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Assimilation of Cellulose-Derived Carbon by Microeukaryotes in Oxic and Anoxic Slurries of an Aerated Soil

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Soil microeukaryotes may trophically benefit from plant biopolymers. However, carbon transfer from cellulose into soil microeukaryotes has not been demonstrated so far. Microeukaryotes assimilating cellulose-derived carbon in oxic and anoxic soil slurries were therefore examined by rRNA-based stable-isotope probing. Bacteriovorous flagellates and ciliates and, likely, mixotrophic algae and saprotrophic fungi incorporated carbon from supplemental [U-^{13}C]cellulose under oxic conditions. A previous study using the same soil suggested that cellulolytic *Bacteria* assimilated ^{13}C of supplemental cellulose. Thus, it can be assumed that ciliates, cercozoa, and chrysophytes assimilated carbon by grazing upon and utilizing metabolic products of *Bacteria* that hydrolyzed cellulose in the soil slurries.

Cellulose is the most abundant organic compound of plant litter (40% of dry weight), is mineralized primarily by soil microorganisms, and contributes significantly to carbon dioxide release of terrestrial ecosystems (1, 2). Bacteria and fungi are generally assumed to be the main consumers of cellulose in terrestrial ecosystems. *Ascomycota*, *Basidiomycota*, *Zygomycota*, and *Chytridiomycota* are important fungal groups for cellulose degradation, since they comprise saprotrophic species (3, 4). Several protistan functional groups, i.e., bacterivorous, fungivorous, and cytotrophic protists, might also benefit from cellulose-derived carbon mainly by consuming products and microorganisms that have utilized cellulose. Protists have a pivotal role in the microbial loop, which facilitates the transfer of carbon from detrital organic material (e.g., cellulose) to higher eukaryotes that feed on soil microorganisms (5, 6). Many cellulolytic *Bacteria* are known (2), and recently, the utilization of hydrolysis and fermentations products of cellulose by noncellulolytic soil prokaryotes was resolved using nucleic acid stable-isotope probing (NA-SIP) (7–9). Such studies demonstrated that also noncellulolytic prokaryotes contribute to and benefit from carbon flow from cellulose (7–9).

Prokaryotic taxa of an agricultural soil involved in cellulose degradation under oxic and anoxic conditions were resolved in a previous study using rRNA SIP (9). The objectives of the current study were to test in the same experimental setup whether microeukaryotes incorporate carbon from cellulose and to identify the actively involved eukaryotes in order to shed light on their putative roles in the cellulose-dependent food web.

Experimental setup. Slurries with samples from the upper 20 cm of an agricultural soil (dystric cambisol) were prepared as previously described (9) and were supplemented with 0.2 g of [U-^{13}C]cellulose and one with [U-^{12}C]cellulose (9). Since substantial labeling of prokaryotes occurred after 35 days (9), samples for analyzing microeukaryotes (i.e., eukaryotes of a higher trophic level) were taken after 35 and 70 days to ensure enough ^{13}C incorporation in rRNA. Tightly closed gas flasks contained 80 ml of slurry in a total volume of 500 ml for anoxic and 1,000 ml for oxic treatments. All flasks were incubated in the dark in an end-over-end shaker (9). Per treatment (i.e., in the absence and presence of oxygen), two replicated flasks were tested. SIP analyses were done from combined RNA extracts at a given time point. In one case (Fig. 1 and 2), SIP gradients from each experimental replicate were conducted separately to evaluate experimental variations. The headspaces were regularly exchanged with sterile air (every 2 days) or dinitrogen (every 4 days). Under oxic incubations, carbon dioxide was the sole carbonaceous product and molecular hydrogen was not detected, suggesting that these treatments were truly oxic (9). Slurries were incubated for 70 days at 15°C in the dark. RNA was extracted from 0.6 g of soil slurry at the start of the experiment and after 35 and 70 days (10). ^{13}C-labeled RNA was separated from nonlabeled RNA by isopycnic centrifugation with a cesium trifluoroacetate-based gradient medium according to previous protocols (9, 11). Fractions with buoyant densities between 1.767 and 1.776 g ml^{-1} were regarded as containing unlabeled RNA, and fractions with buoyant densities between 1.813 and 1.821 g ml^{-1} were regarded as containing ^{13}C-labeled RNA. RNA was precipitated, quantified from these fractions (12), and reverse transcribed (9). To minimize analytical efforts, 18S rRNA-based analyses were conducted from pooled RNAs from two light and two heavy fractions per gradient (9).

18S rRNA libraries. Two 18S rRNA cDNA libraries using the eukaryote-specific primers 20fNS1 and 516r were analyzed to assess shifts in the microeukaryotic community between the start and the end of the experiment (13). RNA was extracted from fractions that represented labeled RNA ("heavy" fractions) and fractions that represented unlabeled RNA ("light" fractions). 18S rRNA libraries from pooled light and heavy fractions at the start were dominated by fungi (*Ascomycota*), chlorophyta (*Chlamydomonadales* and *Scenedesmaceae*), and metazoan phylotypes (*Annelida*), while most protist phylotypes were affiliated with *Alveolata*, *Amoebozoa*, *Cercozoa*, stramenopiles, and yet-uncultured microeukaryotic taxa (Table 1 and see Table S1 in the supplemen-

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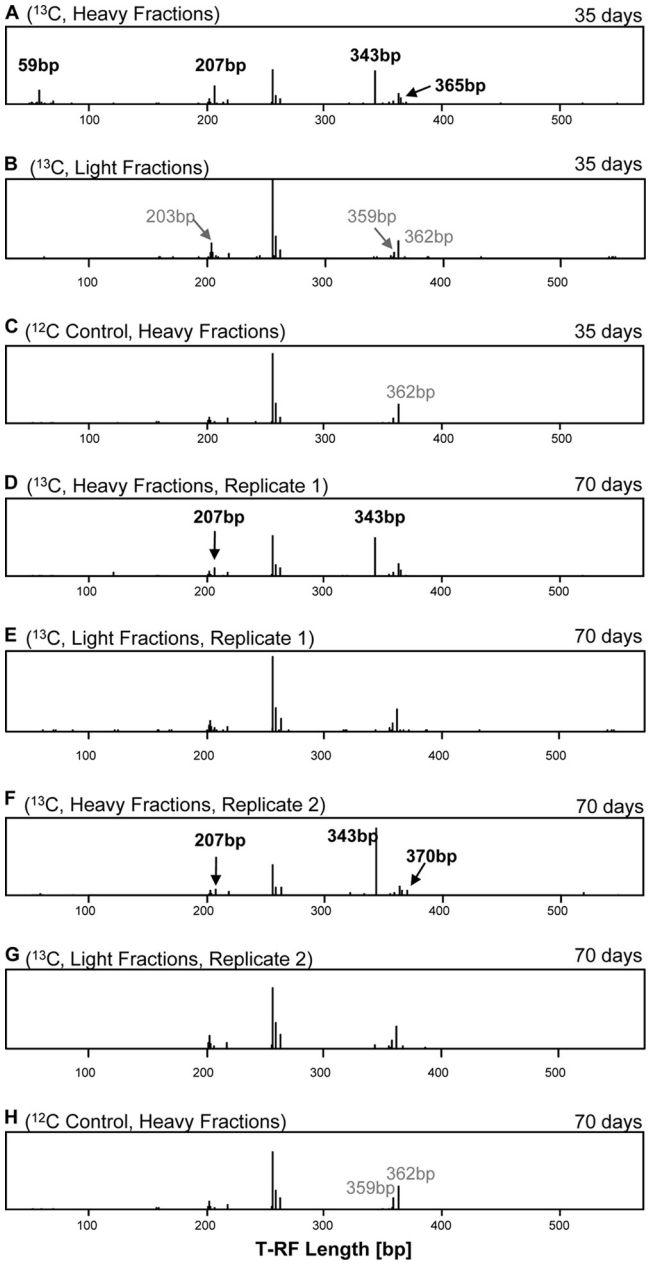

FIG 1 T-RFLP patterns obtained with the eukaryote-targeting primer set from oxic treatments. Anoxic treatments did not reveal any labeling. Patterns are retrieved from heavy fractions of treatments that were supplemented with [U-^{13}C]- and [^{12}C]cellulose. Samples were taken after 35 days (A, B, and C) and 70 days (D, E, F, G, H) of incubation. Black numbers, T-RFs that represented ^{13}C-labeled phylotypes in the heavy fractions. Identification of labeled phylotypes was based on comparison with the T-RFLP patterns of the heavy fractions of the ^{12}C treatment at the same time point and on comparison with the T-RFLP patterns of the light fractions of the same gradient. Gray numbers, nonlabeled T-RFs in the light fractions of the same gradient or in heavy fractions of a ^{12}C control that have a size similar to but different from those of T-RFs that represented labeled phylotypes. The T-RFLP patterns of panels D, E, F, and G are each based on a single gradient from combined RNAs of two experimental replicates.

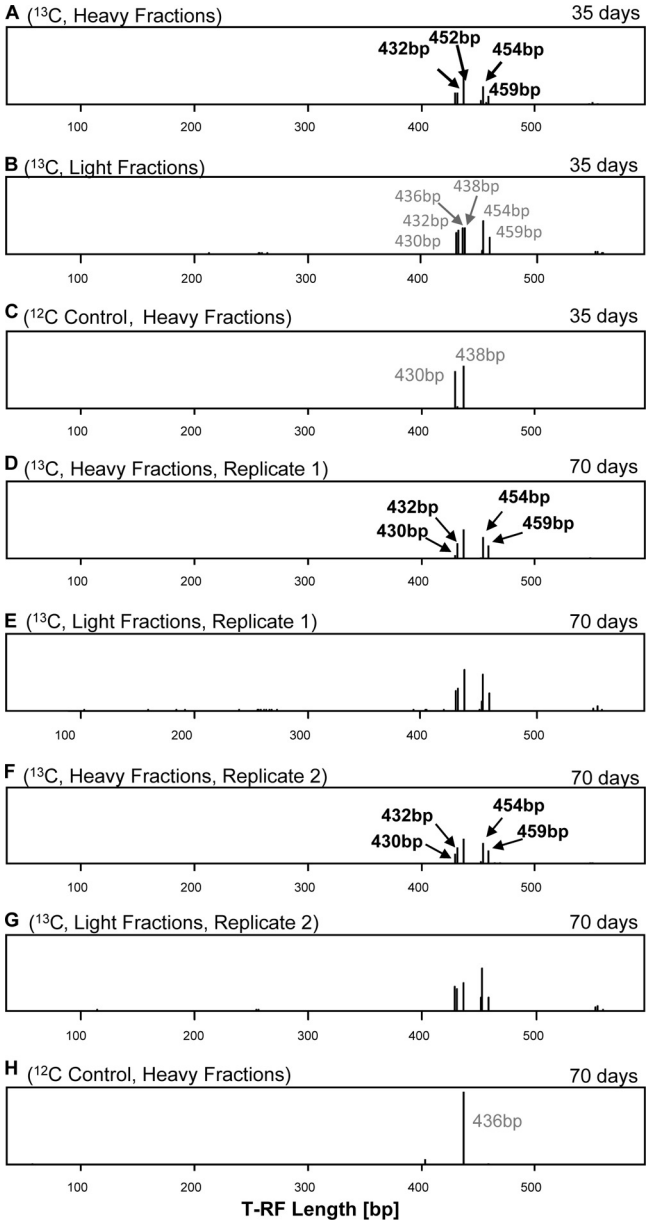

FIG 2 T-RFLP patterns obtained with the *Chrysophyceae*-targeting primer set from oxic treatments. Anoxic treatments did not reveal any labeling. Patterns are retrieved from heavy fractions of treatments that were supplemented with [U-^{13}C]- and [^{12}C]cellulose. Samples were taken after 35 days (A, B, and C) and 70 days (D, E, F, G, H) of incubation. Black numbers, T-RFs that represented ^{13}C-labeled phylotypes in the heavy fractions. Identification of labeled phylotypes was according to comparison with the T-RFLP patterns of the gradient of the heavy fractions of the ^{12}C treatment at the same oxygen availability and time point and by comparison with the light fractions of the same gradient. Gray numbers, nonlabeled T-RFs in the light fractions of the same gradient or in heavy fractions of a ^{12}C control that have a size similar to but different from those T-RFs that represented labeled phylotypes. The T-RFLP patterns of panels D, E, F, and G are each based on a single gradient from combined RNAs of two experimental replicates.

tal material). Since gene libraries were not set up from total RNA, there might have been a bias toward labeled phylotypes. Nonetheless, if substantial changes (i.e., changes of several percent) in relative abundances occurred, they were regarded as true.

Oxic conditions resulted in an apparent increase of *Alveolata* (i.e., peritrich ciliates) and amoebozoan phylotypes affiliating with the naked lobose amoeba group *Euamoebida*. Phylotypes affiliating with the parasitic apicomplexan family *Eimeriidae*, the

TABLE 1 Phylogenetic affiliation and relative abundances of 18S rRNA sequences in clone libraries derived from pooled light and heavy RNAs

Taxon[b]	Relative abundance (%) under indicated condition[a] at:			
	Start		70 days	
	Anoxic (n = 252)	Oxic (n = 254)	Anoxic (n = 120)	Oxic (n = 125)
Alveolata (*Ciliophora*)	0.8	0.0	2.5	44.8
Alveolata (other)	1.2	3.9	5.8	0.8
Amoebozoa	7.1	0.0	6.7	3.2
Apuzoa	0.0	0.4	0.0	0.0
Ascomycota	30.6	24.0	28.3	21.6
Basidiomycota	1.2	3.5	4.2	0.0
Glomeromycota	0.4	0.0	0.0	0.0
Fungi *incertae sedis*	0.8	4.7	0.0	2.4
Fungi (*Chytridiomycota*)	0.0	0.4	0.0	0.0
Zygomycota	1.2	0.0	0.8	0.8
Cercozoa	3.2	5.1	6.7	2.4
Chlorophyta	18.3	15.0	35.0	22.4
Heterolobosea	0.4	0.4	0.0	0.0
Metazoa	30.6	34.3	0.0	0.0
Stramenopiles	2.0	5.5	6.7	0.0
Streptophyta	2.4	1.6	1.7	1.6
Unknown eukaryota	0.0	1.2	1.7	0.0

[a] Values are relative abundances of phylogenetic groups in the respective gene libraries. Incubation conditions, i.e., without (anoxic) and with (oxic) oxygen in the headspace, are noted. Numbers in parentheses are total numbers of analyzed clone insert sequences.

[b] Clone insert sequences had a sequence similarity to known phylotypes of >97% based on BLAST searches in the nucleotide databases of GenBank.

Cercozoa, and the *Basidiomycota* were either reduced in relative abundance or disappeared after 70 days. Unlike in a previous study (14), relative abundances of several stramenopile algae and fungus-like stramenopile plant pathogens (i.e., *Pythium* and *Aphanomyces*) (15, 16) that were present at the start of the experiment decreased in response to cellulose supplementation in the oxic slurries. Members of the chlorophytes changed over the incubation period, and after 70 days, *Scenedesmaceae* were predominant. Anoxic conditions caused shifts within the phylotypes of amoebozoa, alveolata, and chlorophyta (e.g., an enrichment of phylotypes affiliating with the green-algal class *Trebouxiophyceae*).

Fungal phylotypes at the start of incubations belonged mainly to mitosporic *Ascomycota* (i.e., genera *Hyphozyma* and *Tetracladium*) and to several groups within the subphylum *Pezizomycotina*, such as the *Sordariomycetes*. Mitosporic *Ascomycota* and *Pezizomycotina* include parasitic fungi and strains that are decomposers and grow on soil, leaf litter, and wood (see Table S2 in the supplemental material) (17). Cellulases have, for instance, been proved to exist in *Lecythophora* (18). Abundances of phylotypes of mitosporic *Ascomycota* and several saprophytic fungi, such as those of the genus *Acremonium*, increased under oxic conditions, while several genera within the *Sordariomycetes* either decreased in abundance or disappeared after 70 days. The predominant fungi under anoxic conditions were related to the *Dothideomycetes*, which include many plant pathogens (19), or the *Microascales*, which are known as plant and insect pathogens or coprophilous (20). The *Chaetomiaceae*, which are capable of lignocellulose degradation (21), were no longer detected after 70 days.

Identification of ^{13}C-labeled 18S rRNA phylotypes by T-RFLP. Labeled microeukaryotes were examined by 18S rRNA terminal restriction fragment length polymorphism (T-RFLP) analysis. To reduce the likelihood that ^{12}C RNA would be identified as labeled, T-RF patterns of heavy fractions of [U-^{12}C]cellulose-supplemented slurries were compared to those of [U-^{13}C]cellulose (22, 23). T-RFs that were enriched in heavy fractions of ^{13}C treatments but not in ^{12}C treatments were regarded as ^{13}C labeled, even if those T-RFs occurred in the light fractions of ^{13}C treatments. The eukaryotic primer set, as well as primers targeting two ubiquitously distributed bacterivorous flagellate groups, i.e., *Chrysophyceae* and *Kinetoplastidae*, and the ciliates (24, 25) were applied. T-RFLP analysis was conducted as described in a previous study (26) using the restriction enzymes MspI, RsaI, MseI, and CfrI and RsaI for 18S rRNA amplicons of total eukaryotes, ciliates, *Kinetoplastidae*, and *Chrysophyceae*, respectively.

Labeled T-RFs were identified only in oxic incubations using the general eukaryote-targeting (i.e., T-RFs of 59, 207, 343, 365, and 370 bp) and the *Chrysophyceae*-targeting (i.e., T-RFs of 430, 432, 452, 454, and 459 bp) primers (Fig. 1 and 2; see also Fig. S1 and Tables S3 and S4 in the supplemental material). An affiliation of several peaks was possible by comparing the restriction patterns with the T-RFs of cloned sequences originating from the heavy fractions (Table S4). Based on this assignment of T-RFs to phylotypes, the ciliate *Opisthonecta minima* (343 bp), the cercozoan *Proleptomonas faecicola* (370 bp), several chrysophytes related to *Chrysophyceae* sp. strain CCCM41 (430 bp), *Mallomonas peroneides* (432 bp), and a *Leukarachnion* strain (454 bp; i.e., an amoeboid sister group to the *Chrysophyceae*) likely assimilated carbon from supplemental [U-^{13}C]cellulose. A dominant peak at 257 bp belonged to the cellulolytic ascomycete fungus *Lecythophora*; this peak was present in both the ^{12}C- and ^{13}C-labeled fractions at 35 and 70 days, suggesting that members of this group were active, but carbon assimilation from cellulose cannot unambiguously be proven. Overall, the presence of several protistan sequences in the clone libraries from 70 days and the shift in heavy T-RFLP patterns (Tables 1, S1, and S3) suggested that some flagellates, ciliates, and amoebae incorporated cellulose-derived carbon by bacterivory on cellulose-degrading bacteria, confirming that protists contribute to the biopolymer-degrading food webs in soil by top-down control of the bacterial community. Among the flagellates, several sequences affiliating with cercozoa were detected in the 18S rRNA libraries from both oxic and anoxic incubations. The presence of cercozoa under oxygen-limited conditions has recently been shown also in flooded rice soils, indicating that members of the cercozoa are capable of coping with limited oxygen availability (27). Moreover, soil protists include several additional trophic groups that might be directly or indirectly involved in organic matter degradation and, thus, explain the observed assimilation of carbon of supplemental cellulose (28, 29). For instance, the occurrence of phylotypes closely related to the nonphagotrophic cercozoan *Proleptomonas faecicola* (Table S3) (30) and the occurrence of algal phylotypes (i.e., stramenopiles and *Chlorophyceae*) in 18S rRNA libraries of oxic and anoxic slurries suggested that mixotrophic protists contributed to the cellulose-derived carbon flow. The importance of heterotrophy within mixotrophic algal species can vary between species of the same genus and is not related to its taxonomic affiliation (31). Its impact on soil food webs and the distinction between direct degradation of cellulose, degradation

of hydrolysis products, or grazing-mediated incorporation of ^{13}C warrants further studies.

Metabolically active phylotypes belonging to the *Ascomycota* were detected, but unlike in another study (32), ^{13}C labeling was not observed, indicating that the detectable *Ascomycota* were not primarily involved in the hydrolysis of cellulose. Nonetheless, cellulolytic *Ascomycota* were detected in heavy-gradient fractions. Intermediate fractions of SIP gradients were not evaluated. Thus, the possibility that *Ascomycota* assimilated smaller amounts of the ^{13}C cannot be excluded. *Basidiomycota* did not respond to experimental conditions. The experimental setup included shaking, which most likely restricted the growth of hypha-forming members of the *Basidiomycetes* (33), and thus, experimental conditions may have selected for microorganisms that were capable of adaptation to the slurry conditions. Whether the detected microeukaryotes are similarly important in undisturbed and dry soil warrants further investigations. Thus, there is a need to improve the analytic accessibility of metabolically active microorganisms in largely intact soil samples.

Conclusions. Previous studies identified in the same experiment various bacterial taxa that assimilated cellulose-derived carbon under anoxic (e.g., *Kineosporaceae*, *Streptomycetaceae*, *Clostridiaceae*) and under oxic (e.g., *Bacteroidetes*) conditions and demonstrated differential responses to oxygen availability (23), while members of the *Archaea* were not labeled (9). Results of both the previous and the current study indicate that *Bacteria* hydrolyzed primarily cellulose and that ciliates, cercozoa, and chrysophytes assimilated carbon by grazing or utilizing products of cellulolytic bacterial metabolisms in the experiment. The lack of evidence for ^{13}C labeling under anoxic conditions suggests that detectable microeukaryotes played a minor role in carbon flux under oxygen-limited conditions. SIP experiments using ^{13}C-labeled-cellulose-derived hydrolysis and degradation products (9), prey bacteria (34), or litter are promising approaches to reveal further trophic aspects of cellulose degradation with regard to soil microeukaryotes.

Nucleotide sequence accession numbers. Sequences of clone inserts (RNA extracted from heavy and light fractions) were deposited in the GenBank repository under the accession numbers KF356737 to KF357523.

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