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Microbial diversity in alpine tundra wet meadow soil: novel Chloroflexi from a cold, water-saturated environment

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Summary

Cold, water-saturated soils play important biogeochemical roles, yet almost nothing is known about the identity and habitat of microbes active under such conditions. We investigated the year-round microenvironment of an alpine tundra wet meadow soil in the Colorado Rocky Mountains, focusing on the biogeochemistry and microbial diversity of spring snowmelt – a dynamic time for alpine ecosystems. *In situ* measurements revealed spring and autumn periods of long-term temperature stability near 0°C, and that deeper soil (30 cm) was more stable than surface soil, with more moderate summers and winters, and longer isothermal phases. The soil was saturated and water availability was limited by freezing rather than drying. Analyses of bioavailable redox species showed a shift from Mn reduction to net Fe reduction at 2–3 cm depth, elevated SO_4^{2-} and decreased soluble Zn at spring snowmelt. Terminal restriction fragment length polymorphism profiles detected a correlated shift in bacterial community composition at the surface to subsurface transition. Bacterial and archaeal small-subunit rRNA genes were amplified from saturated spring soil DNA pooled along a depth profile. The most remarkable feature of these subsurface-biased libraries was the high relative abundance of novel, uncultivated Chloroflexi-related sequences comprising the third largest bacterial division sampled, and representing seven new Chloroflexi subdivisions, thereby dramatically expanding the known diversity of this bacterial division. We suggest that these novel Chloroflexi are active at near –0°C temperatures, under likely anoxic conditions, and utilize geochemical inputs such as sulfide from upslope weathering.

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Introduction

Permafrost and seasonally frozen ground comprise 24% and 60%, respectively, of the Northern Hemisphere's land surface (Zhang *et al*., 1999). These perennially cold, often snow-covered soils store a quarter to a half of all soil carbon (Post *et al*., 1982; Schlesinger, 1997) and interact with substantial quantities of water destined for human use (Bandyopadhyay *et al*., 1997; Williams *et al*., 2002). Recently, the assumption that biogeochemical cycling ceases at low temperature has been challenged by studies revealing significant microbial activity in frozen or snow-covered soil. Carbon dioxide production (microbial respiration), N_2O and volatile organic hydrocarbon (VOC) efflux, CH_4 uptake, N cycling and peak annual microbial biomass occur in sub–0 C or subnivean soils (Sommerfeld *et al*., 1993; Clein and Schimel, 1995; Brooks *et al*., 1996; 1997; 1998; Lipson *et al*., 1999; Skidmore *et al*., 2000; Schadt *et al*., 2003; Swanson *et al*., 2005), and microbial metabolism proceeds at temperatures as low as –20 C in permafrost (Rivkina *et al*., 2000). Microbial growth under snow is also a biogeochemical sink for N and the release of N from microbial turnover at snowmelt is an important contributor to high primary productivity during the short alpine tundra growing season (Jaeger *et al*., 1999; Lipson *et al*., 1999). Furthermore, under-snow microbial communities are structurally and functionally distinct from summer communities (Lipson *et al*., 2002), and phylogenetically novel, uncultivated microbes appear under winter snowpack and at spring snowmelt (Schadt *et al*., 2003; Lipson and Schmidt, 2004). However, these studies were focused primarily on well-drained, drier alpine tundra soils, while the microbes active in cold, snow-covered or frozen soils remain largely unknown. Particularly absent are studies of the cold, anoxic conditions likely to arise in poorly drained soils and the diversity of associated microbial communities. These studies are crucial because cold saturated soils, including wet or moist tundra, bogs, wetlands and boreal forests store the majority of soil carbon (Oechel, 1989; Shaver *et al*., 1992) and because cold, saturated conditions become more widespread during seasonal snowmelt and as permafrost thawing proceeds. Indeed, arctic and alpine areas are predicted to be critically sensitive to climate change (Shaver *et al*., 1992; Williams *et al*., 2002; Camill, 2005)

and feedbacks are not yet well understood (Giardina and Ryan, 2000; Oechel *et al*., 2000; Campbell *et al*., 2005; Knorr *et al*., 2005).

Here, we investigated the habitat and diversity of microbes from an alpine tundra wet meadow soil at spring snowmelt on Niwot Ridge in the Colorado Front Range (Fig. 1). The Niwot Ridge climate is characterized by long, cold winters and short (1–3 month), cool plant growing seasons. The mean annual air temperature is –3.0 C, and the majority of precipitation falls outside the growing season as snow (Greenland, 1989). Topography directs snow accumulation and meltwater drainage on Niwot Ridge and the resulting snowcover and soil moisture gradients fundamentally structure tundra communities. Wet meadows form in topographic depressions where water flowing downslope from melting snowbanks accumulates (Walker *et al*., 1993). Wet meadow soil development is predominantly governed by aquic and depositional processes (Burns, 1980) and the associated plant community is characterized by bog-like vegetation (May and Webber, 1982). Compared with drier alpine tundra communities, wet meadows have the highest soil moisture, plant productivity, soil organic matter, microbial biomass, microbial activity (Fisk and Schmidt, 1995; Fisk *et al*., 1998), methane efflux (West *et al*., 1999) and free iron content (Burns, 1980). Alpine wet meadows are relatively analogous to arctic wet meadows (Bliss, 1956), although differences include lower arctic plant productivity (Shaver and Chapin, 1991) and higher water movement through alpine soils due to topography, an influence on nutrient supply (Giblin *et al*., 1991). Despite the widespread distribution of soils that experience seasonally cold water-saturated conditions, and numerous previous studies of plant community and biogeochemical aspects of alpine tundra wet meadows, surprisingly little is known about the soil microclimate, redox biogeochemistry, or microbial community of these soils. The purpose of this study was to examine the wet meadow soil chemical and physical microenvironment with emphasis on depth and seasonal trends, and to relate these conditions to microbial community diversity and the presence of novel microbial lineages.

Results

Soil microenvironment

Hourly, year-round datalogger measurements indicate that wet meadow soil temperature and water availability fluctuate dramatically across season, and that responses to climate vary by soil depth. In general, the environment was cold with an annual mean soil temperature of 0.3 C. Subsurface soils (30 cm) were most temperature-stable throughout the year, remaining warmest in winter, coldest in summer, and having the longest 0 C isothermal periods (Fig. 2A). Soil water availability was controlled by thawing and freezing in the spring and autumn, respectively, with saturated conditions in summer and frozen conditions in winter prevailing (Fig. 2B). Winter snowpack was most dense in spring and this period was preceded by episodic snow accumulation and removal via wind or melting in autumn and winter (Fig. 2C).

Four seasons were evident in the wet meadow microenvironment. During the warmest months, soil was thawed and surface temperatures fluctuated diurnally reaching maxima of 8–10 C midsummer. Soil at 30 cm warmed steadily throughout the summer, reaching a high of 5 C, with little response to daily air temperature fluctuations (Fig. 2A). Soil was saturated in early sum-

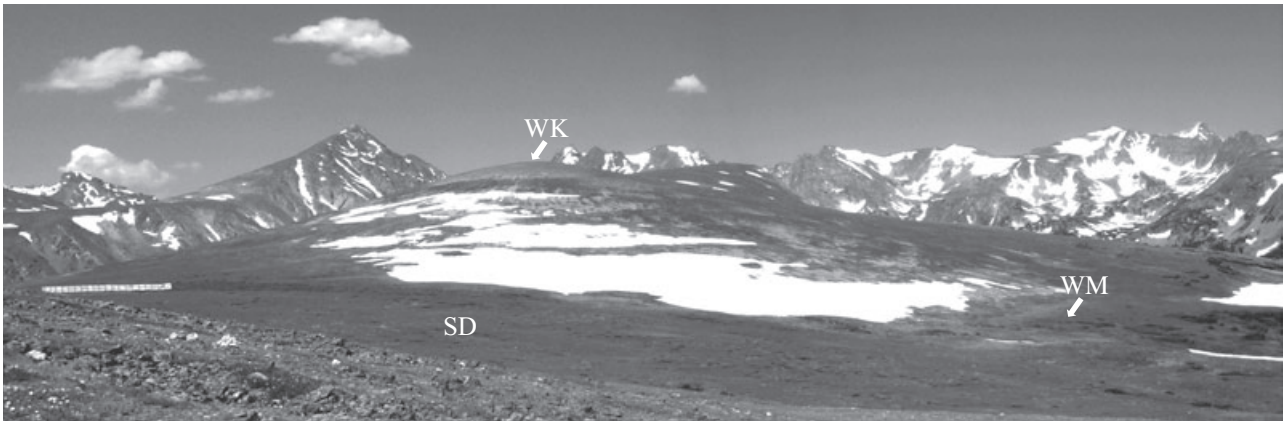

Fig. 1. Location of the sampling site, an alpine tundra wet meadow (WM) on Niwot Ridge in the Colorado Rocky Mountain Front Range. North is to the viewer's right. Photograph was taken on 8 July 2005. The WM plant community occurs where meltwater from the west knoll's (WK) leeward snowbed drains into the saddle area (SD) and collects in a topographic depression.

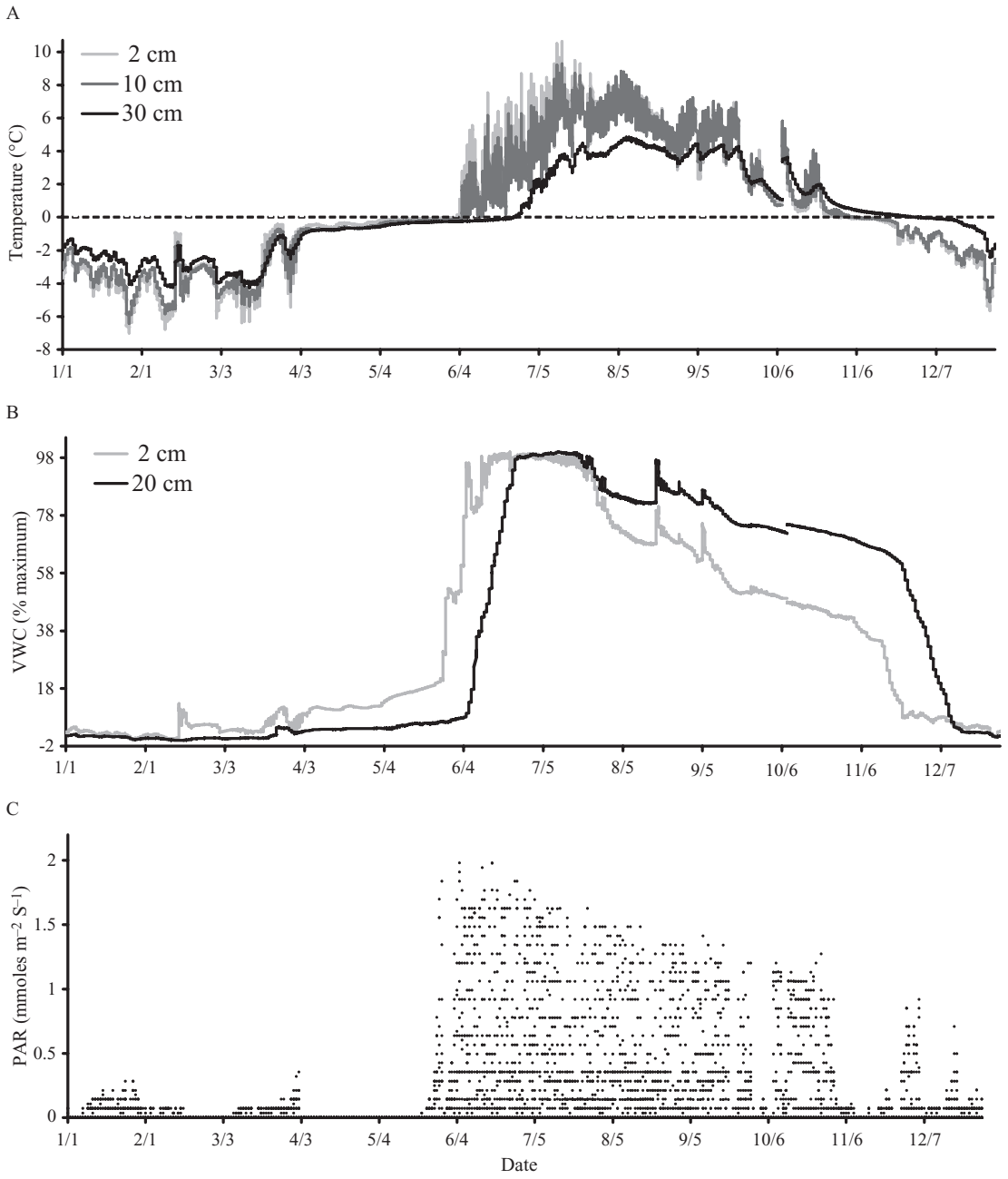

Fig. 2. Wet meadow soil microclimate data for 1 year of hourly datalogger measurements beginning 8 October 2003.
A. Temperature at the soil surface (2 cm depth; light grey line), 10 cm depth (dark grey line) and 30 cm depth (black line). Dashed line is 0 C.
B. Volumetric water content (VWC) at the soil surface (2 cm depth; grey line) and 20 cm depth (black line). Saturated and frozen soils have 100% and 0% VWC respectively.
C. Photosynthetically active radiation (PAR) received at the soil surface. PAR indicates the presence or absence of snowcover at the site.

mer and dried slowly thereafter. In deeper soil, melt occurred later and subsequent drying was less severe (Fig. 2B). The autumn season commenced with episodic snowfall (Fig. 2C), isothermal soil (Fig. 2A), and high initial water availability which dropped precipitously as soils froze (Fig. 2B). Note that while surface soil took an early plunge below 0 C in autumn, soil at 30 cm remained isothermal for nearly 2 months and water remained available (Fig. 2A and B). During the coldest winter months, soil temperatures dipped well below zero (Fig. 2A) and soil water availability was limited by freezing (Fig. 2B). Measurable PAR indicates a shallow snowpack (Fig. 2C) and February snow depth at the site measured 0.5 m. Winter snowpack was not sufficiently insulating to decou-

ple soil and air temperatures, although deeper soil temperatures were warmer and more stable. Late-season snowpack accumulation (Fig. 2C) and the onset of spring melt resulted in isothermal soil temperatures, which persisted for 2 months in surface soil and nearly 3 months at 30 cm depth (Fig. 2A). Soil water availability increased moderately during this period until full melt occurred, earlier in the soil surface and later at 30 cm depth (Fig. 2B).

Soil characteristics along depth and seasonal gradients

Soil cores collected in spring (6 June), autumn (21 October) and winter (13 February) were subjected to laboratory analysis for water content, organic matter and various soluble, bioavailable elements along depth profiles. Soil water content correlated positively with soil organic matter, and this correlation was strongest in spring when saturated conditions prevailed (Fig. 3A). In general, moisture and organic matter were highest in the uppermost layers and decreased with soil depth. In spring, measurable Fe(II) was low in the saturated surface and increased with depth while soluble Mn(II) showed the opposite pattern. The clines of these two redox active species crossed 2–3 cm below the soil surface (Fig. 3B). This pattern was evident but to a weaker degree in the autumn cores (data not shown). Soil SO_4^{2-} levels were unusually high in spring (Fig. 3C) while soluble Zn was markedly decreased (Fig. 3D). Spring sulfate levels in meltwater collected from upslope snowpack and overland flow in this area were generally 1 p.p.m. or lower. Only one water sample collected nearby exhibited high sulfate levels (30 p.p.m.). This sample was from a pool ostensibly receiving water via upslope percolation from melting soil or permafrost (no upslope snowbed). Other elements analysed but not showing significant shifts across season or depth were K^+ (3205 p.p.m.), Mg^{2+} (3120 p.p.m.), Cu (60 p.p.m.) and Al (22 424 p.p.m.).

Bacterial small-subunit (SSU) rDNA gene library

In our preliminary survey of bacterial diversity, 138 sequences representing 14 bacterial divisions were obtained (Fig. 4). Acidobacteria were most abundant (26%), followed by the Proteobacteria (24%) which were dominated by the beta and alpha subdivisions. The most striking feature of this library was the abundance and novelty of sequences related to the division Chloroflexi. Representing the third largest group sampled, 13% of the sequences were novel Chloroflexi (Fig. 4). Intradivisional

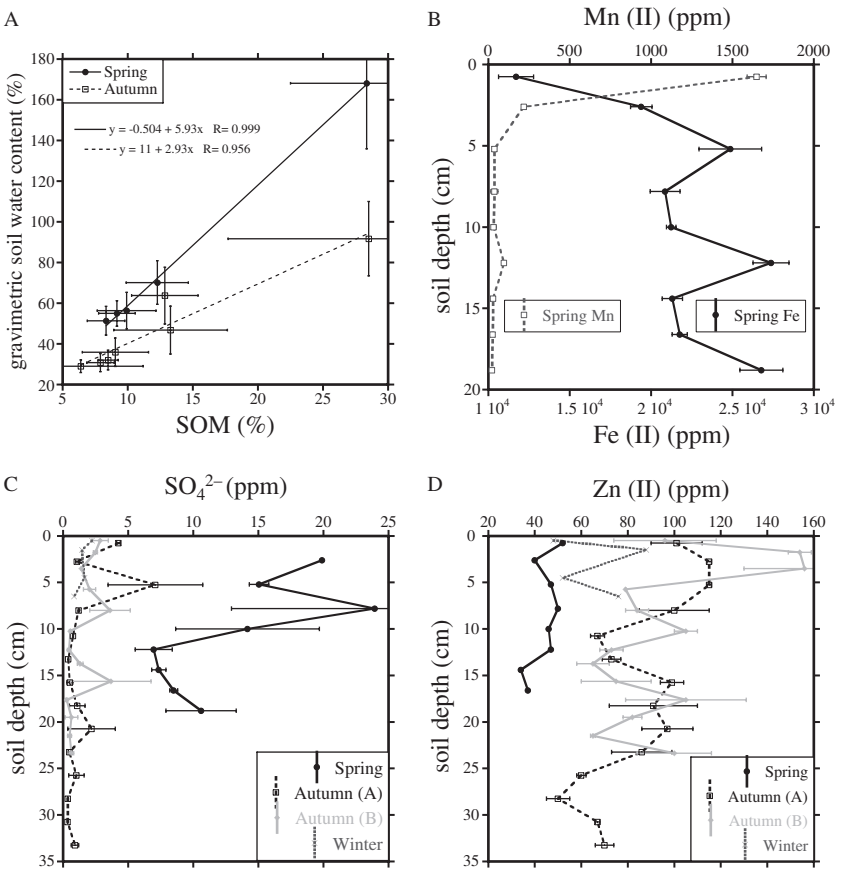

Fig. 3. Soil characteristics of alpine tundra wet meadow cores sampled in spring (6 June), autumn (21 October) and winter (13 February). Panels are (A) linear correlations for organic matter versus soil moisture for spring and autumn cores, (B) Fe(II) and Mn(II) trends with increasing soil depth for the spring core, and (C) sulfate and (D) Zn(II) across depth for each core. Units of p.p.m. are equivalent to ug g^{-1} dry weight soil. Error bars represent variability in unhomogenized soil subsamples.

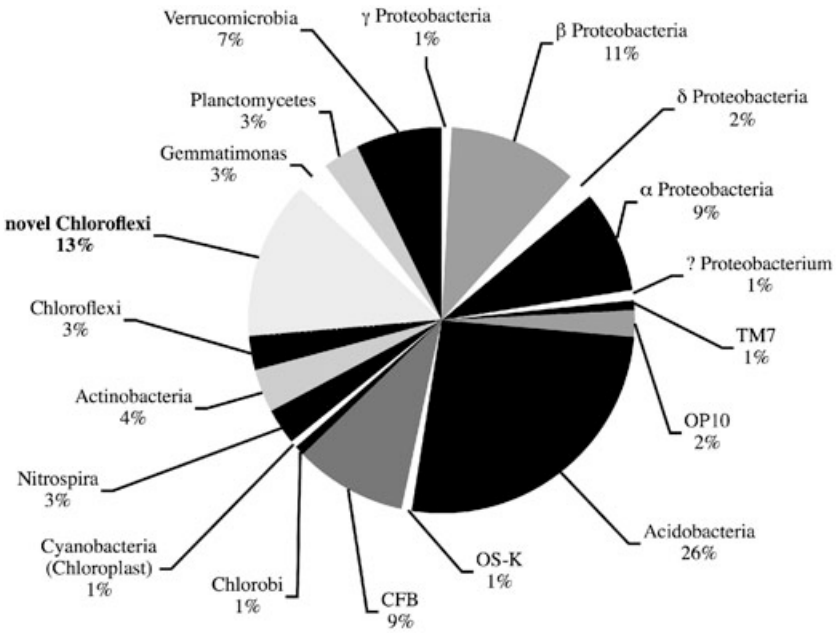

Fig. 4. Pie chart showing proportions of major phylogenetic groups represented in a bacterial clone library from spring alpine tundra wet meadow bulk soil (0–20 cm).

diversity was analysed by counting operational taxonomic units (OTUs) at various sequence difference groupings for a conservative distance matrix (ambiguously aligned regions excluded; uncorrected sequence comparisons) for each bacterial division (Fig. 5). Only the three most frequently sampled divisions, the Acidobacteria, Proteobacteria and Chloroflexi included closely related or exactly repeated sequences (< 1% or no difference). 'Species-level' groups (< 3% difference) were observed within the divisions CFB, Verrucomicrobia and Gemmatimonadetes. In general, within less frequently sampled divisions individual sequences were widely divergent. A lineage-per-time plot generated from a distance matrix with all spring wet meadow bacterial sequences yields a slightly convex curve (Fig. 5 inset centre), suggesting an overall excess of closely related lineages. Rarefaction indicates that sampling of this diverse community was incomplete, with Chao1 richness estimates greatly exceeding the sample size (Fig. 5 inset upper right).

Novel Chloroflexi phylogeny

The most unique feature of the spring wet meadow soil bacterial library was the high frequency, diversity and novelty of division Chloroflexi-related sequences. Comprising the third most abundant group, the 22 sequences related to the Chloroflexi fell into approximately 10 clades (Fig. 6). Only three of these were found to be members of previously defined subdivisions of the Chloroflexi (subdivisions 1, 2 and 4) (Fig. 6). The majority (19 sequences) were novel and unaffiliated with established subdivisions 1–8 of the Chloroflexi (as delineated by Rappé and Giovannoni, 2003). In phylogenetic analyses these novel sequences, their BLAST matches (most of which were unpublished sequences), and Chloroflexi-related sequences not affiliated with previously demarcated subphyla, coalesced into seven new subdivisions of Chloroflexi (Fig. 6). While support for the affiliation of the Chloroflexi subdivisions into a division as a whole and the association of the C05 group in a basal position as shown in Fig. 6 is tenuously supported (as is expected with a large, highly diverse group) the topology was observed repeatedly across alignments varying widely in taxon number, taxon representation, outgroup and phylogenetic inference method (data not shown).

Archaeal SSU rDNA gene library

In contrast to the bacterial library, restriction fragment length polymorphism (RFLP) analysis of 192 archaeal clones revealed frequent repeats and representatives of each group were selected for sequencing. All resultant archaeal sequences (15) were related to the Crenarchaeota as determined via BLAST matching (Table 1) and phylogenetic affiliation with the uncultivated C1b and C1c groups (data not shown). The largest group of archaeal sequences (6) were closely related to a sequence from an unpublished study of uranium mill tailings. Other wet meadow archaeal sequences matched sequences from submerged peat soil from the Okefenokee Swamp, naturally acidic (pH 4.0) subalpine stream sediment collected in summer on Niwot Ridge, and the humus layer of a Finnish boreal forest soil. Notably included in lower-scoring BLAST matches for the wet meadow archaeal sequences were sequences from Oline and colleagues' (2006) study of Crenarchaeota from coniferous forests

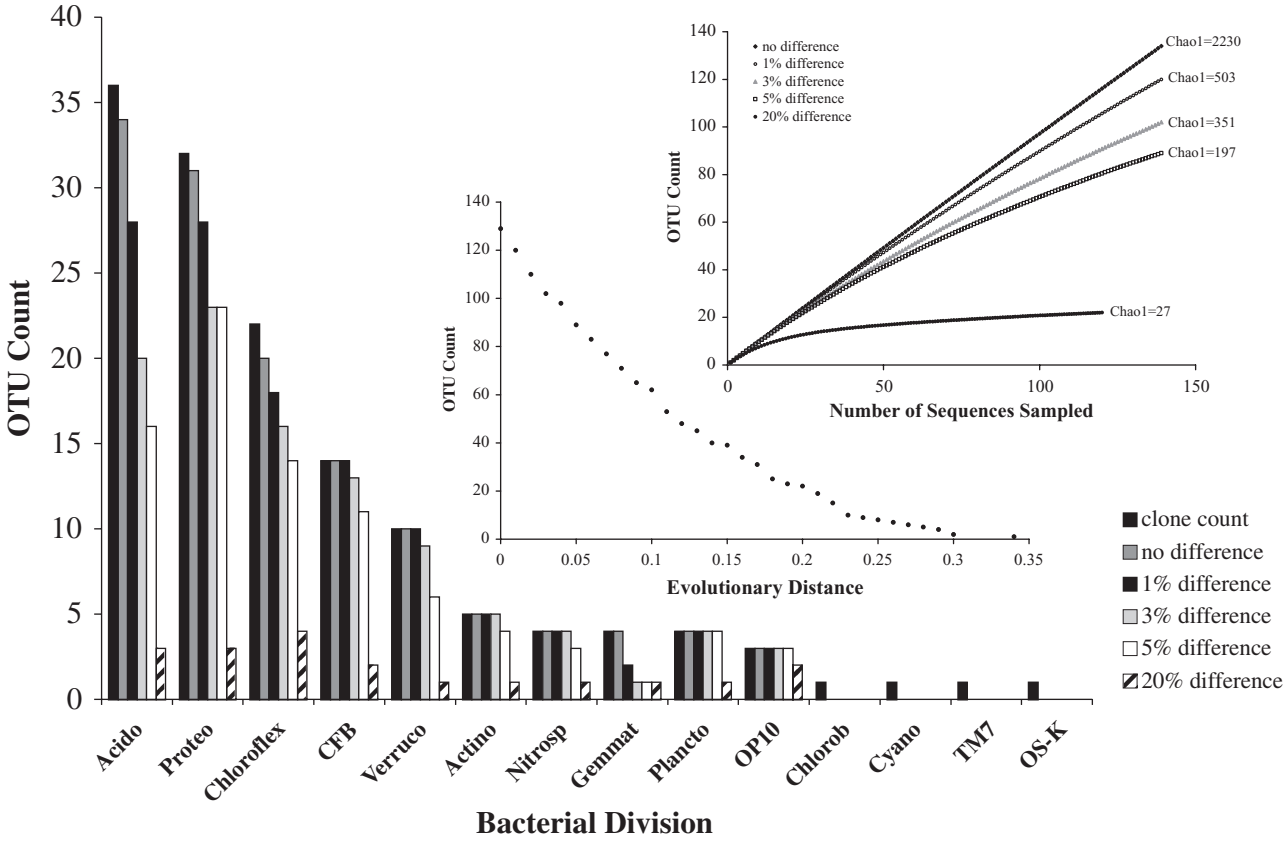

Fig. 5. Summary of 138 bacterial 16S rRNA gene sequences from alpine tundra wet meadow soil at spring snowmelt, grouped by division. Operational taxonomic units (OTUs) were defined at various levels of sequence difference including no difference (100%), species-level (3%), genus-level (5%) and phylum-level (20%) using uncorrected distance matrices and the program DOTUR. Inset centre is a lineage-through-time plot for all sequences. Inset upper right is a rarefaction plot of Chao1 richness estimates at various OTU definitions, with terminal estimates for the full dataset at right. Chao1 confidence intervals were omitted for clarity of presentation. Acido, Acidobacteria; Actino, Actinomycetes; CFB, Cytophaga-Flexibater-Bacteroides; Chlorob, Chlorobi; Cyano, Cyanobacteria; Gemmat, Gemmatimonadetes; Nitrosp, Nitrospira; Plancto, Planctomycetes; Proteo, Proteobacteria; Verruco, Verrucomicrobia.

Table 1. BLAST results for sequences obtained from spring wet meadow archaeal clone libraries.

Wet meadow clone	Closest BLAST match (GenBank accession no.)	BLAST match source	Score (Bits)	*e*-value	Reference
CREN					
C01_ARCH1,	AJ535123	U mining waste pile soil	1261	0.0	S. Selenska-Pobell *et al*., unpublished
D02_ARCH1,			1348	0.0	
C08_ARCH1,			1326	0.0	
F04_ARCH1,			1162	0.0	
H06_ARCH2,			1114	0.0	
H12_ARCH2			892	0.0	
A01_ARCH1	AB056056	Subtropical peat soil	805	0.0	Utsumi and colleagues (2003)
E11_ARCH1	AB056053	Subtropical peat soil	930	0.0	Utsumi and colleagues (2003)
D12_ARCH2	AB056050	Subtropical peat soil	928	0.0	Utsumi and colleagues (2003)
F02_ARCH1,	AY690203	Subalpine stream sediment	1003	0.0	J. Baeseman *et al*., unpublished
D10_ARCH2			1068	0.0	
D01_ARCH1,	X96688	Boreal forest soil	1314	0.0	Jurgens and colleagues (1997)
F10_ARCH2			969	0.0	
BAC					
B01_ARCH1	AJ536876	U mining waste pile soil	571	e^{-159}	G. Satchanska *et al*., unpublished
B10_ARCH2	D86512	*Acidisphaera rubrifaciens*	924	0.0	Hiraishi, 2000
EUK					
A01_ARCH3	AY654886	*Laccaria ochropurpurea*	1227	0.0	P.B. Matheny and D.S. Hibbett, unpublished

BAC, bacteria; CREN, Crenarchaeota; EUK, eukaryotes.

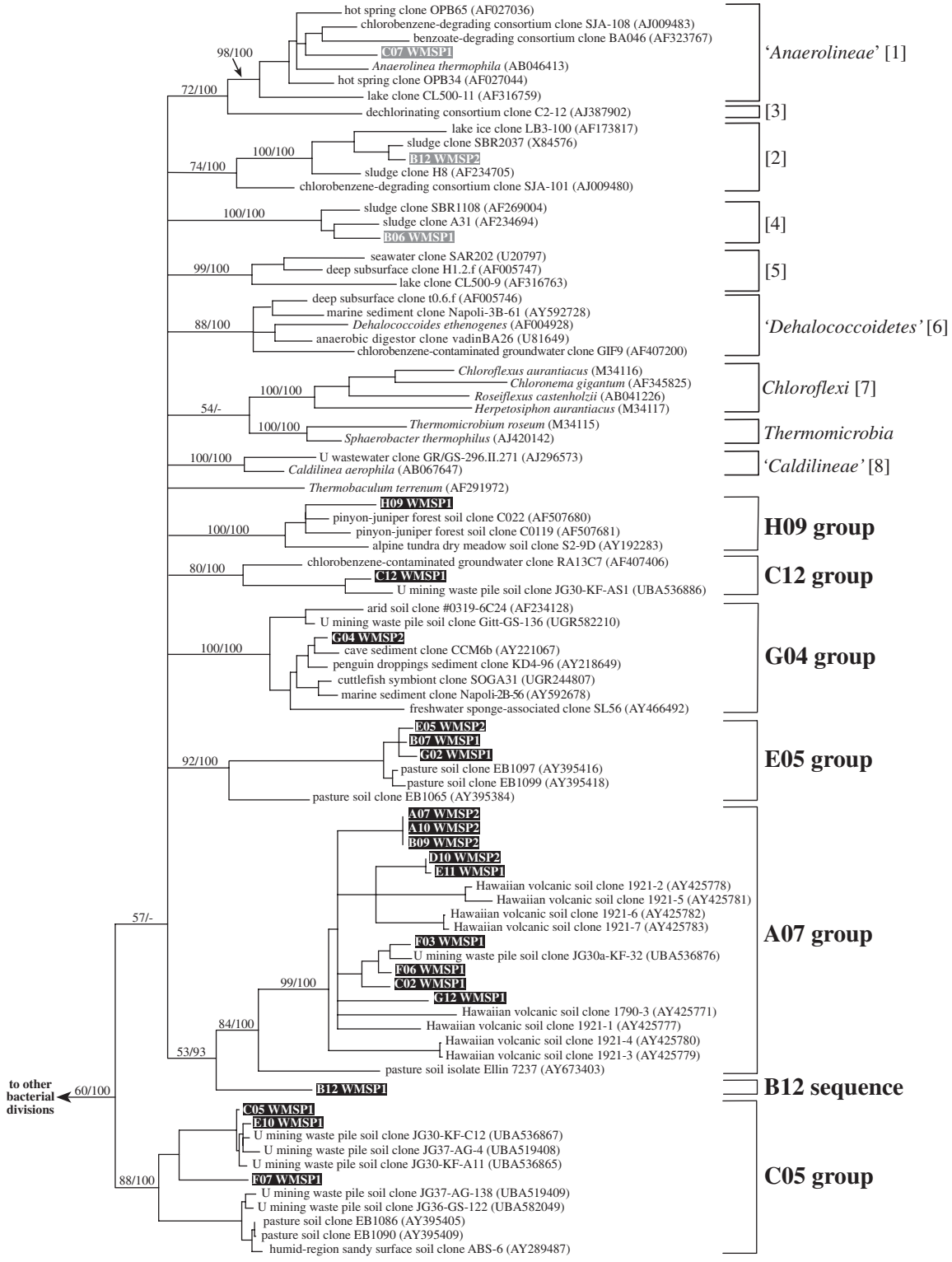

Fig. 6. Division Chloroflexi consensus tree of 100 heuristic search, distance-based bootstrap replicates with starting trees found by neighbour-joining and tree bisection-reconnection (TBR) branch swapping, using a distance matrix generated with an empirically determined GTR + I + G model of sequence evolution. Nodes with <50% support are collapsed. Bayesian posterior probabilities (*100) follow bootstrap support. Wet meadow sequences are highlighted. Reference taxa trimmed from the tree for clarity of presentation include Chloroflexi-related sequences unaffiliated with previously delineated subdivisions (1–8) or the new subdivisions, and representatives of approximately 45 bacterial divisions. New Chloroflexi-related groups are informally named after a representative sequence from the WMSP library. Novel clades include some, but not all, BLAST-related sequences. Relatives of sequence B12_WMSP1 were too short to include in the analysis.

and alpine tundra of the Colorado Front Range. Permissive polymerase chain reaction (PCR) conditions and primer degeneracy led to the concurrent amplification of some bacterial and eukaryotic sequences within the archaeal library, including a sequence related to novel Chloroflexi group A07 (Table 1, Fig. 6).

Spring bacterial terminal RFLP (tRFLP) profile

The discovery of novel bacteria (Fig. 6) and shifts in spring soil biogeochemistry along a depth gradient (Fig. 3B) prompted the investigation of the relationship between bacterial community profile and soil depth in the spring core. The greatest observed shift in bacterial community tRFLP profile occurred at the surface to subsurface transition, between 0.75 cm and 5.2 cm, as shown by a parsimony-based bootstrapped phenogram (Fig. 7A). Interestingly, tRFLP profile complexity and extractable DNA increased with soil depth (Fig. 7B) and tRFLP complexity had a positive linear relationship with extractable DNA ($r = 0.8$) (plot not shown).

Discussion

This study constitutes the first year-round, variable-depth microclimate record for Niwot Ridge alpine tundra wet meadow soil; an example of a cold, water-saturated soil environment. Despite the extreme conditions inherent to alpine areas, our data show that stable temperatures and available water persist for several consecutive months in spring and autumn. Ley and colleagues (2004) and Brooks and colleagues (1997) demonstrated that deep winter snowpacks insulate soil from freezing air temperatures and soil moisture fluctuations, promoting long-term, stable environments for microbial growth near 0 C. These conditions arose in the wet meadow, only later in the year in early April. Nonetheless, there was a sustained 2- to 3-month period of water availability and stable, growth-promoting soil temperatures near 0 C in spring, from early April until June. Furthermore, there was a second isothermal phase in autumn in the wet meadow, and this period was also associated with transitory snow accumulation. In addition to snowpack insulation, we suggest that stable growth conditions are promoted by water saturation and soil depth at our site. Wet meadow microclimate data revealed a moderating effect of soil depth on temperature and moisture. Deeper soils remained cooler in summer and warmer in winter, and did not experience the extreme diurnal temperature fluctuations of surface soils. More importantly, deeper soils exhibited longer isothermal periods in spring and autumn, ostensibly because heat transfer to and away from lower layers is slower, and because deeper soils are wetter during autumn freeze. Thus, deeper soils have temperature and moisture regimes more conducive to cold-temperature microbial growth than surface soils, and the fate of soil organic matter, especially the more recalcitrant forms found in deeper soils, may depend on the activities of such microbes. Moreover, microclimate data from subsequent years (not shown) suggest that wet meadow soil temperature and

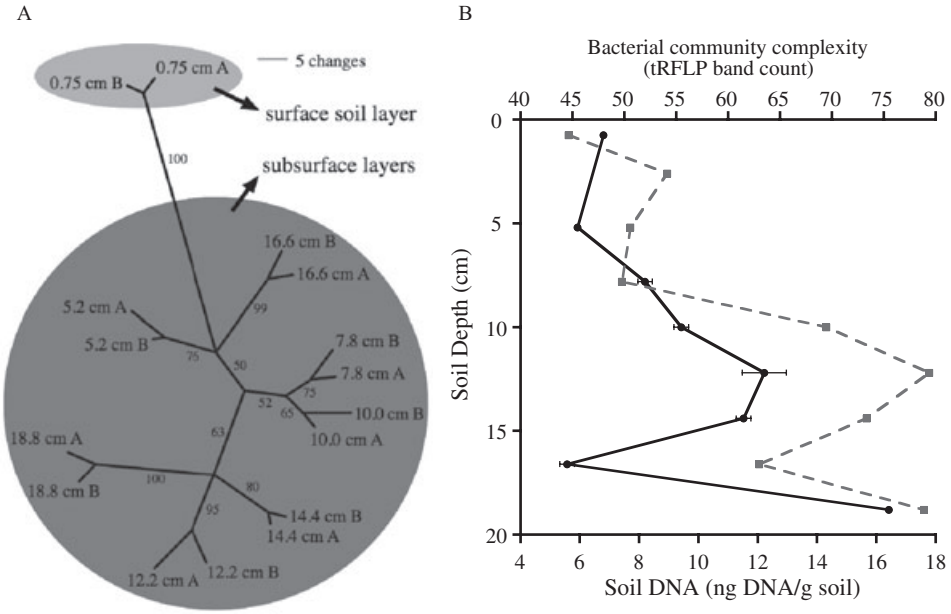

Fig. 7. (A) Phenogram depicting bootstrap supported (≥ 50%) linkages between bacterial tRFLP profiles at various soil depths, with the surface-to-subsurface transition highlighted, and (B) graph showing changes in tRFLP profile complexity (solid line) and soil DNA content (dotted line) with soil depth in saturated alpine tundra wet meadow soil.

moisture regimes are annually consistent, with slight variation in the timing of spring melt-out, and that long phases of near −0 C, water-available conditions arise yearly. These stable but cold periods provide ideal settings for the development of cold-adapted microbial communities.

Upslope snowbed and ground ice melt contributes water to the wet meadow during thaw, and spring soil geochemistry reflects this process. Wet meadow soils are saturated throughout the growing season. Saturated soil behaves like aquatic sediment and rapidly becomes anoxic due to heterotrophic oxygen consumption and limited dissolved oxygen transport (Fenchel and Finlay, 1995). In wetland soils, oxygen diffusion may be so slow that redox potentials decrease rapidly with increasing soil depth (Stolzy *et al*., 1981) and strong redox gradients can form within 2.0 mm of the soil surface (Howeler and Bouldin, 1971; Schlesinger, 1997). Our data show that in the wet meadow, the transition zone between Mn reduction and net Fe reduction occurs near 2.0 cm in depth. We observed little overlap between these processes and it has been shown that Fe(II) is re-oxidized in the presence of Mn-oxides such that net Fe(III) reduction is not observed until Mn-oxides are depleted (Lovley and Phillips, 1988). This zone is often indicative of a transition from mildly oxidizing, facultative anaerobic communities to strongly reducing, obligately anaerobic ones (Schlesinger, 1997).

Bacterial community tRFLP profiles show a correlated shift in community composition at the redox boundary near the soil surface. This may be indicative of a shift from an aerobic or facultatively anaerobic community to a strictly anaerobic one. Concordantly, community complexity and extractable DNA increase in the subsurface. At first this seems to counter a widely held view that bacterial communities are most extensive and complex in surface organic layers where recently senesced plant litter, oxygen and water are all most abundant. However, phylogenetic complexity might be expected to increase in the anoxic subsurface where the hierarchical flow of carbon and energy creates a diverse array of niches. While it is attractive to surmise that population sizes might be larger overall in the subsurface in spring due to the longer temperature-stable period and higher extractable DNA concentrations (as well as studies showing surface-community decline at the spring-to-summer transition) (Lipson *et al*., 2000; 2002) we caution that DNA may simply be better preserved in the cold, anoxic subsurface soil.

In addition to the development of local redox gradients due to soil saturation, wet meadows may be infiltrated by geochemical inputs from upslope weathering or subsnowbed microbial activities. Spring soils were found to be high in SO_2^{4-} and low soluble Zn. Spring wet meadow sulfate levels were approximately 10^3 times higher than reported for local snowpack, where sulfate can accumulate due to atmospheric deposition (Williams *et al*., 2006). However, high sulfate levels (comparable to ours and higher) have been observed in glacial meltwaters (Bottrell and Tranter, 2002) and in rock glacier outflow in the Green Lakes Valley watershed adjacent to Niwot Ridge (Williams *et al*., 2006). Both studies invoke the dissolution of S-bearing parent material (such as pyrite) via freeze-thaw action as the source of the sulfur. Bottrell and Tranter (2002) show that this occurs under anoxic conditions and that Fe(III) was the oxidizing agent under a glacier. Williams and colleagues (2006) indicate that the sulfate is associated with baseflow (interior ice melt), and may result from freeze-thaw along ice–rock interfaces, leading to internal ice melt with enriched geochemical values. Microbial activities may also play a role in sulfate production in rock glaciers (Williams *et al*., 2006). We suggest that the wet meadow may receive sulfur in spring via similar processes, i.e. upslope weathering with subsequent downslope percolation at thaw and microbial oxidation. Indeed, geomorphological cryoturbation features characteristic of ground ice dynamics are present in this area, and ground ice and permafrost are predicted to be extensive on Niwot Ridge (Ives and Fahey, 1971; Janke, 2005). The presence of sulfate may be linked to the reduced soluble Zn levels observed in spring. Labrenz and colleagues (2000) reported low-temperature, biologically mediated sphalerite (ZnS) precipitation in groundwater biofilms. We suggest that if the sulfate present at spring snowmelt arrives in the area as sulfide or is used as a terminal electron acceptor by sulfate reducing bacteria in these likely anoxic soils, the resultant sulfide may precipitate ZnS.

High bacterial community diversity reflects the dynamic nature of the wet meadow soil chemical and physical environment. Wet meadow bacterial sequences were generally unique to the extent that they might be expected to be functionally distinct from each other and known isolates. Overall, wet meadow bacteria were closely related to those involved in iron cycling, nitrogen cycling (fixation, nitrification, denitrification), hydrogen oxidation and complex C breakdown. Interestingly, no close relatives of known sulfate reducers were found. Sequences related to wet meadow bacteria were derived from environments that may share key characteristics with the spring wet meadow. These included peat, wetland, rice field, mine-waste impacted and contaminated soils and sediments. In particular, sequences from uranium mine tailings, volcanic soil and local alpine stream sediments were frequently shared. These results suggest that the spring wet meadow bacterial community may reflect characteristics of both soil and sediment, with inputs from rock weathering. However, we note that phylogenetic inferences about functional characteristics based on close relatives is problematic due to the wide functional diversity at fine phylogenetic scales exhibited by microbes.

Repetitive sampling a sequence from a highly diverse community may indicate activity for the particular lineage at the time of sampling. For the spring wet meadow bacterial library, repeat sampling was rare and occurred for members of the three most abundant divisions only: the Acidobacteria, Proteobacteria and Chloroflexi. Zhou and colleagues (1997) and Neufeld and colleagues (2004), in their studies of Siberian tundra soil and Canadian arctic soil, respectively, found high numbers of Acidobacteria and Proteobacteria, but few Chloroflexi-related sequences. This is a general pattern for many soils including alpine tundra dry meadow soil on Niwot Ridge (Lipson and Schmidt, 2004). Thus, it was surprising that we found novel Chloroflexi-related sequences comprising 13% of the bacterial library from spring wet meadow soil, representing seven new subdivisions of Chloroflexi. Several studies (most unpublished) are notable for their shared high frequency and diversity of novel Chloroflexi. For example, the E05, A07, B12 and C05 groups include numerous sequences from an unpublished study of Hawaiian volcanic soil (Fig. 6; site description in King, 2003) and the groups C12, G04, A07 and C05 consist of related sequences from an unpublished study of soil impacted by uranium mine tailings (site description in Selenska-Pobell *et al*., 2001). Additionally, many short sequences (not shown in Fig. 6) from an unpublished study of alpine stream sediments were related to groups A07 and C05 and several unpublished Alaskan boreal forest soil sequences were included in the G04 group. Also filling out the G04 group are sequences from a Minnesota farm soil (Tringe *et al*., 2005). Australian pasture soil clones were members of several new groups, including a clone related to the A07 group that was recently cultivated. This isolate grew slowly (8–12 weeks for colony appearance) on solid, xylan-containing medium (Davis *et al*., 2005). Also note that group H09 was previously observed in alpine tundra dry meadow on Niwot Ridge, but was phylogenetically classified as a candidate division SAM (Lipson and Schmidt, 2004). In general, the sequences comprising the novel Chloroflexi subgroups originate from soil or sediment-like environments, although some groups like G04 seem to be made up of sequences from more diverse habitats than others. Finally, the number of sequences discovered for each group in this and other studies varies widely across group, with some remaining small (C12, H09) and others much more frequently observed (A07, G04). We suggest from the sample repeats for the A07 group and the overall abundance and diversity of the wet meadow Chloroflexi that these bacteria may play an active biogeochemical role in these cold, anoxic soils. DNA templates used for PCR from the spring core were pooled from extracts of each soil segment, and were consequently biased towards the subsurface (the eight segments below the Mn reduction to net Fe reduction transition). Therefore, libraries likely reflect this bias and are more representative of the subsurface community. Because nearby, drier tundra soils did not reveal the same degree of Chloroflexi diversity or abundance, the role of these bacteria may be unique to the wet meadow soil subsurface compared with drier tundra soils at this site where the subsurface is rarely saturated. However, further studies are certainly necessary to identify the specific spatial, temporal and functional niches of these diverse, novel bacteria. For example, due to our limited sampling, it remains unclear whether populations of novel Chloroflexi arise each spring or are present throughout the year.

The division Chloroflexi was originally described by Woese (1987) as the green non-sulfur bacteria and identified as a deep-branching lineage of the domain Bacteria (Oyaizu *et al*., 1987); a phylum defined at that time by four isolates with widely varying phenotypes and relatively low sequence homology. Despite it's early description, cultivation of Chloroflexi has lagged behind discovery of cosmopolitan environmental sequences, leading to it's designation as an abundant but little-known division (Hugenholtz *et al*., 1998; Rappé and Giovannoni, 2003). The Chloroflexi have been described as a loose but coherent grouping (Gibson *et al*., 1985) and a recent comprehensive phylogenetic analysis delineated eight monophyletic subdivisions (Rappé and Giovannoni, 2003). However, reports of Chloroflexi phylogenies vary, most likely due to the rapid accumulation of new sequences, high sequence diversity exhibited by the group, limitations to phylogenetic resolution using 16S rDNA sequences, and methodological problems associated with large trees. Indeed, phylogenetic uncertainty dogs the cohesiveness among Chloroflexi subdivisions and the relationships between this division and other bacterial phyla. The Chloroflexi topology used as the framework for this article included the eight subdivisions described by Rappé and Giovannoni (2003), the Thermomicrobia/Sphaerobacter group, and numerous sequences not currently affiliated with these groups including the isolate *Thermobaculum terrenum*. This study identifies seven new subdivisions, each having representatives from alpine tundra wet meadow soil at spring snowmelt, as well as numerous members from unanalysed database sequences. These sequences dramatically expand the known diversity of the Chloroflexi, and point toward the need for an intensive database search for more related sequences and phylogenetic re-analysis of the division, as it's current cohesiveness is tenuous.

In contrast to the bacterial library, archaeal RFLP analysis indicated low diversity in the spring wet meadow soil core, yielding only archaeal sequences related to the Crenarchaeota. While methane production from wet meadow soils has been observed at our site (West *et al*., 1999), no

euryarchaeotal sequences were found, and none could be amplified using euryarchaeotal-specific primers. The Crenarchaeota found here clustered with two groups which are consistently found in soil or freshwater sediment environments (while related groups seem to derive mainly from marine environments). Frequent observation and phylogenetic covariance with environment suggests that these Archaea are active in a wide variety of settings. Indeed, recently a free-living low-temperature autotrophic ammonia-oxidizing Crenarchaeote was isolated from a marine environment (Könneke *et al*., 2005). Interestingly, the Crenarchaeota found here are related to sequences found in soil, sediments and uranium mining tailings; echoing the nature of wet meadow soil as a saturated, anoxic environment where geochemical weathering of newly exposed parent material may influence the biogeochemical setting.

Conclusions

Arctic and alpine ecosystems are predicted to be critically sensitive to global change. Arctic tundra soils store a significant portion of terrestrial carbon which is threatened by warming (Shaver *et al*., 1992) and alpine soils interact with large supplies of water from melting winter snowpacks, also in danger from pollution and warming (Williams *et al*., 2002). Arguably, understanding biogeochemistry and predicting future responses to global change in these delicate, cold-adapted communities requires robust assessment of species diversity for those organisms mediating key processes. Indeed, recent work has shown significant phylogenetic shifts corresponding with changes in biogeochemistry in response to climate change and seasonal fluctuations in the environment (Schadt *et al*., 2003; Horz *et al*., 2004; 2005).

An essential first step towards investigating the highly complex relationships between phylogenetic diversity, phenotypic diversity and ecosystem functioning in changing environments is the estimation of species diversity and careful examination of phylogenetic relationships. We have taken the first steps toward this goal in our study of alpine tundra wet meadow soil. We have identified saturation and soil depth as important factors in creating long-term, stable, cold soil environments for microbial communities. Additionally, freeze-thaw processes and saturated soil conditions likely lead to input of nutrients from upslope geochemical weathering and widespread soil anoxia respectively. These conditions are associated with high diversity and shifts in bacterial community structure. Most unique to this dynamic system are abundant representatives of at least seven novel subphyla of the bacterial division Chloroflexi that significantly expand the known diversity of this group.

We acknowledge that our conclusions regarding spatial and seasonal relationships between microbial diversity and environmental factors are limited by our small sample sizes and are therefore preliminary in nature. More comprehensive studies will be necessary to identify the specific niches of the novel Chloroflexi across space and time in these soils and to further resolve the phylogeny of this diverse and environmentally relevant group of bacteria.

Experimental procedures

Site description

Soil for this study was collected from an alpine tundra wet meadow located on Niwot Ridge, a National Science Foundation (NSF) sponsored Long-term Ecological Research (LTER) site and United Nations Educational, Scientific and Cultural Organization Biosphere Reserve. Niwot Ridge encompasses an extensive area of alpine tundra approximately 35 km west of Boulder, Colorado in the Front Range of the Rocky Mountains. Wet meadow snowpack depth averages 60 cm per year and soils have a mean annual temperature near 0 C. The soils are acidic (pH ranging from 4.3 to 5.3) (Burns, 1980; Fisk and Schmidt, 1995) and are classified as loamy-skeletal histic pergalic Cryaquepts (Burns, 1980). The wet meadow site used in this study lies on a turf-banked terrace (or solifluction lobe) (Benedict, 1970) at the NE base of a leeward snowbed in the 'saddle' area of Niwot Ridge [40 03.573′N, 105 35.368′W, 3461 m (11 356′)] (Fig. 1). This extensive snowbed forms yearly on the east-facing slope of the 'west knoll' and persists late into the growing season. This slope exhibits patterned ground and solifluction lobes that are cryoturbation features characteristic of soil movement due to the appearance and disappearance of ground ice. Much of this area may indeed be underlain by permafrost or seasonal ground ice (Ives and Fahey, 1971; Janke, 2005). At our wet meadow site the plant community is largely comprised of a sedge, *Carex scopulorum*, the marsh marigold, *Caltha leptosepala* and a willow, *Salix* sp.

Microclimate

In situ temperature, water content and photosynthetically active radiation (PAR) were measured at the site using a datalogger (model CR10X, Campbell Scientific, Logan, UT). The datalogger was outfitted with two bundles of probes. Each bundle consisted of three thermistors (107, Campbell Scientific), one water content reflectometer (CS616, Campbell Scientific) and one quantum sensor to measure PAR (LI190SB, Campbell Scientific). Photosynthetically active radiation sensors were utilized to document the presence or absence of snow on the site. The datalogger was installed on 8 October 2003. The two probe bundles were placed in the soil ~4 m apart along an east–west axis. For each bundle, temperature was measured at 2 cm, 10 cm and 30 cm depth and PAR was measured at the soil surface. The west reflectometer logged water content at the soil surface (2 cm) and the east reflectometer logged water content at 20 cm soil depth. Data were recorded hourly over the course of several years, and we report only year one. The reflectometers were

not field calibrated and soil water content is reported here as a percent of maximum, where 100% is saturated and 0% is frozen.

Soil collection and analyses

Soil cores were aseptically collected using PVC corers (5.5 cm diameter) in early spring (6 June 2001), autumn (21 October 2002) and winter (13 February 2003). At the time of spring collection the soil was saturated and a standing water layer was present at the soil surface. Although the site had been recently snow-covered, plants were already green and in bloom. At the time of the winter collection snowpack depth was 50 cm, the soil was frozen, and soil could only be collected to a depth of 8 cm. Finally, the cores collected in autumn were from thawed areas of the patchily frozen ground. At this time, plants had senesced. The soil cores from all sampling dates were aseptically packaged, placed on ice, and immediately transported to the University of Colorado where they were frozen intact at −80 C.

Spring, autumn and winter soil cores were subdivided at roughly 2-cm intervals and each segment analysed for moisture content, organic matter, SO_4^{2-}, soluble metals Fe(II), Mn(II), Zn(II), Al(III) and Cu(II) and the cations K^+ and Mg^{2+}. Soil for elemental analyses was extracted using standard methods aimed at capturing soluble, bioavailable species. All anions, cations and metals were analysed at the Laboratory for Environmental and Geological Studies (LEGS), Department of Geological Sciences, University of Colorado Boulder via ion chromatography or atomic absorption spectrometry. Sulfate in water samples collected on Niwot Ridge were analysed by ion chromatography at the Kiowa Environmental Chemistry Laboratory at the University of Colorado's Mountain Research Station. Soil moisture was measured by oven drying to constant weight at 100 C and organic matter was determined by loss on ignition at 550 C.

DNA extraction, purification and quantification

Frozen soil was removed from the PVC corer and subdivided into 2-cm segments prior to DNA extraction. Soil for DNA extraction was removed from the inner portion of each segment, avoiding potential contaminants from the outer rind. DNA was extracted according to a bead-beating method modified from Moré and colleagues (1994). In brief, 0.5 g soil and 0.3 g each of 1.0 mm glass, 0.5 mm silica and 0.1 mm silica beads (Biospec Products) were homogenized in 1.0 ml of phosphate lysis buffer (100 mM $NaPO_4$, 100 mM Tris-HCl, 100 mM NaCl, 10% SDS, pH 8.0) for 2 min on a bead-mill (Biospec Products, Bartlesville, OK). DNA was crudely purified via one extraction with ammonium acetate (7.5 M) and two extractions with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with isopropanol. For each soil segment, three separate 0.5-g extractions were pooled. Humic substances were removed using Sepharose 4D (Sigma-Aldrich, St. Louis, MO) columns constructed according to Jackson and colleagues (1997). DNA concentrations were determined using the PicoGreen fluorimetric assay (Molecular Probes, Eugene, OR) on a Storm 860 Phosphoimager with ImageQuant software (Molecular Dynamics).

Polymerase chain reaction amplification of bacterial and archaeal 16S rRNA genes

In order to obtain a glimpse of microbial diversity across the soil profile that was representative of both surface and subsurface communities, DNA from each 2-cm segment of the spring soil core was pooled for PCR. Bacterial and archaeal community small-subunit ribosomal DNAs (SSU rDNAs) were amplified using the Bacteria-specific primer pair 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1391R (5′-GACGGGCGGTGWGTRCA-3′) (Lane, 1991) and the Archaea-specific primer pair 8Fa (referring to *Escherichia coli* position 8) (5′-TCYSGTTGATCCTGCS-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Bacterial PCRs were performed with 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.4 M each primer, 1 U *Taq* polymerase (Promega, Madison, WI) and buffer supplied with the enzyme using a range of template concentrations. Gradient thermal cycling was carried out for 25 cycles to minimize PCR bias. Amplicons from various reactions were pooled for cloning. Conditions were identical for archaeal PCR except 3.0 mM $MgCl_2$ was used. Archaeal PCR generated two products, a dark band at ~1500 bp (archaeal SSU) and lighter band at ~1800 bp (eukaryotic SSU). The two products were cloned separately. Polymerase chain reaction products were purified on agarose gels and extracted using spin columns (Qiagen, Valencia, CA).

Cloning, RFLP and sequence analysis

Purified PCR products were ligated into TOPO TA cloning vectors (Invitrogen, Carlsbad, CA), transformed into *E. coli,* and transformants were randomly arrayed on 96-well plates. Plasmid inserts were amplified using primers M13F and M13R and subjected to RFLP analysis. Amplicons were digested with 1 U *Msp* I and 1 U *Cfo* I (Promega) at 37 C overnight. Restriction fragments were electrophoresed on 1% agarose, 2% NuSieve (Cambrex, Baltimore, MD) gels. For the bacterial library, few to no RFLP repeats were observed and randomly selected clones were sequenced. For the archaeal library, numerous RFLP repeats were observed and sequencing was performed on selected representatives only.

Prior to sequencing, M13 amplified inserts were cleaned with exonuclease I and shrimp alkaline phosphatase (New England Biolabs, Ipswich, MA). Cycle sequencing was carried out using BigDye Terminator ready reaction mixture (version 3.1; Applied Biosystems, Foster City, CA) according to the manufacturers instructions. Sequencing primers were vector targeted T7 (5′-AATACGACTCACTATAG-3′) and M13R-9 (5′-GCTATGACCATGATTACG-3′) and SSU rDNA targeted 515F (5′-GTGCCAGMGCCGCGGTAA-3′). Only T7 reads were acquired for the archaeal representatives. Sequencing reactions were purified on 96-well Sephadex (Sigma-Aldrich) columns. Sequences were generated on an ABI 3700 capillary DNA sequencer at the Iowa State University DNA Sequencing Facility or MJ Research Base Station 51 at the University of Colorado. Partial sequences were edited and compiled in Sequencher (version 4.2; Gene Codes, Ann Arbor, MI). Putative chimeras were identified using the Bellerophon server (Huber *et al.*, 2004), the Ribosomal Database Project's (RDP) CHIMERA_CHECK program

(Maidak *et al*., 2001), and manually via partial tree analysis. Approximate phylogenetic affiliation and related sequences were found using BLAST (Altschul *et al*., 1990) and the GenBank database (Benson *et al*., 2005).

Alignments and phylogenetic analyses

Non-chimeric 16S rDNA sequences and their BLAST matches were aligned in ARB (Ludwig *et al*., 2004) with an alignment database expanded from Hugenholtz's Combined Alignment available at the RDP website (Hugenholtz, 2002). In our lab, this alignment is continually updated with new bacterial candidate divisions and over 50 divisions were represented. Numerous subalignments were generated for phylogenetic analyses. These alignments varied widely in taxon representation and taxon number. Taxon selection was guided by preliminary trees made in ARB using the Olsen evolutionary distance correction and neighbour-joining tree-building algorithm. In general, alignments included representatives of various divisions of Bacteria, subdivisions of Chloroflexi and unaffiliated Chloroflexi sequences as reference taxa. Alignment sizes varied from 30 to 380 taxa. Out-group taxa were either Archaea or Aquificales. Almost all sequences were >1300 nucleotides in length, with exceptions for several shorter sequences considered important representatives in the phylogenetic analysis. Sequence alignments were exported from ARB using the Lane mask function to remove ambiguously aligned hypervariable regions (Lane, 1991) and truncated at *E. coli* position 1407. Exported alignments consisted of 1205 character positions.

Phylogenetic analyses were aimed at classifying novel wet meadow 16S rDNA sequences by inclusion into (or exclusion from) established bacterial divisions, with particular emphasis on novel Chloroflexi. The suite of phylogenetic inference methods used included distance-based minimum evolution or neighbour-joining, maximum parsimony, maximum likelihood and Bayesian techniques. Distance-based nonparametric bootstrapping and Bayesian techniques were emphasized in estimating support for nodes. Sequence evolution model selection and parameter estimates were made for each alignment using MODELTEST (version 3.5; Posada and Crandall, 1998; Posada and Buckley, 2004). A general time-reversible model with gamma distributed rate heterogeneity and estimated invariant sites (GTR + G + I) was frequently the selected model. For each alignment, the suite of phylogenetic analyses was tailored to accommodate computational constraints while maximizing analysis quality. For instance, maximum likelihood was feasible for the smallest alignments only, and neighbour-joining was reserved for only the largest alignments. Most distance, parsimony and likelihood tree searches were heuristic with tree bisection-reconnection (TBR) branch swapping on each of 10 starting trees found by random stepwise sequence addition. Random sequence addition replicates were reduced, TBR rearrangements were constrained and/or starting trees were found by neighbour-joining in some cases to reduce computing time. For distance trees, the optimality criterion was minimum evolution, except for alignments with 300+ taxa in which the neighbour-joining clustering algorithm was used. Bootstrapping consisted of 100 replicates and nodes with <50% bootstrap support were collapsed. Distance, parsimony and likelihood analyses were performed in PAUP* (version 4.0; Swafford, 2002).

Bayesian posterior probabilities were approximated in MrBayes (version 3.0; Ronquist and Huelsenbeck, 2003) using four-chain Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analysis. GTR + G + I model parameter values were approximated by the analysis. Chain stationarity was determined by plotting the likelihood scores against generation number and discarding the burn-in. In this manner, trees and model parameter values were sampled from a target distribution generated when the chains converged. The number of generations required to achieve the target distribution varied according to alignment size. Thus, Bayesian analyses was limited to 200 taxa or less because larger alignments did not stabilize or produce a full distribution and had the symptom of inflated posterior probabilities for relationships between bacterial divisions. For Bayesian consensus trees, nodes with ≥0.95 posterior probability were considered significant.

All phylogenetic analyses were performed on a dual processor PC running Linux OS. Phylogenetic trees were compared for consistency across analyses, alignments, and with previous studies. Inconsistencies were often attributable to the following problems: (i) long branch attraction (OP11 representatives), (ii) chimerism (database sequences), (iii) data limitation (i.e. resolution problems, inherent and due to bootstrap subsampling), or (iv) inadequate burn-in time for Bayesian analyses of large alignments. The alignment used to generate the phylogeny shown in Fig. 6 consisted of 197 taxa before pruning for presentation. This tree represents the most frequently observed topology across various alignments and phylogenetic analyses.

Determination of OTUs and lineage-per-time plots

We used the program DOTUR (Distance-Based OTU and Richness Determination; (http://www.plantpath.wisc.edu/fac/joh/dotur.html; Schloss and Handelsman, 2005) for OTU determination, rarefaction, Chao1 richness estimation, and to generate lineage-per-time plots as described previously (Schloss and Handelsman, 2004). For the bacterial library and subsets of sequences representing each division, distance matrices were calculated in ARB from alignments using the Lane mask to eliminate ambiguously aligned regions (whose inclusion would cause an overestimation of unique lineages). Also, no distance correction (model of sequence evolution) was implemented when calculating distance matrices and distances were based on absolute sequence similarity. We acknowledge that by excluding hypervariable regions and using absolute similarity, we are presenting conservative estimates of OTUs. In DOTUR, the furthest neighbour sequence assignment method was used.

Bacterial community tRFLP fingerprinting

Bacterial 16S rRNA genes were amplified from each spring soil core segment using IRDye™ 700-labelled 8F primer with 1391R. Polymerase chain reaction was carried out as described above at various template concentrations and

annealing temperatures. Polymerase chain reaction products were pooled for each soil segment. Amplicons were digested with 2.5 U BamHI (Promega) and electrophoresed on a polyacrylamide gel using a LI-COR DNA Analysis System (LI-COR Biosciences, Lincoln, NE). Terminal fragments were identified, sized, and binned using GeneImagIR software (LI-COR). Profiles were generated by scoring absence (0) versus presence (1) of a band for each fragment size class. The profiles were compiled and sample order randomized in MacClade (Sinauer Associates, Sunderland, MA). Parsimony and distance-based bootstrap support for relationships between tRFLP profiles were performed in PAUP.

Nucleotide sequence accession numbers

The SSU rRNA sequences generated for this study were deposited in the GenBank database under Accession numbers DQ450683–DQ450836.

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