Full Paper

Analyses of the cell-wall peptidoglycan structures in three genera *Micromonospora*, *Catenuloplanes*, and *Couchioplanes* belonging to the family *Micromonosporaceae* by derivatization with FDLA and PMP using LC/MS

(Received December 15, 2015; Accepted February 25, 2016; J-STAGE Advance publication date: July 29, 2016)

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It is the major characteristic of the cell-wall peptidoglycan structure in members of the family *Micromonosporaceae* that N-acetylmuramic acid (MurNAc) of glycan strand is replaced with N-glycolylmuramic acid (MurNGlyc). Consequently, it is difficult to use enzymatic methods for their peptidoglycan analyses. We therefore developed analysis method of peptidoglycan without using cell wall lytic enzymes as example to take the 3 genera, *Micromonospora*, *Catenuloplanes*, and *Couchioplanes*, belonging to the family *Micromonosporaceae*, and their peptidoglycans were partially hydrolyzed with 4 M HCl at 60°C for 16 h followed by derivatization with N²-(5-fluoro-2,4-dinitrophenyl)-D-leucinamide (FDLA) or 1-phenyl-3-methyl-5-pyrazolone (PMP) and LC/MS analysis. Peptidoglycan of the genus *Micromonospora* consisted of a MurNGlyc–Gly–D-Glu–meso-Diaminopimelyl (DAP)–D-Ala peptide stem and direct linkage between D-Ala and meso-DAP. In contrast, peptidoglycans of the genera *Catenuloplanes* and *Couchioplanes* consisted of a MurNGlyc–Gly–D-Glu–L-Lys–D-Ala peptide stem, and cross-linkage between D-Ala and L-Lys was mediated by an L-Ser residue. This method can be used to analyze the cell-wall peptidoglycan structure of other bacteria as well. By derivatization with FDLA or PMP followed by LC/MS analysis, the structure can be determined using only 0.2 mg of purified peptidoglycan.

Key Words: *Catenuloplanes*; *Couchioplanes*; FDLA; LC/MS; *Micromonospora*; peptidoglycan; PMP

Introduction

The cell-wall peptidoglycan of bacteria consists of two parts, a glycan strand in which N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) are alternately linked and a crosslinking peptide bridge to form a mesh-like structure. Peptidoglycan is generally characterized by the variation of diamino acids and interpeptide bridge crosslinking positions 3 and 4 (Schleifer and Kandler, 1972). Although peptidoglycan structure is highly correlated to bacterial taxonomy, data for peptidoglycan structure are not sufficient because it is very difficult to determine the amino acid sequences of the peptidoglycan. Consequently, peptidoglycan type is generally only estimated based on the molar ratio of amino acids.

In early stage of determination of peptidoglycan structure, purified peptidoglycan was partially hydrolyzed with HCl, and then the hydrolysate was separated by two-dimensional paper chromatography, with subsequent identification of partially hydrolyzed peptides in comparison with the known peptides by Rf values (Schleifer and Kandler, 1972). However, this method needed known peptides and experiences. Recent studies on peptidoglycan structure introduce liquid chromatography/mass spectrometry (LC/MS) and tandem mass spectrometry (MS/MS) for the analysis of muropeptides hydrolyzed with...
cell wall lytic enzymes, such as lysozyme, mutanolysin, and/or lysostaphin for the genera *Staphylococcus* (de Jonge et al., 1992), *Bacillus* (Attri et al., 1996, 1999), *Salmo-
nella* (Quintela et al., 1997), *Lactobacillus* (Billot-Klein et al., 1997), and *Streptomyces* (Hugonnet et al., 2014). However, some strains are resistant to these enzymes (Vollmar, 2008). As some species of the genus *Bacillus* contain nonacetylated glucosamine or muramic acid in the peptidoglycan, these strains could exhibit resistance to lysozyme and mutanolysin. Furthermore, some strains of the genera *Staphylococcus* and *Campylobacter* are resistant to lysozyme due to O-acetylation of MurNAc in the peptidoglycan. To solve this problem, we attempted to develop the method of analysis for peptidoglycan structure without using cell wall lytic enzymes.

Preparation of partial hydrolysate of peptidoglycan was carried out by using HCl and they were detected by LC/MS after derivatization of the hydrolysate with N-α-(5-fluoro-2,4-dinitrophenyl)-D-leucinamide (FDLA) or 1-phenyl-3-methyl-5-pyrazolone (PMP) which binds to the amino group of amino acids and the aldehyde moiety of reducing sugars, respectively. These enantiomer derivatives can be separated by reverse-phase column chromatography (Fujii et al., 1997; Tang et al., 2009). LC/MS analysis has the advantage of enabling the detection of a small amount of hydrolysate and to unambiguous identification of partial structures based upon their molecular weight.

Peptidoglycans of 3 genera *Micromonaspora*, *Catenuloplanes*, and *Couchioplanes* of the family *Micromonosporaceae* were analyzed by partial hydrolysis followed by derivatization with FDLA or PMP and subsequent LC/MS analysis. The peptidoglycan of members of the family *Micromonosporaceae*, which includes strains that produce antibiotics such as gentamicins, salinosporamides, and teichomycins (Tiwari and Gupta, 2012), cannot be analyzed by enzymatic methods due to resistance of the cell wall to lytic enzymes. It is the major characteristic of the cell-wall peptidoglycan structure in members of the family *Micromonosporaceae* that MurNAc of glycan strand is replaced with N-glycolylmuramic acid (MurNGlyc) (Genilloud, 2012). Due to the resistance to lysozyme imparted by this substitution, it is difficult to determine the peptidoglycan structure of these actinomycetes using enzymatic methods.

**Materials and Methods**

**Bacterial strains and cultivation.** *Micromonaspora aurantiaca* ICM 3232T, *Catenuloplanes japonicus* NBRC 14176T, and *Couchioplanes caeruleus* subsp. *caeruleus* NBRC 13979T were used for analysis of peptidoglycan after culture for 1 week at 27°C in YD broth (1.0% yeast extract (Difco, Detroit, MI, USA), 1.0% dextrose (Difco)).

**Purification of peptidoglycan.** Peptidoglycan was purified using a modification of the method described by Kawamoto et al. (1981). Cells were harvested by centrifugation and washed twice with distilled water. Ten grams of wet cells were suspended in 30 mL of 0.05 M phosphate buffer (pH 7.2) and then disrupted using an XL2020 ultrasonic liquid processor (Misonix Inc., Farmingdale, NY, USA) on ice for 45 min with alternating 10-s pulses and 10-s cooling intervals. Unbroken cells were removed by centrifugation at 900 × g for 15 min. The supernatant was centrifuged at 10,000 × g for 30 min and then the precipitates washed twice with 0.05 M phosphate buffer (pH 7.2). The precipitates were resuspended in 6 mL of buffer containing 4% sodium dodecyl sulfate and heated at 100°C for 40 min, and then washed 3 times with 0.05 M phosphate buffer (pH 7.2, 50°C) by centrifugation at 30°C. The precipitates were treated with 0.2 mg of pronase E (Merck, Darmstadt, Germany) in 6 mL of 0.05 M phosphate buffer (pH 7.6) at 37°C for 2 h and washed twice with 0.05 M phosphate buffer (pH 7.6). The insoluble material was suspended with 4 mL of 5% trichloroacetic acid, heated at 100°C for 20 min, and then washed twice with distilled water to remove sugars. After centrifugation (10,000 × g, 30 min, room temperature), the precipitates were suspended with 6 mL of ethanol and then diethylether to remove fatty acids. Pure peptidoglycan was collected by centrifugation and desiccation.

**Hydrolysis of peptidoglycan.** One milligram of purified peptidoglycan was hydrolyzed with 1 mL of HCl. The conditions for the preparation of complete hydrolysates were treatment with 6 M HCl at 100°C for 3 h. For partial hydrolysis, various the HCl concentrations (1, 2, 3, 4, or 6 M), reaction temperatures (60, 70, 80, 90, or 100°C), and reaction times (10 min, 20 min, 40 min, 1 h, or 16 h) were tested. After hydrolysis, distilled water was added to the solution and evaporated to remove HCl, and then the solution volume was adjusted to 1 mL by adding distilled water.

**FDLA derivatization.** Hydrolyzed samples were derivatized with FDLA (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) according to the method of Fujii et al. (1997). Fifty microliters of 1% FDLA in acetone and 20 μL of 1 M sodium bicarbonate were added to 50 μL of hydrolysate solution or 5 mM amino acids as standards, hydrolyzed samples were derivatized with FDLA or PMP were injected into the electrospray ion source of a QSTAR Elite ESI quadruple time-of-flight (Q-TOF) MS instrument (AB Sciei, Framingham, MA, USA) coupled to an Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA) chromatography system. Chromatographic separation was performed on an Inertsil ODS-4
column (3.0 × 250 mm, GL Sciences Inc., Tokyo, Japan) at 40°C. As for gradient elution, eluent A was LC/MS-grade water with 2 mM ammonium acetate, and eluent B was methanol with 2 mM ammonium acetate. The gradient elution was programmed as follows: 0–5 min, 5% B; 5–30 min, 5–100% B; 30–35 min, 100% B. The flow rate was 0.5 mL/min, the injection volume was 5 μL, and UV spectra were recorded using a photodiode array detector. Fragmentation was recorded for 30 min in the m/z region from 100 to 2,000 Da with the following instrument parameters: ion spray voltage = 5,500 V, source gas = 50 L/min, curtain gas = 30 L/min, declustering potential = 50 V, focusing potential = 250 V, temperature = 450°C, detector voltage = 2,300 V, and cone voltage = 20, 35 and 50 V. LC/MS analysis was performed by a high-resolution ESIMS (HRESIMS; R ≥ 10,000, the tolerance for mass accuracy was 10 ppm).

**Results**

**Determination of the molar ratio of amino acids composition from the peptidoglycan hydrolysate**

Completely hydrolyzed and FDLA-derivatized peptidoglycan of *Micromonospora aurantiaca* JCM 3232T appeared as four peaks upon LC-UV analysis. Comparison with the amino acid standards indicated that the peaks represented d-glutamic acid, glycine, d-alanine, and diaminopimelic acid (DAP) isomers. These results were also supported by LC/MS analysis (Fig. 1) and DAP derivatized with two FDLA molecules ([M+H]+, m/z 779.2959), as the molecular weight of the amino acids adducted with FDLA increased by 294 Da. Interestingly, L-alanine, which is generally the N-terminal amino acid in the peptide moiety of peptidoglycan, was not detected. The intensities of LC/MS peaks showed that the molar ratio of glycine, d-glutamic acid, DAP, and d-alanine was 1.1:1.0:1.1:0.8. Although a trace amount of L-DAP was detected, meso-DAP was the major diaminopimelic acid. These results were in agreement with those reported by Kawamoto et al. (1981).

**Peptidoglycan hydrolysis conditions for LC/MS analysis**

The HCl concentration, temperature, and time of the peptidoglycan hydrolysis required to obtain different lengths of peptides, were investigated. Peptidoglycan hydrolysates were detected as FDLA derivatives. The quantity of hydrolysate increased in an HCl concentration-dependent manner (1, 2, 3, 4, or 6 M HCl at 100°C for 10 min), and treatment with 4 M HCl was found to be optimal. The optimal temperature and hydrolysis time were examined under the condition of 4 M HCl. Consequently, the treatment of peptidoglycan with 4 M HCl at 60°C for 16 h was found to be optimal for obtaining the various peptides.

**Detection of peptides derivatized with FDLA**

Peptidoglycan of *M. aurantiaca* JCM 3232T was hydrolyzed with 4 M HCl at 60°C for 16 h, and the resulting hydrolysate was derivatized with FDLA and analyzed by LC/MS (Fig. 2 and Table 1). Derivatives of four amino acids in the peptidoglycan were detected. The following peptide derivatives were detected by LC/MS: three dipeptides, Gly–Glu (m/z 499.1775), Glu–DAP (m/z 908.3364), and DAP–Ala (m/z 850.3312); four tripeptides, Gly–Glu–DAP (m/z 965.3610), Glu–DAP–Ala (m/z 979.3786), Ala–DAP–Ala (m/z 921.3678), and DAP–Ala–DAP (m/z 1316.5141); and three tetrapeptides, Gly–Glu–DAP–Ala (m/z 1036.3964), Glu–DAP–(Ala)2 (m/z 1050.4173), and Ala–DAP–Ala–DAP (m/z 1387.5545). It was speculated that the m/z 1387 ion corresponded to Ala–DAP–Ala–DAP, as DAP–DAP or Ala–Ala derivatives were not observed. This tetrapeptide without FDLA (m/z 505) was then identified by MS/MS analysis. Since the binding of DAP and Ala could be deduced from five fragments (m/z 190, 315, 244, 262, and 434), the structure of the tetrapeptide was found to consist of an alternating arrangement of DAP and Ala (Fig. 3).

**Detection of glycopeptides derivatized with PMP**

Peptidoglycan of *M. aurantiaca* JCM 3232T was hydrolyzed with 4 M HCl at 60°C for 16 h, derivatized with PMP, and analyzed by LC/MS (Table 2). Derivatives of GlcNAc ([M+H]+, m/z 552.2476) and MurNGlyc (m/z 640.2633) in the peptidoglycan were detected, and derivatives of glucosamine (GlcN, m/z 510.2325) and muramic acid (MurN, m/z 582.2536), which were derived from hydrolysis, were also detected. The derivatives of GlcNAc–MurN (m/z 785.3346) and GlcN–MurN (m/z 743.3264) were detected as disaccharides composed of these sugars. Moreover, glycoamino acids and glycopeptide derivatives were also obtained: N-glycolylmuramyl glycin (MurNGlyc–Gly, m/z 697.2804), muramyl glycin (MurN–Gly, m/z 639.2717), and muramylglycyl glutamate (MurN–Gly–Glu, m/z 768.3180).
Peptidoglycan structure of the genus Micromonospora

The cell-wall peptidoglycan structure of the strain *M. aurantiaca* JCM 3232\(^T\) according to our results is shown in Fig. 4. PMP derivatization of hydrolyzed peptidoglycan of the genus *Micromonospora* indicated that the glycan strand consists of an alternating arrangement of GlcNAc and MurNGlyc, as the PMP derivatives of MurNAc or dimers of GlcNAc or MurNGlyc (such as GlcNAc–GlcNAc, GlcN–GlcN, MurNGlyc–MurNGlyc or MurN–MurN) were not detected. The carboxylic acid residue of MurNGlyc was bound to amino group of glycine.

The peptide stem of Gly–D-Glu–meso-DAP–D-Ala could be deduced from the FDLA derivatives of hydrolysates. Internal peptide of cross-linkage did not exist, and the C-terminal D-Ala residue in the peptide stem of the acyl donor was bound directly to the amino group of meso-DAP of the acceptor in the peptidoglycan. The molar ratio of D-Ala was lower than that of the other amino acids, indicating that the non-crosslinked D-Ala residue may be lost in the peptidoglycan.

Peptidoglycan structure of the genera Catenuloplanes and Couchioplanes

The peptidoglycans of *Catenuloplanes japonicus* NBRC 14176\(^T\) and *Couchioplanes caeruleus* subsp. *caeruleus* NBRC 13939\(^T\) were analyzed using the same method for *M. aurantiaca* JCM 3232\(^T\). Complete hydrolysis and FDLA derivatization indicate that the peptidoglycans of the genera *Catenuloplanes* and *Couchioplanes* have similar structures to that of *M. aurantiaca* JCM 3232\(^T\).
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strains NBRC 14176T and NBRC 13939T consisted of L-serine, D-glutamic acid, glycine, D-alanine and L-lysine. For both of these strains, the molar ratio of L-serine, D-glutamic acid, glycine, D-alanine and L-lysine was approximately 1:1:1:0.7–0.8:1.

Peptidoglycans of the strains NBRC 14176T and NBRC 13939T were hydrolyzed with 4 M HCl at 60°C for 16 h, derivatized with PMP (data not shown) or FDLA (Table 3), and analyzed by LC/MS. As the pattern of PMP derivatization of hydrolyzed peptidoglycans for strains NBRC 14176T and NBRC 13939T was similar to that of the strain *M. aurantiaca* JCM 3232T, the glycan strand was determined to consist of an alternating arrangement of GlcNAc and MurNGlyc, with MurNGlyc bound to glycine.

FDLA derivatives of the dipeptides Gly–Glu ([M+H]+, m/z 499), Glu–Lys (m/z 864), Lys–Ala (m/z 806), Lys–Ser (m/z 822), and Ser–Ala (m/z 471) were detected. Moreover, four different tripeptides and three different tetrapeptides were detected in each sample (Table 3). These data indicate that the peptide stem consisted of Gly–Lys–Ala–D-Ala. With respect to cross-linkage, the d-Ala of the peptide stem of the acyl donor is bound to the l-Lys of the acceptor, mediated by an L-Ser residue.

| Aminosugar or glycopeptide | m/z [M+H]+ | Derivatives with PMP [M+H]+ | ppm error |
|---------------------------|-------------|-----------------------------|-----------|
| GlcNAc                    | 222.0972    | 552.2453                    | 4.2       |
| MurNGlyc                  | 310.1133    | 640.2613                    | 3.1       |
| GlcN                      | 180.0867    | 510.2347                    | 4.4       |
| MurN                      | 252.1078    | 582.2559                    | 3.9       |
| GlcNAc–MurN               | 455.1872    | 785.3352                    | 0.8       |
| GlcN–MurN                 | 413.1766    | 743.3247                    | 2.3       |
| MurNGlyc–Gly              | 367.1347    | 697.2828                    | 3.5       |
| MurN–Gly                  | 309.1293    | 639.2773                    | 8.8       |
| MurN–Gly–Glu              | 438.1719    | 768.3199                    | 2.5       |

Strains *M. aurantiaca* JCM 3232T, as determined from these results, is shown in Fig. 5. Similar to the genus *Micromonospora*, the non-crosslinked d-Ala residue may be lost in peptidoglycan.
The structure was deduced from results of this study.

The diamino acid at position 3 of the peptidoglycan of the genus *Micromonospora*, but the genera *Catenuloplanes* and *Couchioplanes* possess one L-Ser molecule. These results indicate that the peptidoglycan structure can be applied to the classification of organisms within the family *Micromonosporaceae*.

Members of the family *Micromonosporaceae* have traditionally been classified based on various spore morphologies, whether the organism produces single spores, long spore chains, sporangia, or motile spores, in addition to other characteristics (Genilloud, 2012). The number of organisms for which 16S rRNA gene sequences are available has increased greatly since 1990s, consequently, new genera with same phenotypic characteristics have been proposed by the phylogenetic analysis. For instance, morphological and chemotaxonomical characteristics of the genus *Salinispora* are consistent with those of the genus *Micromonospora*, except for the requirement for seawater (Maldonado et al., 2005). Although the whole genome of the genus *Salinispora* has been analyzed (Nett et al., 2009), the amino acid composition of its peptidoglycan remains unknown. Peptidoglycan amino acid composition of approximately half of the 29 genera belonging to the family *Micromonosporaceae* has not been determined. It will be of value to accumulate the data of peptidoglycan structure of various actinomycetes to demonstrate them as chemotaxonomic criteria for actinomycetes. The method that we have developed in this study enables to determine the peptidoglycan structure by derivatization with FDLA or PMP followed by LC/MS from only 0.2 mg to determine the peptidoglycan structure by derivatization with FDLA or PMP followed by LC/MS from only 0.2 mg.

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**Table 3.** FDLA derivatives of peptidoglycan hydrolysates of *C. japonicus* NBRC 14176 and *C. caeruleus* subsp. *caeruleus* NBRC 13939.

| Amino acid or peptide | Calculated m/z [M+H]+ | Catenuloplanes | Couchioplanes |
|-----------------------|------------------------|----------------|--------------|
| Gly                   | 370.1357               | 370.1367 2.6   | 370.1364 1.8 |
| Glu                   | 442.1569               | 442.1571 0.5   | 442.1575 1.4 |
| Lys                   | 735.3057               | 735.3063 0.9   | 735.3069 1.7 |
| Ala                   | 384.1514               | 384.1513 0.3   | 384.1516 0.5 |
| Ser                   | 400.1463               | 400.1468 1.2   | 400.1457 1.5 |
| Gly–Glu               | 499.1783               | 499.1783 0.2   | 499.1783 0   |
| Glu–Lys               | 864.3483               | 864.3464 2.2   | 864.3483 0   |
| Lys–Ala               | 806.3428               | 806.3432 0.5   | 806.3443 1.9 |
| Lys–Ser               | 822.3377               | 822.3375 0.2   | 822.3390 1.6 |
| Ser–Ala               | 471.1834               | 471.1824 2.2   | 471.1830 0.9 |
| Gly–Glu–Lys           | 921.3697               | 921.3704 0.7   | 921.3697 0   |
| Glu–Lys–Ala           | 935.3854               | 935.3846 0.8   | 935.3827 2.9 |
| Glu–Lys–Ser           | 951.3803               | 951.3807 0.4   | 951.3796 0.7 |
| Lys–Ser–Ala           | 893.3748               | 893.3736 1.4   | 893.3740 0.9 |
| Gly–Glu–Lys–Ala       | 992.4068               | 992.4010 5.9   | 992.4184 11.7|
| Glu–Lys–Ala–Ser       | 1022.4174              | 1022.4203 2.8  | 1022.4187 1.3|
| Lys–Ala–Ser–Lys       | 1315.5662              | 1315.5601 4.6  | 1315.5604 4.4|

The structure was deduced from results of this study.
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