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Compound-Specific Nitrogen Isotope Analysis of D-Alanine, L-Alanine, and Valine: Application of Diastereomer Separation to δ^{15}N and Microbial Peptidoglycan Studies

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We have developed an analytical method to determine the compound-specific nitrogen isotope compositions of individual amino acid enantiomers using gas chromatography/combustion/isotope ratio mass spectrometry. A novel derivatization of amino acid diastereomers by optically active (*R*)-(−)-2-butanol or (*S*)-(+)-2-butanol offers two advantages for nitrogen isotope analysis. First, chromatographic chiral separation can be achieved without the use of chiral stationary-phase columns. Second, the elution order of these compounds on the chromatogram can be switched by a designated esterification reaction. We applied the method to the compound-specific nitrogen isotope analysis of D- and L-alanine in a peptidoglycan derived from the cell walls of cultured bacteria (Firmicutes and Actinobacteria; *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus staphylolyticus*, *Lactobacillus acidophilus*, *Bacillus subtilis*, *Micrococcus luteus*, and *Streptomyces* sp.), natural whole bacterial cells (*Bacillus subtilis* var. *natto*), (pseudo)-peptidoglycan from archaea (*Methanobacterium* sp.), and cell wall from eukaryota (*Saccharomyces cerevisiae*). We observed statistically significant differences in nitrogen isotopic compositions; e.g., δ^{15}N (‰ vs air) in *Staphylococcus staphylolyticus* for D-alanine (19.2 ± 0.5‰, $n = 4$) and L-alanine (21.3 ± 0.8‰, $n = 4$) and in *Bacillus subtilis* for D-alanine (6.2 ± 0.2‰, $n = 3$) and L-alanine (8.2 ± 0.4‰, $n = 3$). These results suggest that enzymatic reaction pathways, including the alanine racemase reaction, produce a nitrogen isotopic difference in amino acid enantiomers, resulting in ^{15}N-depleted D-alanine. This method is expected to facilitate compound-specific nitrogen isotope studies of amino acid stereoisomers.

Since the first successful separation of chiral amino acids by gas chromatography (GC) with a chiral stationary phase nearly 40 years ago,[1] numerous analytical studies of chiral molecules have been performed (e.g., refs 2−7). To date, two chromatographic methods for chiral separation have been used as common applications. The first, enantiomer separation using chiral stationary phase, is a typical procedure. This method has been developed for stable isotope studies of D- and L-amino acids by gas chromatography (GC) techniques using columns of chiral polysiloxanes (Chirasil-type) stationary phases with a valine diamide selector,[7] later known as Chirasil-Val. Recently, Levkin et al. have shown an inversion of the elution order of the D- and L-amino acids on GC with a Chirasil-Val-C_{11} column.[8] The second method is diastereomer separation, in which chemical derivatization with optically active reagents can be performed without a chiral stationary phase.[9]

Previous studies report that stationary phase containing nitrogen compounds should be used with caution for the precise determination of ^{15}N/^{14}N ratios.[10] For instance, Macko et al. noted a possible inconsistency in δ^{15}N data in the case of a large discrepancy between D- and L-glutamic acids, possibly resulting from a minor contribution of nitrogen from the stationary phase, as these two components elute at the maximum temperature (<220 °C) for Chirasil-Val.[11]

In the present study, we aimed to establish the diastereomeric separation of representative D- and L-amino acids for compound-specific nitrogen isotope ratio analysis without using a chiral stationary-phase column, to ensure a nitrogen-free procedure. It is convenient in compound-specific nitrogen isotope studies to use gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) with a nonpolar achiral column phase that can tolerate high temperatures, such as the Ultra-2 capillary column (Agilent Technologies), which can be used at temperatures up to

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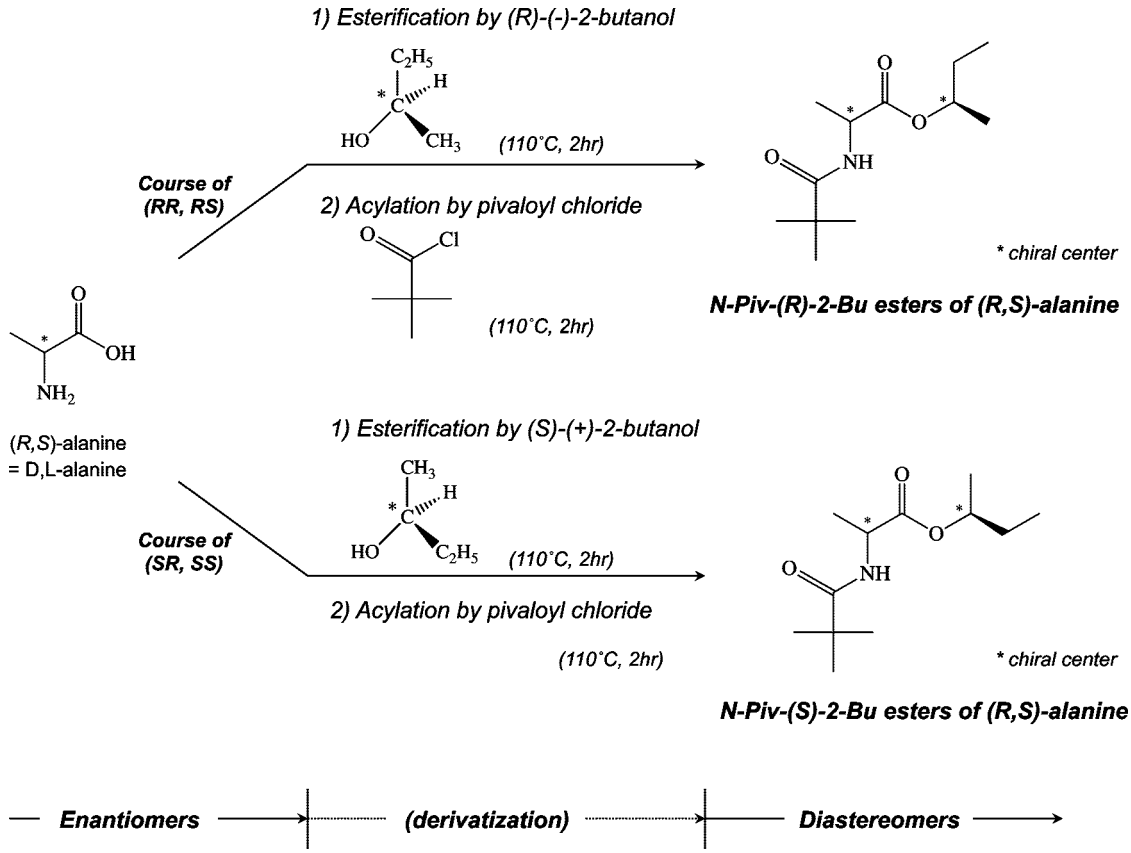

Figure 1. Reaction pathway of diastereomeric (*R*,*S*)-alanine (D,L-alanine) derivatives using (*R*)-(−)- or (*S*)-(+)-2-butanol and pivaloyl chloride for esterification and acylation, respectively. As noted by Metges and Petzke,[10] fluorinated compounds may cause a rapid deterioration of the combustion catalyst and oxidants. Since fluorine forms extremely stable fluorides with Cu (CuF_2) and Ni (NiF_2) thus irreversibly reducing combustion efficiency of the CuO/NiO system,[34] we used pivaloyl chloride for acylation reagent.

325 °C. The experiment described above allows us to discuss the reliability of output data originating only from nitrogen sources within the samples. We applied our method to the chiral amino acids alanine (C_3) and valine (C_5) and representative natural samples of D-amino-acid-containing peptidoglycan derived from various bacteria, (pseudo)-peptidoglycan from members of the archaea, and cell wall from eukaryota.

D-Alanine is considered to be a bacterial biomarker, specific for peptidoglycan.[12−19] The cell wall of Gram-positive bacteria consists of a thick and uniform peptidoglycan layer that includes D-amino acids, forming up to ~90% of the cell wall. Laboratory studies of the degradation of peptidoglycan in cultured bacteria[19] and seawater samples[14] showed it to decompose more slowly than proteins, indicating a semilabile compound in nature. ^{15}N-Labeling studies of peptidoglycan have documented the degradation pathway of peptidoglycan in aquatic cycles.[16] Here, we also show the compound-specific nitrogen isotopic ratios of D- and L-alanine in microbial peptidoglycan.

EXPERIMENTAL SECTION

Samples and Reagents. We used optically active amino acid standards (L-alanine, purity 99%; D-alanine, 98%; L-valine, 99%; D-valine, 99%) purchased from Wako Pure Chemical Industries, Ltd. (hereafter, Wako Chemical). We also used a laboratory alanine standard provided by the Center of Ecological Research, Kyoto University, Japan. Optically active (*R*)-(−)-2-butanol (purity 99%; boiling point 99−100 °C) and (*S*)-(+)-2-butanol (purity 99%; boiling point 99−100 °C) were obtained from Wako Chemical and Sigma-Aldrich Co., respectively. Therefore, the impurities of the amino acid diastereomers should be less than 2%. Other derivatization reagents, thionyl chloride (boiling point 79 °C) and pivaloyl chloride (trimethylacetyl chloride, boiling point 105−106 °C), were also obtained from Wako Chemical.

Peptidoglycan from bacteria (*Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus staphylolyticus*, *Lactobacillus acidophilus*, *Bacillus subtilis*, *Micrococcus luteus*, and *Streptomyces* sp.), (pseudo)-peptidoglycan from members of the archaea (*Methanobacterium* sp.), and cell wall from eukaryota (*Saccharomyces cerevisiae*) were obtained from Wako Chemical and Fluka Sigma-Aldrich Co. Whole cells of natural *Bacillus subtilis* var. *natto* were purchased as a commercial food at a market in Yokohama, Japan.

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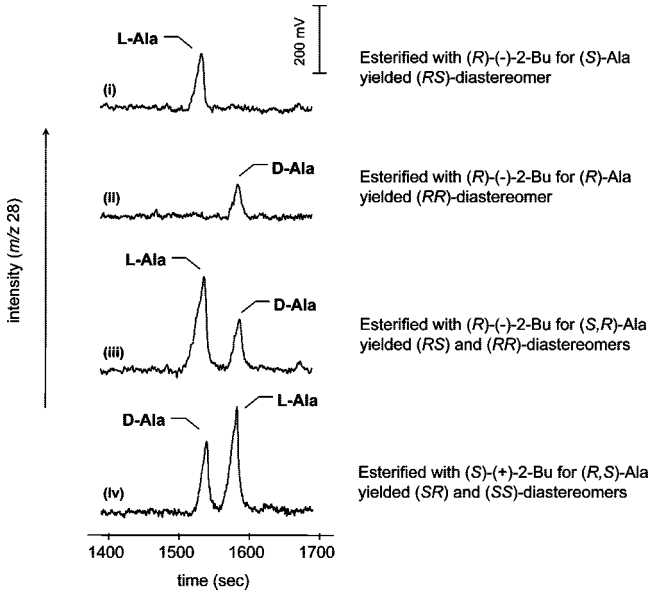

Figure 2. Representative GC/C/IRMS chromatograms of the chiral separation of D- and L-amino acids. Various component analyses were performed to determine the accuracy of the δ^{15}N data in terms of chiral molecules. (i) Single analysis of only L-alanine with (*R*)-(−)-2-butanol; (ii) single analysis of only D-alanine with (*R*)-(−)-2-butanol; (iii and iv) mixed analysis of L-alanine and D-alanine (a predominant amount of L-alanine was intentionally added to distinguish the elution order). Although significant signals does not appear in GC/C/IRMS chromatogram, the D/L ratio fluctuation of hydrothermal racemization occurred during acid hydrolysis (ref 35; less than 3% of initial D/L ratio for 32 h of acid hydrolysis).

Derivatization Procedure for Stereoisomers of Alanine and Valine. The derivatization processes were performed using a modified method described previously[20−22] to yield *N*-pivaloyl-(*R*,*S*)-2-butyl esters (NP/2Bu) of the amino acid diastereomers (Figure 1). The initial esterification step is a diastereomeric reaction: (*R*)-(−)-2-butanol yields (*R*)-(−)-2-butyl esters of (*R*,*S*)-amino acid diastereomers (*RR*, *RS*), and (*S*)-(+)-2-butanol yields (*S*)-(+)-2-butyl esters of (*R*,*S*)-amino acid diastereomers (*SR*, *SS*), as expressed in the Cahn−Ingold−Prelog (CIP) priority rule. The elution order of the amino acid diastereomers can be switched by an initial esterification step. First, 500 μL aliquots of the amino acid standard (10 μmol of total amino acids per sample) or the sample solution were dispensed into 16 × 100 mm reaction vials with PTFE-lined caps and evaporated to dryness under a nitrogen flow. The esterification reaction was performed with 500 μL of a thionyl chloride/(*R*)-(−)- or (*S*)-(+)-2-butanol mixture (1:4, v/v) at 110 °C for 2 h. After the solution had been cooled to ambient temperature, it was evaporated to dryness under a gentle nitrogen flow at ∼80 °C. The acylation reaction was then performed with 500 μL of a pivaloyl chloride/dichloromethane mixture (1:1, v/v) at 110 °C for 2 h. After the solution was cooled, it was again evaporated to dryness with a gentle nitrogen flow at ∼80 °C. The NP/2Bu esters of the amino acid diastereomers were extracted by liquid/liquid separation in 0.5 mL of distilled water and 1.0 mL of a hexane/dichloromethane (6:5, v/v) mixture. The hexane/dichloromethane mixture fraction containing the NP/2Bu esters of the amino acid diastereomers was recovered and dried under a gentle nitrogen flow. Then, 200 μL of dichloromethane was added to the final fraction. The NP/2Bu esters of the amino acid diastereomers were identified by GC/mass spectrometry (GC/MS; Agilent Technologies 6890N/5973MSD). The capillary column used for GC was an HP-5 (30 m × 0.32 mm i.d., 0.52 μm film thickness; Agilent Technologies). The GC oven temperature was programmed as follows: initial temperature 40 °C for 4 min, ramped up at 10 °C min^{-1} to 90 °C, and ramped up at 5 °C min^{-1} to 220 °C, where it was maintained for 10 min. The MS was scanned over m/z of 50−550 with the electron-impact mode set at 70 eV. All glassware was heated at 450 °C for 4 h before use to eliminate any possible contaminants.

Separation of Amino Acid Fraction. Peptidoglycans are polymers of repeating units of *N*-acetylglucosamine and *N*-acetylmuramic acid[12] and may produce complex organic matter during acid hydrolysis. Before GC/C/IRMS analysis, the amino acid fractions were separated from interfering organic matter by cation-exchange chromatography. The experimental scheme and the purification of the amino acid fraction from the hydrolyzed peptidoglycan samples by cation-exchange chromatography were shown in the flow chart (see Figure S-1 in the Supporting Information). Briefly, an aliquot of dried sample powder (peptidoglycan, (pseudo)-peptidoglycan, cell wall, or cultured bacterial whole cells) was acid hydrolyzed with 6 M HCl at 110 °C for 12 h. After drying under an N_2 flow, the samples were adjusted to pH 1 with 0.1 M HCl and the amino acid fraction was isolated with cation-exchange column chromatography. The purification of amino acid fractions via application to an AG-50W-X8 (200−400 mesh; Bio-Rad Laboratories) cation exchange resin column has been validated elsewhere (see instruction manual and literatures within, Bio-Rad Laboratories). A slurry of resin in deionized water was poured into a disposable glass pipet column plugged with quartz wool. Before the application of the sample to the column, the resin was cleaned by passing three bed volumes (resin/carrier, 1:3, v/v) of 1 M HCl, H_2O, 1 M NaOH, and H_2O through the column in succession (i.e., 2 mL of AG50 resin requires 6 mL of 1 M HCl for the first prewash). Immediately before the application of the sample, the resin was reactivated to the H^+ form with three bed volumes of 1 M HCl and then rinsed with three bed volumes of H_2O. The sample solution was loaded and then eluted with three bed volumes of H_2O to retain only the amino acid fraction. Finally, the amino acid fraction was eluted with three bed volumes of 10% NH_3 aqueous solution and then dried by nitrogen flow for the next derivatization procedure.

Nitrogen Isotope Analysis. The nitrogen isotopic compositions of the amino acid standards before derivatization were determined with an isotope ratio mass spectrometer (IRMS; ThermoFinnigan Delta Plus XP) coupled to a Flash elemental analyzer (EA; ThermoFinnigan EA1112) via a Conflo III interface.[23] The nitrogen isotopic composition is expressed as the per

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Table 1. Comparison of $\delta^{15}N$ Data for Each Amino Acid Stereoisomer Identified Using the EA/IRMS and GC/C/IRMS Methods[a]

analysis	amino acid	component	esterification	diastereomer derivatives	diastereomer abbreviation	$\delta^{15}N_{EA}$ before derivatization (bulk)	$\delta^{15}N_{GC}$ after derivatization	1σ	n	difference ($\delta^{15}N_{EA} - \delta^{15}N_{GC}$)
1	(*R*)-alanine (D-alanine)	single	(*R*)-(−)-2-butanol	*N*-pivaloyl-(*R*)-2-butyl-ester of (*R*)-alanine	(*RR*)	−1.4	−1.4	0.3	3	0.0
2	(*S*)-alanine (L-alanine)	single	(*R*)-(−)-2-butanol	*N*-pivaloyl-(*R*)-2-butyl-ester of (*S*)-alanine	(*RS*)	−1.7	−1.7	0.4	3	0.0
3	(*R*)-alanine	mixture	(*R*)-(−)-2-butanol	*N*-pivaloyl-(*R*)-2-butyl-ester of (*R*)-alanine	(*RR*)	−1.4	−1.6	0.5	7	−0.2
	(*S*)-alanine		(*R*)-(−)-2-butanol	*N*-pivaloyl-(*R*)-2-butyl-ester of (*S*)-alanine	(*RS*)	−1.7	−1.4	0.6	7	0.3
4	(*R*)-alanine	mixture	(*S*)-(+)-2-butanol	*N*-pivaloyl-(*S*)-2-butyl-ester of (*R*)-alanine	(*SR*)	−1.4	−1.5	0.3	3	−0.1
	(*S*)-alanine		(*S*)-(+)-2-butanol	*N*-pivaloyl-(*S*)-2-butyl-ester of (*S*)-alanine	(*SS*)	−1.7	−1.8	0.5	3	−0.1
5	(*R*)-alanine	racemic	(*R*)-(−)-2-butanol	*N*-pivaloyl-(*R*)-2-butyl-ester of (*R*)-alanine	(*RR*)	−1.7	−1.8	0.7	2	−0.2
	(*S*)-alanine		(*R*)-(−)-2-butanol	*N*-pivaloyl-(*R*)-2-butyl-ester of (*S*)-alanine	(*RS*)		−1.9	0.4	2	−0.3
6	(*R*)-valine	mixture	(*S*)-(+)-2-butanol	*N*-pivaloyl-(*S*)-2-butyl-ester of (*R*)-valine	(*SR*)	3.8	3.6	0.4	3	−0.2
	(*S*)-valine		(*S*)-(+)-2-butanol	*N*-pivaloyl-(*S*)-2-butyl-ester of (*S*)-valine	(*SS*)	3.5	3.7	0.3	3	0.2

[a] The amino acids are conventionally expressed (*R*)-alanine, (*S*)-alanine, (*R*)-valine, and (*S*)-valine according to the Cahn−Ingold−Prelog (CIP) priority rule. Here, the D- and L-amino acids are expressed as the (*R*)-form and (*S*)-form based on the CIP priority rule; e.g., the abbreviation (*RR*) represents the *N*-pivaloyl-(*R*)-2-butyl-ester of (*R*)-alanine. $\delta^{15}N_{EA}$ represents EA/IRMS data verified without amino acid derivatization, and $\delta^{15}N_{GC}$ represents GC/C/IRMS data verified with the diastereomeric amino acid derivatization method using optically active *R*-(−)- or *S*-(+)-2-butanol and pivaloyl chloride. "Difference" indicates subtraction of the $\delta^{15}N_{EA}$ and $\delta^{15}N_{GC}$ data by multiple analysis. Since the (*R*)-alanine and (*S*)-alanine in the analysis runs 1 to 4 (single or mixture) and run 5 (racemic mixture) are different standard reagents (see bulk $\delta^{15}N_{EA}$ column), we verify the consistency between the $\delta^{15}N_{EA}$ and $\delta^{15}N_{GC}$ data in terms of single alanine enantiomers (chiral separated reagent for D- and L-alanine; D/L = 100:0 and D/L = 0:100 within 99% purity) and racemic chiral enantiomers (nonseparated reagent for D- and L-alanine; D/L = 50:50).

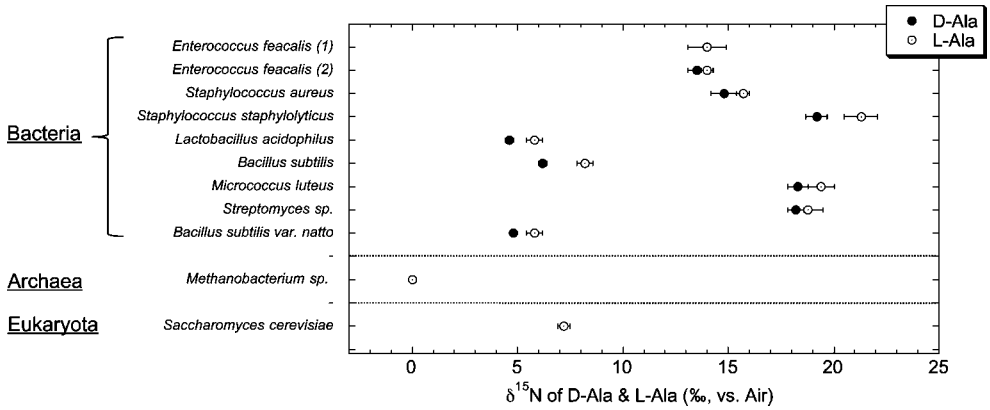

Figure 3. $\delta^{15}N$ (‰ vs air) of D-alanine and L-alanine in peptidoglycan (*Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus staphylolyticus*, *Lactobacillus acidophilus*, *Bacillus subtilis*, *Micrococcus luteus*, and *Streptomyces* sp.), archaeal cell wall (pseudo)-peptidoglycan (*Methanobacterium* sp.), eukaryotic cell wall (*Saccharomyces cerevisiae*), and natural whole bacterial cells (*Bacillus subtilis* var. *natto*).

mil (‰) deviation from the standard (air), as defined by the following equation: $\delta^{15}N = [(^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{standard} - 1] \times 1000$ (‰).

The nitrogen isotopic compositions of the individual amino acids were determined by GC/C/IRMS using a ThermoFinnigan Delta Plus XP combined with an Agilent Technologies 6890N GC with an Ultra-2 capillary column (5% phenyl 95% methyl polysiloxane; 25 m × 0.32 mm i.d., 0.52 μm film thickness; Agilent Technologies) in combustion and reduction furnaces.[22] Combustion was performed in a microvolume ceramic tube with CuO, NiO, and Pt wires at 1000 °C. Reduction was performed in a microvolume ceramic tube with a Cu wire at 550 °C. The GC oven temperature was programmed as follows: initial temperature of 40 °C for 4 min, ramped up at 15 °C min^{-1} to 130 °C, ramped up at 1 °C min^{-1} to 160 °C, and ramped up at 30 °C min^{-1} to 260 °C, where it was maintained for 10 min. The carrier gas (He) flow was 1.3 mL min^{-1}. The CO_2 generated in the combustion furnace was eliminated with a liquid nitrogen trap. We verified procedural blank and single run analysis by using single amino acid enantiomer, but we did not observe any detectable amount of amino acids in the procedure (Figure 2). The standard deviations (1σ) of the $\delta^{15}N$ values for the D- and L-amino acids ranged from ±0.3‰ to ±0.7‰ (Table 1) with a minimum sample amount of 30 ng of N.[22]

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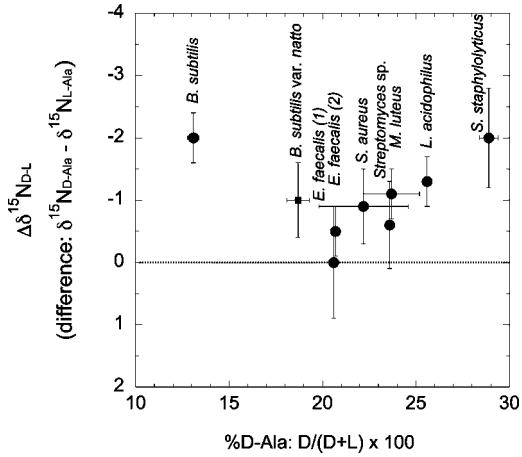

Figure 4. Diagram between D-Ala% (defined as D/(D + L) × 100) and the $\Delta\delta^{15}N_{D\text{-}L}$ (difference [Δ] between $\delta^{15}N_{D\text{-}Ala}$ and $\delta^{15}N_{L\text{-}Ala}$). The error bar for $\delta^{15}N$ is based on the maximum standard deviations in $\delta^{15}N_{D\text{-}Ala}$ and $\delta^{15}N_{L\text{-}Ala}$. ● and ■ represent peptidoglycan (*Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus staphylolyticus*, *Lactobacillus acidophilus*, *Bacillus subtilis*, *Micrococcus luteus*, and *Streptomyces* sp.) and natural whole bacterial cells (*Bacillus subtilis* var. *natto*), respectively.

RESULTS AND DISCUSSION

We achieved consistent $\delta^{15}N$ values in this study between the amino acids before cation-exchange column chromatography (without cation-exchange) and those after cation-exchange column chromatography. We conclude that there was no isotopic fractionation of nitrogen isotopes in protein and nonprotein amino acids, even when we used a 10% NH_3 aqueous solution in the cation-exchange chromatography (Figure S-1 in the Supporting Information).

The separation of the amino acid diastereomers was performed with baseline resolution. The compound-specific nitrogen isotope compositions of the amino acids were consistent when determined with the EA/IRMS (before derivatization) and GC/C/IRMS (after derivatization) method. The differences between the EA/IRMS and GC/C/IRMS values were within analytical error (<0.3‰), indicating an absence of nitrogen isotopic exchange or fractionation in the pathway of derivatization, GC separation, and IRMS, even when we switched the elution order of the enantiomers (Table 1).

Although it is a very minor reaction, individual enantiomers may produce opposite stereochemical forms of amino acids as a result of impurities in the permanent reagents (amino acids, optically active alcohol) or thermal racemization reactions (stereochemical conversion via α-hydrogen elimination). To check for possible impurity signals, the single analysis of only L-alanine or D-alanine by the same method was performed. However, as shown in Figure 2, no signal for such impurities or the racemization reaction during the procedure was apparent on the GC/C/IRMS chromatograms, meaning that any stereochemical conversion of the chiral amino acids during the derivatization process (from monomeric D- or L-amino acids to NP/2Bu esters) can be disregarded on the GC/C/IRMS system.

We used this method to analyze peptidoglycan and (pseudo)-peptidoglycan derived from members of the bacteria, archaea, and eukaryota. Representatively %D-alanine (vs total D,L-alanine; D/(D + L) × 100) in *Micrococcus luteus* and *Lactobacillus acidophilus* showed 23.7 ± 1.5% ($n = 3$) and 25.6 ± 0.1% ($n = 3$), respectively. No significant nitrogen signals from D-alanine were detected when (pseudo)-peptidoglycan from the archaea (*Methanobacterium* sp.) or cell wall from eukaryota (*Saccharomyces cerevisiae*) was analyzed with this GC/C/IRMS system.

$\delta^{15}N_{D\text{-}Ala}$ for peptidoglycan tended to be depleted in ^{15}N relative to $\delta^{15}N_{L\text{-}Ala}$ among the bacterial cultures tested. For instance, D-alanine of *Staphylococcus staphylolyticus* and *Bacillus subtilis* were significantly depleted in ^{15}N relative to L-alanine, by 2.0‰ in both bacteria. Only *Enterococcus faecalis* (culture 1) showed homogeneous values for $\delta^{15}N_{D\text{-}Ala}$ and $\delta^{15}N_{L\text{-}Ala}$ (Figure 3). The mole fractions of D-alanine (% D-alanine vs total alanine) correlated with the $\Delta\delta^{15}N_{D\text{-}L}$ (difference [Δ] between $\delta^{15}N_{D\text{-}Ala}$ and $\delta^{15}N_{L\text{-}Ala}$), except for *Bacillus subtilis* (Figure 4), suggesting some enzymatic control of the incorporation process during the formation of glycan chains.[24,25] The structure of the in vitro polymerized peptidoglycan may differ from that formed in the native in vitro process.[25,26] However, alanine racemase (Enzyme Commission, EC; 5.1.1.1) that interconvert L-alanine to D-alanine, one of isomerases for chiral amino acids, previously indentified in a biosynthetic pathway,[27] participates in one potential enzymatic reaction before D-alanine-D-alanine ligase (EC; 6.3.2.4) pathway in peptidoglycan metabolism. In this context, the function of alanine racemase has been reported to be an essential limiting factor for the growth of some bacteria, including *Lactobacillus plantarum*.[28] Molecular and bacterial culture surveys of the deep-sea subsurface environment detected a predominance of Gram-positive bacteria from benthic microbial communities.[29] This method for the analysis of $\delta^{15}N_{D\text{-}Ala}$ and $\delta^{15}N_{L\text{-}Ala}$ should facilitate microbial cell wall research. However, further experimental approaches using stable isotope techniques will be required for other kinds of D- and L-amino acids.

CONCLUSIONS

In this study, we developed an "online technique" for determining compound-specific nitrogen isotope analysis of D- and L-amino acid enantiomers. The resulting data are consistent with those of an EA/IRMS analysis that included intentional inversion of the D- and L-amino acids. By applying this method, we determined the nitrogen isotope compositions of D- and L-alanine in the peptidoglycan and (pseudo)-peptidoglycan from members of bacteria, archaea, and eukaryota. These results suggest that some enzymatic reaction pathways, including the alanine racemase reaction,[27,28] induce a difference in the nitrogen isotopes included in amino acid enantiomers. Our knowledge of the compound-specific nitrogen isotope ratios of other important chiral amino acids in peptidoglycan and other natural samples is extremely limited. This method will be useful

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in microbial cell wall biochemistry[29−32] and in the analysis of other geochemical samples, including ancient fossils and extraterrestrial materials.[33]

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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