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Differential acquisition of amino acid and peptide enantiomers within the soil microbial community and its implications for carbon and nitrogen cycling in soil

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ABSTRACT

L-isomeric amino acids and oligopeptides are thought to represent a key nitrogen (N) source for plants and soil microorganisms, bypassing the need to take up inorganic N, whilst self-cycling of D-enantiomers within peptidoglycan-containing bacteria may provide a further short circuit within the N cycle. Here we use stable isotope profiling (SIP) to identify the fate of organic N within soil microbial communities. We followed the incorporation of ^{13}C-labelled D- or L-labelled amino acids/peptides into phospholipid fatty acids (PLFAs). L-alanine and its peptides were taken up more rapidly than D-enantiomers by Gram-positive bacteria with ^{13}C incorporation being predominantly into anteiso- and iso-fatty acids typically associated with Gram-positive bacteria. D-enantiomer uptake was found not to differ significantly between the microbial groups, providing little support for the view that soil bacteria may self-cycle D-forms of amino acids and peptides. There was no consistent association between peptide chain length and incorporation. The concentrations of L- and D-isomeric amino acids in soil solution were 866 nM and 72 nM, respectively. We conclude that Gram-positive bacteria appear to be the primary competitors for L-enantiomeric forms of amino acids and their peptides, but that both D- and L-enantiomers are available N and C sources for bacteria and fungi.

1. Introduction

Polar regions present unique challenges to life, with organisms experiencing low temperatures and limited bio-availability of nutrients, in particular nitrogen (N) (Atkin, 1998). Direct inputs of N are primarily from excreted animal waste and dead soil organic matter (SOM) in the form of proteins, urea and uric acid, although in regions away from animal and bird colonies, protein is the predominant N input (Erskine et al., 1998). However, slow decomposition rates owing to low temperatures and limited water availability in polar regions frequently result in low availability of inorganic N (Nadelhoffer et al., 1992; Atkin, 1998). The ability to access alternative sources of N such as amino acids and peptides, located further up the decomposition chain, would confer a competitive advantage over organisms reliant on inorganic sources, in essence short circuiting the N cycle (Hill et al., 2011a). It has been found that L-amino acids are a key component of this cycle, with non-mycorrhizal plants such as arctic sedge, wheat and other Arctic plants found to be capable of utilizing this organic N source (Chapin et al., 1993; Näsholm et al., 2009; Hill et al., 2011c), as well as the identification of several amino acid membrane transporters in roots (Tegeder and Rentsch, 2010). Furthermore, it is now thought that the primary competition point for N is higher up the SOM decomposition chain (i.e. at the point at which oligopeptides are created from proteins), with the identification of several plant peptide transporters, and the confirmation of L-peptide uptake by both plants and micro-organisms (Miranda et al., 2003; Komarova et al., 2008; Tegeder and Rentch, 2010; Hill et al., 2011a, 2011b; Farrell et al., 2013). D-enantiomers of amino acids are another source of N that has been shown to be utilized by both plants and micro-organisms (Hopkins et al., 1997; O'Dowd and Hopkins, 1998; Hill et al., 2011b, 2011c, 2012). Short peptides of D-amino acids are also utilized by micro-organisms, but do not appear to be available as an N source for plants (Hill et al., 2011b, 2011c, 2012).

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All soils investigated appear to have microbial communities that are able to rapidly take up and assimilate both L- and D-amino acids and their short peptides (Jones et al., 2009; Hill et al., 2011b, 2012; Farrell et al., 2013; Wilkinson et al., 2014). There does appear to be some soil specificity in mineralization rates, and microbial communities in soils from the maritime Antarctic appear to vary most in their capacity to metabolise D-peptides (Hill et al., 2011b; Wilkinson et al., 2014). However, current evidence has not attributed this variation to either the size or the composition of the soil microbial community (Hill et al., 2011b). Although D-amino acids may arise from the abiotic racemisation of L-amino acids (Schroeder and Bada, 1976; Amelung et al., 2006), a large proportion enter the soil as short peptides from the peptidoglycan of bacterial cell walls (Schleifer and Kandler, 1972; Holtje, 1998). As D-isomers are prevalent within the bacterial cell wall (Roberts et al., 2009), and can contain D-peptides up to three amino acids in length (Schleifer and Kandler, 1972), it may provide bacteria with an exclusive, self-cycling mechanism that excludes competition by fungi and plants. This source of N may be a significant pool, with bacteria being shown to be capable of assimilating exogenous sources of D-amino acid and incorporating them into peptidoglycan (Lam et al., 2009), as well as demonstrating pathways for their conversion into more metabolically available forms (Yonaha et al., 1975; Inagaki et al., 1986; Martinez-Rodriguez et al., 2010).

The aim of this study was to ascertain the functional role of an Antarctic soil microbial community in the uptake of amino acids and oligopeptides. The influence of amino acid chain length and enantiomeric form, and the subsequent incorporation of these C and N sources as ^{13}C into phospholipid fatty acids (PLFAs), were studied. We tested whether bacteria exclusively use D-enantiomers, and whether other short circuits in the N cycle exist through the uptake of di- and tri-peptides by soil microbes. PLFAs were utilized because of their rapid rate of turnover (Tunlid and White, 1992), which excludes dead biomass, and their ability to identify broad changes in the microbial population through characteristic fatty acids (Frostegård et al., 1993). Alanine was selected as the amino acid because both of its enantiomers are found regularly within Antarctic soil, and the D-form is a predominant peptidoglycan amino acid (Roberts et al., 2009). D-alanine di-peptides are found regularly within the bacterial cell wall, with D-amino acid trimers having been identified in *Butyribacterium rettgeri* and *Corynebacterium* spp. (Schleifer and Kandler, 1972). Although Antarctic bacterial soil community composition has been previously studied using DNA-based methods (e.g. Yergeau et al., 2007a), this, to our knowledge, is the first time that a PLFA profiling method has been coupled with stable isotope tracer methodology to study Antarctic soils. This combination allows for a more detailed evaluation of the fate of organic nitrogen in soil, as well as a validation of previous microbial community estimates of Signy Island.

2. Materials and methods

2.1. Soils and stable isotope labelling

Soils for use in the study were sampled (*c.* 0–8 cm depth) from replicate experimental areas (0.5 m × 0.5 m; $n = 4$) at Deschampsia Point on Signy Island in the South Orkney Islands (60.43°S, 45.38°W) (Collins et al., 1975; Smith, 1984). The soils were frozen within 2 h of collection at −20 °C, to which soil temperatures decline on the island during winter (Walton, 1982), prior to transfer to the UK at this temperature. Vegetation at or immediately adjacent to the sampling point was dominated by the angiosperm *Deschampsia antarctica* Desv., and the bryophytes *Sanionia uncinata* (Hedw.) Loeske and *Chorisodontium aciphyllum* (Hook. f. & Wilson) Broth. Soils had a water content (determined by drying at 80 °C) of 1.1 ± 0.04 g g^{-1} DW, and C and N contents (determined by elemental analyser; Carlo Erba, Wigan, UK) of 196 ± 4 and 17 ± 0.6 mg g^{-1} DW (mean ± SEM; $n = 3$), respectively.

The four replicate soil samples were first thawed at 4 °C overnight, at rates similar to those recorded in soils on Signy Island (Holdgate et al., 1967), and were then split into eight 2 g (FW) sub-samples. The sub-samples from each of the four soils were then each labelled with one of eight peptides, consisting of 2 ml of 1 mM uniformly ^{13}C-labelled (98 atom %) L-mono-, L-di-, L-tri-, L-tetra or L-pentaalanine or D-mono-, D-di- or D-tri-alanine solutions (L-enantiomers from CK Gas Products, Hook, UK; D-enantiomers from Sigma–Aldrich, Poole, UK). Control samples were prepared in the same manner, except that 2 ml of doubly distilled water was added to a sub-sample from each of the four soils in place of the peptide solutions. The 36 sub-samples of soils were incubated at room temperature (20 °C) for 1 h and were then frozen at −20 °C and freeze-dried overnight.

2.2. PLFA extraction and derivatization

Lipids for ^{13}C analysis were extracted from the freeze-dried soils using a modified Bligh and Dyer extraction, using glassware that had been heated in a muffle furnace at 450 °C for 4 h. Freeze dried soil (*c.* 1 g) was extracted using 3 ml of a mono-phasic solvent mixture (4:5:10 v/v/v) of potassium dihydrogen phosphate buffer (0.05 M, pH 7.2), chloroform and methanol. The samples were sonicated for 15 min, centrifuged (2200 *g*) for 5 min, and the supernatant decanted into 50 ml glass tubes. The mono-phasic extraction was carried out twice more, with the supernatant fractions being pooled. The organic and aqueous phases were partitioned by the addition of 2 ml of potassium dihydrogen phosphate buffer (0.05 M, pH 7.2) and 2 ml of chloroform. The bi-phasic mixture was vigorously mixed, centrifuged (2200 *g*) for 5 min, and the lower organic phase removed and placed in fresh glass tubes. The aqueous phase was washed twice with 2 ml of chloroform, which was pooled with the organic phase. The organic phase was dried under a stream of N_2.

Individual phospholipid classes were separated using Discovery DSC-Si solid phase extraction (SPE) columns (Sigma–Aldrich, Poole, UK). Columns were conditioned following the manufacturer's instructions. The total lipid extract was re-dissolved in 1 ml of chloroform, and was then applied to the SPE column. The column was washed with 4 ml of chloroform, 12.5 ml of acetone, and the bound phospholipids were eluted using 4.5 ml of methanol. The phospholipid fraction was dried down under a stream of N_2.

Phospholipid bound fatty acids were trans-esterified using acid catalysed anhydrous methanol (Christie, 1989). The dried phospholipid fraction was dissolved in 5 ml of anhydrous HCl in methanol (5% v/v) and heated at 50 °C for 2 h. Once cool, 5 ml of NaCl solution (5% w/v) followed by 1 ml of n-hexane was added, and the solution was vigorously mixed. The mixture was briefly centrifuged, and the upper layer of the bi-phasic mixture was separated into a clean tube. A further two aliquots of n-hexane were added, mixed and separated, then pooled. The hexane was evaporated under a gentle stream of N_2. The samples were re-suspended in 100 μl of n-hexane and transferred to 2 ml vials for analysis. Lipids for the PLFA profiling of soils prior to peptide spiking were extracted, separated and derivatized according to Buyer and Sasser (2012).

2.3. GC-MS and GC-C-IRMS analysis

Identification of individual PLFAs was carried out using gas chromatography mass spectrometry (GC-MS) using an Agilent Technologies 5973 Mass Selective Detector (electron impact

ionisation 70 eV, scan mode) coupled to an Agilent Technologies 6890 GC fitted with a CP-SIL 5CB fused silica capillary column (50 m × 0.32 mm i.d. × 0.25 μm). The temperature program was an initial isothermal step at 50 °C for 5 min, followed by a ramp to 150 °C at 10 °C min^{-1}, which was increased to 270 °C at a rate of 3 °C min^{-1} and finally, 320 °C at a rate of 20 °C min^{-1}.

$\delta^{13}C$ values of individual PLFAs were analysed using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Compounds were separated using an Agilent Technologies 6890 series gas chromatograph (splitless mode) fitted with a CP-SIL 5CB column (50 m × 0.32 mm × 0.2 μm). The temperature program was held isothermally at 50 °C for 5 min and was then ramped to 150 °C at 10 °C min^{-1}, followed by an increase to 270 °C at 3 °C min^{-1} and then to 340 °C at 20 °C min^{-1}, with the final temperature being held for a further 5 min. Helium was used as the carrier gas. The GC effluent was diverted via a heart split union to a ceramic combustion furnace (650 mm × 0.3 mm i.d.) packed with a copper oxide/platinum/nichrome catalyst wire heated to 940 °C. Water was subsequently removed from the combustion products by passing the effluent through a nafion membrane, prior to the CO_2 entering the isotope ratio mass spectrometer (Isoprime Ltd). The PLFA $\delta^{13}C$ values were corrected for the addition of the extra C atom introduced to the molecule during methylation, using a correction factor obtained by CF-EA-IRMS measurement on the derivatizing methanol and the application of the mass balance equation of Jones et al. (1991). Following the recommendations of Ellert and Rock (2008), $\delta^{13}C$ values for both samples and blanks were converted to atom% using the following formula for each fatty acid:

$$\text{atom\%} = 100 \times [R_{\text{standard}} \times (1 + (\delta/1000))]/ [1 + R_{\text{standard}} + (R_{\text{standard}} \times \delta/1000)]$$

where R_{standard} is equal to 0.0112372, the $^{13}C/^{12}C$ ratio of V-PDB. The averaged background atom% values for each fatty acid component in the blanks were subtracted from fatty acid atom% values within the samples, to give the atom percent excess (APE) using the following equation:

$$\text{APE} = \left(F_{\text{postdose}} - F_{\text{blank}}\right)$$

where F_{postdose} is the individual fatty acid atom% in the sample, and F_{blank} is the averaged background atom% for each fatty acid found within the blanks (Ellert and Rock, 2008).

2.4. HPLC enantiomeric amino acid analysis

Soil solution was extracted from soils under vegetation dominated by *D. antarctica* and *S. uncinata* using Rhizon soil solution samplers (Rhizosphere Research Products, Wageningen, Netherlands) at three locations on Signy Island. Samples from Berntsen Point (60°42.4′ S, 45°35.6′ W), North Point (60°40.4′ S, 45°37.50′ W) and Foca Cove (60°41.8′ S, 45°38.9′ W) were pooled to give sample volumes of c. 20 ml ($n = 3$), and were concentrated by freeze drying. The dry soil solution residues were re-suspended in 500 μl of 0.01 M HCl with 1.875 pmol μl^{-1} of L-homoarginine as the internal standard. Following the method of Bruckner et al. (1998), samples were filtered through 0.2 μm regenerated cellulose filters (Whatman), and 8 μl of sample was automatically derivatized using the autosampler (Ultimate 3000, Dionex). Briefly, 8 μl of 0.25 M borate buffer (pH 10.4) was mixed with 8 μl of sample. Following this, 1 μl of a 260 mM N-isobutyryl-L-cysteine and 112 mM o-phthaldialdehyde in 10% (v/v) methanol and 0.25 M borate buffer was added to the mixture. Finally, 2 μl of 1 M acetic acid was added and mixed before the reaction mixture was injected onto the column. The Hypersil ODS column (250 × 4.0 mm, 5 μm, Thermo Scientific) and the Hypersil ODS guard column (10 × 4 mm, 3 μm, Thermo Scientific) were kept at 30 °C throughout the run. Elution solvent A comprised of 17.4 mM sodium acetate trihydrate buffer, pH adjusted to 6.05 using 1 M acetic acid. Elution solvent B was methanol:acetonitrile (12:1 v/v). The initial composition of the eluants was 95% A and 5% B. The concentration of solvent B was increased linearly to 20.5% over 30 min, followed by an increase to 21.0% at 32 min, then to 53.5% at 92 min, then decreased to 5% by 93 min, with the flow rate set at 1 ml min^{-1}. The fluorescence excitation wavelength was set at 230 nm and the emission wavelength was set at 445 nm. The detector sensitivity was set to 7 with a detector response time of 0.2 s. Amino acids were quantified using the internal standard, using response factors calculated from individual amino acid standards (Sigma, UK). The HPLC system used was an Ultimate 3000 system (Dionex). The system control and data handling software used was Chromeleon 6.8.

2.5. Statistical analyses

The main and interactive effects of enantiomeric form and peptide length on ^{13}C incorporation into PLFAs were determined using general linear models and oneway ANOVA with LSD post-hoc test in MINITAB v15 (Minitab Inc., State College, PA). Because we did not label soils with D-tetra- and D-pentaalanine, it was not possible to achieve a balanced design suitable for ANOVA, and so the measurements for ^{13}C incorporation from L-tetra- and L-pentaalanine were excluded from these analyses. Pearson correlations were also used to determine whether or not there were associations between ^{13}C incorporation and peptide length. These correlative analyses included incorporation data from L-tetraalanine and L-pentaalanine. In order to test whether or not there were differences in ^{13}C incorporation between Gram-positive bacteria, Gram-negative bacteria and fungi, class specific PLFAs APE values were averaged across the L-peptides ($n = 12$, excluding tetra- and pentapeptides) and D-peptides ($n = 12$). The averaged class specific PLFAs used were i15:0, a15:0, i16:0, a17:0 and i17:0 (indicative of Gram-positive bacteria), 16:1 ω7, 16:1 ω5, 17:0 cyclo, 18:1 ω7 and 19:0 cyclo (indicative of Gram-negative bacteria) and 18:2 ω6 (indicative of fungi; Leckie, 2005; Ruess and Chamberlain, 2010).

3. Results

3.1. Incorporation of ^{13}C into microbial PLFAs

ANOVA indicated significant effects of enantiomeric form on the incorporation of ^{13}C from alanine into the PLFAs i15:0, a15:0, i16:0, a17:0, i17:0, 18:1 ω9, 18:1 ω7 and 18:0 (Table 1). In each case, there was more uptake of the L-form of alanine and its peptides into each PLFA, compared with the D-form (Fig. 1). Considering peptide length as a variable independent from enantiomeric form, the averaged L- and D- monomer to trimer showed a significant effect for 18:1 ω9 (Table 1), with the averaged L- and D-dipeptide showing the greatest incorporation. There was also a marginally significant effect of length on the uptake of 18:2 ω6 (Table 1), with the dipeptide showing the greatest incorporation (data not shown). Significant effects of peptide length on other PLFAs were not recorded (Table 1). Pearson correlations calculated for L-enantiomers only, paired with ANOVA, indicated a significant positive association between peptide length and the incorporation of ^{13}C into C18:1 ω9 ($r^2 = 0.65$; $P < 0.05$). No other associations between peptide length and ^{13}C incorporation were found.

Table 1
F ratios from general linear models testing for the main and interactive effects of enantiomeric form (D- or L-isomers) and peptide chain length (mono- to trimer) on the incorporation of ^{13}C-labelled alanine into 17 phospholipid fatty acids (PLFAs). Significant effects are denoted by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Error d.f. in all analyses were 18. There were no significant enantiomeric form × length interactions.

PLFA	Enantiomeric form	Length
i15:0	19.17***	0.05
a15:0	6.80*	0.17
i16:0	12.85**	2.70
16:1 ω7	0.05	0.13
16:1 ω5	0.01	0.93
16:0	0.11	1.57
17:0, 7 me	0.00	0.29
17:0 br	1.16	1.12
a17:0	55.67***	0.29
i17:0	15.11**	1.78
17:0 cyclo	1.80	1.79
17:0	0.15	3.05
18:2 ω6	1.48	2.99[a]
18:1 ω9	4.91*	5.06*
18:1 ω7	5.51*	1.06
18:0	8.40*	1.18
19:0 cyclo	0.09	2.12

[a] Significant at $P < 0.10$.

3.2. Preference for different N forms within the microbial community

PLFA profiles of soils prior to labelling with ^{13}C peptides (Fig. 2) indicated a microbial (bacteria and fungi) community dominated by bacteria, with Gram-negative and Gram-positive bacteria comprising 65.9% and 32.5% of the community, respectively. Fungi formed 1.6% of the community (Fig. 2). Analysis of the mean incorporation of ^{13}C into the PLFAs indicative of Gram-positive bacteria, Gram-negative bacteria and fungi showed that there was significantly more incorporation into fatty acids representing Gram-positive bacteria than into those representing the other two groups of microbe ($F_{2,66} = 15.64$; $P < 0.001$; Fig. 3). There was also a significant effect of enantiomeric form on the incorporation of ^{13}C into these PLFAs ($F_{1,66} = 15.99$; $P < 0.001$), with the L-form being incorporated to a greater extent into PLFAs than the D-form (Fig. 3). There was also a significant interactive effect of micro-organism group and enantiomeric form on ^{13}C incorporation ($F_{2,66} = 7.42$; $P = 0.001$), with more ($P < 0.001$) incorporation of ^{13}C from the L-forms of alanine into PLFAs indicative of Gram-positive bacteria than from the L- or D- forms of alanine into PLFAs indicative of Gram-negative bacteria or fungi. Similarly, total recovery of ^{13}C supplied as L-enantiomers in PLFA markers for Gram-positive

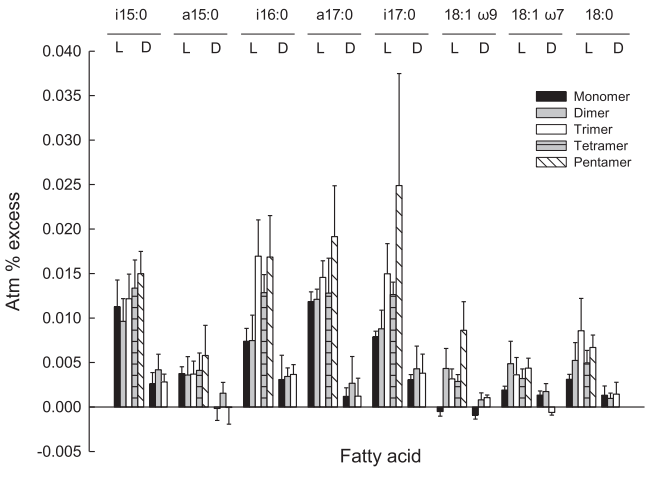

Fig. 1. Incorporation of ^{13}C from L- and D-enantiomers of alanine into eight PLFAs. Values shown, which are means ± SEM ($n = 4$), are atom percent excesses. See Table 1 for the main effects of enantiomeric form and peptide length on ^{13}C incorporation from D- and L-mono-, di- and trialanine into the PLFAs.

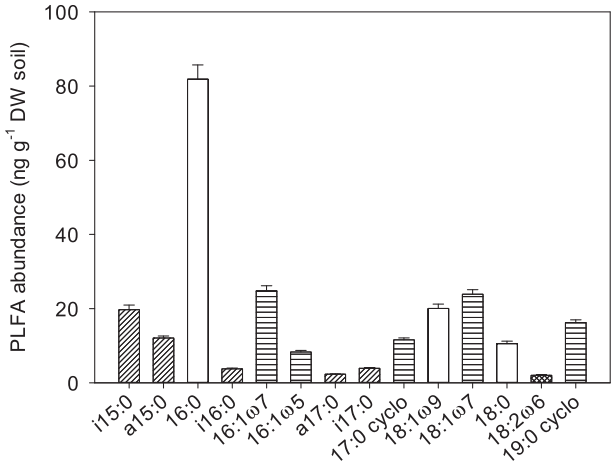

Fig. 2. Abundances of PLFAs in Signy Island soils. Fatty acids ascribed to Gram-positive bacteria, Gram-negative bacteria and fungi are indicated by diagonal shading, horizontal shading or cross-hatching, respectively. Those not ascribed to a specific group are unshaded. Values are means ± SEM ($n = 4$).

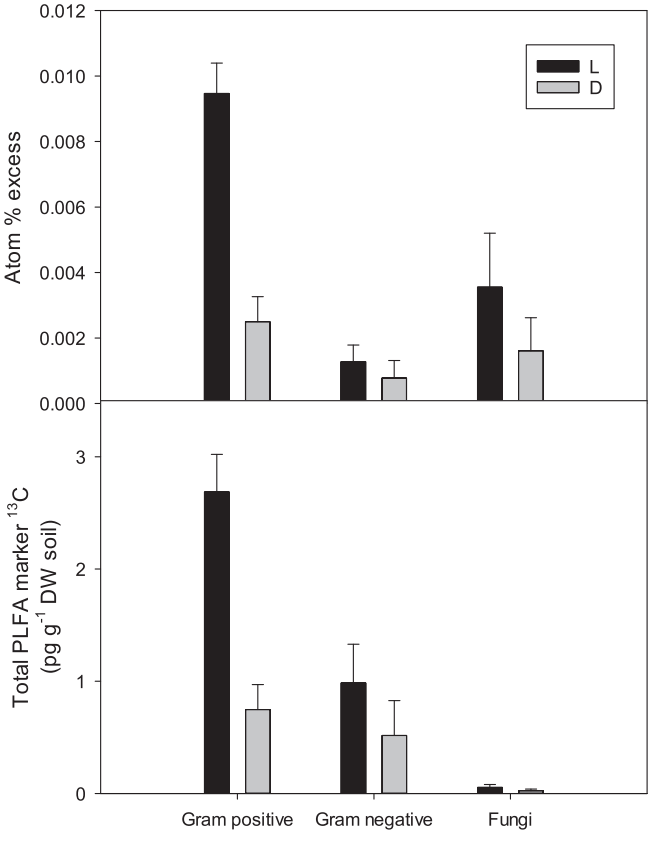

Fig. 3. Incorporation of ^{13}C from L- and D-forms of alanine into PLFAs indicative of Gram-positive bacteria, Gram-negative bacteria and fungi. Data are shown as averages of class specific PLFAs atom percent excesses (upper panel) and total ^{13}C recovered in class specific PLFAs markers (lower panel). Values are means across L- and D- mono- to tripeptides ± SEM ($n = 12$).

bacteria was greater ($P < 0.001$) than for that supplied as D-enantiomers and for ^{13}C recovered in markers for Gram-negative bacteria and fungi. The lower abundance of the fungal PLFA marker also meant that, although APE was not different, total ^{13}C supplied as L-enantiomers recovered in Gram-negative markers exceeded that in fungal markers ($P \leq 0.01$). However, we suggest that some caution should be exercised in the interpretation of these data as ^{13}C recovery in PLFA markers may not be entirely representative of total substrate uptake and utilisation by any group of soil microorganism, particularly as lower numbers of PLFA markers were attributed to fungi than to bacteria.

3.3. Amino acid availability in soil solution

Concentrations of individual amino acids in soil solutions were variable (Fig. 4). L-enantiomers were generally more abundant, with 17 of the 20 proteogenic amino acids detected and L-glutamate having the highest concentration. Other L-amino acids which formed a significant proportion of the soluble free amino acid pool were L-glutamic acid, L-serine, L-glutamine, L-threonine, L-alanine and L-methionine (Fig. 4). In addition, 10 D-amino acids were also detected, with D-alanine, D-phenylalanine and D-leucine having the highest concentrations. Ratios of D- to L-enantiomers ranged from 0.01 ± 0.007 (mean ± SEM; $n = 2$) for glutamine to 1.9 ± 1.3 ($n = 2$) and 1.6 ± 0.7 ($n = 3$) for leucine and phenylalanine, respectively. The D-alanine concentration was 15 ± 3% ($n = 3$) that of L-alanine.

4. Discussion

4.1. General patterns of isomeric form use by the soil microbial community

The addition of ^{13}C-labelled alanine and its short chain peptides to Antarctic soils resulted in higher incorporation of L-enantiomeric forms into microbial PLFAs relative to the D-isomeric forms. The highest incorporation of ^{13}C was into iso- and anteiso-fatty acids, which are predominantly associated with Gram-positive bacteria, such as the Actinobacteria and Firmicutes that inhabit Signy Island soils (P.G. Dennis, pers. comm.). The ^{13}C incorporation profiles, especially from L-alanine and its peptides,

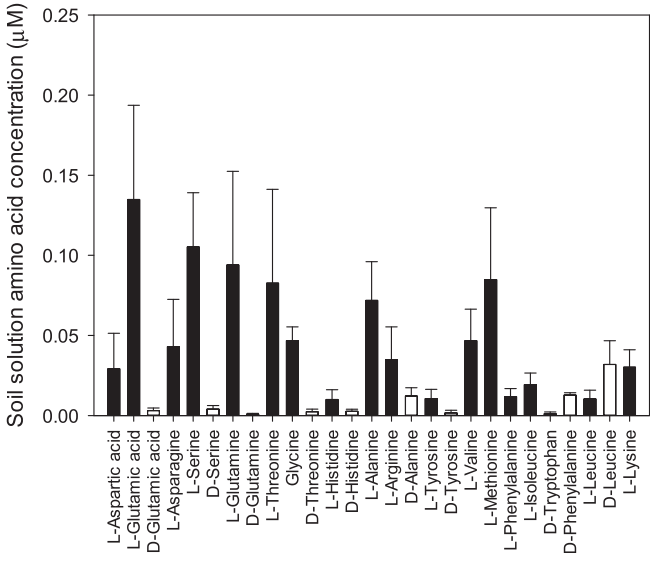

Fig. 4. Amino acid enantiomeric composition of soil solution averaged across three locations on Signy Island. Values are means ± SEM ($n = 3$).

suggest that Gram-positive bacteria are capable of responding more rapidly to exogenous sources of C and N compared with Gram-negative bacteria. Other nucleic acid and fatty acid based studies of the soil microbial communities of Signy Island have also found Gram-negative and Gram-positive bacteria in similar abundances (Dennis et al., 2013), with significant numbers of Gram-negative Proteobacteria in the communities (Yergeau et al., 2007a). As concluded by previous studies (Yergeau et al., 2007b), the soil PLFA profiles here indicated that fungi were less frequent than bacteria in Signy Island soils. Although only comprising <2% of the soil microbial PLFAs, fungi incorporated a significant concentration of ^{13}C into the single fungal PLFA marker compared with Gram-negative bacteria, which appeared to form a much larger proportion of the community. The role of peptide chain length was less clear with regards to uptake and incorporation into PLFAs, with only one fatty acid, 18:1 ω9, showing statistical significance when ^{13}C incorporation for both D- and L- forms were combined. Taking into account individual enantiomeric forms and a peptide length from monomer to pentamer, only C18:1 ω9 showed a statistically significant result with regard to L-amino acid peptide length. There was no apparent correlation between D-enantiomer peptide length and incorporation.

4.2. Relative use of D- and L-isomeric forms of nitrogen

The apparently greater uptake of L-amino acids and peptides over D-enantiomers reported here is not unexpected, primarily because of the integral role of L-forms in protein biosynthesis, with higher L-enantiomer uptake or mineralisation having been found in Antarctic and other soil microbiomes (Hopkins et al., 1997; Hill et al., 2011b, 2012). However, the relatively limited apparent uptake of D-alanine and its peptides by Gram-positive bacteria is surprising considering the central role of D-isomeric amino acids and peptides in cell wall peptidoglycan (Holtje, 1998). The ability to self-cycle such amino acids could provide these microbes with an exclusive source of C and N, especially relative to plants that appear to have a limited capacity to assimilate D-isomeric peptides (Hill et al., 2011c). However, it has been found that microbial communities can adapt to repeated exposures of organic N sources (O'Dowd and Hopkins, 1998), with increased respiration rates on subsequent exposures. It is therefore possible that the metabolism of Gram-positive bacteria may be primed on first exposure to D-enantiomers, allowing for faster and more efficient utilisation on subsequent exogenous additions. It was also noted that there was a lag period before the maximal respiration rate was achieved, when soils were amended with D-amino acids (O'Dowd and Hopkins, 1998), suggesting that cells needed to modify gene expression to cope with D-isomers when added at high exogenous concentrations. This might be expected from the lower availability of D- than L-enantiomers in soil, with L-amino acids comprising on average 87% (excluding glycine) of the free amino acid pool, whilst D-amino acids formed just 8% (15% for D-alanine relative to L-alanine) in soil solutions. Therefore, the peak uptake and utilisation of D-amino acids may not have been reached within the time period of this study. Under the conditions used here, Gram-positive bacteria did not incorporate more ^{13}C from D-isomeric forms into marker PLFAs compared with both Gram-negative bacteria and fungi, providing little evidence of a bacterial self-cycling process. However, it is not currently clear whether incorporation of ^{13}C delivered as D-alanine into bacterial (or fungal) PLFAs provides a good indication of utilisation *per se*.

Two mechanisms exist to convert D-alanine into more metabolically available forms. The first of these is the direct conversion to L-alanine (Inagaki et al., 1986), whilst the second provides a more direct route with respect to primary metabolism, through

the conversion of D-alanine to pyruvate by an enantiomer-specific trans-aminase (Yonaha et al., 1975). Both pathways highlight the discrimination between D- and L-utilisation. The proposed mechanisms involve either converting D-alanine to L-alanine, or to pyruvate, which is non-enantiomeric. Therefore, when comparing D- with L-alanine as a substrate, any metabolic steps post-conversion of D-alanine to L-alanine or pyruvate should be equally as efficient. This suggests that either the rate of the additional conversion of D- to L-alanine, or the direct conversion to pyruvate, is much slower than the equivalent reaction of converting L-alanine to pyruvate, or that the rate of reaction is limited by the uptake of D-alanine.

4.3. Incorporation of ^{13}C into PLFAs

A further question to be addressed is the large incorporation of ^{13}C into both anteiso- and iso-fatty acids. It is recognised that these fatty acids are diagnostic of Gram-positive bacteria, although their *de novo* synthesis requires precursors derived from the amino acids leucine, isoleucine and valine as primer molecules (Supplementary Fig. S1). Therefore, it is assumed that alanine is converted to 3-methylbutyryl-CoA and 2-methylbutyryl-CoA for the production of iso- and anteiso-, odd carbon number fatty acids, and 2-methylpropanyl-CoA for even numbered, iso-fatty acids. However, this pathway is longer than the direct utilisation of acetyl-CoA from pyruvate, which can enter directly into fatty acid synthesis to form saturated fatty acids. Therefore one might expect a much higher incorporation into the saturated fatty acids and monounsaturates, due to the much shorter biological pathway. Indeed, soil PLFA profiling revealed that C16:0 was the most abundant single fatty acid, comprising 25.3 ± 0.8% (mean ± SEM; $n = 4$, data not shown) of the total phospholipids. C16:0 is also the termination point for the fatty acid synthase complex, and one might have expected greater ^{13}C incorporation than was recorded (APE 0.0027 ± 0.0007; mean ± SEM; $n = 32$), suggesting that the anteiso- and iso-pathway is the favoured fatty acid synthesis mechanism, at least in Signy Island soils. The reason for the incorporation into branched chain fatty acids may be to maintain membrane fluidity, as the branched methyl groups of anteiso-fatty acids prevent ordered packing between the fatty acids, whilst iso-branched fatty acids decrease membrane fluidity relative to anteiso-fatty acids (Zhang and Rock, 2008). It is generally thought that low temperatures stimulate the production of unsaturated and anteiso-branched chain fatty acids to maintain the cell's membrane viscosity. Therefore the prevalence of branched chain fatty acids may be an adaptation to the low temperatures to which organisms are exposed in the Antarctic, with bacteria isolated from the region having been shown to produce large proportions of iso-, anteiso- and unsaturated fatty acids (Nichols et al., 1993), which may help to regulate membrane viscosity at low temperatures. The Gram-positive bacterium *Listeria monocytogenes* has also been shown to grow at low temperatures, owing partly to its production of anteiso-fatty acids (Annous et al., 1997; Zhu et al., 2005), with a decrease in growth temperature resulting in an increase in anteiso-fatty acid production (Nichols et al., 2002). The incubation of soil at temperatures above freezing point may have resulted in Gram-positive bacteria modifying their fatty acid profiles, resulting in an increase in the proportional amounts of the longer chain fatty acids (a17:0 and i17:0), an increase in i15:0, and a decrease in a15:0, decreasing membrane fluidity in Gram-positive bacteria.

4.4. Conclusions

The data reported here indicate that Gram-positive bacteria are the primary competitors for alanine and its peptides in Signy Island soils, and that L-enantiomers are utilised to a greater extent by both Gram-positive bacteria and fungi. This supports the view of Hill et al. (2011a, 2011b), who suggest that the highest competition point between plants and micro-organisms is at the oligopeptide level. However, we found little support for the idea of self-cycling of D-enantiomers by bacteria. ^{13}C incorporation with regard to the D-enantiomers differed little due to peptide length, suggesting that there may be a rate limiting step in their utilisation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.05.003.

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