown).

Discussion

The results obtained in this study suggest that 16S rRNA heterogeneity is typical of bacteria isolated from the environment, thus implying that it is sequence diversity, including that of intraspecies variation that is reflected in the 16S rRNA banding pattern. Sequence diversity will change depending on how many bands the individual species of the community give rise to and will therefore, not necessarily reflect the true changes in species diversity. Conversely, only one band was observed for each bacterial isolate and the DGGE banding pattern from the mixture could clearly be related to single isolates. The use of the *rpoB*–DGGE was better and more straightforward, resolving much better than the 16S rRNA profiles. It suggests that the single copy genes are better biomarkers than the heterogeneous, multiple copy genes and should be used when performing such type of community analysis, thus supporting the assertion of (Costa et al. 2006). The adoption of good, new biomarkers is important, given that no single biomarker is without limitation and multiple approaches result in a more balanced assessment of the bacterial communities on the leaf. Mantel tests (Kropf et al. 2004) showed significant comparisons amongst the restriction enzyme digestion results of the T-RFLP (data not shown).

MLST analysis of the housekeeping genes, compared to quantitative (T-RFLP) and semi-quantitative (DGGE) methods, could provide a more reliable indicator of the effect of recombination on the genome as a whole and the local influences of homologous recombination. It is, thus, an ideal strategy for analyzing the long-term evolutionary


0.50 0.65 0.70 0.75 0.80 0.85 0.90 1.00
20 Aug 2007
01 Oct 2007
02 July 2007
09 July 2007
06 July 2007
27 July 2007
10 Sep 2007
16 July 2007
16 Sep 2007
13 Aug 2007
20 Sep 2007
15 Oct 2007
09 Oct 2007
23 July 2007
22 Oct 2007
01 Sep 2007
(a) B.thuringiensis
0.85 0.86 0.88 0.90 0.92 0.94 0.96 0.98 1.00
23 July 2007
22 Oct 2007
16 July 2007
15 Oct 2007
02 July 2007
09 July 2007
10 Sep 2007
20 Sep2007
09 Oct 2007
01 Sep 2007
27 Aug2007
13 Aug2007
01 Oct 2007
20 Aug 2007
16 Sep 2007
(b) B. cereus
248-S-1
169-S-5
219-S-1
286-S-6
1710-S-3
I
127-S-1
286-S-1
216-S-2
65-S-2
II
67-S-1
127-S-3
65-S-47
Bc1230-88
III
1710-S-2
BC186-S-1
2810-S-8
4D-1
2810-S-4
169-S-16
158-S-3
1710-S-1
IV
2810-S-3
164-S-1
158-S-2
V
(c) B.thuringiensis
UPGMA
219-S-1
158-S-2
127-S-3
Bc186-S-1
2810-S-3
169-S-16
1710-S-2
248-S-1
I
65-S-2
1710-S-1
286-S-1
2810-S-8
2810-S-4
II
127-S-3
158-S-3
216-S-2
67-S-1
65-s-47
1710-S-3
III
286-S-6
4D-1
Bc1230-88
164-S-1
169-S-5
IV
0.04 0.2 0.36 0.52 0.68 0.84 1
Pearson Coefficient
(d) B.thuringiensis

◀ **Fig. 3** Dendrogram showing the relationship between the **a** *B. thuringiensis* and **b** *B. cereus* isolates sourced from leaves and used for the seasonal variation study using DGGE for the *rpoB* gene, the sample name corresponds to the date and year of collection.; **c** *B. thuringiensis* vegetative isolates as used in the MLST study (Bizzarri et al. 2008) using DGGE for the 16S rRNA; **d** T-RFLP analysis (*Rsa*I) of 16S rRNA using MLST isolates. The cluster analysis was based on the Pearson's correlation index and the unweighted pair group method (*UPGMA*) with arithmetic averages

relationships in bacterial populations (Coelho et al. 2007) and the detection or quantification of target organisms in complex environmental samples.

Although the T-RFLP has potential benefits in terms of reproducibility, automation for processing multiple samples and the ability to track phylogenetically representative TRFs over a period of time it equally has drawbacks. Conversely, although DGGE is practically useful for monitoring changes in temporal community dynamics, the identification of ribotypes requires expensive and labour intensive PCR-amplification and DNA sequencing. Nevertheless, the ultimate phylogenetic resolution achievable through DGGE analysis is superior to T-RFLP due to the possibility of sequence analyses of excised bands. It follows, that studies combining both techniques (DGGE and MLST), even in the absence of genetic libraries, would generate more comprehensive and integrated datasets and this approach should be adopted in future studies.

The resolution of a limited region of genome, the presence of intragenomic heterogeneity and a lack of universal threshold sequence identity value limits the 16S RNA-sequence based identification. MLST, however, can reveal multiple ecotypes with excellent discrimination and high resolution and reproducibility, but is an expensive process as compared to rapid fingerprinting techniques such as DGGE and T-RFLP.

In terms of the ecology of the bacteria on the phylloplane, as sampled at different times, there was no relationship between the genetic similarity of the dominant PCR ribotypes and the time at which they appeared (Fig. 3c). Microheterogeneity in several bacterial genomes has been displayed by 16S rRNA genes with the highest variation being for *Bacillus* species (Felske et al. 2003). Conversely, in the previous studies using MLST (Bizzarri et al. 2008), a clonal population structure was indicated and greater variation in STs was discovered than in previous collections of *B. cereus*/*B. thuringiensis*. There would not seem to be any reason why the dominant PCR ribotype of the *B. thuringiensis* community at one sampling time should have similar 16S rRNA sequences to that which was present at the subsequent sampling time. In agreement with Redford and Fierer (2009), this study shows that change in the community structure does occur. It is possible that there are multiple successional sequences occurring throughout the growing season that are responsible for these changes or differences. On a more practical note, this study provides more insight into phylloplane studies, as the leaf surface offers the potential to replicate the successional process as new leaves emerge each year.

The next generation molecular techniques (Margulies et al. 2005) such as high throughput sequencing, genomics, metatranscriptomics and proteomics should be exploited to assess the links between community structure,

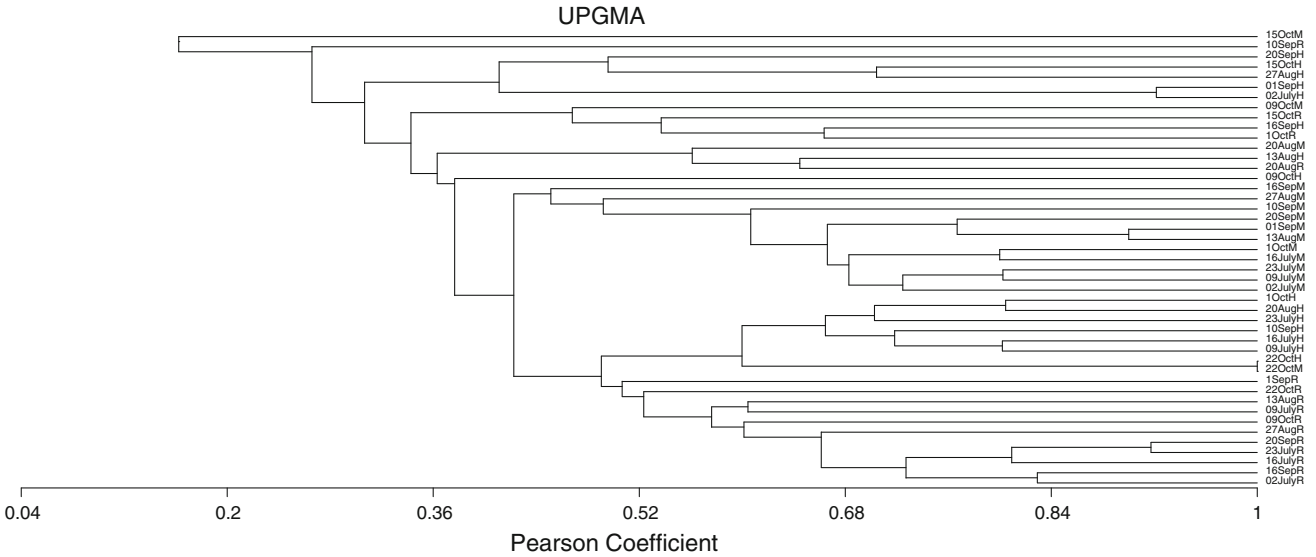


Fig. 4 The complete relationship between the bacterial community structures of *B. thuringiensis* based on T-RFLP analysis of 16S rRNA using the samples digested with the use of the three restriction enzymes. *Hha*I(H), *Msp*I(M) and *Rsa*I(R). The cluster analysis was based on the Pearson's correlation index and the unweighted pair group method with arithmetic averages. The initials *R*, *H*, *M* indicate the respective restriction enzyme *Rsa*I, *Hha*I and *Msp*I

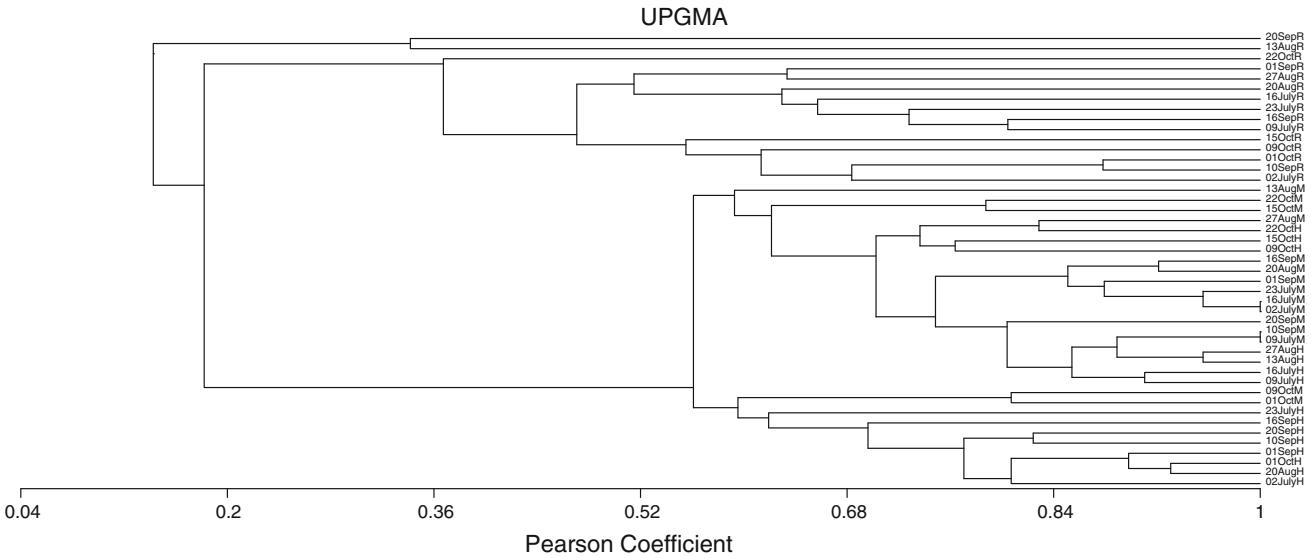


Fig. 5 The complete relationship between the bacterial community structures of *B. cereus* based on T-RFLP analysis of 16S rRNA using the samples digested with the use of the three restriction enzymes. *HhaI*(H), *MspI*(M) and *Rsa*I(R). The cluster analysis was based on the Pearson's correlation index and the unweighted pair group method with arithmetic averages. The initials *R*, *H*, *M* indicate the respective restriction enzyme *Rsa*I, *Hha*I and *Msp*I

physiological diversity and ecosystem function rather than characterising the identity of the strains. The problems and biases associated with the cultivation-based and molecular studies can be used to advantage to understand the mechanisms driving microbial diversity which includes signalling, spatial scale and horizontal gene transfer on the evolution of bacterial species. Furthermore, detection by such a global approach largely depends on its abundance, necessitating the screening of a large number of natural samples from different environments outside to reach saturated resolution and to detect the competing and low abundant organisms. The use of high resolution genetic markers may give a better description of population structures and help to understand the local and global patterns of migration and dissemination.

Conclusion

In conclusion, the results obtained suggest that bacterial 16S rRNA gene fragments amplified from the bacterial isolates on the phylloplane could be separated using the three techniques and the characteristic dominant ribotypes and sequence types were obtained for each of the samples. In this study, the application of *rpoB* PCR-DGGE with DNA was straightforward and resolved differences better than 16S rDNA profiles. DGGE allows an easier access to characterize differentiating bands by sequencing and cloning, whereas T-RFLP seems to be more advisable for routine analysis because difficulties resulting from gel-to-gel variation do not exist. In the present study the comparative power of DGGE and T-RFLP to unravel the diversity of bacterial community from samples collected over a period of time, showed a similar clustering of the samples. Whereas DGGE can be coupled with other additional approaches such as MLST to identify differentiating bands, T-RFLP approach scores better in terms of automation and reproducibility. Future work could examine the potential of other distance metrics and other methods of data analysis such as correspondence analysis, use of artificial neural networks, as well as more complex methods of defining the fluorescence baseline. Possibly the use of quantitative analysis coupled with molecular methods creates new opportunities for addressing applied and ecological problems in microbial community analysis.

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