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REGULAR ARTICLE

Effect of hydrogen on soil bacterial community structure in two soils as determined by terminal restriction fragment length polymorphism

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Abstract The metabolism of hydrogen evolved from HUP^- legume nodules can alter bacterial community structures in the rhizosphere. Our earlier experiments demonstrated increased hydrogen uptake and appearance of white spots within bacterial colonies in H_2-treated soil. We were also able to isolate hydrogen-oxidizing bacteria from soil samples exposed to hydrogen, but not from samples exposed to air. To further understand the effect of hydrogen metabolism on soil microbial communities, in this study 16S rRNA terminal restriction fragment (TRF) profiles of different soil samples exposed to hydrogen gas under laboratory, greenhouse, and field conditions were analyzed. Relationships between soil bacterial community structures from hydrogen-treated soil samples and controls, illustrated by UPGMA (unpaired group mathematical averages) dendrograms, indicated a significant contribution of hydrogen metabolism to the variation in bacterial community. The intensity variation of TRF peaks includes both hydrogen-utilizing bacteria, whose growth were stimulated by hydrogen exposure, and other bacterial species whose growth was inhibited. Comparison of TRF profiles between laboratory and greenhouse samples showed that T-RFLP is a useful technique in the detection of root-related effects on soil bacterial community structure.

Keywords Bacterial communities · *Burkholderia* · *Flavobacterium* · H_2−oxidizing bacteria · HUP status · T-RFLP · *Variovorax*

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Introduction

A recent study showed hydrogen emission rates from soybean nodules lacking an uptake hydrogenase (HUP^-) of 215,000 L/ha (Peoples et al. 2008). Most hydrogen released from legume nodules is absorbed by soil (La Favre and Focht 1983; Dong and Layzell 2001). The soil surrounding hydrogen-releasing nodules showed higher H_2 oxidation capacity and greater bacterial population densities (La Favre and Focht 1983; Popelier et al. 1985; Cunningham et al. 1986). McLearn and Dong (2002) demonstrated that bacteria were responsible for the hydrogen metabolism in soil. Thus, the increase in hydrogen uptake rate in H_2-treated soil or soil around HUP^- nodules could be an indicator of increased bacterial activity correlating with hydrogen oxidization. Dean (2004) found that the diversity of white spots within a group of bacterial colonies was increased in H_2– treated soil, and the soil

possessing white spots had higher H_2 uptake ability compared to controls. The fact that three genera of hydrogen-oxidizing bacteria were isolated only from hydrogen-treated soil or soil adjacent to HUP^- legume nodules also showed the influence of hydrogen metabolism on the abundance of hydrogen-oxidizing bacteria in soil (Maimaiti et al. 2007). Thus, it has been experimentally shown that hydrogen metabolism alters rhizobacterial community structure.

Terminal restriction fragment length polymorphism (T-RFLP) is a fingerprinting technique that follows the same principle as restriction fragment length polymorphism (RFLP) and amplified ribosome DNA restriction analysis (ARDRA), except that one PCR primer is labeled with a fluorescent dye, such as TET (4,7,2',7'-tetrachloro-6-carboxyfluorescein) or 6-FAM (phosphoramidite fluorochrome 5-carboxyfluorescein) (Liu et al. 1997). T-RFLP analysis has become increasingly popular and has been utilized in many studies to investigate complex bacterial communities in the environment because the use of capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection makes it an automated and sensitive technique. T-RFLP has been proven to be an effective method to study complex bacterial communities in water, soil, and feces (Moeseneder et al. 1999; Dunbar et al. 2000; Kaplan et al. 2001; Osborne et al. 2006).

In the present study, 16S rRNA TRF (terminal restriction fragment) profiles of different soil samples were used to better understand the effects of hydrogen metabolism on rhizobacterial population changes; the results showed that hydrogen metabolism did induce significant variation of bacterial community structure in soil.

Materials and methods

Preparation of soil samples

Laboratory conditions Soil samples were collected from Lawrencetown, Annapolis County, Nova Scotia (N44°53'; W65°09'). The soil was sandy clay loam from a fallow field. Soil was mixed with fine clear filter sand at a ratio of 2:1 (vol.:vol.) and treated by hydrogen gas (labeled as H) and air (labeled as A) as described in Dong and Layzell (2001) for 30 days. Samples were frozen at −20°C after hydrogen uptake rate measurements.

Greenhouse conditions The mixture of field soil and sand was used to grow soybean plants (Cat. 32601R, First Line Seeds Ltd., Guelph, Ontario) inoculated by two common commercial strains of *Bradyrhizobium japonicum*: USDA110 (HUP^+) and 532C (HUP^-). After 10 weeks of growth in pots, soil was sampled within 10 mm of the nodules. Soil samples adjacent to HUP^- nodules (532C) were labeled from G-1 to G-8, and those from around HUP^+ nodules (USDA110) were labeled from G+1 to G+4. Soil samples were frozen at −20°C after their hydrogen uptake rates were measured.

Field conditions Two soil samples (F1 and F2) adjacent to HUP^- soybean nodules, and three bulk soil samples (B1, B2, and B3) were taken from a soybean garden in Halifax, Nova Scotia in the spring of 2006 and then frozen at −20°C after their hydrogen uptake rates were measured.

Bacterial samples

Strains of hydrogen-oxidizing bacteria isolated by Maimaiti et al. (2007) were used for spiking the 16S rRNA TRF profiles of different soil samples exposed to hydrogen gas.

Measurement of soil hydrogen uptake

Based on the system described by Dong and Layzell (2001), the hydrogen uptake capability of each soil sample was calculated from the concentrations of hydrogen before and after subjecting the soil samples to hydrogen treatment; these were measured with a hydrogen sensor (Model S211, Qubit System Inc., Kingston, Ontario).

DNA extraction

The total genomic DNA of each soil sample was extracted from 0.5 g of soil by using the Ultraclean Soil DNA Isolation Kit (MO BIO Laboratory, Inc., Solana Beach, CA). For maximum yields, the alternative protocol suggested by the manufacturer was followed. Genomic DNA of isolates was extracted by using the modified protocol described by Lechner and Conrad (1997). For Gram-negative isolates (JM01, JM63, JM110, JM162, and JM162a),

cells were lysed by using Protease K (20 mg/ml) and SDS (0.5%) at 37°C for 30 min; cells of Gram-positive isolates such as JM120 were lysed by passing the cell suspension through a French pressure cell (pre-chilled at 4°C) thrice at the pressure of 16,000 psi (Thermo Electron Co., Waltham, MA, USA). Supernatant containing genomic DNA was collected after a centrifugation of the lysed cell suspension at 22,000 g (14,000 rpm in a JA21 rotor in a Beckman Avanti J-E centrifuge) for 1 hr at 4°C.

Amplification of 16S rRNA genes

Part of the 16S rRNA gene of soil samples and isolates, with an expected length of 527 base pairs (bp), was amplified with a pair of bacterial universal primers fluorescently labeled at the 5' terminus of the forward primer, BSF 8/20 (6-FAM-5' - AGAGTTT GATCCTGGCTCAG - 3'), with unlabelled BSR 534/18 (5' - ATTACCGCGGCTGCTGGC - 3') acting as the reverse primer. Each 50-μl reaction mixture contained the following: 36.6μl PCR water (Sigma-Aldrich Canada Ltd, Oakville, Ont., CA); 5 μl of 10× ThermoPol Reaction Buffer (New England Biolabs Ltd., Pickering, Ont., CA); 5μl of 2 mM dNTP (New England Biolabs Ltd., Pickering, Ont., CA); 1μl each of 20μM 6-FAM-5'-BSF8/20 and BSR534/18 (Applied Biosystems, Foster City, CA); 1μl soil DNA solution and 0.4μl of 5U/μl Taq DNA polymerase (New England Biolabs Ltd., Pickering, Ont., CA). Amplified reactions were carried out in a Bio-rad iCycler thermal cycler (Bio-rad Laboratories, Inc., Hercules, CA) with the following cycling conditions: three minutes of initial denaturation at 94°C, 35 cycles of 75 s at 94°C for denaturation, 45 s at 55°C for annealing, and 45 s at 72°C for extension, with a final primer extension at 72°C for 10 min. Six PCR reactions from a single sample were pooled together to minimize PCR-induced random biases. PCR products were purified with the Qiaquick PCR Purification Kit (QIAGEN Inc., Mississauga, Ont., CA).

Generation of TRF profiles and data sets

Four separate TRF profiles for each sample were obtained by using four restriction endonucleases (REs). Approximately 200 ng of purified PCR product was digested with 20 U of one of the following restriction endonucleases, which were also used in previous T-RFLP analyses: *Bst*UI, *Hae*III, *Hinf*I, and *Msp*I (New England Biolabs Ltd., Pickering, Ont., CA) in a 50-μl reaction system (Osborne et al. 2006; Dunbar et al. 2000; Kitts 2001; Lui et al. 1997). Each 50-μl reaction mixture was incubated at the optimal temperature of the restriction endonuclease overnight. For each restriction digestion, three replicates were set up and pooled together to minimize artificial biases. Digested PCR products were then purified with the QIAquick Nucleotide Removal Kit (QIAGEN Inc., Mississauga, Ont., CA). Finally, 6-FAM-TRFs (6-FAM- labeled terminal restriction fragments) in digested amplicons were separated and recorded by an ABI 3730 DNA sequencer (University Core DNA Services, University of Calgary, Alberta, Canada). The fragment length in nucleotides, the peak height at apex and the area under the peak in fluorescence units (FU) of each TRF in a given pattern were calculated by GeneMarker V. 1.4 software (SoftGenetics LLC, USA).

To assess the contribution of hydrogen-oxidizing bacteria isolated by Maimaiti et al. (2007) to the variation of bacterial community structure in soil samples exposed to hydrogen gas, spiked TRF profiles were generated by using mixed DNA from hydrogen-treated soil samples (G-2 and G-6; H2 and H4), and from isolates JM01 (*Variovorax*), JM120 (*Burkholderia*) and JM162a (*Flavobacterium*) at the ratio of 3 to 2. Peaks that were spiked in TRF profiles of combined PCR products were possibly contributed by isolates.

Data analysis

All TRF profiles generated by the same restriction endonuclease were incorporated into one dataset and these were standardized by the variable percentage threshold method (Osborne et al. 2006) before further analysis. A series of gradient divisors was set up and checked by using TRFLPdemo, a Matlab based program (Zhang 2006). Divisors started at 100 times the mean total area of all profiles belonging to the same dataset and increased by intervals of $1x10^6$ to generate percentage thresholds. The number of peaks remaining was plotted against the total area of the original profiles. For each divisor, the program generated a linear regression trend line fitted as a power function and the R square (R^2) of the curve. The optimal divisor was picked as the one resulting in the smallest R^2 value, indicating the weakest

relationship between the number of peaks remaining and the total area of the original profile.

Following normalization, derivative TRF profiles within a dataset were aligned and TRFs showing synonymous fragment sizes were identified and binned together based on the function of the Bin Table report in the GeneMarker V. 1.4 software. All TRFs within a bin represented the same peak, which was assigned the average size of all TRFs combined. A single, composite list of the binned peaks (fixed within ± 0.4 bp) was generated for all samples within a dataset. For each sample, the presence or absence of the binned peaks in the composite list was represented by a binary vector, with (1) representing presence and absence represented by (0). The dataset was then transformed into a binary matrix whose rows represented binned peaks and whose columns represented samples. Based on the 'pdist' function in Matlab (V.7.1), the Jaccard coefficient was used to generate a matrix with an upper triangular or square form to show the similarity and dissimilarity between each two samples. Then, the Jaccard coefficient was applied to carry out the agglomerative hierarchical clustering under the rule of unweighted average distance (UPGMA) by using the function of 'linkage' in Matlab. Finally, the hierarchical, binary cluster tree created by the linkage function was plotted by using the 'dendrogram' function. To measure how well the cluster tree reflected the data, the cophenetic correlation coefficient for the hierarchical cluster tree was calculated based on the 'cophenet' function; the closer the value of the correlation coefficient is to 1, the more accurately the clustering solution reflects the data.

The significantly changed T-RFLP peaks after hydrogen treatment were analyzed using the T-RFLP phylogenetic assignment tool (PAT) (Kent et al. 2003). Peaks used in the PAT analysis were generated by three restriction enzymes BstUI, HaeIII and MspI. The bacterial community composition determined by PAT was grouped at the phylum level.

Results

Hydrogen uptake of different soil samples

H_2-treated soil samples (H) showed significantly higher H_2 uptake rates than that of air-treated soil samples (A) (Fig. 1). Soil samples adjacent to HUP^- nodules (F and G-) had significantly higher hydrogen uptake rates than those adjacent to HUP^+ nodules (G+) and bulk soil samples (B). Thus, it was ensured that all the samples were qualified as subjects for studying the effects of hydrogen metabolism on the soil bacterial community structure because the hydrogen uptake rate of all soil samples exposed to hydrogen gas showed a significant increase compared to their controls.

Assay of TRF profiles of different soil samples

A total of 104 TRF profiles were generated from 13 samples with 2 replicates (13 samples × 2 replicates × 4 restriction endonucleases). The optimal divisors generated by the TRFLPdemo program are 4.49×10^7 for the BstUI dataset, 4.70×10^7 for HaeIII, 4.37×10^7 for HinfI, and 3.75×10^7 for MspI. The curves of the number of peaks remaining *vs*. the total area of original profiles resulting from those optimal divisors became horizontal lines after they were fitted as a power function. The R^2s of the power curves for the above mentioned 4 restriction endonucleases are 1.8×10^{-5}, 3.6×10^{-6}, 8.4×10^{-6}, and 1.5×10^{-4} respectively. The thresholds for each profile ranged from 0.06% for F1 (b) with HaeIII digestion and G+2 with MspI digestion to 0.75% for H4 (a) with HaeIII digestion.

All profiles were compiled into a single complex dataset and were also separated into 4 datasets based on the restriction endonucleases used. The distance

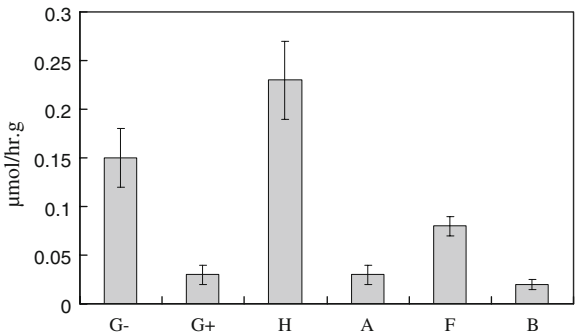


Fig. 1 Hydrogen uptake rates of different soil samples: G-: Greenhouse soil adjacent to HUP^- nodules; G+: Greenhouse soil adjacent to HUP^+ nodules; H: Soil treated by hydrogen gas (3000 ppm) in lab; A: Soil treated by air in lab; F: Soil adjacent to HUP^- nodules in field; B: Bulk soil in field. Data are means ± standard deviations from duplicated experiments with three replicates in each

between each pair of TRF profiles within each dataset was calculated using the Jaccard coefficient. Dendrograms were constructed to show the similarities between TRF profiles of different samples (Fig. 2). The cophenetic correlation coefficients of dendrograms were as follows: 0.95 for the BstUI dataset, 0.93 for the HaeIII dataset, 0.91 for the HinfI dataset, 0.88 for the MspI dataset, and 0.96 for the dendrogram of the combined datasets. The high coefficients suggest that these dendrograms are reliable. Dendrograms of different datasets showed high similarity and all dendrograms showed that the four REs used (*Bst*UI, *Hae*III, *Hinf*I, and *Msp*I) have the ability to group most replicates (data not shown). Relationships of different soil samples reflected by TRF profiles generated by all four restriction endonucleases also matched well, as expected.

All soil samples were first divided into two large groups: Group X and Group Y. Group X included all samples derived from the soil collected from the farm in the Annapolis Valley, Nova Scotia (G-2, G-6, G+1, G+2, H2, H4, A2, and A3), and Group Y included all the samples taken from the field in Halifax, Nova Scotia (B1, B2, B3, F1, and F2).

Group X was then further divided into two subgroups, Group X1 and Group X2. Group X1 included all greenhouse soil samples (G+1, G+2, G-2, and G-6), while all soil samples treated in the laboratory (H2, H4, A2, and A3) were located in Group X2. In the group of lab-treated soils (Group X2), all hydrogen-treated soil samples with high hydrogen uptake rates (H2 and H4) were separated from their controls, which were the air-treated soil samples with very low hydrogen uptake rates (A2 and A3). In the group of greenhouse soils (Group X1), soil samples adjacent to HUP^- nodules with high hydrogen uptake rates (G-2 and G-6) were separated from soil samples around HUP^+ nodules with low hydrogen uptake rates (G+1 and G+2). As for the group of field soils, all bulk soils (B1, B2, and B3) and soil samples adjacent to HUP^- nodules (F1 and F2) were completely separated into two different subgroups.

Hydrogen-induced variation of bacterial community structure in soil samples

It was found that hydrogen metabolism resulted in not only an intensity increase in some TRF peaks but also an intensity decrease in some others in TRF profiles from soil samples exposed to hydrogen gas, such as the greenhouse samples and soil samples treated with hydrogen in the laboratory (Fig. 3).

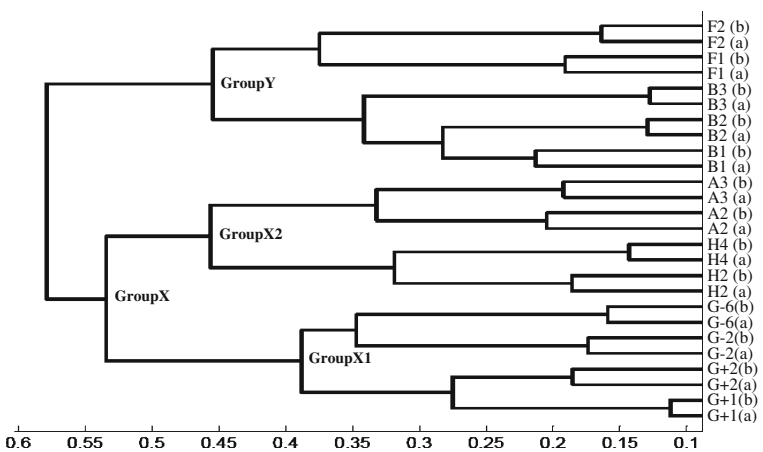


Fig. 2 Relationships between soil bacterial community structure from lab-treated soil samples, greenhouse soil samples, and field soil samples, illustrated by unweighted average distance (UPGMA) dendrograms generated from Jaccard distance matrices of TRF profiles by using Matlab7.1 on combined datasets including all 4 restriction enzyme digestions. The X axis is the Jaccard distance measure. The samples are indicated by letter codes at the branch termini: G– (soil samples adjacent to HUP^- nodules grown in greenhouse); G+ (soil samples adjacent to HUP^+ nodules grown in greenhouse); B (bulk soils in field); H (soil samples treated by hydrogen gas in laboratory); A (soil samples treated by air in laboratory); F (soil samples adjacent to HUP^- nodules grown in field). Replicate samples were indicated as (a) and (b). (Data: normalized & binary; cophenetic correlation coefficient=0.96)

Most of the intensity-increased TRF peaks only appeared in the TRF profiles from soil samples exposed to hydrogen gas (soil treated by hydrogen gas under laboratory conditions (H2 and H4), and soil adjacent to HUP^- nodules under greenhouse conditions (G-2 and G-6) as compared with their controls (A2, A3; and G+1, G+2).

To study the hydrogen-induced variation of the soil bacterial community structure, the total differences of mean intensity (% of total area) of TRF peaks whose intensity increased or decreased significantly in soils exposed to hydrogen gas compared to their controls, Si and Sd, were calculated and are listed in Table 1. It was found that the mean Si from laboratory samples was markedly higher than that from greenhouse samples (38.3±3.4 to 18±2.8), which matched the comparisons of hydrogen uptake ability between laboratory hydrogen-treated samples and greenhouse HUP^- nodule samples (0.23±0.04 to 0.15±0.03; see Fig. 1).

It was found that TRF profiles from soils exposed to hydrogen gas always included a few TRFs whose intensity variation was predominant, and these contributed a large percentage of the hydrogen-induced bacterial community structure variation. The top five peak increases (Si top5) represented approximately 48% to 81% of the total increase calculated by the percentage of total area (Table 1). The top five peak decreases also represented a large portion of the total peak area decrease.

Most peaks whose intensity varied significantly in TRF profiles from greenhouse samples did not match those in TRF profiles from laboratory samples. Thus, most of the bacteria responsible for hydrogen-induced bacterial community structure variation in greenhouse samples (G-2 and G-6) were different from those observed in laboratory samples (H2 and H4). Only a few of them were common in soil samples from both the greenhouse and the laboratory. They contributed to both the intensity increases (B109.5, B375.5, B391.9, Ha209.5, Hi298.8, Hi312.7, Hi313.8, Hi329, and M453.1) and intensity decreases of TRF peaks (B234.6, B400.5, Ha187.2, Ha188.5, Ha199.3, Ha202.6, Ha222.5, Ha225, Hi310.6, Hi316.8, Hi322.7, Hi326.6, Hi330.4, Hi337.2, M151.4, and M486). Binned TRF peaks are indicated by letter and number: B, Ha, Hi, and M represent peaks generated by using *Bst*UI, *Hae*III, *Hinf*I, and *Msp*I respectively; the number is the average of the sizes of all peaks in the bin fixed within ±0.4 bp).

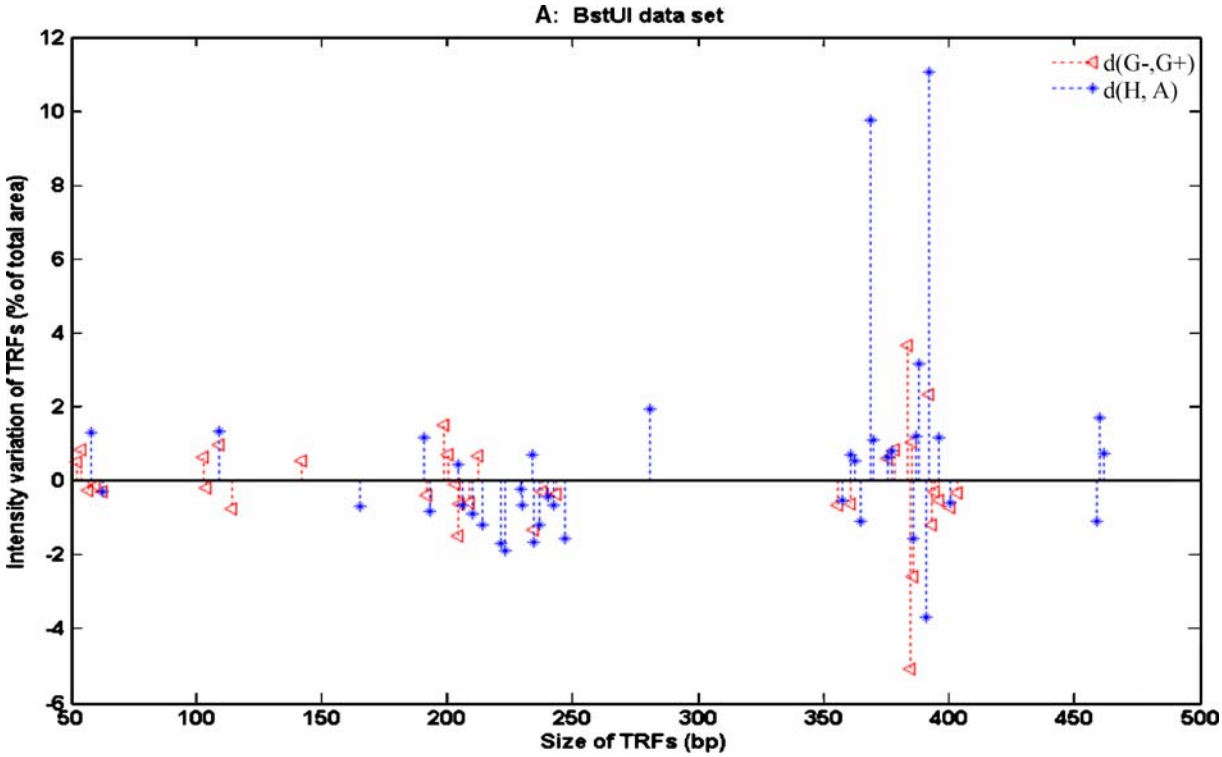
Fig. 3 The effect of H2 treatment on the intensity variation of TRF peaks: d(G-, G+): difference of the mean intensity (% of total area) of TRF peaks between soil samples adjacent to HUP^- (G-2 and G-6) and HUP^+ (G+1 and G+2) nodules in greenhouse; d(H, A): difference of the mean intensity (% of total area) of TRF peaks between soil samples treated by hydrogen gas (H2 and H4) and air (A1 and A2) in the laboratory. Points contributed by peaks whose intensity increased after H_2 treatment are above the X axis; points contributed by peaks whose intensity decreased after H_2 treatment are below the X axis. (**a**) Normalized data generated by *Bst*UI digestion. (**b**) Normalized data generated by *Hae*III digestion. (**c**) Normalized data generated by *Hinf*I digestion. (**d**) Normalized data generated by *Msp*I digestion

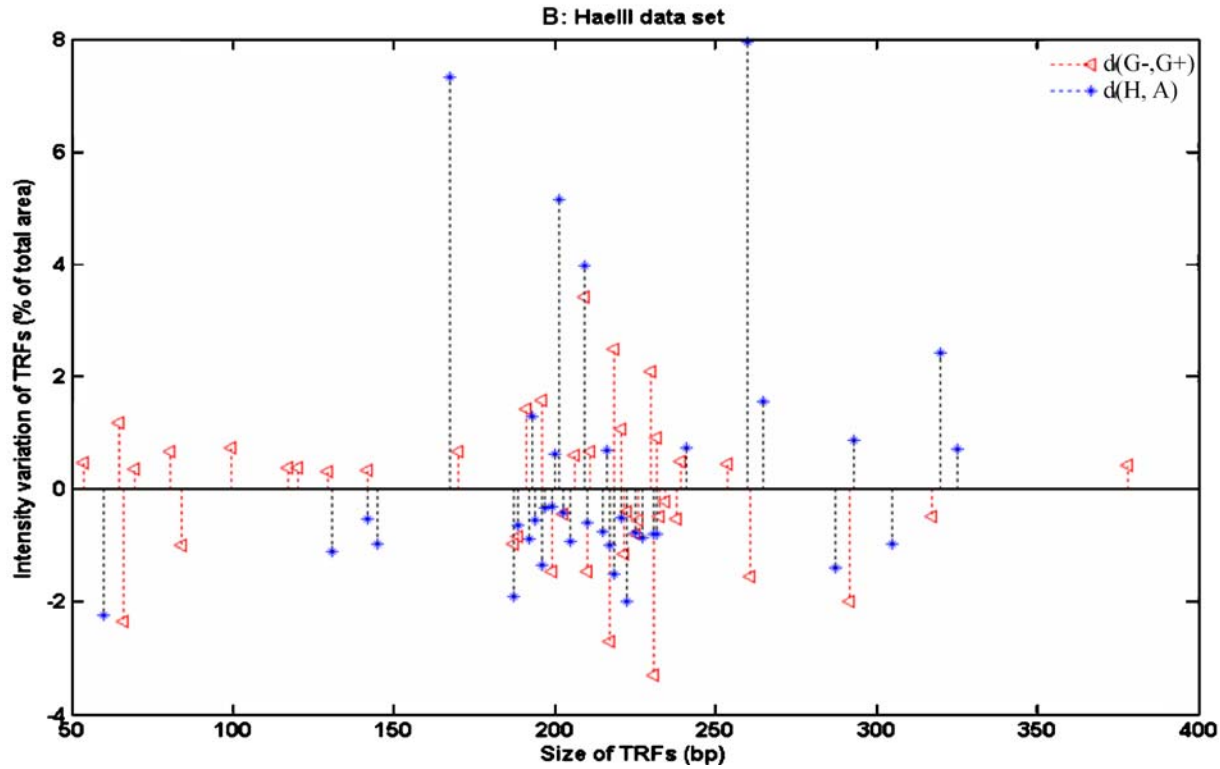
Contribution of isolates to hydrogen-induced bacterial community structure variation

It was found that all TRF peaks of mixed DNA from hydrogen-treated soil samples (G-2 and G-6; H2 and H4) and from isolates JM01 (*Variovorax*), JM120 (*Burkholderia*), and JM162a (*Flavobacterium*) showed a general intensity decrease compared with those in profiles from hydrogen-treated soil samples alone. TRF peak increases were observed at the predicted sites: B68, Ha220, Hi325, and M491 for *Variovorax*; Ha222 and Hi327 for *Burkholderia*, and B106, Ha77, Hi324, and M86 for *Flavobacterium*. These spiked peaks in the TRF profiles of mixed PCR products were contributed by isolates and are considered to be an indication of the contribution of hydrogen-oxidizing bacteria similar to isolates in hydrogen-induced bacterial community structure variation. However, only two of the spiked peaks are close to the significant increased TRF peaks in the profiles from hydrogen-treated soil samples (Hi320 and M484).

Phylogenetic assignment analysis

Although the diversity of bacterial phyla identified by PAT varied among peaks, the communities in the hydrogen-induced increased peaks appear to be dominated by species classified as Gammaproteobacteria and Firmicutes in both lab-treated and greenhouse-treated soil samples. The dominant bacteria in the significantly decreased peaks appear to be Actinobacteria, Alphaproteobacteria, and Firmicutes.


A: BstUI data set
d(G-,G+)
d(H, A)
Intensity variation of TRFs (% of total area)
Size of TRFs (bp)
12
10
8
6
4
2
0
-2
-4
-6
50
100
150
200
250
300
350
400
450
500


B: HaeIII data set
d(G-,G+)
d(H, A)
Intensity variation of TRFs (% of total area)
Size of TRFs (bp)
8
6
4
2
0
-2
-4
50
100
150
200
250
300
350
400

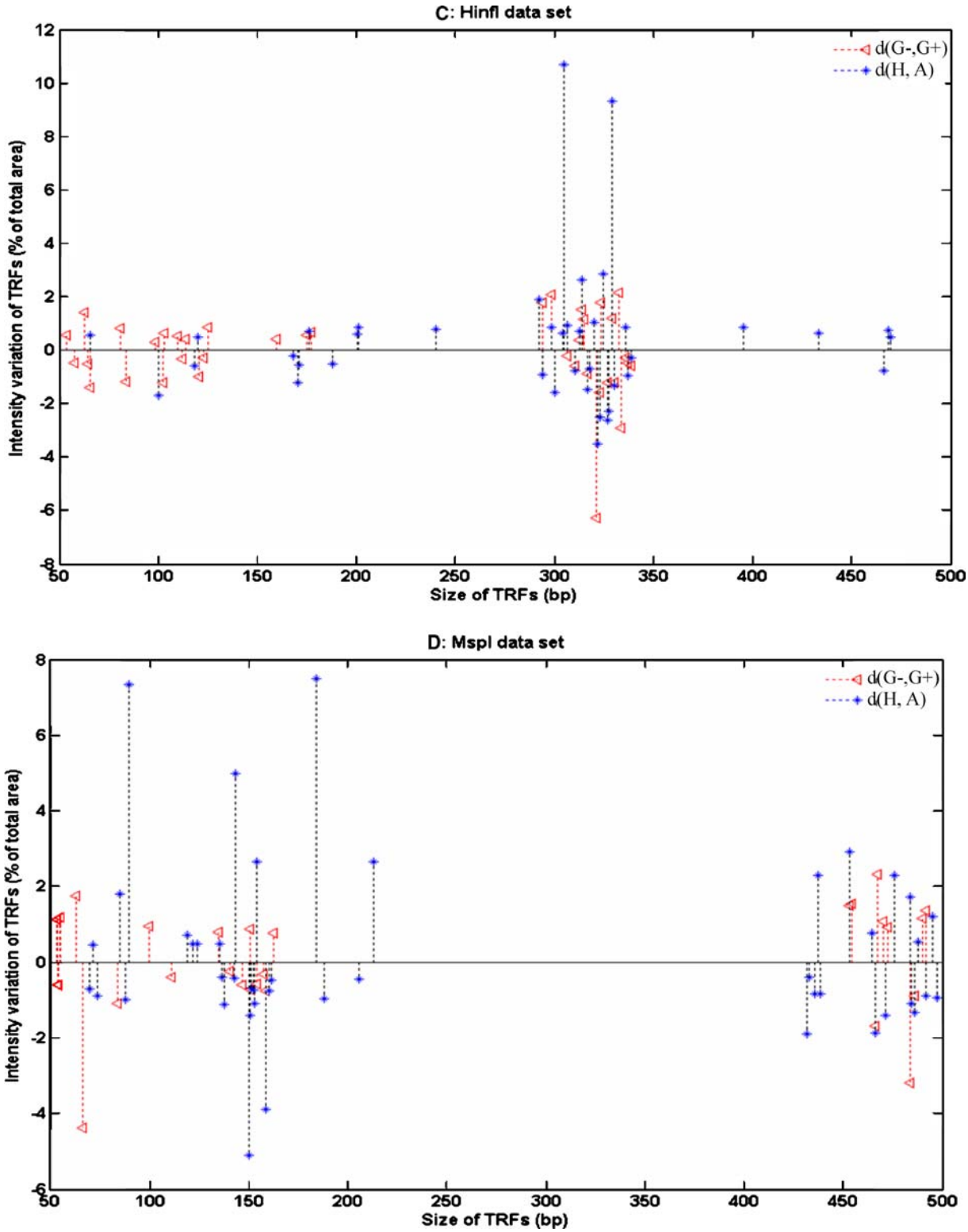


Fig. 3 (continued)

Table 1 Intensity variation of TRF peaks whose intensity increased or decreased significantly in soils exposed to hydrogen gas compared to the controls

		Si	Si_top5	Si_top5/Si	Sd	Sd_top5	Sd_top5/Sd
*Bst*UI dataset	Greenhouse samples	14.4	9.5	0.66	18.9	11.7	0.62
	Laboratory samples	39.5	27.7	0.70	23.1	10.5	0.46
*Hae*III dataset	Greenhouse samples	21.1	12.8	0.61	22.1	11.9	0.56
	Laboratory samples	33.4	26.9	0.81	24.1	9.0	0.37
*Hinf*I dataset	Greenhouse samples	19.3	9.3	0.48	22.6	13.4	0.59
	Laboratory samples	39.0	27.3	0.70	24.5	12.7	0.52
*Msp*I dataset	Greenhouse samples	17.4	8.4	0.48	15.5	11.3	0.73
	Laboratory samples	41.3	25.4	0.62	21.7	14.2	0.65

Si means total differences of the mean intensity (% of total area) of TRF peaks whose intensity increased significantly in soil exposed to hydrogen gas compared to the controls. Si_top5 is the total difference of the mean intensity (% of total area) of the five TRF peaks that showed the largest intensity increase. Sd represents the total differences of the mean intensity (% of total area) of TRF peaks whose intensity decreased significantly in soil exposed to hydrogen gas compared to the controls. Sd_top5 is the total difference of the mean intensity (% of total area) of the five TRF peaks that showed the largest intensity decrease

Discussion

TRF profiles always contained numbers of small peaks resulting from either artifacts or differences in the amount of DNA loaded into the sequencer and this cannot be accurately controlled (total area of peaks in each TRF profile). These could exert negative effects on the similarity analysis of bacterial community structure in different soil samples. To limit the negative influence of false peaks on similarity analysis, TRF datasets should be normalized by using an artificial threshold. Peaks below the threshold are considered as background noise and removed from the dataset. Methods to generate constant percentage thresholds or constant baseline thresholds have been proposed (Sait et al. 2003; Dunbar et al. 2001). Even though both methods are relatively easy to use, the increase in their ability to filter false peaks normally results in the loss of more small peaks possessing useful information during normalization. The method based on the variable percentage threshold reported by Osborne et al. (2006) was used for normalizing TRF profiles in this study due to the wide variation in total area of each profile within a dataset. This is a unique percentage threshold for each profile calculated by dividing the total area of that profile by the optimal divisor; this helped normalization to reach a reasonable trade-off between removing false peaks and keeping peaks with useful information.

Since the number of peaks should have no correlation with the total area of each profile, the optimum divisor should be the one that results in the weakest relationship between the two. To search for the optimum divisor, we calculated a correlation coefficient (R^2) of linear regression for each divisor and picked the one resulting in the smallest R^2 value. This is a practical method that can be easily programmed to process large numbers of datasets.

One gram of soil may contain up to ten billion cells of possibly 4000–7000 different bacterial species (Bianchi and Biachi 1995). However, the number of peaks remaining in normalized TRF profiles from 0.5 g soil samples was no more than a hundred. Therefore, it was inferred that each TRF peak possibly represented more than one species. It was not possible to accurately depict relationships of bacterial community structure between different soil samples based on TRF profiles generated from one restriction endonuclease alone. Therefore, four REs were applied in our study to gain more information about the soil bacterial community structure by increasing the possibility of grouping these species into different peaks within profiles belonging to different datasets. The similarity of dendrograms between datasets generated with different REs suggests that soil samples were grouped at a sufficiently high confidence level on the basis of bacterial community structure.

It was interesting to see the separation of greenhouse soil samples (Group X1) from soil samples treated in laboratory (Group X2). Soil from a farm in the Annapolis Valley, Nova Scotia was mixed with sand before being divided into two parts for the greenhouse and lab treatments. The only difference

here was that the greenhouse soil samples were collected adjacent to root nodules, while the lab soil samples were artificially treated in plastic tubes by air, or by hydrogen gas mixed in air, without actual plant contact. The separation of greenhouse samples from lab-treated soil samples suggests that the activity of soybean roots exerted significant effects on the rhizobacterial community structure.

The gas used to treat soil samples was the only difference between two subgroups of lab-treated soil, which suggests that the metabolism of electrolytic hydrogen in soils is the main reason for the significant variation of bacterial community structure in hydrogen-treated soil samples (H2 and H4). In Group X1, hydrogen treatment was also the only known difference between the subgroups G- and G+ because HUP^- nodules released hydrogen gas to rhizosphere soil, while little hydrogen was released from HUP^+ nodules. Therefore, it can be deduced that metabolism of hydrogen in soils was responsible for the significant variation of bacterial community structure in greenhouse soils adjacent to the HUP^- nodules (G-2 and G-6).

Although the intensity variation (% of total area) of TRF peaks is not always linked with the quantitative variation of bacterial communities, the especially large intensity differences could be used to reflect population changes. The fact that some peak areas increased by 10% of the total area (Fig. 3) after exposure to hydrogen gas suggests that those TRF peak increases are very likely the results of hydrogen-induced bacterial population changes, responsible for hydrogen metabolism in soil, because these variations were repeatable and related to the hydrogen treatment and hydrogen uptake rate increase of soil samples. The hydrogen-oxidizing bacterial populations must have been small before they were exposed to hydrogen gas. During the hydrogen treatment, they were activated and consumed hydrogen to support their growth (Dong and Layzell 2001), which increased the hydrogen uptake rates of the soil samples.

Nocardia was suggested to be responsible for the main hydrogen uptake in hydrogen-treated soils by McLearn and Dong (2002). Five strains of *Pseudonocardia* isolated from hydrogen-treated soils by Osborne are known to utilize hydrogen and promote plant growth (personal communication 2006). However, the PAT (phylogenetic assignment tool) analysis showed that the major increased peaks induced by hydrogen are dominated by the Gammaproteobacteria and Firmicutes groups, while the dominant bacteria in the significantly decreased peaks appear to be the Actinobacteria and Alphaproteobacteria. This supports the observation that Gammaproteobacteria are stimulated by hydrogen treatment (Stein et al. 2005).

Although the PAT analysis extended the utility of T-RFLP as a community fingerprinting method for comparisons of bacterial community composition between treatments, this method is complicated for capillary electrophoresis, in particular by the fact that observed and expected T-RF sizes often differ (Kaplan and Kitts 2003), making definitive prediction problematic. Since hydrogen-induced T-RFs have been identified, it is possible to use large clone libraries to identify bacteria that are representative of the significantly changed T-RFs in a total community profile. This approach could also be used to discover some other previously unknown and unpublished species that are also involved in the hydrogen-induced variation of bacterial community structures.

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