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Domain-level identification and quantification of relative prokaryotic cell abundance in microbial communities by Micro-FTIR spectroscopy

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Summary

Domain-level identification of microbial cells or cell-like structures is crucial for investigating natural microbial communities and their ecological significance. By using micro-Fourier transform infrared (micro-FTIR) spectroscopy, we established a technical basis for the domain-level diagnosis and quantification of prokaryotic cell abundance in natural microbial communities. Various prokaryotic cultures (12 species of bacteria and 10 of archaea) were examined using micro-FTIR spectroscopic analysis. The aliphatic CH_3/CH_2 absorbance ratios ($R_{3/2}$) showed domain-specific signatures, possibly reflecting distinctive cellular lipid compositions. The signatures were preserved even after chemical cell fixation (formaldehyde) and nucleic acid staining (DAPI) processes – techniques that are essential in studying microbial ecology. The micro-FTIR technique was successfully applied for quantification of the bacteria/archaea abundance ratio in an active microbial mat community in a subsurface hot aquifer stream. We conclude that the micro-FTIR $R_{3/2}$ measurement is both fast and effective for domain-level diagnosis and quantification of first-order prokaryotic community structures.

Domain-level microscopic analysis of microorganisms and quantification of bacteria/archaea abundance in living prokaryotic communities are the most fundamental steps in researching natural microbial communities and their ecological and biogeochemical significance. For instance, on the basis of the results of microscopic fluorescence in-situ hybridization (FISH) analysis targeting ribosomal RNA (rRNA) molecules in living microbial cells, Karner and colleagues (2001) showed the dominance of a specific archaeal species in deep-sea water environments. Archaea (*Nitrosopumilus* spp.) (Könneke *et al.*, 2005) were then recognized to include the greatest number of species among the extant life of this planet (Karner *et al.*, 2001). This exemplifies that microscopic FISH analysis can serve as an excellent technique for the domain-level diagnosis and quantification of prokaryotic communities. However, the domain-level diagnosis and quantification by microscopic FISH analysis is not always applicable to any natural microbial community, because there is difficulty in design of the domain-specific FISH probes that fully cover all the archaeal or bacterial phylotypes in the community, and many natural samples of microbial communities contain functionally inert populations and a number of inorganic and organic substances that would prevent the detection and quantitative estimation by the FISH-probe-specific signatures.

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Since a calculation by Whitman and colleagues (1998) that pointed to the enormous biomass potential of deep terrestrial and oceanic subsurface environments, there has been an increasing interest in the microbial components and functions of the deep biosphere. Some studies have described the widespread functional and phylogenetic diversity of prokaryotic populations, most of which are previously uncultivated from the surface habitats, in global deep sub-seafloor sedimentary environments (D'Hondt *et al*., 2004; Parkes *et al*., 2005; Schippers *et al*., 2005; Biddle *et al*., 2006; 2008; Inagaki *et al*., 2006). In these studies, microscopic FISH analysis has not yet effectively estimated the microbial community composition (Schippers *et al*., 2005). Instead, quantitative PCR (Q-PCR) techniques targeting *16S rRNA* genes have indicated that besides a few exceptional examples (Biddle *et al*., 2008), bacterial phylotypes predominate many of the sub-seafloor microbial communities (Schippers *et al*., 2005; Inagaki *et al*., 2006). Conversely, by quantifying bacterial and archaeal intact polar lipid (IPL) components extracted from the sediments, Lipp and colleagues (2008) have speculated that most of the subseafloor microbial communities are likely to be dominated by archaeal populations. These disparate conclusions are largely the result of using technically incomparable analytical methods, which employ different extracted molecular markers (Pearson, 2008). Thus, methods that involve direct observation and reliable domain-identification of intact cells and that are (unlike FISH) useful in analysing natural populations are required.

Fourier transform infrared spectroscopy (FTIR) is a powerful technique for characterizing the chemical compositions of microorganisms. This technique provides vibrational characteristics of all subcellular constituents, including DNA/RNA, protein, membrane and cell-wall components. Combined with statistical methods, FTIR has been shown to have great potential for use in the chemical classification of bacterial cells at the species, subspecies, and even strain levels (e.g. Helm *et al*., 1991a,b; Naumann *et al*., 1991a,b; Mariey *et al*., 2001; Burgula *et al*., 2007); however, archaeal species have been rarely investigated. Hedrick and colleagues (1991) showed that specific IR absorption bands of ester (C = O) and aliphatic methyl (CH_3) groups in cellular lipid extracts were useful for distinguishing archaea from bacteria. However, further domain-level diagnoses of prokaryotic cells by using FTIR have not yet been conducted.

Igisu and colleagues (2006; 2009) showed that specific IR absorption peaks for aliphatic methylene (CH_2) and aliphatic methyl (CH_3) groups can be potential chemical indicators for domain-level identification of prokaryotes. From the domain-specific lipid components (e.g. main hydrocarbon chain species) known so far, it was hypothesized that the ratios of aliphatic CH_3/CH_2 absorbance ($R_{3/2}$ values) of cells and lipid components would be similar within domains, but distinct between domains (Igisu *et al*., 2009). Indeed, when several extant eukaryotic, bacterial and archaeal microorganisms, as well as two bacterial lipid extracts, were examined, the diagnostic potential of $R_{3/2}$ values in domain-level identification of extant and fossilized cells was confirmed (Igisu *et al*., 2009). However, neither the systematic standardization of domain-specific $R_{3/2}$ values nor the standardization of methodological configurations for investigating extant microbial communities has occurred. Therefore, rather than first applying FTIR to long-aged geologic samples (e.g. microfossils), which are influenced by diagenesis and consist of unknown microbial species with varied origins in the geologic time scale, experimental foundations should be established via investigation of presently thriving prokaryotic cells. Thus, this study aimed to examine the applicability of FTIR to domain-level diagnosis and quantification of bacteria/archaea abundance ratios through experimental studies of extant prokaryotic specimens, including a variety of cultured prokaryotes and a dense thermophilic microbial community sample.

First, 22 different bacterial and archaeal species, representing a wide spectrum of phylogenetic diversity, were characterized by micro-FTIR spectroscopic analysis (Fig. 1 and Table S1). Cell assemblages were prepared in three different ways (untreated, fixed and stained cells; Appendix S1). Although untreated cells are likely the best samples for analysis, nucleic acid staining and fluorescence-imaging might be necessary in future applications of this technique in natural samples, because it is critical to distinguish microbial cells from microbial cell-like particles such as minerals and debris that contain organic compounds (non-cell organic matter). Hence, we directly compared between the IR signatures and microscopic images of fixed and stained cells.

Representative infrared transmission spectra of bacterial and archaeal cell assemblages are shown in Fig. S1. The IR absorption spectra of both bacterial and archaeal cells have specific bands around 3300 cm^{-1} (O-H and N-H), 2960 cm^{-1} (aliphatic CH_3: end-methyl), 2925 and 2850 cm^{-1} (aliphatic CH_2: chain-methylene), 1650 cm^{-1} (C = O: amide I), and 1540 cm^{-1} (N-H and C-N: amide II) (assignments are based on Bellamy, 1954). From these IR spectral profiles, the $R_{3/2}$ values of representative bacterial and archaeal cell assemblages were calculated and are summarized in Fig. 1 and Table S1. With the exception of the $R_{3/2}$ values of fixed and stained *Methanopyrus kandleri* cells, all the $R_{3/2}$ values were similar within domains, but distinct between domains (Fig. 1 and Table S1). In addition, the potential domain-specific $R_{3/2}$ values were not affected by fixing/staining of cell assemblages; the mean values for bacterial untreated, fixed, and stained cells were 0.66 $\pm$ 0.04 ($n = 9$), 0.65 $\pm$ 0.07 ($n = 9$)

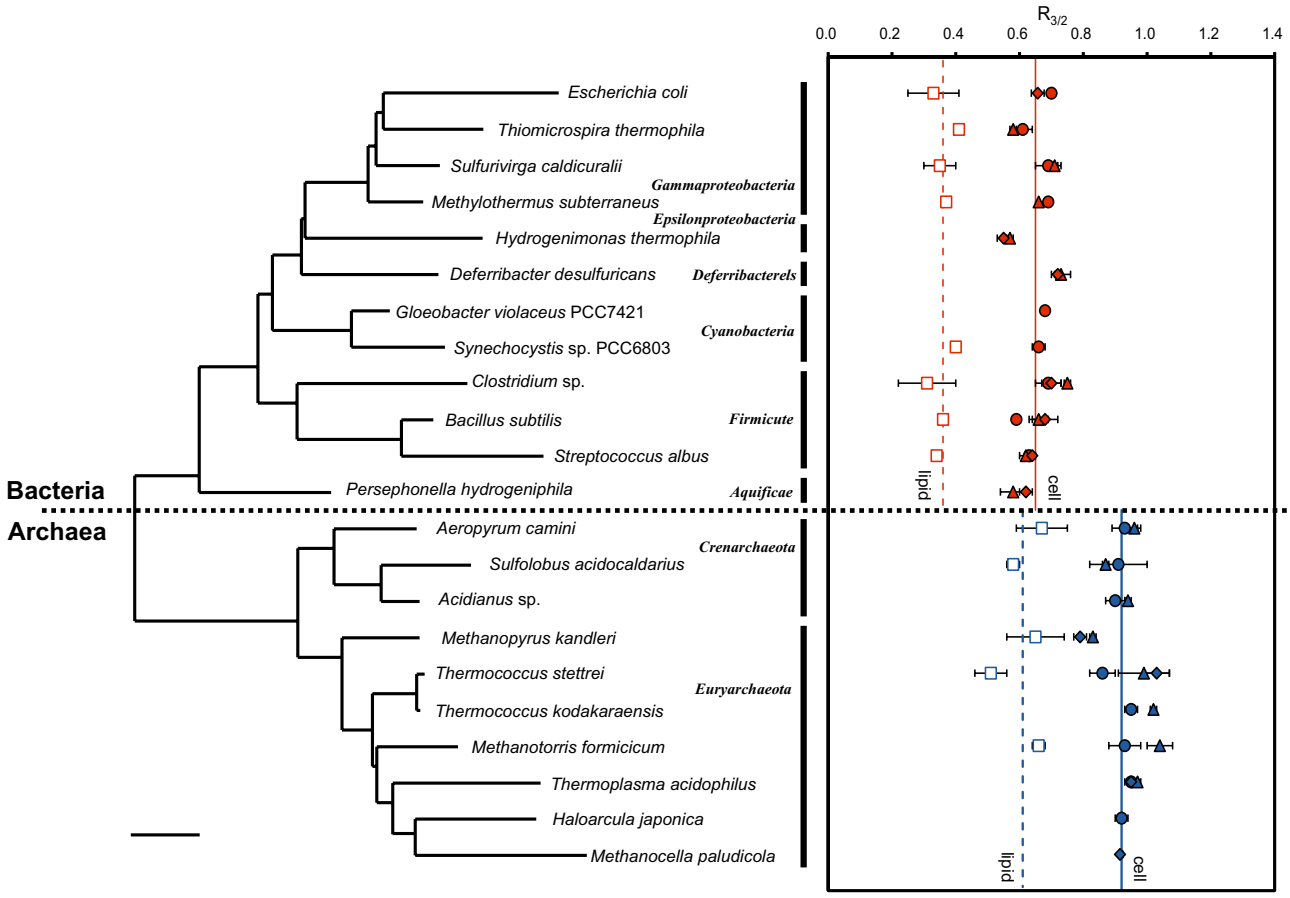

Fig. 1. Phylogenetic tree and $R_{3/2}$ values of cell assemblages and lipids of representative bacterial and archaeal species and strains. (Left panel) The tree was reconstructed by the Maximum Likelihood method using 1057 unambiguously aligned nucleotide positions. Bar indicates 0.1 substitutions per nucleotide. (Right panel) The $R_{3/2}$ values of bacterial and archaeal cells and lipids are shown by red and blue respectively. The $R_{3/2}$ values of untreated cells, fixed cells, stained cells and lipids are indicated by the circle, triangle, diamond and square respectively. Solid lines represent mean $R_{3/2}$ values of stained bacterial and archaeal cells, and dashed lines indicate those of bacterial and archaeal lipids.

and 0.65 ± 0.06 ($n = 7$) respectively, whereas the mean values for archaeal untreated, fixed and stained cells were 0.92 ± 0.03 ($n = 8$), 0.95 ± 0.07 ($n = 8$) and 0.92 ± 0.10 ($n = 4$) respectively. There were no significant differences in $R_{3/2}$ values between the untreated, fixed and stained cells ($P > 0.05$ by one-way analysis of variance for bacteria; $P > 0.05$ by Kruskal–Wallis test for archaea) (Appendix S1). The $R_{3/2}$ values of bacterial untreated, fixed and stained cells were significantly different than the $R_{3/2}$ values of their corresponding archaeal counterparts ($P < 0.01$ by Student's *t*-test) (Appendix S1). These results strongly suggest that the $R_{3/2}$ values determined by micro-FTIR spectroscopy are effective for domain-level diagnosis of microbial cell assemblages, even after formaldehyde fixation and DAPI staining (Fig. 1 and Table S1).

Why does micro-FITR spectroscopy yield domain-specific $R_{3/2}$ values for microbial cell assemblages? Igisu and colleagues (2009) hypothesized that domain-specific $R_{3/2}$ values are a result of the varied amount of aliphatic CH_3/CH_2 groups of the main hydrocarbon chains in cell membrane lipids (e.g. fatty acids or isoprenoid alcohols in bacterial or archaeal membranes respectively). To verify this hypothesis, total lipid components were extracted from representative bacterial and archaeal cultures and were characterized by micro-FTIR spectroscopy (Fig. 1 and Table S1). Extracted bacterial and archaeal total lipids have different IR absorption spectra than do the previously examined cell assemblages. IR spectra of total lipid extracts had clear signatures around 2960 cm^{-1} (aliphatic CH_3) and around 2925 and 2850 cm^{-1} (aliphatic CH_2). Notably, as observed in cell assemblages, the $R_{3/2}$ values of lipid extracts were similar among domains but were significantly distinct between domains (Fig. 1 and Table S1). The mean $R_{3/2}$ values of bacterial and archaeal lipids were 0.36 ± 0.03 ($n = 8$) and 0.61 ± 0.07 ($n = 5$) respectively ($P < 0.01$ by Student's *t*-test) (Appendix S1). The variance between mean $R_{3/2}$ values of bacterial and

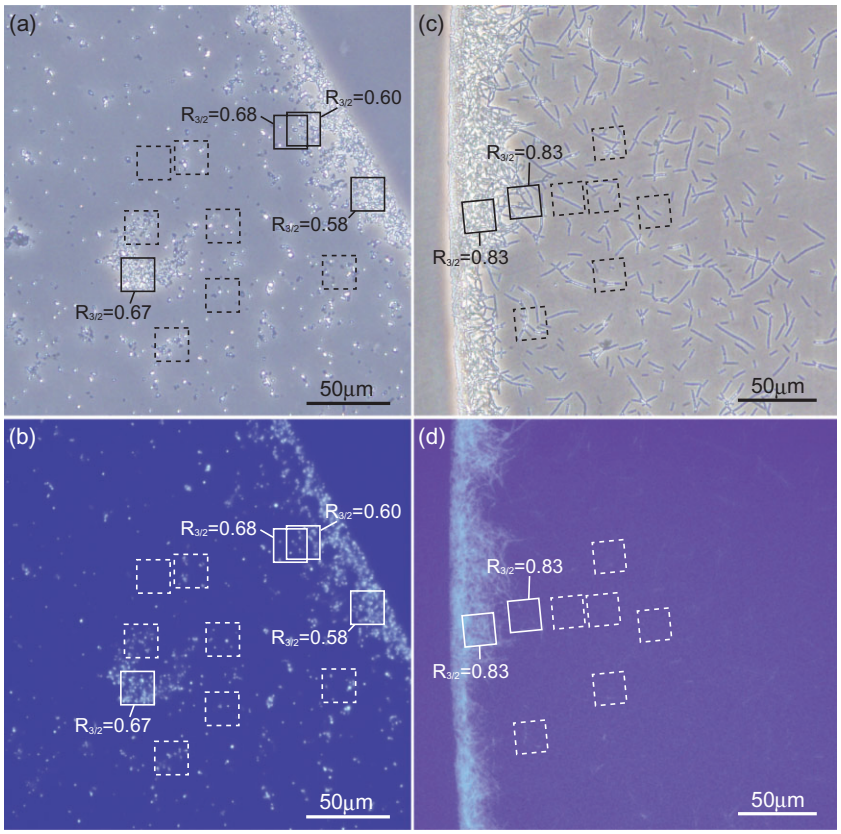

Fig. 2. Optical and epifluorescence micrographs of stained cells used for micro-FTIR spectroscopy.
A and B. Micrographs of *Hydrogenimonas thermophila*.
C and D. Micrographs of *Methanopyrus kandleri*. Analysed areas (solid squares) for micro-FTIR spectroscopic analysis and the $R_{3/2}$ values are indicated. Dotted squares show the areas where aliphatic CH signals were not detected. Scale bars represent 50 μm.

archaeal lipids (0.25) was nearly equivalent to the variance between the mean $R_{3/2}$ values of bacterial and archaeal cell assemblages (0.26–0.30). This result substantially supports the conclusion that domain-specific $R_{3/2}$ values of microbial cell assemblages result from unique, domain-specific cellular lipid compositions.

According to the IR signatures used previously (Hedrick *et al.*, 1991), the ratios of peak height of ester C = O bands to that of aliphatic CH_3 bands (C = O/CH_3) were calculated for our data. The peak height ratios were 0.76 ± 0.44 (n = 8) for bacterial lipid extracts and 0.13 ± 0.06 (n = 5) for archaeal lipid extracts (P < 0.05 by Student's t-test). These values are consistent with the previously reported results of Hedrick and colleagues (1991), i.e. 0.695 ± 0.113 for bacterial lipids and 0.096 ± 0.027 for archaeal lipids. These ratios show a significant difference between bacterial and archaeal lipids. The IR spectra of some bacterial and archaeal cell assemblages, however, did not have distinctive peaks corresponding to ester (C = O) groups around 1740 cm^{-1}. The mean values of C = O/CH_3 for bacterial untreated, fixed and stained cells were 0.26 ± 0.24 (n = 9), 0.15 ± 0.14 (n = 9) and 0.34 ± 0.17 (n = 7) respectively, whereas those for archaeal untreated, fixed and stained cells were 0.11 ± 0.20 (n = 8), 0.02 ± 0.07 (n = 8) and 0.07 ± 0.13 (n = 3) respectively. These results are possibly attributable to the presence of an amide bond, which produces an IR band around 1650 cm^{-1} that interferes with the ester band, and/or to analytical differences such as total lipid extraction method and experimental conditions. Although a significant difference may not be necessary for domain-level diagnosis of prokaryotic cells, our newly developed technique provides highly accurate and reliable IR signatures that can be used to distinguish bacterial cell assemblages and lipids from those of the archaeal domain.

The analytical limits of employing our FTIR assay in domain-level diagnosis were examined. By using cultured, DAPI-stained bacterial and archaeal cells, we investigated the minimum number of sample cells necessary for the assay. By choosing detection spots within the micro-FTIR spectroscopic field that included 1–20 cells, the minimum numbers of cells providing confident domain-specific $R_{3/2}$ values were estimated (Fig. 2). The lower limit varied among the different species tested. Larger cells tended to yield a confident signature with fewer numbers. For example, a methanotrophic species (*Methylothermus subterraneus*) having intra-cytoplasmic membrane structures provided a bacteria-specific $R_{3/2}$ value when only a few cells were examined. Typically, the minimum number of cells needed to provide domain-specific $R_{3/2}$ values for bacterial and archaeal species was determined to be more

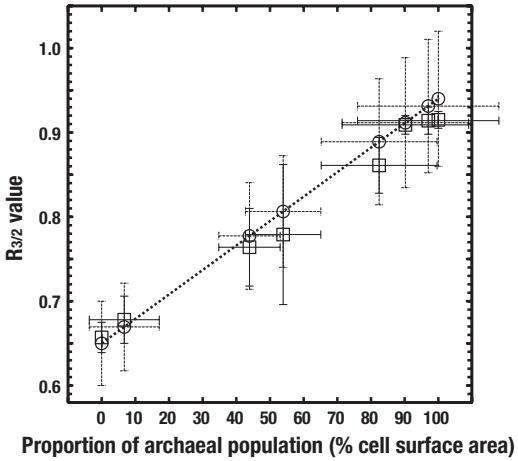

Fig. 3. Relationship between $R_{3/2}$ values and ratios of bacterial and archaeal cellular populations (*Escherichia coli* and *Methanocella paludicola*). Squares indicate the $R_{3/2}$ values of bacterial and archaeal cell mixtures. Circles and the dotted line represent the $R_{3/2}$ values predicted by the standardized domain-specific $R_{3/2}$ values and the mixing ratios of bacterial and archaeal populations along with the relative cell surface areas. Error bars indicate the standard deviations of measured and estimated $R_{3/2}$ values and the standard deviations of proportions estimated from the bacterial and archaeal cell surface areas.

than 10 (Fig. 2). Because cultured microbial cells are better nourished than their natural counterparts, more than several tens of microbial cells may be required for the confident FTIR spectroscopic estimation of $R_{3/2}$ values for natural microbial communities. The sensitivity of commercial micro-FTIR spectroscopy equipment, however, has been greatly improved during the last decade. If the sensitivity is improved by 1 order of magnitude higher in the near future, the domain-level diagnosis of a single cell among natural microbial communities may become possible. Nevertheless, even with the current sensitivity and resolution, it is possible that some $R_{3/2}$ values were obtained from natural microbial communities that contain heterogeneous bacterial and archaeal components, if the cell assemblages are densely prepared on the CaF_2 disks.

Next, we examined whether the $R_{3/2}$ value of a heterogeneous prokaryotic cell assemblage can be an indicator of the ratio of bacteria to archaea in the population. DAPI-stained cells of cultured *Escherichia coli* (bacterium) and *Methanocella paludicola* (archaeon) were mixed in various proportions and were examined by micro-FTIR spectroscopy (Fig. 3 and Table S2). The calculated $R_{3/2}$ value of bacterial and archaeal cell mixtures was expected to be a ratio of the values that correspond to standardized bacterial and archaeal cell cultures (Fig. 1 and Table S1). Because the $R_{3/2}$ value of prokaryotic cell assemblages is closely related to the composition and abundance of cellular lipids (as discussed above), it is possible that the $R_{3/2}$ value of a heterogeneous cell assemblage is related to the ratio of membrane volumes of bacterial to archaeal cellular populations. In fact, the $R_{3/2}$ values obtained most strongly correlated with the ratio of bacterial and archaeal cellular surface areas in the cell mixtures (Fig. 3 and Table S2). These results indicate that the relative abundance of bacterial and archaeal components of natural microbial communities can be estimated by treating the $R_{3/2}$ value of the population as a ratio of the bacterial and archaeal $R_{3/2}$ standard values.

For a case study, we applied this technique to a filamentous microbial community growing at a subsurface geothermal aquifer stream in a Japanese gold mine (Takai *et al*., 2002; Inagaki *et al*., 2003; Hirayama *et al*., 2005; 2011). The thermophilic filamentous microbial organisms that thrive along the geothermal water stream have been extensively studied (Hirayama *et al*., 2005; 2011; Nunoura *et al*., 2005; 2011). In several samples of microbial mat communities, the major cellular and phylotypic components were previously uncultivated archaeal phylotypes (HWCG-I and HWCG-III), along with some bacterial entities such as *Sulfurihydrogenibium*, *Methylothermus*, *Thiobacter*, *Nitrospira* and OP1 members (Hirayama *et al*., 2005; 2011; Nunoura *et al*., 2005; 2011). A portion of the filamentous community was newly sampled (details in Appendix S1), and the ratio of bacterial and archaeal cell abundance, extracted IPLs, 16S rRNA gene numbers, and cellular components were estimated. Micro-FTIR spectroscopy yielded an $R_{3/2}$ value, which was used to determine the ratio of bacterial to archaeal components (Table 1).

To distinguish microbial cells from extracellular assemblages of organic compounds, we used DAPI-stained cells for micro-FTIR spectroscopy. Because a naturally occurring dense microbial community usually contains not only microbial cells but also large amounts of extracellular organic compounds (e.g. polysaccharides), a method of

Table 1. Estimation of abundance ratio of bacterial and archaeal cells, extracted intact polar lipids (IPL), 16S rRNA gene number and cellular components in a microbial mat community of a Japanese subsurface geothermal aquifer stream by different techniques.

Estimation technique	Abundance ratio of bacteria : archaea
Extracted IPL amount[a]	39:61
16S rRNA clone composition[a]	59:41
Quantitative PCR[a]	87:13
Whole cell FISH	72 (± 18):28 (± 7)[b]
Micro-FTIR	
Dispersive cell fraction sample	73 (± 17):27 (± 17)[c]
Whole community sample	
Microbial-cell-dominating part	78 (± 16):22 (± 16)[c]
Mineral-dominating part	52 (± 18):48 (± 18)[c]

a. The methods and results are described in Supporting Information.
b. The standard deviation between the samples in parenthesis.
c. The analytical error in parenthesis.

physical separation and concentration of the microbial cells was required during sample preparation. This step was critical to our assay, because excessive amounts of extracellular organic compounds can interfere with the determined $R_{3/2}$ values and therefore lead to inaccurate estimation of bacterial and archaeal population ratios. To investigate these effects, two different methods of sample preparation were used, dispersive cell fraction sampling and whole community sampling. In the whole community sample, microbial cell assemblage components were not easily distinguished from mineral aggregate components (e.g. Fe-oxides associated with extracellular organic compounds) during epifluorescence microscopic observation – most likely because of the complex microbial–mineral associations. However, $R_{3/2}$ values were obtained from each of the strong DAPI-derived fluorescence regions (microbial-cell-dominating) and the weakly fluorogenic areas (mineral-dominating). In contrast, by using dispersive cell fraction sampling, microbial cell assemblages could be clearly identified by microscopic observation. The dispersive cell fractions and the microbial-cell-dominating areas of the whole community samples had $R_{3/2}$ values corresponding to lower archaeal abundance ratios (27% and 22% respectively), whereas the mineral-dominating areas of whole community sample had higher ratios (48%). These results imply the potential interference caused by extracellular organic matter during domain-level quantification of prokaryotic populations and determination of bacterial/archaeal population ratios by micro-FTIR.

When using other methods for quantifying population ratios, all estimates suggested the significant abundance of archaea (13–61%) in the thermophilic microbial community of our case study (Table 1). Of these estimates, the ratio of bacterial and archaeal IPL amounts suggested the highest abundance ratio of archaea, whereas Q-PCR of prokaryotic and archaeal 16S rRNA genes gave the lowest ratio (Table 1). Although the detailed methods and the targeted microbial communities used were completely different from those used in this study, these two techniques were adopted for the estimation of archaeal abundance in subseafloor sediments (Lipp *et al.*, 2008). The estimates reflect technique-dependent variation of archaeal abundance: IPL analysis generally gave higher archaeal abundance estimates than did Q-PCR (Lipp *et al.*, 2008). Thus, technique-dependent variation is likely unavoidable in the quantification of bacteria/archaea abundance ratios in natural microbial communities. Nevertheless, the newly established domain-level quantification technique by micro-FTIR spectroscopy yields plausible data that are comparable to other frequently applied, but time-consuming quantification techniques (Table 1). This strongly suggests that micro-FTIR spectroscopy analysis can be applied as a rapid and easy option for estimating abundance ratios of bacteria/archaea in a variety of microbial communities.

Natural microbial communities consist of not only prokaryotes, but also eukaryotic microorganisms such as fungi and algae. A series of micro-FTIR measurements were conducted using four different cultured micro-eukaryotic cell assemblages and extracted lipids (Table S1). The mean $R_{3/2}$ values for the eukaryotic untreated, fixed and stained cells were 0.58 ± 0.09 ($n = 4$), 0.54 ± 0.10 ($n = 2$) and 0.53 ± 0.13 ($n = 2$) respectively. There were no significant differences in $R_{3/2}$ values among the eukaryotic untreated, fixed and stained cells ($P > 0.05$ by one-way analysis of variance). In addition, the eukaryotic cell assemblages had $R_{3/2}$ values that could be distinguished from the values of bacterial and archaeal assemblages ($P < 0.05$ by one-way analysis of variance). The mean $R_{3/2}$ value of eukaryotic lipids was 0.48 ± 0.07 ($n = 4$) (Appendix S1) and, similarly, was significantly different from the $R_{3/2}$ values of bacterial and archaeal samples ($P < 0.05$ by one-way analysis of variance). However, the variance between the mean $R_{3/2}$ values of bacterial and eukaryotic cells was relatively unclear when compared with the variance between the bacterial and archaeal mean values. In addition, the variance between the mean $R_{3/2}$ values of eukaryotic cells and lipids significantly differed from the variances between the bacterial and archaeal cells and lipids. Therefore, it would be difficult to differentiate organisms of all three domains in natural microbial samples on the basis of only the $R_{3/2}$ value. For samples obviously containing eukaryotic cells or organic compounds, pretreatments such as physical separation or DAPI-staining should be carried out to eliminate eukaryote-derived material before IR spectroscopy.

In summary, we established the basics of a new micro-FTIR spectroscopic technique and showed that the resulting $R_{3/2}$ values can be used as a powerful index for domain-level diagnosis and quantification of prokaryotic microorganisms. The application of this technique to identify single cells is challenging and may be possible by further improvements in sensitivity and spatial resolution. Finally, in order for this technique to more effectively be applied to geological samples, further characterization of the intra- and extra-cellular diagenesis of organic compounds during fossilization is essential.

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Supporting information

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

Fig. S1. Representative transmission IR absorption spectra of bacterial and archaeal species.
Table S1. $R_{3/2}$ values and numbers of analyses of prokaryotes and micro-eukaryotes.
Table S2. Relationship between $R_{3/2}$ values and relative proportion of bacterial and archaeal populations in bacterial and archaeal culture cells (*Escherichia coli* and *Methanocella paludicola*).
Appendix S1. Experimental procedures.

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