Presence and potential role of thermophilic bacteria in temperate terrestrial environments

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Abstract

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Organic-sulfur and nitrogen are major reservoirs of these elements in terrestrial systems although their cycling remains to be fully understood. Both sulfur and nitrogen mineralization are directly related to microbial metabolism. Mesophiles and thermophiles were isolated from temperate environments. Thermophilic isolates were

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classified within the Firmicutes, belonging to the *Geobacillus*, *Brevibacillus* and *Ureibacillus* genera, and showed optimum growth temperatures between 50°C and 60°C. Sulfate and ammonium produced was higher during growth of thermophiles both for isolated strains and natural bacterial assemblages. Sulfate and ammonium produced were positively related to organic nutrient load. Temperature also affected the release of

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sulfate and ammonium by thermophiles. Quantitative, real-time RT-PCR on environmental samples indicated that the examined thermophilic Firmicutes represented up to 3.4% of total bacterial community RNA. Temperature measurements during summer days showed values above 40°C for over 10 h a day in soils from Southern Spain. These results support a potential role of thermophilic bacteria in temperate

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terrestrial environments by mineralizing organic sulfur and nitrogen ruled by the existence and length of warm periods.

Introduction

50 Microorganisms are known to play essential roles in the biogeochemical cycling of
elements (Torsvik et al. 2002; Crawford et al. 2005; Allison and Martiny 2008).
Element cycling and the availability of inorganic nutrients for plant growth in terrestrial
environments are topics of great interest. Understanding the potential sources of these
nutrients as a function of environmental conditions is of critical importance for the
55 management of sustainable environments and agriculture.

A high fraction of the nitrogen in terrestrial environments is bound to organic matter
(Gonzalez-Prieto and Carballas 1991). Nitrogen is one of the limiting elements for plant
growth and fertilization is often required (Gruber and Galloway 2008). Most fertilizers
60 contain ammonium as a source of nitrogen which can be both incorporated and
transformed by microorganisms. Nitrification is carried out by several microorganisms
(e.g., *Nitrosomonas*, *Nitrospira*) (Rowan et al. 2003) leading to nitrate which can be
readily utilized by plants. One fate of inorganic nitrogen compounds from diverse
sources is the synthesis of biomolecules and so, organic nitrogen (e.g., proteins)
65 represents a major fraction of nitrogen in the environment. Organic matter also forms
part of non-living matter (i.e., humic acids and dead biomass). Ammonium is an
important source of nitrogen for microorganisms and plants and can be released into the
environment, for instance, as a result of organic matter mineralization by ammonifying
bacteria (Jarvis et al. 1996). Besides, the complexity of the nitrogen cycle is increasing
70 as a result of recent investigation (Kartal et al. 2006). Organic content in soils and
sediments constitute a nutrient reservoir for terrestrial system productivity which has
been related to the capacity of soils to sustain plant growth (Gruber et al. 2008; Kreutzer
et al. 2009).

75 Sulfur is another element required to maximize crop yields and quality (Tabatabai 1984;
Wen et al. 2003). Most fertilizers rely on additions of elemental sulfur (Wen et al.
2003) although soluble sulfate is the major source of sulfur for plants. Sulfur
mineralization is the conversion of organically bound sulfur to inorganic sulfate which
is available to plants use (Eriksen 2008). Sulfur immobilization is the opposite process
80 in which inorganic sulfur is assimilated by the biota and incorporated into organic
matter becoming unavailable to plant roots (Eriksen 2008; Schoenau and Malhi 2008).

The processes responsible for sulfur transformations in soils, such as mineralization, immobilization, oxidation, and reduction, are mainly microbially mediated (Sparling and Searle 1993; Niknahad-Gharmakher et al. 2009). Factors affecting the microbial activity involved in these processes, such as pH, temperature and nutrient availability have been scarcely studied.

In nature, sulfur is either in organic or inorganic forms. Organic sulfur represents most of the sulfur in soils with estimates of over 90% of total sulfur (Schlesinger 1997; Anandham et al. 2008). In unfertilized environments, mineralization is supposed to be the dominant input of sulfate for plant growth (Schoenau and Malhi 2008). However, under temperate conditions (around 20°C), organic sulfur mineralization to sulfate has been reported to be highly limited (Ghani et al. 1993; Eriksen 1996). Heterotrophic sulfur-oxidizing bacteria have also been proposed as dominant in some soils, especially in the rhizosphere (Grayston and Germida 1990), although the mesophilic microorganisms tested so far utilized mostly elemental sulfur, rather than organically bound sulfur, as source to generate sulfate (Grayston and Germida 1990; Kelly et al. 1997). Most studies of microbial sulfur cycling have been centered on transformations of inorganic sulfur by microorganisms, including sulfate-reducing (e.g., *Desulfovibrio*, as the most cited genus) and inorganic sulfur-oxidizing (e.g., *Beggiatoa*, *Sulfurimonas*) bacteria (Kelly et al. 1997; Madigan et al. 2003) often related to the use of inorganic sulfur as fertilizer (Wen et al. 2003).

The presence of thermophilic bacteria in temperate environments has been previously reported (Wiegel et al. 1979; Mutzel et al. 1996; Rahman et al. 2004). However, their function and possible ecological role remains to be understood. Huber et al. (Huber et al. 2009, 2010) reported the presence of thermophilic anaerobes in cold arctic marine sediments. Their existence in this cold environment was explained by the proximity of warm subsurface petroleum reservoirs. These thermophiles showed sulfate-reducing activity around 50°C and were classified as Gram-positive bacteria related to the genus *Desulfotomaculum*. Marchant et al. (2002, 2008) have reported on the presence of thermophilic aerobic bacteria, mainly belonging to the genus *Geobacillus*, in cool temperate soils from North Ireland. These thermophiles grew above 40°C and showed optimum growth temperatures around 70°C. Their function is unknown but they were proposed to be endemic species at the studied sites (Marchant et al. 2002) in spite of the

high rate of arrival of soil bacteria from the Sahara Desert (Portillo and Gonzalez, 2008; Perfumo and Marchant, 2010).

In this study, we report on a potential role for some thermophilic bacteria present in temperate terrestrial environments. We analyzed the physiological capabilities of some thermophiles on the mineralization of organic sulfur and nitrogen, producing sulfate and ammonium. Both isolates and natural bacterial communities were used throughout this study. The presence of active thermophiles and some of the opportunities to exploit their metabolism in temperate terrestrial environments are discussed.

Materials and Methods

Sampling. Samples for the isolation of microorganisms were collected from soil in Southwestern Spain (Carmona, Seville; 37° 28' N, 5° 37' W), from a pine forest soil and a nearby soil partially covered by weeds located in Northeastern Spain (Teruel, Aragon; 40° 24' N, 1° 26' W). Seville represents one of the hottest areas of the Iberian Peninsula and Teruel shows a continental climate and is among the coldest Spanish provinces. These soils have been previously described (Navas and Machin 2002; Ordóñez-Fernández et al. 2007). Samples for RNA extraction and quantification were also collected from an outlying area of Seville. Samples for RNA extraction and quantification were preserved in RNeasy (Ambion, Inc., Austin, TX) following the manufacturer's recommendations.

Experiments with thermophilic isolates. Cultures were carried out in nutrient broth (NB) which composition, per liter, was: 3 g beef extract and 5 g peptone. Nutrient agar had, in addition, 20 g l⁻¹ agar. Environmental samples were suspended in 0.9% NaCl saline solution and vortexed; 100 µl suspension were inoculated in nutrient agar and incubated for 3 days at 25°C or 50°C, for mesophiles and thermophiles, respectively. Colony forming units (CFU) of aerobic heterotrophic thermophiles were enumerated on nutrient agar plates incubated at 50°C for 3 days. Single colonies were transferred to fresh nutrient agar following three passes of isolation picking single colonies and following standard microbiological procedures. Isolates were identified by sequencing the small subunit of the rRNA gene. For bacterial isolates, the 16S rRNA genes were

150 PCR amplified with the 27F (5'-AGA GTT TGA TYM TGG CTC AG) and 907R (5'-
 CCC CGT CAA TTC ATT TGA GTT T) primer pair (Gonzalez et al. 2003). For the
 fungal isolate, the primer pair EukA (5'- AAC CTG GTT GAT CCT GCC AGT) and
 EukB (5'-TGA TCC TTC TGC AGG TTC ACC TAC) was used (Diez et al. 2001). The
 influence of temperature on the growth of the thermophiles was analyzed by OD
 155 determinations at 600 nm during incubation in NB with shaking (100 rpm) at different
 temperatures (from 20°C to 85°C). The effect of temperature on the production of
 sulfate and ammonium by thermophilic isolates was examined as above at 50°C, 60°C
 and 70°C.

160 Aliquots were collected overtime to estimate soluble sulfate concentration according to
 the method of Kolmert et al. (2000). Ammonium concentration was estimated according
 to Kartal et al. (2006). The produced sulfate and ammonium were estimated as the
 difference with respect to the initial concentration. Sterilized controls were carried out
 and used to estimate final levels of sulfate and ammonium during growth. All
 165 experiments were performed in duplicate collecting triplicate aliquots for each
 measurement. Nitrate and nitrite were measured during preliminary experiments and we
 observed no significant increase of concentration during the growth of the isolated
 thermophiles. The concentration of nitrate was at the lowest limit of detection (0.5 mM)
 for the method used (Cataldo et al. 1975).

170 Thermophilic isolates were grown in NB with and without supplemented sodium
 thiosulfate (3 mM) to test its influence on sulfate production. Thiosulfate is a major
 intermediate in the inorganic oxidation of sulfur and sulfide to sulfate for a number of
 bacteria (Kelly et al. 1997; Madigan et al. 2003). To evaluate the production of H₂S as a
 175 potential source for sulfate after its oxidation the concentration of H₂S was determined
 in cultures at exponential and stationary phases of growth using H₂S electrodes
 (Unisense, Aarhus, Denmark). H₂S was undetectable during the whole duration of the
 experiments and the initial concentration of sulphate in NB medium was 1 mM.

180 Thermophilic isolates were grown at different concentrations of NB (from 0.01x to 2x)
 to assess the effect of organic nutrients on sulfate and ammonium production. Standard
 concentration of NB (1x) contained 5 g peptone and 3 g beef extract per liter. Biomass
 obtained during growth was estimated as cellular protein content and determined by the

Bradford method (Bradford Protein Assay, BioRad, Hercules, CA). Sulfate and
185 ammonium concentrations were normalized by the biomass produced during growth.

Experiments with natural bacterial assemblages. Samples (5 g) were incubated in 10
ml nutrient broth (NB) in a shaker (250 rpm) at 25°C and 50°C. Treatments using these
natural bacterial assemblages were performed in duplicate. Aliquots (in triplicate) were
190 collected overtime to estimate soluble sulfate and ammonium concentration as described
above.

***In situ* relative rRNA quantification.** Relative quantification of ribosomal RNA
(rRNA) from thermophilic Firmicutes (represented in the genera *Brevibacillus*,
195 *Geobacillus* and *Ureibacillus*) was performed from environmental samples. The rRNA
has been used as indicator of metabolic activity by bacteria (Molin and Givskov 1999;
Portillo et al. 2009). Quantification of RNA belonging to the selected thermophilic
Firmicutes with respect to total bacterial RNA was carried out by quantitative, real-time
RT-PCR. Total RNA was extracted with the RNAqueous4-PCR kit (Ambion, Inc.,
200 Austin, TX). The protocol for total RNA extraction included DNaseI treatment to
remove any DNA remaining in the final RNA extract.

Reverse-transcription PCR (RT-PCR) was performed using oligonucleotides targeting
the 16S-23S rRNA gene internal transcribed spacer (ITS) of the genus *Geobacillus*
205 (Kuisiene et al. 2007) and newly designed oligonucleotides targeting the 16S rRNA
genes of the known thermophilic representatives of the genera *Brevibacillus*,
Geobacillus and *Ureibacillus*. The primers targeting the ITS were GeobacF (forward;
5'-TAA GCG TGA GGT CGG TGG TTC) and GeobacR (reverse; 5'-GCG CTC TCG
GCT TCT TCC TT) (Kuisiene et al. 2007) which resulted in amplicons of about 300 bp.
210 The 16S rRNA genes of the thermophilic Firmicutes belonging to the three selected
genera were targeted by amplifications combining the forward primers Geo485F (5'-
CTG ACG GAG CGA CGC CGC GTG AGC) and B-U485F (5'-CTG ATG GAG CAA
CGC CGC GTG ARC) and the reverse primer 518R (5'-ATT ACC GCG GCT GCT
GG). The forward primers Geo485F and B-U485F were designed to target *Geobacillus*,
215 and the thermophilic representatives of the genera *Brevibacillus* and *Ureibacillus*,
respectively. The forward primer mix was composed by 50% of each primer, Geo485F

and B-U485F. Total bacterial RNA was amplified by using the primer pair 341F (5'-CC TAC GGG AGG CAG CAG) (Lane 1991) and 518R (Neefs et al. 1990).

Reverse transcription reactions were carried out using Thermoscript (Invitrogen, Carlsbad, CA) at 55°C using the reverse primers. Quantitative PCRs were performed with the SensiMix SYBR & Fluorescein Kit for real-time PCR (Quantace, Germany) on a thermocycler iQ iCycler (Bio-Rad, Hercules, CA). Triplicate reactions were processed under the following cycling conditions: 95°C for 5 min and 50 cycles of a denaturing step at 95°C for 15 s, annealing at 62°C for 15 s, extension at 72°C for 30 s, and a final step for the measurement of SYBR Green fluorescence at 80°C for 10 s. Relative quantitative PCR estimates were performed according to the sigmoidal non-linear curve-fitting method described by Rutledge (2004). Relative quantitative estimates of thermophilic Firmicutes RNA were normalized with those from total bacterial RNA.

Environmental temperature measurements. Environmental temperatures were measured using a K-thermocouple thermometer (Hanna Instruments, Ann Arbor, Michigan, USA). Surface and 2 cm depth measurements were performed throughout a day period at a soil of an outlying area of Seville (Spain). Air temperature was determined at about 1 m from soil surface.

Statistical analysis. Significant differences ($P < 0.05$) among treatments were compared through ANOVA (Sokal and Rohlf 1981).

Results

Different isolates were obtained from sediments and soils at distinct Spanish locations at Southwestern and Northeastern areas showing significant climatic differences. A list of these isolates is shown in Table 1 and they were among the most common culturable bacteria at 25°C and 50°C from the studied sites. The mesophilic bacterial isolates were identified by 16S rRNA gene sequencing and belonged to the Actinobacteria (*Cellulomonas* and *Streptomyces*), Firmicutes (*Paenibacillus*), and Gammaproteobacteria (*Enterobacter* and *Klebsiella*). A fungus (*Neocosmospora*) was also isolated. The isolated thermophiles were all members of the Order Bacillales

(Firmicutes) and were represented by the genera *Bacillus*, *Brevibacillus*, *Geobacillus*, and *Ureibacillus*. No growth was observed under anaerobic conditions. Culturable thermophiles averaged $4.5 \pm 1.3 \times 10^5$ CFU/g for the studied samples. Thermophilic isolates were used as models to study the microbial capacity to release sulfate and ammonium during growth.

In general, cultures of thermophilic isolates produced higher levels of soluble sulfate than the mesophilic strains (Fig. 1). The growth of the mesophiles (25°C) showed low to insignificant levels of soluble sulfate in the medium along their growth curve. Although leading to much lower soluble sulfate concentrations, some of the mesophiles resulted in a significant increase in the release of sulfate during the stationary phase of growth (i.e., strains 1 and 6). Other bacterial mesophiles (i.e., strain 4) showed a consumption of soluble sulfate during the stationary phase of growth. Bacterial strain 2 and the fungal strain 5 did not show significant increase of soluble sulfate during the growth curve.

The concentration of sulfate varied along the growth curve of the thermophilic isolates and it was strain dependent. Maximum growth yields reach in the cultures of the thermophilic isolates ranged from 10^7 - 10^8 cells ml⁻¹; mesophilic isolates resulted in yields of at least 10^8 cells ml⁻¹. Figure 1 shows the increase of sulfate in thermophilic cultures incubated for 1 day (exponential phase of growth) and 8 days (stationary phase of growth). Some of the thermophiles (i.e., strains 7, 10, and 12) presented an increase of soluble sulfate during their stationary phase of growth. Other thermophiles (strains 8, 9, and 11) showed no significant changes in the amount of sulfate released during the stationary phase of growth. These results suggested a production of soluble sulfate during the growing phase and, in addition, some strains were able to continue the release of sulfate to the medium during the stationary phase of the growth curve.

In order to verify the thermophily of the high temperature isolates from this work, the influence of temperature on growth rate was examined. The optimum growth rates for these isolates were between 50°C and 60°C. No growth was detected in cultures of these thermophilic isolates below 40°C and above 80°C.

Temperature had a significant influence on the release of soluble sulfate by thermophiles. Figure 2 showed the concentration of soluble sulfate in the cultures of the

isolated thermophiles at different temperatures. The release of sulfate was strain dependent. Strains 7 and 9 showed a significant increase in the concentration of soluble sulfate at 70°C about 10°C above the optimum for these two strains. No significant release of soluble sulfate was detected for strains 7 and 9 at 50°C. Strains 8 and 10 showed maximum production of soluble sulfate at 60°C, approximately at their optimum growth temperature. However, strain 10 showed a significant reduction of sulfate production at 50°C and 70°C and strain 8 maintained an elevated sulfate release at 70°C. Strain 11 showed no production of sulfate at 70°C and strain 12 showed maximum production of soluble sulfate at 50°C and poor to null sulfate released at 60°C and 70°C.

An intermediate of the oxidation of inorganic sulfur is thiosulfate (Kely et al. 1997; Madigan et al. 2003). Most bacteria carrying out inorganic sulfur-oxidation are able to further oxidize thiosulfate to sulfate. To test if the thermophilic isolates analyzed in this study were inorganic sulfur oxidizing bacteria, sulfate concentrations were monitored during growth with and without addition of thiosulfate. Results showed similar sulfate concentrations in supplemented and unsupplemented cultures (Fig. 3). The production of H₂S by the thermophilic isolates (which could be chemically oxidized to sulfate) was ruled out because none of these isolates were H₂S producers.

Environmental samples were incubated at 25°C and 50°C to determine if bacterial assemblages, rather than isolates, also resulted in similar effects on the concentrations of soluble sulfate. Figure 4A shows that higher concentrations of soluble sulfate were reached at 50°C than at 25°C. The concentration of sulfate produced at 50°C was from 2- to 5-fold the levels measured at 25°C.

The release of ammonium during growth of natural bacterial assemblages resulted in similar or higher concentrations at 50°C than at 25°C (Fig. 4B). The ammonium produced by communities of thermophiles was from non-significant differences up to over 2-fold the levels reached by the mesophilic assemblages.

To evaluate the influence of nutrient concentration on the release of sulfate and ammonium by thermophiles, two isolates were selected (strains 10 and 12; Table 1). In general, the lowest nutrient concentrations showed the lowest levels of sulfate and

ammonium (Fig. 5). Produced sulfate and ammonium were related to nutrient
availability (Fig. 5). The values of organic carbon (8 g organic nutrients L⁻¹) in the
growth experiments were close to the range of environmental estimates of organic
carbon in soils (from 10 to 200 g C Kg⁻¹) recently reported (Calderon et al. 2011;
Martínez-Lladó et al. 2011; Sleutel et al. 2011). Higher concentration of sulfate and
ammonium was measured during the stationary phase of growth (6 days incubation)
than during the strains exponential phase. The production of sulfate showed maximum
yield levels at 1.5x NB and a slight inhibition at higher nutrient loads (Figure 5A and
C). About the production of ammonium, similar results were observed for strain 10
(Figure 5B) although strain 12 showed a continuous increase of produced ammonium
for the evaluated range of nutrient concentrations (Figure 5D).

In environmental samples, the presence of active thermophiles of the studied group was
determined by quantitative RT-PCR. The relative fraction of RNA from these
thermophiles was detectable in the studied samples. A maximum proportion of
thermophilic Firmicutes RNA of 3.14% was detected in the studied bacterial
communities (Table 2). Fractions from 0.029% to 3.14% of thermophilic Firmicutes
RNA (within the studied genera) with respect to total bacterial RNA were measured in
different samples. Although different factors could be involved in the levels of RNA
from these thermophiles in natural environments, an exponential relationship was
observed between RNA of these thermophiles and soil temperature for most of the
studied communities. This corroborates a positive effect of temperature on the activity
of thermophilic Firmicutes. This relationship was represented by the equation ($r^2 = 0.97$;
 $n = 6$): $Y = 0.1115 \exp(0.053 T)$, where T is temperature (°C) and Y is the percentage of
RNA from the studied thermophilic Firmicutes with respect to total bacterial
community RNA. However, two samples showing the lowest thermophilic Firmicutes
RNA content appeared to present a parallel relationship, likely due to the effect of other
environmental factors inducing low levels of Bacillales RNA.

The possibility of reaching temperatures adequate for the growth of the analyzed
thermophiles in natural ecosystems was examined by measuring surface and 2 cm depth
temperatures along a 24 h period for a soil in Southwest Spain (Carmona, Seville,
Spain) (Fig. 6). These measurements were carried out during a mild summer day at the
studied location. Maximum air temperature was around 40°C during the early afternoon.

Temperatures of and above 40°C were measured in the soil for about 10 h a day.
Maximum temperature at this occasion was 62°C and 57°C at the soil surface and 2 cm
355 depth, respectively.

Discussion

360 The current concept of microbial communities in the environment highlights the
elevated diversity existing in natural systems (Curtis et al. 2002; Pedrós-Alió 2006).
According to our understanding of these communities, they are composed by low
number of highly abundant microbial taxa and an unimaginable high number of poorly
represented microbial phylotypes (Pedrós-Alió 2007). Thermophilic microorganisms
365 might be a group among those low abundant microbial taxa and they could play a role
in some temperate terrestrial, as well as arid, systems, at least, within a seasonal
periodicity.

At present, there are numerous reports on the cultivation, isolation, and classification of
370 thermophilic strains from temperate environments (Wiegel et al. 1997; Mutzel et al.
1996; Rahman et al. 2004). However, few studies have actually dealt with the presence,
biogeography, and potential impact of thermophiles in temperate environments.
Marchant et al. (2002) analyzing North Ireland soils detected culturable thermophiles in
every sampled site although their environmental role remained to be elucidated. Studies
375 by Huber et al. (Huber et al. 2009, 2010) reported on the stable presence of sulfate-
reducing thermophiles in cold arctic sediments. They could originate from nearby warm
petroleum reservoir systems (Huber et al. 2009). Our study confirms the existence of
culturable thermophiles in different Spanish soils. The cultured thermophiles were
classified as members of the order Bacillales (Firmicutes), specifically within the genera
380 *Bacillus*, *Brevibacillus*, *Geobacillus* and *Ureibacillus*, which is in agreement to the
strains of *Geobacillus* reported by Marchant et al. (2002) in Irish soils. The abundance
of culturable thermophiles was about one order of magnitude higher in the present study
than in a previous report (Marchant et al. 2001) which can be a consequence of the
lower temperatures in North Ireland soils (up to 15°C) (Marchant et al. 2002) than at
385 some of the soils in Southwestern Spain.

Environmental soil bacterial assemblages and different bacterial isolates were analyzed for soluble sulfate and ammonium production during growth. At the thermophilic temperature range higher soluble sulfate and similar to higher ammonium were produced than by mesophiles. The final concentrations of ammonium obtained in the experiments with thermophilic isolates were at similar levels than those reported for hyper-ammonia producing bacteria (Eschenlauer et al. 2002; Whitehead and Cotta 2004) suggesting a similar high potential for N mineralization. The origin of the sulfate and ammonium was the organic matter consumed during the metabolism of these thermophiles. Most heterotrophic sulfur-oxidizing bacteria are able to further oxidize thiosulfate to sulfate (Kelly et al. 1997; Madigan et al. 2003). Experiments of cultures supplemented with thiosulfate failed to show differences in soluble sulfate concentrations for different thermophilic strains. These results suggest that the isolated thermophiles did not produce sulfate oxidizing inorganic sulfur sources rather it represents a dissimilative organic-sulfur oxidizing process that mineralizes organically bound sulfur producing sulfate. The ammonium produced by the studied thermophiles is a result of the decomposition of organic matter (e.g., proteins) as reported for the heterotrophic ammonifying bacteria within the mesophiles (Eschenlauer et al. 2002; Whitehead and Cotta 2004).

Growth of the studied thermophiles under different concentrations of nutrients showed that soluble sulfate and ammonium was produced at higher concentrations as the availability of organic nutrients increased. As well, sulfate and ammonium production occurred both during the exponential and the stationary phase of growth suggesting that organic matter consumed for growth and the organic molecules processed during maintenance/survival stages lead to the release of the exceeding sulfur and nitrogen as sulfate and ammonium, respectively. The effect of temperature on sulfate and ammonium production by these thermophiles was strain dependent. Some strains produced higher sulfate at near their maximum growth temperature (i.e., 70°C), others achieved maximum sulfate concentrations at temperatures close to their optimum for growth (50°C and 60°C) and some were unable to reach detectable production of sulfate under high temperature stress conditions (i.e., 70°C). The above suggests that the diversity of these thermophiles could complement environmental requirements allowing changes in soil communities as a function of temperature and availability of organic nutrients during heated periods.

Sulfur and nitrogen are elements often limiting plant growth (Tabatabai 1984; Wen et al. 2003). Interestingly, most sulfur (>90%) in soils is bound to organic matter (Schlesinger 1997; Anandham et al. 2008). As well, a large fraction of nitrogen (>95%)
425 form part of biomolecules and humic acids (Gonzalez-Prieto and Carballas 1991; Madigan et al. 2003). Thus, the mineralization of sulfur and nitrogen represents major sources of inorganic nutrients for plant growth (Gruber and Galloway 2008; Schoenau and Malhi 2008). Previous studies carried out in the mesophilic temperate range (around 20°C)
430 have shown that bacterial mineralization of organic-sulfur as sulfate is poor in soils (Eriksen 1996; Ghani et al. 2001). As a result, the potential role of thermophiles as sulfur and nitrogen mineralizers during warm seasonal periods should be considered a pathway for the natural fertilization of soils, above all, in warm and arid environments.

Bacterial thermophiles were expected to only represent a minority within temperate soil
435 bacterial communities. Their presence as detectable metabolically active bacteria in the environment suggests that their mechanisms of persistence and participation in environmental processes should be considered. The detection of RNA from a specific bacterial group in complex communities have been reported to be a direct indicator of the occurrence of that microbial group as a metabolically active component of the
440 bacterial community (Molin and Givsov 1999; Portillo et al. 2009). In this study, the RNA of thermophiles within the Bacillales was targeted by quantitative RT-PCR. Results confirmed that these thermophiles represented detectable fractions of the bacterial RNA in soil communities, reaching up to 3.14% of total bacterial community. This relative quantification was analyzed as a function of sample temperature indicating
445 that the portion of thermophilic RNA in soil bacterial communities directly depends on temperature even if other factors can also influence the activity of these thermophiles. Although the analysis of the factors (besides temperature) affecting the level of RNA from thermophiles in soils needs to be studied in detail, we suggest that the availability of organic nutrients might significantly affect the metabolism of the group of
450 thermophilic microorganisms under analysis.

To assess if thermophiles could have a chance to develop in temperate terrestrial environments, it is important to evaluate whether these soils can reach the temperature range of growth for these thermophiles. Results presented in this study, as well as those

by Marchant et al. (2002), report no visible growth below 40°C. Whereas Marchant et al. (2002) reported that North Ireland soil temperatures reach at most 15°C, higher latitudes such as southern European countries surrounding the Mediterranean Sea, and areas at similar latitude, could easily present soil temperatures above 40°C during warm seasons. More extreme environments such as arid and desert locations have reported even higher temperatures. For instance, temperatures above 50°C are common in arid regions of Africa and America (McCalley and Sparks 2009). Mojave Desert mid-day soil temperatures are >65°C and can exceed 90°C (McCalley and Sparks 2009). These results confirm that soil temperatures at a wide range of latitudes can be within the temperature range required for the reported thermophiles to grow.

It is known that spore-forming bacteria (as is the case for many Bacillales) can survive for extremely long periods. However, previous discussion (Marchant et al. 2002, 2008) as well as the RNA results presented in this study confirm that at least a fraction of these thermophiles actually exist as vegetative forms. The possibility of very slow but continuous growth of thermophiles at low temperatures has been recently proposed (Marchant et al. 2008) and can account for the maintenance of viable thermophiles in cold temperate environments (Jones and Lennon 2010). In cold temperate environments, such as low latitude locations, these survival strategies (Marchant et al. 2008; Jones and Lennon 2010) offer an explanation to the ubiquitous presence of thermophiles (Boyd and Boyd 1962; Wiegell et al. 1979; Rahman et al. 2004). This mechanism of survival and the detection of these thermophiles as metabolically active cells within the bacterial communities indicate that the thermophiles under study show vegetative stages and metabolic activity even at temperatures lower than their typical range for growth. Thus, thermophiles inhabiting low-medium latitude and medium-high latitude locations might be showing different survival strategies. Those at the cold temperate sites could base their survival on the slow, long term, growth alternative. However, thermophiles at warmer places could have the chance to grow at higher rates at least during short time periods annually. Under both scenarios, thermophiles might likely be adapted to thrive under adverse environments (the dominating low temperatures) waiting until adequate conditions (i.e., temperature increase) permit their normal development allowing them to compete with, or partially replace, mesophiles. The occurrence of minority groups, such as the thermophiles, within the rare biosphere (Huber et al. 2010; Jones and Lennon 2010; Pedrós-Alió 2007) could represent a

microbial seed bank able to redraw the community if a change of the environmental
490 conditions arises.

An extensive debate can be maintained about the ultimate origin of the thermophiles,
mainly Firmicutes, in temperate environments. Some results point to the existence of
both short and long distance dispersal from hot environments by fluids, such as wind or
495 water currents (Kellogg and Griffin 2006; Portillo and Gonzalez 2008; Huber et al.
2009; Perfumo and Marchant 2010). Current studies on the dispersal of particles from
the Sahara Desert to European and America (Kellogg and Griffin 2006; Hervás et al.
2009; Perfumo and Marchant 2010) represent a serious support for this theory. The
possibility of an onsite speciation and adaptation to exploit specific spatial and temporal
500 niches as a result of a long period of evolution could also be argued in some cases, such
as this study. Independently of the actual mechanisms that allowed thermophiles to
reach these environments and in agreement to these two hypothesis and previous reports
(Marchant et al. 2002), different lines of evidence support that, at present, these
thermophiles represent endemic taxa and established minorities within bacterial
505 communities in temperate environments. The potential for thermophiles to incorporate
into the mineralization of organic matter and the sulfur and nitrogen cycling during
warm seasons (i.e., under a seasonal periodicity) is proposed.

Because temperature is a decisive factor for the proposed role of thermophiles in
510 temperate terrestrial environments, the expected increase in periodicity of extreme heat
events and average temperature due to the on-going global climate change (IPCC 2007;
Battisti and Naylor 2009) points towards an enhancement of the actual significance of
thermophiles in temperate environments. An increase of mean annual temperatures will
imply an increasing frequency of extreme heat events and high temperature conditions
515 during summer periods (Melillo et al. 2002; Battisti and Naylor 2009) which should
favor conditions for the development of thermophiles and their metabolic activity.
Besides, there is a major lack of knowledge on the potential consequences of climate
change on the environment (Melillo et al. 2002; Lobell and Burke 2008), and
specifically on microbial communities and their implications on the ecosystems.
520 Thermophilic Firmicutes could represent model microorganisms that can be identified
to evaluate future global warming changes on microbial communities.

Conclusions

Results presented in this study suggest that thermophiles could represent an important fraction of the microbial communities that should be considered to fully understand the functioning of temperate terrestrial environments. These thermophiles could actively participate in promoting organic matter mineralization producing sulfate and ammonium during warm periods. In this scenario, thermophiles could have a niche and opportunity to actively participate in sulfur and nitrogen cycling from organic matter in temperate soil environments during summer periods when mesophiles would be either dormant or under maintenance/survival stages of their living cycle.

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Figure legends

- 705 **Figure 1.** Sulfate concentrations during the growth of mesophilic (strain numbers 1 to 6) and thermophilic (strain numbers 7 to 12) isolates. Results correspond to samples at exponential phase (1 day; white bars) and stationary phase (8 days; grey bars) of growth.
- 710 **Figure 2.** Effect of temperature on the production of soluble sulfate by thermophilic isolates (strain numbers 7 to 12) at 50°C (grey bars), 60°C (black bars) and 70°C (white bars).
- 715 **Figure 3.** Effect of the addition of thiosulfate to the cultures of thermophilic isolates (strain numbers 7 to 12). Cultures with (grey bars) and without (white bars) supplemented thiosulfate were compared at their stationary phases of growth (8 days).
- 720 **Figure 4.** Soluble sulfate (A) and ammonium (B) concentration produced by natural bacterial soil assemblages incubated at 25°C and 50°C from three different Spanish locations: soil sample 1, Southwest (Carmona, Seville); soil sample 2, Northeastern pine forest (Aragon); soil sample 3A, Northeastern soil partially covered by weeds (Aragon); soil sample 3B, outlying area of Seville.
- 725 **Figure 5.** Sulfate (A and C) and ammonium (B and D) produced during the growth of thermophilic strain numbers 10 (A and B) and 12 (C and D) as a function of nutrient load (nutrient broth concentration) after 1 (white squares) and 6 (black squares) days of incubation at 50°C.
- 730 **Figure 6.** Environmental measurements of temperature at surface (black squares), 2 cm below surface (grey squares), and air (white triangles) during a summer day in an Andalusian soil (Seville, Spain).

Table 1. List of mesophilic and thermophilic isolates obtained during this study, their taxonomic classification, accession number for their 16S rRNA gene sequences, similarity to closest homologue, sequence length, identification numbers, temperature of incubation during isolation, and optimum growth temperature for the thermophilic isolates. Strains numbers 10 and 12 show their accession numbers at the Spanish Type Culture Collection (CECT).

Strain number	Accession number	Incubation temperature	Taxonomic affiliation Genus (Phylum)	Closest homologue species (Acc. No.)	Similarity %	bp	Optimum Growth Temperature
1	HQ730480	25°C	<i>Paenibacillus</i> (Firmicutes)	<i>P. lautus</i> (EU834247)	97	863	
2	HQ730481	25°C	<i>Klebsiella</i> (Gammaproteobacteria)	<i>K. oxytoca</i> (GQ496665)	100	855	
3	HQ730482	25°C	<i>Cellulosimicrobium</i> (Actinobacteria)	<i>C. cellulans</i> (GQ496666)	100	804	
4	HQ730483	25°C	<i>Streptomyces</i> (Actinobacteria)	<i>S. variabilis</i> (EU741200)	100	815	
5	HQ730484	25°C	<i>Neocosmospora</i> (Fungi)	<i>N. vasinfecta</i> (AB302212)	94	558	
6	HQ730479	25°C	<i>Enterobacter</i> (Gammaproteobacteria)	<i>E. hormaechei</i> (HQ238703)	99	757	
7	HQ730487	50°C	<i>Bacillus</i> (Firmicutes)	<i>B. thermoamylovorans</i> (FN397520)	100	966	55-60°C
8	HQ730488	50°C	<i>Geobacillus</i> (Firmicutes)	<i>G. kaustophilus</i> (JN036603)	99	650	60°C
9	HQ730489	50°C	<i>Geobacillus</i> (Firmicutes)	<i>G. thermoleovorans</i> (EU214614)	99	782	60°C
10; CECT7629	HQ730490	50°C	<i>Brevibacillus</i> (Firmicutes)	<i>B. thermoruber</i> (DQ011668)	99	903	50-60°C
11	HQ730485	50°C	<i>Geobacillus</i> (Firmicutes)	<i>G. thermoleovorans</i> (EU214614)	99	645	60°C
12; CECT7628	HQ730486	50°C	<i>Ureibacillus</i> (Firmicutes)	<i>U. thermosphaericus</i> (AF403019)	97	656	50°C

Table 2. Relative RNA analysis by quantitative RT-PCR targeting thermophilic Firmicutes of the studied genera (thermophilic *Bacillus*, *Brevibacillus* and *Ureibacillus*, and *Geobacillus*) in samples collected at different temperatures from several Spanish locations.

Sample ¹	Temperature (°C)	Primer set	Percentage of bacterial RNA ²
Pine forest, Aragon (NE)	38	GeobacF-GeobacR	0.730 (0.143)
Soil partially covered by weeds, Aragon (NE)	40	GeobacF-GeobacR	0.029 (0.001)
Carmona, Seville (SW)	58	GeobacF-GeobacR	0.134 (0.022)
Outlying area, Seville (SW)	62	GeobacF-GeobacR	3.140 (0.494)
Outlying area, Seville (SW)	22	Geo485F, B-U485F, 518R	0.330 (0.033)
Outlying area, Seville (SW)	44	Geo485F, B-U485F, 518R	1.060 (0.068)
Outlying area, Seville (SW)	31	Geo485F, B-U485F, 518R	0.691 (0.042)
Outlying area, Seville (SW)	34	Geo485F, B-U485F, 518R	0.728 (0.016)

¹ The location area within Spain is indicated between brackets. NE, Northeast; SW, Southwest.

² Percentage of RNA corresponding to thermophilic Firmicutes (thermophilic Bacillales) with respect to total bacterial RNA in the natural communities. SD between brackets.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6