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RESEARCH ARTICLE

Links between sulphur oxidation and sulphur-oxidising bacteria abundance and diversity in soil microcosms based on *soxB* functional gene analysis

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Abstract

Sulphur-oxidising bacteria (SOB) play a key role in the biogeochemical cycling of sulphur in soil ecosystems. However, the ecology of SOB is poorly understood, and there is little knowledge about the taxa capable of sulphur oxidation, their distribution, habitat preferences and ecophysiology. Furthermore, as yet there are no conclusive links between SOB community size or structure and rates of sulphur oxidation. We have developed a molecular approach based on primer design targeting the *soxB* functional gene of nonfilamentous chemolithotrophic SOB that allows assessment of both abundance and diversity. Cloning and sequencing revealed considerable diversity of known *soxB* genotypes from agricultural soils and also evidence for previously undescribed taxa. In a microcosm experiment, abundance of *soxB* genes increased with sulphur oxidation rate in soils amended with elemental sulphur. Addition of elemental sulphur to soil had a significant effect in the *soxB* gene diversity, with the chemolithotrophic *Thiobacillus*-like *Betaproteobacteria* sequences dominating clone libraries 6 days after sulphur application. Using culture-independent methodology, the study provides evidence for links between abundance and diversity of SOB and sulphur oxidation. The methodology provides a new tool for investigation of the ecology and role of SOB in soil sulphur biogeochemistry.

Introduction

Sulphur (S) oxidation and reduction are biogeochemical processes central to the energy metabolism of a phylogenetically diverse range of microorganisms across many habitats. In some ecosystems, such as acid mine drainage, hydrothermal vents, sediments and sulphide-rich metallomorphic systems, S-based reactions provide the primary energy source supporting microbial communities and allow increasingly complex ecosystems to establish (Baker & Banfield, 2003; Chivian *et al.*, 2008; Pedersen *et al.*, 2010; Burton *et al.*, 2011). The evolution of chemoautotrophic iron–sulphur metabolism is thought to have originated in ecosystems typical of those present on early Earth (Wächtershäuser, 2006) and likely supported the development of life based on energy-gaining processes other than S-cycling.

In functionally complex soil ecosystems, biogeochemical processes associated with cycling of C and N are the major energy flow systems. Sulphur, although considered a 'minor element', is vital for life, and biogeochemistry of S can affect ecosystem productivity. This is often seen in naturally sulphur-deficient soils, where addition of S-fertiliser can dramatically increase primary production (Bunemann & Condron, 2007) and affect rates of processes of coupled biogeochemical cycles such as C, N and P cycling (McGill & Cole, 1981; Chapman, 1997; Kirkby *et al.*, 2011). In soil, sulphur exists in oxidation states ranging from −2 to + 6 in both organic and inorganic forms (Bunemann & Condron, 2007). By far the largest pool is organic S (Banerjee & Chapman, 1996), which must undergo mineralisation to inorganic S to become biologically available for microbial and plant uptake (mainly as sulphate). Alleviation of S deficiency in soils

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and increasing the S content of the organic pool is often achieved through addition of elemental S-fertiliser, which again must be oxidised by microorganisms before becoming plant available. Despite the importance of sulphur cycling for soil fertility and primary productivity across many terrestrial ecosystems, little is known about the microbial taxa involved in the process, nor their wider ecology in supporting ecosystem function.

Oxidation of reduced inorganic sulphur compounds (ISC; including sulphide, sulphur, sulphite and thiosulphate) to sulphate is driven by an extensive range of photo- and chemolithotrophic sulphur-oxidising prokaryotes, occupying very diverse, extreme and moderate environments and having evolved different pathways for sulphur oxidation (review by Ghosh & Dam, 2009). For sulphur-oxidising bacteria (SOB), at least three different biochemical pathways have been described. These include the *Paracoccus* sulphur oxidation pathway for which a complete and functional Sox enzyme system has been described, mainly associated with the facultative chemolithotrophic *Alphaproteobacteria*; the branched thiosulphate oxidation pathway, involving the interaction of a truncated Sox and reverse dissimilatory sulphite reductase (rDSR) enzyme systems, characteristic of photolithotrophic sulphur oxidisers; and the tetrathionate intermediate (S4I) pathway typical for obligate chemolithotrophic *Thiobacillus* and *Acidithiobacillus* spp. (Friedrich *et al.*, 2005; Hensen *et al.*, 2006; Grimm *et al.*, 2008; Ghosh *et al.*, 2011; Gregersen *et al.*, 2011). Advances in detecting and understanding the role of microorganisms in sulphur oxidation in marine habitats (Meyer & Kuever, 2007; Loy *et al.*, 2009; Lenk *et al.*, 2011, 2012), in freshwater and marine sediments (Pham *et al.*, 2008), in sulphide removing bioreactors (Luo *et al.*, 2011), in hypersaline and soda lakes (Tourova *et al.*, 2013) and in terrestrial sulphidic springs (Headd & Engel, 2013) have occurred using functional genes involved in sulphur oxidation biochemical pathways as molecular markers. However, functional gene tools developed to date are specific to groups of sulphur-oxidising taxa present in habitats that differ considerably from agricultural soils, and their potential application for soil is limited.

The aim of this study was to develop a functional gene method, to allow quantification and characterisation of sulphur-oxidising bacteria in response to elemental sulphur application in soil ecosystems and particularly agricultural soils. We selected the *soxB* gene, encoding the SoxB subunit of the Sox enzyme system, which is considered a fundamental and primordial molecular mechanism for sulphur oxidation (Friedrich *et al.*, 2001; Meyer *et al.*, 2007; Ghosh & Dam, 2009). *soxB* gene homologues have been detected in representatives of the major bacterial phyla containing sulphur oxidisers irrespective of the pathway they are using for sulphur oxidation (Friedrich *et al.*, 2001, 2005; Petri *et al.*, 2001; Meyer *et al.*, 2007). In addition, the *soxB* gene phylogeny of 121 phylogenetically and physiologically divergent SOB strains is largely congruent to the 16S rRNA gene-based phylogenetic lineages (Meyer *et al.*, 2007). This allowed the development of primers focusing on detection of *soxB* gene of nonfilamentous chemolithotrophic SOB and excluding lineages not typically found in soil. In a soil microcosm experiment, oxidation of elemental sulphur was accompanied with increases in the *soxB* gene abundance, an increase mostly associated with a specific group from among the diverse *soxB* gene pool detected in the soils, the obligate chemolithotrophic *Thiobacillus*-like *Betaproteobacteria*. The PCR-based method opens up new opportunities to study the biogeochemistry of soil sulphur cycling and also for understanding the ecology of sulphur-oxidising bacteria in soil providing new insights into this functionally significant group of soil microbiota.

Materials and methods

Soil collection and analysis

Soils were collected from under livestock-grazed pasture at three locations in New Zealand; Leslie Hills (LH), Dacre (D) and Southbridge (SB). The locations, grazing systems and soil types are given in Supporting Information, Table S1. Samples were collected to 10 cm depth, sieved to 2 mm and stored at 4 °C until use. The physicochemical properties of each soil were assessed on a dried fraction; analysis was conducted by Hill Laboratories (NZ), and a description of the methods is available at http://www.hill-laboratories.com/file/fileid/15530.

Soil sulphur oxidation

For each of the three soils (LH, D and SB), four soil microcosms were used to assess kinetics of elemental sulphur oxidation. Soils were treated with and without elemental sulphur at 1 mg S g^{-1} dry wt. soil, and assays were carried out in duplicate. The amount of sulphur added was selected to be comparable to applications reported for laboratory soil incubation experiments that are usually in the range of 0.5–2.5 mg S g^{-1} soil (Dick & Deng, 1991; Pepper & Gerba, 2004). Each microcosm consisted of 50 g of soil in a 250-mL Erlenmeyer flask, and these were incubated for 30 days at 25 °C. Over the course of the incubation, the soil water content, *c.* 60% of MWHC, was maintained stable through routine application of water to original mass. Subsamples of soil, 4 g for later chemical analysis (sampled at days 1, 6, 12, 21 and 30) and *c.* 1 g for later DNA extractions (sampled at

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days 1, 6, 21 and 30) were stored at −20 and −80 °C respectively. Sulphate concentrations were measured using a modification of the protocol described by Pepper and Gerba (2004). Briefly, 10 mL of 0.1 M NaCl was added to 4 g of soil, and samples were shaken for 30 min. Activated charcoal was added to each sample (0.1 mL of a 20% (w/v) charcoal suspension) and shaking resumed for 3 min. After settling for 10 min, the suspension was filtered through a syringe filter (0.45 μM; 30 mm diameter; nylon, Thermo fisher), and 1 mL transferred to a clean tube followed by addition of 0.2 mL of 0.5% (w/v) gum Arabic and 0.3 mL of 1 M $BaCl_2$ solution. Tubes were vigorously shaken, and an aliquot (0.2 mL) was transferred into a 96-well microtitre plate for turbidity measurements at 470 nm using a microtitre plate photometer (Versa max, Molecular devices). A standard curve was prepared using eight serial 1 : 2 dilutions of a stock solution of 32 mM K_2SO_4 (concentrations from 16 to 0.125 mM). One millilitre from each dilution was transferred into a clean tube and was treated the same way as the samples.

For soils LH and D, the trend in sulphate production (SO_4) over time (x) was modelled using an exponential decay curve fitted in GenStat 14. The curve is given by ($SO_4 = a + b\, r^x$) where a denotes the asymptote, b the amplitude and r the rate of exponential change. Here $0 < r < 1$, and the smaller r the faster the asymptote is reached. Fitting a curve was not possible for the SB soil as the sulphate production did not reach asymptote during the incubation period.

DNA from 0.25 g soil samples was extracted using the PowerSoil DNA isolation kit (MO BIO Laboratories Inc.) according to manufacturer's instructions, and quantification was performed using a spectrophotometer (NanoDrop 2000c, Thermo fisher). Extracted DNA was stored at −20 °C.

Primers for *soxB* gene abundance and diversity estimates in soil

A total of 143 *soxB* gene sequences of described SOB were downloaded from NCBI and aligned using CLUSTALW. The alignment was used to design several primer pairs using primer3 (Rozen & Skaletsky, 2000) to be tested in real-time PCR assays. Primer selection criteria included as follows: amplicon size of *c.* 200 bp, but not > 600 bp; length of 17–22 bp; melting temperature of 57–65 °C; mole % G + C content of 40–80%; and absence of predicted secondary structure. No primer sites conserved across all lineages of sulphur-oxidising bacteria could be found, as such the alignment of reference sequences was progressively refined focusing on those sequences typical for the soil habitat, primarily the nonfilamentous chemolithotrophic sulphur oxidisers. All *soxB* gene sequences of photolithotrophic sulphur oxidisers (including green and purple sulphur bacteria and *Roseobacter* clade bacteria) were excluded. In addition, we excluded the sequences of *Aquificae*, *Deinococcus-Thermus* and *Spirochaeta* phyla, including organisms associated with either marine or extremophilic environments and also sequences of the filamentous sulphur bacteria associated with sulphide-rich marine and freshwater environments. All *Alpha*-, *Beta*- and *Gammaproteobacteria* *soxB* gene sequences of nonfilamentous chemolithotrophic sulphur oxidisers were included for primer design with the exception of the gammaproteobacterial *Thermithiobacillus* and *Halothiobacillus soxB* gene sequences because of low sequence similarity of this group with the rest of the sequences and because these organisms are found in environments not typical of most agricultural soils. A total of 18 primers were designed, and combinations evaluated for efficacy.

The primer pair 710F/1184R, generating a 511-bp fragment (including primer sites), was selected for qPCR assays. The alignment of these primers against sequences of *Alpha*-, *Beta*- and *Gammaproteobacteria* is presented in Table S2. DNA extracted from *Thiobacillus thioparus* DSM-505, a typical soil sulphur-oxidising bacterium, was used to create standards for absolute quantification of *soxB* genes in real-time PCR assays. The strain was obtained from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ, Germany) and was grown in DSM Medium # 36 according to the DSMZ instructions (http://www.dsmz.de). Based on the *soxB* gene sequence of this strain available from GenBank (accession AJ294326), primers were designed (forward: 5′-GGATACTTGGCAGGGTTCG-3′ and reverse: 5′-GTAATAGGGGTCGGCGTTG-3′) that amplified a 1009-bp fragment, which was subsequently cloned into the pCR4-TOPO vector (Invitrogen). Plasmid DNA was isolated (Plasmid Mini Kit, Bioline, UK), linearised in restriction digests using the NotI restriction enzyme (Fermentas, Thermo fisher) and quantified by comparison with DNA standards using agarose gel electrophoresis and spectrophotometrically using a NanoDrop spectrophotometer. The number of copies of *soxB* ng^{-1} plasmid DNA was calculated using the molecular weight of the plasmid + insert and Avogadro's constant; eight serial dilutions of this DNA (ranging from 10^8 down to 10 copies) were used to create a standard curve (Fig. S1).

Quantitative PCR of *soxB* gene was conducted on DNA samples extracted from the soil microcosm experiment. Amplifications were performed in Mastercycler ep realplex (Eppendorf, Germany). Each reaction was conducted in 20 μL volume containing 10 μL SensiFAST SYBR mix (Bioline), 0.7 μM of each primer (710F and 1184R), 0.2 mg mL^{-1} BSA (Fermentas, Thermo fisher) and 15 ng

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of template DNA. Amplification conditions included an initial denaturation step for 3 min at 95 °C followed by 35 cycles of denaturation for 5 s at 95 °C, annealing for 10 s at 55 °C and extension for 20 s at 72 °C. Melting curve analysis (Fig. S1) was performed at the end of all qPCR runs to indicate amplification of specific products only, before confirmation by standard agarose gel electrophoresis. Efficiency of real-time PCR assays was 91% and r^2 was 0.99. All qPCR data presented were from independent extractions from duplicate microcosms and duplicate independent PCR amplifications.

Cloning and sequencing of soil *soxB* genes

Four clone libraries from two soils (LH and D) amended with elemental sulphur were constructed for samples collected after 1 and 6 days of incubation. The four clone library names start with the soil origin (LH or D), followed by S1 (sulphur-amended microcosm, replicate1), followed by the time of sampling (D1 or D6 for sampling after 1 and 6 days of incubation, respectively) and followed by the number of clone. To construct the libraries, end-point PCR products of *soxB* genes were generated using the primer pair 710F/1184R. The reaction mixture (25 μL) consisted of 0.2 U Taq polymerase (MyTaq, Bioline), 1x reaction buffer, 0.5 μM of forward and reverse primer, and 0.2 mg mL^{-1} BSA. Amplification conditions included an initial denaturation step for 2 min at 95 °C followed by 35 cycles of denaturation for 15 s at 95 °C, annealing for 15 s at 55 °C and extension for 30 s at 72 °C, followed by a final extension step for 5 min at 72 °C. PCR products were purified and cloned into the pCR4-TOPO vector (Invitrogen). Clones were PCR-amplified using the primers M13 forward and reverse according to standard procedures. Purification of PCR products and sequencing using the vector primer T7 were carried out by Macrogen Inc. (Seoul, Korea). Sequence data were supplied as chromatographs, and manual refinement was carried out using Sequencer 4.1 (Genes Codes Corp., Ann Arbor, MI).

Phylogenetic analysis

The placement of *soxB* gene sequences into an evolutionary tree was conducted using the maximum likelihood method based on the JTT matrix-based model (Jones *et al.*, 1992). The analysis involved 126 amino acid sequences, with the coding data translated assuming a standard genetic code. There were a total of 174 positions in the final data set. Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log-likelihood value. The tree with the highest log likelihood is shown. Bootstrap support (100 replicates) above 45 is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

Nucleotide sequence accession numbers

The *soxB* gene clone sequences reported in this study have been deposited in GenBank under accession numbers KC332936–KC333036. The *soxB* gene sequence of *T. thioparus* NZ strain (included in the presented phylogenetic analysis) and the 16S rRNA gene of the same strain have also been deposited in GenBank under accession numbers KC542801 and KC542803, respectively. This strain was previously isolated from two different agricultural soils.

Results

Primers for *soxB* gene abundance and diversity estimates in soil

A total of 18 primers were designed and 34 primer combinations were tested using DNA extracted from *T. thioparus* DSM-505 (data not shown). Six primer combinations gave positive results and were further evaluated using DNA extracted from two different types of agricultural soil (Fig. 1). The primer pair 710F/1184R was the most sensitive for both *T. thioparus* and soil extracts and was subsequently used for real-time PCR assays (amplification efficiency of 91%) and cloning/sequencing. Details of all primers evaluated in Fig. 1 are described in Table 1. All pairs of primers were searched against the NCBI nucleotide collection (nt) database; all were highly specific to the *soxB* gene. Alignment of primers 710F and 1184R with the respective region of the *soxB* gene of various *Alpha-*, *Beta-* and *Gammaproteobacteria* is shown in Table S2.

Sequences of *soxB* gene were downloaded from the NCBI nucleotide database after searching with the query 'soxB[Title] AND bacteria[ORGN]'. This yielded 1556 results. To test primers 710F and 1184R for specificity within metagenome data sets, the SRR606448 and ERR059353 data sets were downloaded from the NCBI short read archive. The metagenomic reads were searched against the NCBI *soxB* nucleotide sequences using BLAST. Only seven and three sequences from data sets SRR606448 and ERR059353, respectively, contained matches (e-value cut-off of 1E-20) to the 1556 NCBI *soxB* nucleotide sequences. Of these, only two sequences from

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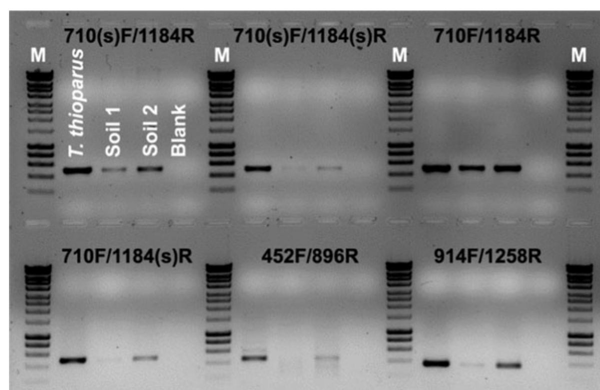

Fig. 1. Agarose gel electrophoresis of PCR products generated from six different primer combinations. For each primer combination, four samples are included; DNA from *Thiobacillus thioparus*, DNA from two different types of agricultural soil and negative control. Marker (M) is included in between the different primer combinations. The primer pair 710F/1184R was subsequently used for real-time PCR assays and cloning/sequencing.

each data set matched any of the primer. The primers were searched against the 1556 NCBI *soxB* nucleotide sequences using FUZZNUC (Rice *et al.*, 2000), allowing for a maximum mismatch of one nucleotide over the length of the primer (when the primer sequences were searched against the 101 *soxB* sequences recovered in this study, primers matched the sequences with a maximum mismatch of one nucleotide over the length of the primer). Both primers matched 187 of the 1556 NCBI *soxB* sequences (53 of the 153 annotated sequences) encompassing a wide range of *Alpha*-, *Beta*- and *Gammaproteobacteria* believed to be present in soils. These results demonstrate high level of primer specificity especially because no non-*soxB* gene regions were identified when the *soxB* primers were compared with the wider metagenome. It should be noted that even within a modest 454-generated soil genome, the total numbers of *soxB* genes are likely to be relatively low, and this finding was borne out by the analysis conducted.

Soil sulphur oxidation

For all soils, sulphate concentration in the control microcosms was negligible but increased through time in microcosms amended with elemental sulphur (Fig. 2). The kinetics of sulphur oxidation varied considerably between the three soils. By fitting exponential curves to the data for soils from LH and D, the rate (r) and the asymptote (a) were calculated (Table S1). This was not possible for the soil from SB, as the asymptote was not reached and sulphate production kept increasing throughout the experiment. Soil from LH had the highest S-oxidation rate with substantial sulphate production after only 6 days of incubation. However, it had a lower asymptote compared with soil D, which was reached approximately after 12 days of incubation. Sulphur oxidation in soil from SB was generally slower, and substantial increases in sulphate production occurred after a lag phase of 6 days. After that time, sulphate concentration kept increasing and asymptote was not reached during the incubation period.

Quantification of soil *soxB* genes

The mean *soxB* gene copy number in the control microcosms, estimated at the beginning of the experiment (day 1), varied among the different soils: LH soil had 7.9×10^7 *soxB* gene copies g^{-1} dry soil, while soil D and SB had approximately an order of magnitude greater copy numbers (9.2×10^8 and 9.4×10^8, respectively). In the microcosms with no addition of S, no significant

Table 1. Position, sequence and other characteristics of primers used in this study

Primer	Position*	Sequence (5′–3′)	Length	Melting temperature (average)	% GC (average)	Self matches	Number of bases that could form a hairpin
452F	436–452	TGGCAGGGYTCVGCVAC	17	56.3	69.6	3	0
710(S)F	694–710	GGYCAGGCYTTYCCSTA	17	61.3	61.8	4	0
710F†	691–710	ATCGGYCAGGCYTTYCCSTA	19	57.7	57.5	3	0
914F	896–914	TCGGBGGNCAYACSCACGA	18	61.9	66.7	3	3
896R	896–914	TCGTGSGTRTGNCCVCCGA	18	61.9	66.7	4	3
1184R†	1184–1202	MAVGTGCCGTTGAARTTGC	18	61.23	52.6	3	2
1184(S)R	1184–1201	AVGTGCCGTTGAARTTGC	18	50.3	50.9	3	0
1258R	1258–1277	CCCCARCGGAANCCSGGNGA	18	67.0	72.5	4	4

*Positions are according to *soxB* gene of *Thiobacillus denitrificans* ATCC 25259 (GenBank: CP000116.1; after removing the first 90 bp) (Beller *et al.*, 2006).

†Primers used for subsequent qPCR and cloning/sequencing analysis.

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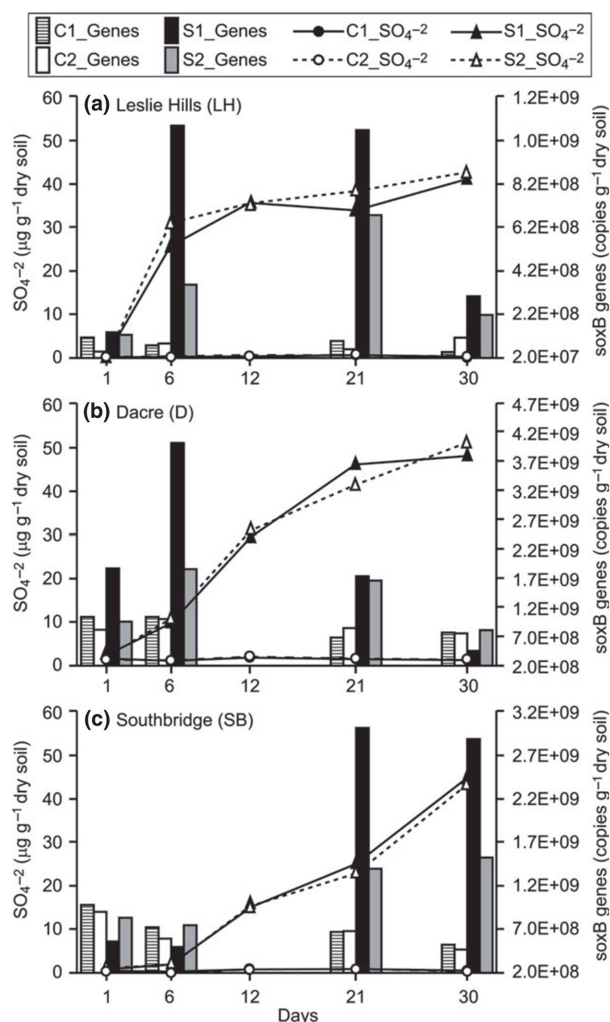

Fig. 2. Relationship between sulphate production in duplicate soil microcosms amended with elemental sulphur (S1_SO_4^{-2} and S2_SO_4^{-2}) or not (C1_SO_4^{-2} and C2_SO_4^{-2}), and *soxB* gene abundance in soil microcosms amended with elemental sulphur (S1_Genes and S2_Genes) or not (C1_Genes and C2_Genes) during incubation for 30 days. Experiment was performed for three different types of soil, (a, b and c) for soils LH, D and SB, respectively.

change in *soxB* gene copy numbers was observed across the three soils and for the duration of the incubation (Fig. 2). In the sulphur-amended microcosms, the *soxB* gene copy number increased for all soils. For soils from LH and D, increases were observed early in the experiment (6 days of incubation), and this was concomitant with a substantial increase in sulphate production (Fig. 2). After 21 days of incubation, gene copy numbers decreased in the D soil, and this decrease was also observed in the LH soil after 30 days of incubation, by which time sulphate production had plateaued (Fig. 2). An increase in gene copies in the SB soil was only observed after 21 days of incubation, again coupled with substantial sulphate production.

Diversity of *soxB* genes from soils

Observation of the melting (dissociation) curves following the qPCR assays indicated a dramatic shift in the diversity (based on GC content differences) of the *soxB* genes in soil microcosms in which S-oxidation was occurring. To investigate further, clone libraries from the LH and D soils (sulphur-amended microcosms) were constructed at day 1 and after 6 days of incubation, the later coinciding with increases in *soxB* gene abundance and active sulphur oxidation as assessed by increases in sulphate production. A total of 101 clone sequences were generated and all had homology to *soxB* genes, confirming absolute specificity of primers to the gene of interest. Phylogenetic analysis of the *soxB* gene clone sequences alongside those of described bacteria (available from NCBI) revealed substantial diversity of *soxB* genes amplified using the new primer set (Fig. 3). Within the *Betaproteobacteria*, 29 clones are affiliated with the *Thiobacillus*-like cluster, including *T. thioparus* and other well-characterised sulphur chemolithotrophs. Within both Alpha- and *Betaproteobacteria*, a substantial number of clones (at least 27 clones within *Alphaproteobacteria* and 12 clones within *Betaproteobacteria*) were not closely related to bacteria currently described or available on NCBI databases. Phylogenetic analysis presented in Fig. 3 includes several compressed clusters including closely related clone sequences; the expanded phylogenetic analysis is presented in Fig. S2. Although phylogenetic analysis was performed using an *c.* 500-bp fragment of the *soxB* gene, phylogenetic clusters are in accordance with those obtained by Meyer *et al.* (2007) using full-length *soxB* gene sequences.

The distribution of clones within the four clone libraries (Fig. 4) reveals a significant shift in the structure of the *soxB* genotypes present in soil after 6 days of incubation in the presence of elemental sulphur. In both soils at day 1, all *soxB* clone sequences (49 in total) were affiliated with *Alpha*- and *Betaproteobacteria* and no *Thiobacillus*-like *Betaproteobacteria* were detected. However, at day 6 and when sulphate production was substantial, *Thiobacillus*-like *Betaproteobacteria soxB* gene clone sequences were detected, and in both soils, they dominated the clone libraries. The expanded phylogeny of the *Thiobacillus*-like group within *Betaproteobacteria* (Fig. S2) indicates that different soils may harbour different *Thiobacillus*-like populations. The LH soil is dominated by a strain closely related to *T. plumbophilus,* while in soil D, there is greater overall diversity but clones affiliated to *T. thioparus* predominate.

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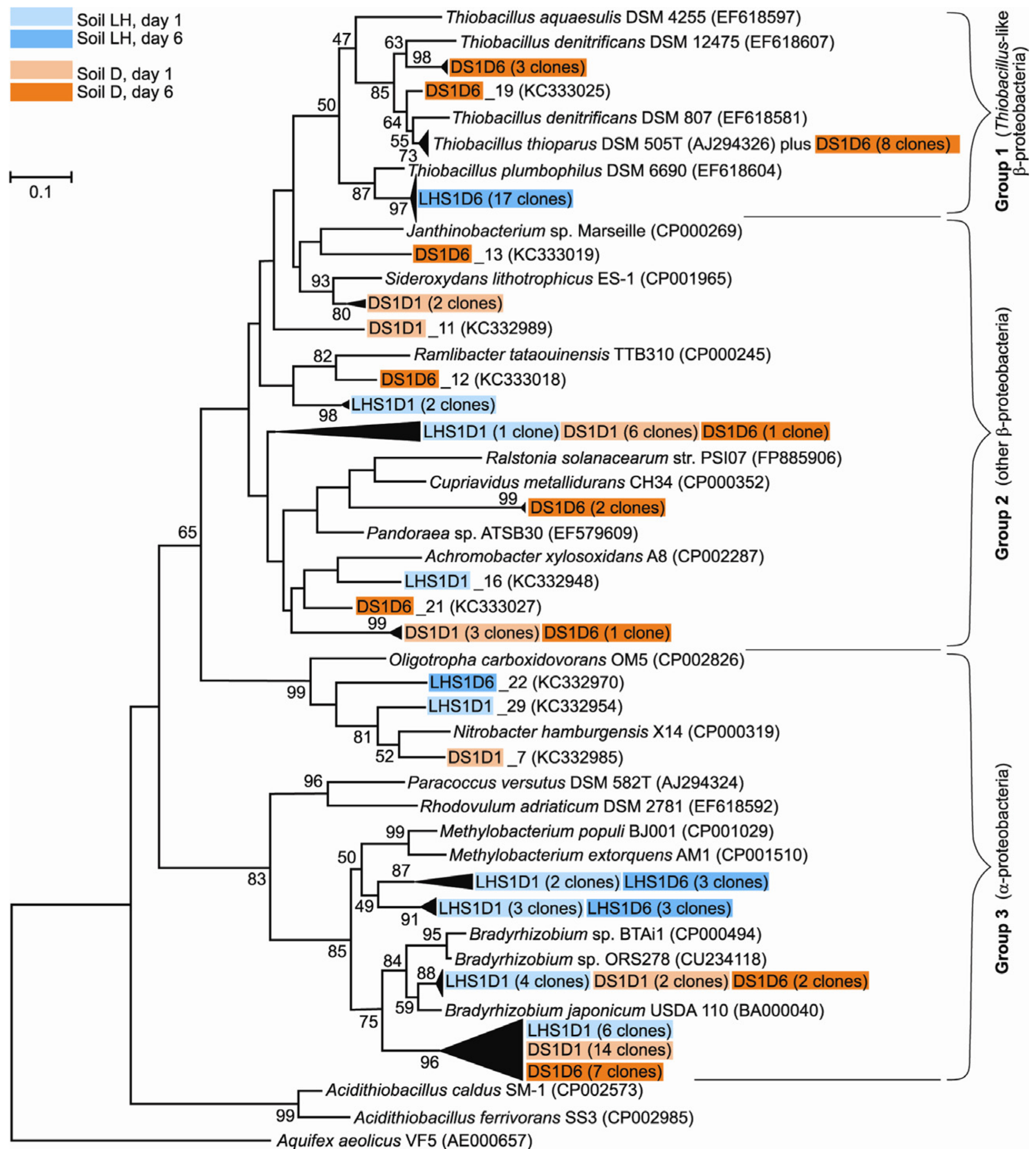

Fig. 3. Phylogenetic analysis of 101 clone sequences recovered from four clone libraries plus SoxB sequences of described SOB downloaded from NCBI (a total of 126 SoxB amino acid sequences). The four clone library names (shown in four different colours) include the number of the soil (LH or D), followed by S1 (sulphur amended microcosm, replicate1), followed by the time of sampling (D1 or D6 for sampling after 1 and 6 days of incubation respectively), followed by the number of clone (when individual clones are presented). The coding data were translated assuming a Standard genetic code table. There were a total of 174 positions in the final data set. Bootstrap support (100 replicates) above 45 is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree was rooted with the sequence of *Aquifex aeolicus*. *Acidithiobacillus* strains belong to *Gammaproteobacteria*. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

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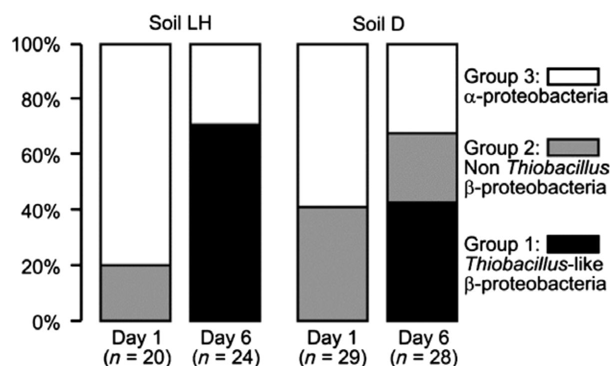

Fig. 4. Distribution of clones into groups within each library. For each soil (LH and D), two libraries were constructed after 1 and 6 days of incubation in the presence of elemental sulphur. Number of clones recovered from each library (*n*) is shown in the graph. The three groups (as shown in phylogenetic analysis of Fig. 3) include the *Alphaproteobacteria* (white bars), the *Betaproteobacteria* (grey bars) and the *Thiobacillus*-like *Betaproteobacteria* (black bars).

Discussion

Oxidation of reduced ISC to sulphate is a crucial step regulating fertility and primary productivity in many terrestrial ecosystems. This biogeochemical process is carried out by SOB; however, the ecology of these bacteria in soils is poorly understood as there is a lack of suitable molecular markers to specifically detect these bacteria. For other ecosystems, functional gene-based methods have been used to provide insights into the structure of SOB, such as use of *aprA* and *dsrAB* genes in marine environments (Meyer & Kuever, 2007; Loy *et al.*, 2009; Lenk *et al.*, 2011, 2012), *sqr* gene detection in freshwater and marine sediments (Pham *et al.*, 2008), and *soxB* gene for targeting taxa present in hypersaline lakes (Tourova *et al.*, 2013) and terrestrial sulphidic springs (Headd & Engel, 2013). More recently, real-time PCR assays have been developed and applied for a better understanding of factors affecting community size or activity in environments such as mangrove sediments and corals (Bourne *et al.*, 2013; Varon-Lopez *et al.*, 2014). However, these tools are specific to clades of sulphur-oxidising taxa that differ to those most abundant in the soil environment. In this study, we have developed *soxB*-specific primers allowing for assessment of both abundance (qPCR) and diversity (cloning and sequencing) of SOB in soil. This new tool for functional microbial ecology will allow investigations into the dynamics of particular metabolic types of SOB and associated S-biogeochemistry in terrestrial ecosystems.

The development of molecular methods to specifically detect SOB is challenging, particularly as the SOX enzyme system is not associated with a monophyletic group of bacteria or archaea, but rather present across a wide range of taxa harbouring various genotypes (Meyer *et al.*, 2007). The first *soxB* primers reported in the literature (Petri *et al.*, 2001) were designed based on only a few *soxB* gene sequences available at that time. The primers opened the way for studying SOB and were used by Meyer *et al.* (2007) who demonstrated congruence between *soxB* and 16S rRNA gene phylogenies for 121 strains, representative of the major bacterial phyla containing sulphur oxidisers. However, the primers were highly degenerated and included three different primer pairs because not every primer pair worked for every SOB strain. When these primer pairs were applied to DNA directly sourced from environmental samples, amplification of *soxB* genes was not always possible, the efficacy of each primer pair was different and the composition of clone libraries differed considerably (Luo *et al.*, 2011; Tourova *et al.*, 2013). These findings are not surprising considering the taxonomic and physiological diversity of sulphur oxidisers.

Given the strong level of congruence between *soxB* and 16S rRNA gene phylogenies, we adopted a phylogenetic-based approach to progressively exclude from our *soxB* gene sequence alignments, *soxB* lineages not typically associated with soil ecosystems, based on species descriptions. The goal was to develop primers that would detect as many taxa as possible. The final alignment included all *Alpha*-, *Beta*- and *Gammaproteobacteria soxB* gene sequences of nonfilamentous, chemolithotrophic sulphur-oxidising bacteria. The alignment did not include *Gammaproteobacteria*, *Thermithiobacillus* and *Halothiobacillus soxB* gene sequences as they had low sequence similarity to the majority of other sequences, and because these organisms are found in environmental conditions not typical of most agricultural soils. Although primer development focused on taxa predominant in soil ecosystems, they can certainly be used in other environments for the detection of the nonfilamentous chemolithotrophic sulphur-oxidising bacteria. As more soil-associated *soxB* gene sequences are recovered, we anticipate that the primers will be refined providing extended coverage or modified (in terms of degeneracies) favouring *Halothiobacillus* or other genera depending on the environment under investigation. It should be noted that we have been conservative in the primer design including few degeneracies because of concerns that increasing degeneracies could decrease efficiency of real-time PCR assays. However, taking into account the reasonably high efficiency of the real-time PCR assays (91%) and high specificity of the primers (all amplicons were *soxB* gene specific), we believe sensitivity of the primers could be increased by replacing the Y (C or T) degeneracy in the 6th and 12th position of the forward primer (Table S2) by B degeneracy (C or G or T) without sacrificing efficiency. We anticipate that ongoing use of these primers will result in

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evolution of primer design and open new insights into S-oxidising bacteria in soil, and perhaps other, ecosystems.

Concomitant increases in sulphur oxidation and *soxB* gene abundance were observed in three agricultural soils amended with elemental S. After 6 days of incubation, at a point when sulphate production was substantial, an increase in *soxB* gene numbers was observed in soils LH and D followed by a substantial decrease after 30 and 21 days, for soils LH and D, respectively. This drop in the *soxB* gene number was not observed for soil SB, but sulphur oxidation was slower in this soil and sulphate concentration kept increasing within the incubation period. The drop in *soxB* gene number as soon as, or soon after, sulphate concentration reached the asymptote may be due to inhibition of SOB due to a substantial decrease in soil pH (Table S1).

Using culture-independent methodology, a relationship between abundance and function for sulphur oxidisers in soil has been demonstrated. In this experiment, because of limited number of time points when increases in both sulphate production and *soxB* gene abundance occurred, regression analysis between the two lacked statistical power to provide useful insights on the data trends. Future work, closely monitoring *soxB* gene abundance and sulphate production over time, will enable this relationship to be fully determined. This may be further expanded to the assessment of expression of *soxB* genes within the soil metatranscriptome, allowing for more sensitive assessment of environmental factors controlling community size, regulating activity and how these interact to affecting sulphur oxidation rates in soil. In addition, *soxB* gene expression studies could help explain the observed difference between the soils in lag time for S-oxidation and accompanied *soxB* gene numbers. Soil LH had the lowest *soxB* gene copy number but still the highest rate of sulphur oxidation, and soil SB had the highest gene number, but sulphate production was characterised by a long lag phase. Further studies are needed to assess how good a predictor of S-oxidation the overall *soxB* gene abundance could be, and if expression of *soxB* genes or abundance of *soxB* genes of specific groups could better predict S-oxidation.

Elemental sulphur application had a dramatic effect in the diversity of *soxB* genotypes, resulting in dominance of specific taxa during sulphur oxidation. At the time of S-application, all *soxB* sequences were affiliated among the *Alpha-* and *Betaproteobacteria* with no *Thiobacillus*-like *Betaproteobacteria* detected. However, 6 days after S-application, and when sulphate production was actively progressing, gene sequences affiliated to *Thiobacillus* genus dominated the clone libraries. This indicates that *Thiobacillus*-like bacteria are most likely responsible for the sulphur oxidation of the inorganic elemental sulphur applied to these soils; however, these bacteria constituted a relatively low proportion of the naturally occurring sulphur-oxidising community. Furthermore, the soils tested harboured different *Thiobacillus*-like strains providing evidence for habitat selection (ecophysiological variation among strains) or biogeographic separation of community composition. In soil from Leslie Hills (LH), strains closely related to *T. plumbophilus*, an acidophilic organism originally isolated from a uranium-contaminated soil (Drobner *et al.*, 1992), were dominant. The overall richness of *soxB* genes from the Dacre soil (D) was greater, but clones affiliated to the neutrophilic *T. thioparus* strain dominated. This may further explain the rapid drop in *soxB* gene numbers observed for soil D as soon as sulphate production was reaching stationary phase (e.g. these organisms could be more sensitive to the drop in the pH due to sulphate accumulation). Across both soils, many *soxB* gene sequences did not cluster with known SOB. These either represent taxa not yet known to contain *soxB* genes, or as yet unknown microbial clades. Despite the groupings shown in Fig 3, these may not belong to *Alpha-* and *Betaproteobacteria*, as lateral *soxB* gene transfer events have been reported (Meyer *et al.*, 2007). Regardless, these represent new taxa most likely capable of sulphur oxidation.

The majority of the nonfilamentous chemolithotrophic SOB belong to the *Proteobacteria* phylum and were traditionally known as Thiobacilli. Phylogenetic analysis based on the 16S rRNA gene (Kelly & Wood, 2000; Boden *et al.*, 2012) resulted in major renaming and restructuring of Thiobacilli. Within *Gammaproteobacteria*, three phylogenetically distinct clades became the *Acidithiobacillus*, *Halothiobacillus* and *Thermithiobacillus* genera including organisms with respective physiologies; within *Betaproteobacteria*, the genus *Thiobacillus* was maintained including obligate chemolithotrophs; *Alphaproteobacteria* sulphur oxidisers are mainly facultative autotrophic and sometimes mixotrophic or chemolithoheterotrophic. Based on competition experiments between obligate and facultative sulphur chemolithotrophs in chemostats, Kuenen and Beudeker (1982) constructed a hypothetical model predicting the occurrence of chemolithotrophic sulphur bacteria in freshwater environments in relation to the ratio of ISC to organic substrates (OS). During simultaneous limitation or ISC and OS, growth of facultative chemolithotrophs will be most successful. Obligate chemolithotrophs will thrive in environments where the ratio of reduced ISC is high relative to that of organic compounds; vice versa, facultative chemolithotrophs will flourish under conditions of a high ratio of OS relative to that of ISC. In addition, obligate chemolithotrophs are very resistant to starvation periods maintaining their

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autotrophic ability and being able to respond very quickly to the appearance of a sulphur compound. Although in different environments including soils, conditions other than the energy source may be growth-limiting, our results are in accordance with this model. Addition of elemental sulphur resulted in quick response and dominance of the obligate chemolithotrophic *Thiobacillus*-like sulphur oxidisers. It would be interesting to explore this model further and to follow dynamics of SOB populations in soil under different substrates and different ratios of ISC to OC.

No *soxB* gene sequences associated with the *Acidithiobacillus* genus were detected in our libraries; this was unexpected as *Acidithiobacillus* has been reported in soil receiving flowers of sulphur, composts of soils and acidic soil adjacent to sulphur stockpiles (Ghosh & Dam, 2009). Further research using the current primers, or with further modifications to closely target these organisms, could elucidate their role, if any, in agricultural and other soil ecosystems. In addition, experiments testing the primers against representative isolates would enable confirmation as to whether the primers can amplify *soxB* genes from taxa identified to have the greatest amount of mismatches as shown in Table S2.

Conclusion

In this study, we show links between abundance and diversity of SOB and S-oxidation rates in soils. This was achieved using our novel molecular approach based on design of primers for both quantitative and qualitative characterisation of SOB in soil ecosystems, targeting the *soxB* functional gene, a component of the Sox enzyme molecular mechanism for S-oxidation. Oxidation of elemental S in soil microcosms was accompanied with increases in the *soxB* gene abundance, an increase mostly associated with a specific group, from among the diverse *soxB* gene pool detected in the soil, the obligate chemolithotrophic *Thiobacillus*-like *Betaproteobacteria*. The *soxB* functional gene-based method opens up new opportunities to study the ecology of SOB and their role in biogeochemistry of soil S-cycling (e.g. species involved, factors determining their activity, responses to environmental change, niche partitioning, variation according to soil type or use) which in turn will enable a better understanding of processes affecting ecosystem function.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Origin and physicochemical and other properties of soils used in this study.

Table S2. Alignment of primers 710F and 1184R (the reverse complement is presented; rc) with the respective regions of *soxB* gene sequences of various sulphur-oxidising, *Alpha*-, *Beta*- and *Gammaproteobacteria*.

Fig. S1. (a) Standard curve, created using a dilution series with 10^8–10 copies, generated by the real-time

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PCR software; (b) melting curve profile of standards and blank sample (blue line) generated by the real-time PCR software; (c) melting curve profile of a subsample of soil samples.

Fig. S2. Detailed phylogenetic tree with expanded clusters of (a) *Betaproteobacteria* and (b) *Alphaproteobacteria* SoxB amino acid sequences.

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