Physiological and Community Responses of Established Grassland Bacterial Populations to Water Stress

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The effects of water stress upon the diversity and culturable activity of bacterial communities in the rhizosphere of an established upland grassland soil have been investigated. Intact monoliths were subjected to different watering regimens over a 2-month period to study community adaptation to moisture limitation and subsequent response to stress alleviation following rewetting. Genetic diversity was analyzed with 16S-based denaturing gradient gel electrophoresis (DGGE) of total soil-extracted DNA (rRNA genes) and RNA (rRNA transcripts) in an attempt to discriminate between total and active communities. Physiological response was monitored by plate counts, total counts, and BIOLOG-GN2 substrate utilization analyses. Controlled soil drying decreased the total number of CFU on all the media tested and also decreased the substrate utilization response. Following rewetting of dried soil, culture-based analyses indicated physiological recovery of the microbial population by the end of the experiment. In contrast, DGGE analyses of community 16S rRNA genes, rRNA transcripts and cultured communities did not reveal any changes relating to the moisture regimens, despite the observed physiological effects. We conclude that the imposed moisture regimen modulated the physiological status of the bacterial community and that bacterial communities in this soil are resistant to water stress. Further, we highlight the need for a reexamination of rRNA transcript-based molecular profiling techniques as a means of describing the active component of soil bacterial communities.

Bacteria play key roles in soil ecosystem processes by breaking down plant exudates and litter and are therefore important mediators of nutrient cycling in the plant-soil system. However, there is a lack of understanding as to how the activity and diversity of these communities in soils respond to perturbation. This issue needs particular attention in threatened ecosystems, such as grasslands, due to their importance in global carbon cycling and agricultural production. Many studies have examined the consequences of natural and anthropogenic perturbations on the soil microbiota, but these have mainly focused on the measurement of total biomass or gross microbial processes in crop soils. Molecular techniques based upon the analysis of 16S rRNA allow for assessment of genetic diversity (23, 35), which may provide a more sensitive and less biased indicator of changes in soil communities. Using such methods, we have previously observed both temporal and spatial changes in the diversity and activity of bacterial communities in an upland grassland soil (17). Here we extend this research by investigating the role of soil moisture content in regulating community structure and activity in grassland soil ecosystems.

Soil moisture directly affects the physiological status of bacteria (21). Water availability affects the osmotic status of bacterial cells and can indirectly regulate substrate availability, diffusion of gases, soil pH, and temperature. Further, moisture deficit will stress plants and, as a result, may affect bacterial communities through changes in rhizodeposition and nutrient allocation below ground (29). Ultimately, periods of moisture limitation may affect bacterial communities through starvation, induced osmotic stress, and resource competition, eliciting a strong selective pressure on the structure and functioning of soil bacterial communities.

The involvement of soil moisture in controlling fluxes of important greenhouse gases has been shown for methane (19, 41), nitrous oxide (4, 24), and carbon dioxide (9, 12, 32, 38). While these processes are not entirely the result of microbial activity, it has recently been suggested that the underlying mechanisms may be due to changes in the diversity and activity of bacterial populations (8, 40). In bioremediation studies, degradation rates have been correlated to the culturable activities of specific organisms, which are in turn influenced by moisture availability (7, 20). The role of moisture in regulating the activity of specific culturable populations has also been observed following inoculation of marked bacteria into soil (31). However, such studies on culturable bacteria may not adequately reflect changes occurring in the total community.

Many studies have attempted to analyze microbial responses to drying and rewetting by using chemical determinants of biomass or microbial activities. These studies have generally revealed that drying or drying and rewetting causes a decrease in the total soil biomass (5, 27, 45). Furthermore, rewetting of dried soils is known to cause increased mineralization of carbon (28) and nitrogen (1, 6) coupled with a flush of CO2 efflux (14). The exact roles of microbes in mediating these processes is still largely unresolved, since biomass estimations may be hampered by methodological constraints (13), and so it is difficult to determine whether the effects are biological or physically driven. However, CO2 evolution, as a general measure of gross microbial activity, is known to reflect changes in water potential (36). While these studies have revealed the effect of
moisture on broad-scale microbial properties, no inference has been made to changes in diversity of the total bacterial community which underlie these processes. The use of molecular methodologies currently offers the most potential for assessing the diversity of soil communities, yet only a single article was found applying such approaches in investigating moisture effects on soil bacterial populations (22).

Our present study aimed to monitor the molecular diversity and physiological status of soil bacteria subjected to drying and rewetting in intact soil monoliths collected from the Natural Environment Research Council (NERC) Soil Biodiversity field site located at Sourhope (Scotland, United Kingdom). The experimental regimen was applied to allow examination of the bacterial community response to soil drying and recovery following rewetting. The activity of culturable bacteria was measured by performing plate counts on different media, and the substrate utilization potential was assessed by using BIOLOG GN-2 plates (15, 39). To examine culture-independent effects on the extractable cell biomass, total cell counts were compared by flow cytometric analysis of SYBR II-stained cell preparations. Community structure was assessed by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes and 16S rRNA directly extracted from the soil (18). It was anticipated that the RNA transcript might be a more responsive biomarker in this study, due to the known relationship between RNA abundance and the physiological status of bacterial cells (3, 34).

MATERIALS AND METHODS

Experimental set-up and sampling. Intact grassland monoliths were excised to a depth of approximately 15 cm from the NERC Soil Biodiversity Program field site at Sourhope on 4 July 2001 and transported immediately back to the laboratory. The soil is classified as an organic rich brown forest soil (pH 4.5 to 5.0) and hosts a diverse range of grass species typical of unimproved grazed pastures, with the most dominant being Agrostis and Festuca species. Monoliths were trimmed to a cylindrical core with a 45-cm diameter and planted in close-fitting plastic pots to minimize edge effects. Prior to planting, the pots had been weighed for dry weight and plastic bags, and large roots and plant material were extracted by hand. Subsequently, 0.5-g (wet weight) aliquots of soil were then weighed for dry weight determination and microbiological analyses.

Culturability and total cell count of bacterial community. Soil (0.5 g wet weight) was dispersed in 10 ml of phosphate-buffered saline (PBS) with 1.5 g of sterile 5-mm-diameter glass beads and mixed by vortexing for 1 min. Samples were decimally diluted, and 100-µl aliquots were spread onto full-strength tryptic soy agar (TSBA; Difco), Oxoid, and a 1/10-strength TSBA plate, and pseudomonad-selective agar (PDA; Difco). All media were supplemented with 100 µg of cycloheximide/ml to suppress fungal growth. Plates were incubated for 10 days at 18°C prior to colony counting. The diversity of cultured bacteria was also assayed on the 1/10-strength TSBA plates by extracting all of the colony biomass from plates of the same dilution. Three milliliters of PBS was added to each plate, and the biomass was dispersed in the appropriate buffer. Approximately 1.5 ml of cell suspension was then transferred to 1.5-ml Microfuge tubes and stored at −0°C for nucleic acid extraction.

Total bacterial cells were enumerated according to the methods of Whiteley et al. (46). Briefly, 0.5 g of soil was dispersed in PBS, and the resulting supernatant was loaded onto a 1.3-g/ml Nycodenz density cushion. Following centrifugation, cell preparations were extracted, washed, and then fixed with a 1% final concentration of paraformaldehyde. Cells were stained with 0.3 µl of the nucleic acid stain SYBR Green II (Molecular Probes) for 20 min in the dark. Positively stained bacterial cells were then enumerated with a FACSCalibur sorting flow cytometer (Becton Dickinson Immunocytometry Systems, Oxford, United Kingdom).

Substrate utilization analyses. Nine milliliters of a 0.5% (wt/vol) soil suspension was prepared and washed twice by dilution to 50 ml in sterile PBS, mixing, and centrifugation (Jouan BR4) for 5 min at 4,000 × g. Following the cell washes, pelleted cells were resuspended in 20 ml of sterile PBS, and 100-µl aliquots were dispensed into each of the 96 wells of the BIOLOG-GN plates (Oxoid). Plates were incubated at 18°C and were manually scored at daily intervals to determine the number of substrates utilized per day. For each reading, a well was scored as positive based on visual inspection of color change.

Statistical comparison of counts and substrate utilization. All quantitative data were statistically analyzed to address three hypotheses. (i) Does short-term drying decrease bacterial counts? (ii) Does rewetting of temporarily dried soils reverse the effects of drying? (iii) Are there significant differences between the three treatments at the termination of the imposed regimen? To address the first two questions, the change over time for each monolith was first calculated, followed by comparison of the mean differences in change between treatments by analysis of variance. This form of analysis was chosen to circumvent difficulties in repeated measurement designs where there may be differences between replicate monoliths prior to the start of the experiment. For the count data, the estimate used to address the effects of drying was calculated by subtracting the mean of counts from samples S2 and S3 (before drying) from the mean of samples S5 and S6 (after drying). To test rewetting effects, the means of samples S6 and S7 were subtracted from the means of samples S10 and S11. For the substrate utilization data, only rewetting effects were tested, and the change over time here was taken to be the difference between samples S7 and S11. To assess whether the treatments had an overall effect at the end of the experiment, analysis of variance analysis was performed on data from the final sample date (S11). Here, Fisher’s least-significant difference method was used to ascertain differences between the three treatment means. Prior to statistical analysis, count data were log transformed, whereas the percentages of substrates utilized were arcsine transformed. All analyses were performed within the MINITAB statistical software package (version 13.32; Minitab, Inc., State College, Pa.).

Nucleic acid extraction and amplification. Total nucleic acids were extracted for DGGE analyses by the method of Griffiths et al. (18). Briefly, 0.5 ml of cetrimidimethylammonium bromide extraction buffer and 0.5 ml of phenol-chloroform-isooamyl alcohol (25:25:1 [pH 8.0]) were added to 0.5 g of soil sample or 0.5 ml of cell culture in BIO-101 bead-beating tubes. Following mechanical lysis and subsequent solvent extraction, nucleic acids were precipitated from the extracted aqueous layer with two volumes of 30% polyethylene glycol 6000 (Fluka BioChemika)–1.6 M NaCl. Pelleted nucleic acids were washed in ice-cold 70% (vol/vol) ethanol, air dried, and resuspended in 50 µl of RNase-free Tris-EDTA buffer (pH 7.4). Extracted nucleic acids were then inspected by gel electrophoresis prior to enzymatic separation of DNA and RNA and PCR or reverse transcription-PCR amplification (total soil-extracted nucleic acids only). Following DNase treatment, 16S rRNA was reverse transcribed with the universal 16S primer 519r (Oligo-TTA CCG CCG CTG CTG-3) as described previously (18). RNA and cDNA were then PCR amplified with the GC-clamped forward primer 33HR (S-CGG CCG CCG CCG CCC GGC CCC CCG CCG CCG CCC CCG CCG CCG CCG CCG CCG CCG CCG) and the reverse primer according to the method of Griffiths et al. (18).

DGGE. DGGE was performed by using the De-Code system (Bio-Rad) with a 10% (wt/vol) acrylamide gel with a 30 to 60% (wt/vol) denaturing gradient (urea

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and formamide) running for 6 h at 200 V. All solutions and procedures were standardized before the running of each gel to optimize consistency between gels. Gels were stained with SYBR Gold (Molecular Probes, Inc.) and visualized by UV transillumination. Gel images were analyzed densitometrically with the Phoretix one-dimensional software package (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom), and profiles were compared by using the multivariate statistical package MVSP (Kovach Computing, Anglesey, United Kingdom).

RESULTS

Soil moisture content. The effects of the imposed watering regimen on soil moisture contents, determined by oven drying, are shown in Fig. 1. During the experimental period, dried treatments reached a minimum of approximately 15% moisture per g of soil (fresh weight), whereas continually wetted pots fluctuated around 50%. For dried and rewetted treatments, soil moisture content dropped to a minimum of 18% but subsequently recovered to 44% by the end of the treatment regimen.

Bacterial culturability and total cell count. The effect of the three treatment regimens on the total numbers of bacteria able to form colonies (CFU) on agar plates was determined on two nonselective media and one semiselective medium (Fig. 2). The highest overall counts were observed on 1/10 TSBA, with wetted treatments consistently being over \(10^8\) CFU g (dry weight) of soil \(^{-1}\), whereas continually wetted pots fluctuated around 50%. For dried and rewetted treatments, soil moisture content dropped to a minimum of 18% but subsequently recovered to 44% by the end of the treatment regimen.

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FIG. 2. Responses of culturable bacteria to moisture treatments on three different culture media: 1/10 TSBA, TSBA, and PSA. Sampling points are indicated at the base of the graph. Error bars represent standard errors of the means ($n = 3$). Treatments are indicated by symbols as follows: •, continually wetted; ○, dried; ▼, dried and rewetted. Jul, July; Aug, August; Sep, September; Oct, October; Nov, November.
The rates of substrate utilization also appeared to increase in the rewetted treatments with continued water application. To assess the effect of the moisture treatments on substrate utilization, the percentage of substrates utilized after 4 and 7 days of incubation were plotted and are shown in Fig. 3a and 3b, respectively. These plots reveal that immediately following rewetting (sample S7) wetted samples appear to utilize more substrates regardless of incubation time. Continued rewetting increased the number of substrates utilized between sample dates S7 and S11 compared to continually dried monoliths (P < 0.05). However, full recovery at the final time point (S11) could only be ascertained when plate readings after 7 days of incubation were compared. This may be due to the decreased sensitivity of the assay for detecting differences between samples when longer incubations are used.

**DISCUSSION**

Culture-based methods indicated that microbial physiological response was modulated by moisture content, with total culturability and substrate utilization response being maintained in continually wetted treatments, depressed in dried treatments, and cycled between these extremes with the drying and rewetting regimen. Previous studies on unplanted sieved soils found drying and rewetting to significantly reduce microbial biomass to a greater degree than observed in our study (5, 27). The less-pronounced effect observed here may be due to

**TABLE 1.** Total cell counts for all treatments and sample dates determined by flow cytometric counting of SYBR II-stained cells

<table>
<thead>
<tr>
<th>Date (mo.day.yr)</th>
<th>Sample</th>
<th>Total cell count (10⁶) per g (dry weight) of soil (mean ± SEM) for regimen:</th>
<th>Wetted</th>
<th>Dried</th>
<th>Dried and rewetted</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.23.01</td>
<td>S1</td>
<td>87.1 ± 15.6</td>
<td>85.2 ± 7.1</td>
<td>82.7 ± 5.9</td>
<td></td>
</tr>
<tr>
<td>8.2.01</td>
<td>S2</td>
<td>106.6 ± 24.4</td>
<td>133.8 ± 9.1</td>
<td>92.7 ± 11.2</td>
<td></td>
</tr>
<tr>
<td>8.16.01</td>
<td>S3</td>
<td>89.7 ± 8.5</td>
<td>88.5 ± 11.7</td>
<td>83.8 ± 14.8</td>
<td></td>
</tr>
<tr>
<td>8.23.01</td>
<td>S4</td>
<td>179.2 ± 9.9</td>
<td>123.6 ± 8.9</td>
<td>102.9 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>9.5.01</td>
<td>S5</td>
<td>103.0 ± 4.8</td>
<td>71.6 ± 11.6</td>
<td>58.1 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>9.13.01</td>
<td>S6</td>
<td>166.4 ± 9.3</td>
<td>94.7 ± 7.2</td>
<td>86.3 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>9.21.01</td>
<td>S7</td>
<td>127.6 ± 29.6</td>
<td>127.1 ± 20.0</td>
<td>138.1 ± 13.4</td>
<td></td>
</tr>
<tr>
<td>9.28.01</td>
<td>S8</td>
<td>127.1 ± 14.0</td>
<td>94.2 ± 2.0</td>
<td>91.1 ± 33.5</td>
<td></td>
</tr>
<tr>
<td>10.4.01</td>
<td>S9</td>
<td>268.8 ± 20.7</td>
<td>161.1 ± 11.9</td>
<td>224.1 ± 11.8</td>
<td></td>
</tr>
<tr>
<td>10.11.01</td>
<td>S10</td>
<td>493.1 ± 8.3</td>
<td>137.3 ± 11.9</td>
<td>414.8 ± 69.9</td>
<td></td>
</tr>
<tr>
<td>10.26.01</td>
<td>S11</td>
<td>177.1 ± 13.0</td>
<td>244.9 ± 50.0</td>
<td>165.2 ± 26.5</td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 3.** Recovery in substrate utilization following rewetting. Plots show the percentage of the 95 substrates in BIOLOG plates utilized after 4 and 7 days of incubation of plates at 18°C (a and b, respectively). Sampling points are indicated at the base of the graph. Error bars represent standard errors of the means (n = 3). Treatments are indicated by symbols as follows: ⚫, continually wetted; ○, dried; ▼, dried and rewetted. Sep, September; Oct, October; Nov, November.
FIG. 4. DGGE analyses of total extracted rRNA genes (a), rRNA transcripts (b), and total culturable bacteria (c) for the dried and rewetted treatment only. Sampling points are indicated above each gel, and the three lanes for each time point represent the individual profiles obtained from each replicate pot. Marker lanes comprising amplicons from bacterial isolates are located at either side of each gel.
more-gradual soil drying (44), as would be the case in the environment, and/or the free draining system preventing cell death caused by rapid changes in moisture potential (21). Our data therefore implicate the inherent resistance of natural grassland-soil bacterial communities to environmentally realistic gradual changes in moisture content.

In contrast to the physiological methods, no moisture-related changes were observed following molecular profiling of the culturable diversity or total nucleic acids extracted from soil. The lack of change in diversity profiles is surprising in light of the effect of moisture upon the community physiology discussed above. For the analysis of culturable diversity, moisture perturbation may not have sufficiently impacted the total community to influence the reported high variation in species assembly on agar plates (11). The absence of change in community profiles based upon extracted rRNA genes or the rRNA transcripts may be partly explained by the large genotypic diversity of bacteria present in soil (42) and the fact that only the dominant templates are detected in DGGE profiles (16). Assuming the total diversity to be log normally distributed (10), it is conceivable that variation within diverse populations of low numerical abundance may not be detected by primers targeting the whole community. This may have been the case for the rRNA gene-based analysis, since no consistent changes in total cell counts were observed, indicating negligible cell growth or death in response to the moisture treatments (as was found in reference 47).

The similarity of rRNA transcript-based community profiles cannot be explained by the lack of variation in total cell counts, since rRNA transcript concentrations should vary independently of biomass and in relation to cellular physiological state (34) and growth stage (3). However, aside from pure culture studies, there is little information on the variation of rRNA content in bacterial cells present in natural environments such as soils. Therefore, it may be possible that small increases in the rRNA content of active cells are masked by more abundant rRNA from quiescent cells. Furthermore, recent research on marine isolates has revealed that RNA levels may not always relate to growth rate, especially during non-steady-state growth (26). Additionally, RNA/DNA ratios have been shown to not always relate to microbial activity in heterogenous environmental samples such as sediment (25). These findings therefore raise questions on the relative advantages of using rRNA transcript analysis over rRNA gene analysis as a more-responsive biomarker to study soil bacterial communities.

Despite these methodological constraints, our data enforce the belief that soil bacteria may be preadapted to resist moisture variations by regulation of cellular activity (31). The ability of soil bacteria to withstand such perturbations may relate to the so-called starvation state (2). This state is thought to represent a survival strategy for bacterial persistence in harsh, low-nutrient environments and may be mediated by starvation gene expression, cell shrinkage, or sporulation. Specific responses to osmotic stress include sensing mechanisms coupled with the uptake or synthesis of compatible solutes to reestablish cell turgor pressure (33, 48). If our diversity assessments are representative, then it may be that the predominant soil community responds to moisture availability in the same manner, i.e., all the dominant bacterial species are equally capable of surviving drying and no competition occurs after rewetting.

While this does not agree with the concepts of copiotrophy and oligotrophy (43), it does reinforce the idea that soil bacteria are able to cope with both high and low nutrient conditions equally well (34).

To conclude, our data implicate the marked resistance of soil bacteria to water stress based upon physiological criteria (culturability and substrate utilization analyses). However, we did not observe significant changes within the total community from a molecular standpoint when directing the analyses at the population level, with both rRNA gene- and rRNA transcript-based 16S profiling. Under the experimental regimen employed (controlled slow perturbation), these latter approaches may lead to an unrepresentative picture of what is occurring in the natural environment. This is likely to be a facet of the large diversity present within these environments, diversity changes occurring within small fractions of the community, and potential physiological adaptations which have yet to be resolved. Indeed, we have recently shown that differences could be detected in the community structure of active cells by prior isolation by using cell sorting of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC)-stained cells (46). It may therefore be likely that changes occurring in these operationally defined active communities may be more relevant in terms of ecosystem functioning. Further, technologies which directly link the activity of microbes with ecosystem processes, such as the labeling of plant material with $^{13}$CO$_2$ (37) and phylogenetic analysis of isotopically enriched rRNA (36), may be a more appropriate way of subdividing the community for more-resolved analyses of the response to perturbation.

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