Novel Glycosidic Linkage between Arabinogalactan and Peptidoglycan in the Cell Wall Skeleton of *Nocardia rubra* AN-115

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A glycosidic linkage between the arabinogalactan and peptidoglycan has been demonstrated for the first time in cell walls from *Nocardia rubra* AN-115. Two oligosaccharides, Rha-(1→3)-GlcNAc and Gal-Rha-(1→3)-GlcNAc, were identified by gas chromatography-mass spectrometry in acid hydrolysates of arabinogalactan containing defective peptidoglycan polymer obtained from stepwise alkaline degradations of *N. rubra* AN-115 walls. The arabinogalactan of *N. rubra* AN-115 walls contained not only Ara and Gal but also Rha, while peptidoglycan contained glycan polymers composed of GlcNAc-MurNGI units. These findings strongly suggest that arabinogalactan was linked glycosidically to peptidoglycan through a Rha-GlcNAc linkage in *N. rubra* AN-115 walls.

INTRODUCTION

Corynebacterium, *Mycobacterium* and *Nocardia* have similar cell wall structures, which consist of mycolic acid, arabinogalactan and peptidoglycan (Lederer, 1971; Michel & Bordet, 1976; Barksdale & Kim, 1977). Arabinogalactan is a polysaccharide composed of D-arabinose and D-galactose together with, in some cases, small amounts of neutral sugars such as mannose, glucose and rhamnose (Cummins, 1962; Yamaguchi, 1965; Abou-Zeid *et al.*, 1982). The peptidoglycan comprises a glycan moiety composed of disaccharide units, *N*-acetyl-D-glucosaminyl-*N*-glycolyl (or -acetyl)-muramic acid and a tetrapeptide moiety composed of D- and L-alanine, D-glutamic acid and *meso*-diaminopimelic acid.

Mycolic acid is bound to the arabinose residue of arabinogalactan by an ester linkage, while the nature of the linkage between arabinogalactan and peptidoglycan remained unknown. Since Liu & Gotschlich (1967) demonstrated the presence of a phosphodiester linkage between arabinogalactan and peptidoglycan by the isolation of a muramic acid 6-phosphate in walls of *Mycobacterium butyricus*, many studies have been made of the phosphodiester linkage in various bacterial walls (Kanetsuna, 1968; Cunto *et al.*, 1969; Kotani *et al.*, 1970). Muramic acid 6-phosphate has been identified from *N. rubra* AN-115 walls by ¹³C nuclear magnetic resonance studies, and suggests the presence of a phosphodiester linkage between the arabinogalactan and the peptidoglycan (A. Miyamae & M. Shigi, unpublished results).

On the other hand, Kanetsuna & San Blas (1970) reported that a glycosidic linkage between the *N*-acetyl-D-glucosamine residue of peptidoglycan and arabinogalactan in walls of *Mycobacterium* might be present in addition to the phosphodiester linkage. However, there has been insufficient evidence so far to show the presence of this glycosidic linkage.

Abbreviations: Ala, alanine; Ara, arabinose; CI, chemical ionization; Dap, diaminopimelic acid; EI, electron impact; Gal, galactose; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; Glu, glutamic acid; Mur, muramic acid; MurNGI, *N*-glycolylmuramic acid; Rha, rhamnose.

We have found that alkaline treatment of *N. rubra* AN-115 walls liberated not only an arabinogalactan fraction but also large amounts of an additional polymer fraction composed of both arabinogalactan and peptidoglycan constituents. Since the phosphodiester linkage was readily broken by alkaline treatment, this finding indicates the presence of a further linkage between the arabinogalactan and the peptidoglycan besides the phosphodiester linkage.

The present report deals with a novel glycosidic linkage between the arabinogalactan and peptidoglycan in *N. rubra* AN-115.

METHODS

Organism and culture conditions. The walls were prepared from *Nocardia rubra* AN-115. This strain is preserved in the Research Laboratories of Fujisawa Pharmaceutical Co. It was grown in a medium containing the following: 10 g polypeptone, 5 g yeast extract and 1 g detergent (Adecanol; Asahi Denka Kogyo Co., Japan), in 800 ml distilled water, adjusted to pH 7.0 with 1 M-NaOH. The cultures were incubated at 30 °C on a rotary shaker at 220 r.p.m. for 3 d and then used to inoculate a medium containing 1.66 kg dextrose, 20.0 kg polypeptone, 10.0 kg yeast extract, 0.415 kg KH_2PO_4 , 0.415 kg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.15 kg Adecanol and 760–770 l distilled water, adjusted to pH 7.0 with 50% (w/v) NaOH solution, in a jar fermenter. The cells were cultured at 35 ± 0.5 °C for 90 h with mechanical stirring (175 ± 5 r.p.m.). After cooling to below 10 °C, the cells were harvested by centrifugation and washed with distilled water.

Preparation of cell walls. The cells were suspended in distilled water and disrupted mechanically in a mill (Dyno-mill type KDL; Willy A. Bachofen Manufacturing Engineers, Basel, Switzerland: 0.1 mm diameter glass beads were used). The disrupted cell suspension was mixed with 800 ml Veronal buffer pH 7.5 (572 mg Veronal, 375 mg Veronal sodium, 27 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 109 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, in 1000 ml distilled water) containing 70 mg deoxyribonuclease and 105 mg ribonuclease and stirred at room temperature for 30 min. The enzyme treated cell suspension was centrifuged at 1200 g for 15 min. The supernatant fluid was centrifuged at 16950 g and the cell wall fraction was collected. This fraction was washed with acetone, 20% (v/v) Triton X-100 solution and ethanol/distilled water (2:3, v/v), successively. The residue was collected by centrifugation at 16950 g, resuspended in distilled water, and the pH adjusted to 5.5 with 6 M-HCl. After stirring for 60 min, the suspension was centrifuged at 16950 g to obtain the crude cell walls. The walls were then incubated with 1.75 g protease (pronase; Kaken Kagaku Co., Japan) at room temperature for 24 h in Veronal/HCl buffer pH 9.5 [35 l 0.1 M-Veronal sodium solution was adjusted to pH 9.5 with 1 M-HCl]. The walls were sedimented at 16950 g, washed with distilled water and 1,2-dichloroethane/ethanol (1:2, v/v), and finally freeze-dried. This material was disrupted in a mill (Dyno-mill type KD5), passed through a sieve (125 μm), and was designated as 'N-CWS'.

Isolation of arabinogalactan-peptidoglycan complex. About 30 g N-CWS was hydrolysed with 1000 ml 0.5% (w/v) KOH/methanol solution at 37 °C for 48 h under nitrogen. After neutralization with concentrated HCl, the mixture was evaporated to a syrup, and then suspended in 50 ml water. The suspension was extracted three times with 50 ml ether. The residual suspension was filtered and washed with water, ethanol/ether (1:1, v/v), chloroform and chloroform/methanol/water (10:10:1, by vol.) successively. The residue was resuspended in water and freeze-dried to yield the arabinogalactan-peptidoglycan complex (22 g).

Isolation of arabinogalactan containing defective peptidoglycan and arabinogalactan. The above freeze-dried material was hydrolysed with 600 ml 1 M-NaOH at 70 °C for 24 h under nitrogen. After neutralization with acetic acid, the mixture was evaporated to a small volume and passed successively through filters (3.0, 0.8 and 0.45 μm pore size). The filtrate from the latter filter was dialysed against water for 4 d and evaporated to a small volume. This solution was applied to a column of Dowex 50W X8 (H^+ form, 2.5×15 cm), and eluted with 1000 ml water. The eluate was again evaporated to a small volume and applied to a column of DEAE-cellulose DE 52 (OH^- form, 2.5×15 cm). The arabinogalactan fraction was eluted with 1000 ml water and freeze-dried to yield 150 mg. The arabinogalactan containing the defective peptidoglycan fraction was eluted with 1500 ml 0.01 M-phosphate buffer (pH 7.0) and evaporated to a small volume. The solution was dialysed against water for 4 d and freeze-dried to yield 1.2 g.

Analytical methods. Quantitative determinations of each constituent of the three polymers and N-CWS were performed by the following methods.

Lipids. The sample was hydrolysed with 5% (w/v) KOH/methanol solution at 100 °C for 3 h in a sealed tube. The mixture was acidified with 1 M-HCl and extracted three times with ether. The combined extract was evaporated, dried over P_2O_5 *in vacuo* for 90 min at room temperature, and weighed.

Sugars. The sample was hydrolysed with 1 M-HCl at 90 °C for 6 h in a sealed tube. The hydrolysate was applied to an ion-exchange column (Diaion WA 30, OH^- form), and eluted with water, and evaporated to dryness. The residue was then dissolved in water and reduced with sodium borohydride for 1 h. The solution was acidified with acetic acid and evaporated to dryness. Boric acid present in the residue was removed by co-distillation with methanol/HCl (100:1, v/v) and methanol successively. The residue was treated with ethyl acetate/trifluoroacetic

anhydride (1:0.3, v/v) for 30 min. For gas chromatography, a sample of the solution was injected into a Shimadzu GC-7A instrument fitted with a 5% silicon XF-1105 on Gaschrom Q column (3 mm $\times$ 2 m).

Amino sugars and amino acids. The sample was hydrolysed with 6 M-HCl at 100 °C for 20 h in a sealed tube. The hydrolysate was evaporated to dryness and redissolved in 0.02 M-HCl. A sample of the solution was injected into a Hitachi 835 amino acid analyser equipped with a Hitachi Custom ion-exchange resin no. 2617 column (4 mm $\times$ 25 cm). Citrate buffers of pH 3.3, 4.3 and 4.9 were used stepwise for the eluents.

Phosphorus. The sample was pyrolysed in HNO₃/H₂SO₄ (10:3, v/v) by wet combustion. After cooling, the solution was heated again with additional HNO₃. The resulting phosphorus containing compound in the solution was converted to orthophosphoric acid by H₂O₂ and determined by the molybdenum-blue method (Boltz & Lueck, 1958).

Losses on drying. These were determined after heating at 105 °C for 5 h.

Gas chromatography-mass spectrometry of arabinogalactan containing defective peptidoglycan. Arabinogalactan containing defective peptidoglycan (about 100 mg) was partially hydrolysed with 10 ml 0.2 M-H₂SO₄ at 100 °C for 30 min in a sealed tube saturated with nitrogen. The hydrolysate was neutralized with barium carbonate, filtered and the filtrate was evaporated to dryness. The residue was dissolved in 20 ml water and reduced with 200 mg sodium borodeuteride. After acidification with acetic acid, the mixture was applied to a column of Dowex 50W X8 (H⁺ form, 2 $\times$ 10 cm), and then eluted with 150 ml water. The eluate was evaporated to dryness. Boric acid present in the residue was removed by co-distillation with methanol. The reduced product was dried over P₂O₅ *in vacuo* and then dissolved in 20 ml dimethyl sulphoxide saturated with nitrogen gas. Methyl sulphinyl carbanion (1 ml) prepared by the reaction of sodium hydride and dimethyl sulphoxide (Hakomori, 1964) was added to the solution. The reaction mixture was stirred for 5 h, and 1 ml methyl iodide was added dropwise. The mixture was poured into 50 ml water and extracted three times with 30 ml chloroform. The combined chloroform extract was rinsed with water and evaporated to dryness. The methylated product was analysed by GC-MS. A Hitachi M-80 GC-MS instrument was used for electron impact (EI) study and a Finnigan 4000 GC-MS instrument with methane gas was used for chemical ionization (CI) study.

RESULTS AND DISCUSSION

The chemical compositions of N-CWS and the three polymers obtained are summarized in Table 1. The composition of N-CWS was very similar to that obtained from walls of other *Nocardia* reported by Azuma *et al.* (1975, 1976). This similarity allowed us to deduce that N-CWS has a structure common to all walls from *Nocardia*, that is, a mycolic acid-arabinogalactan-peptidoglycan complex having the following constituents: lipid, nocardomycolic acid; arabinogalactan, Ara, Gal, Rha (2.5:1.0:0.08, by molar ratio); peptidoglycan, GlcN, Mur, Ala, Glu, Dap (1:1:2:1:1, by molar ratio) (Table 1).

Rhamnose is one of the intrinsic constituents of arabinogalactan in N-CWS. The molar ratio of Rha to Gal in the three polymers as well as in N-CWS was almost always 0.1:1.0 (Table 1) in spite of some degradation occurring during the purification of the arabinogalactan from N-CWS (see Methods).

In N-CWS, the content of phosphorus which was presumed to be present as muramic acid 6-phosphate was about 0.1%. The molar ratio of phosphorus to muramic acid was about 1:9. The low phosphorus content suggested that even if all of the phosphorus was present in phosphodiester linkages between arabinogalactan and peptidoglycan, the number of linkages may not be sufficient to maintain binding between them. In addition, alkaline treatment of N-CWS liberated lipid fractions from N-CWS, and gave an arabinogalactan-peptidoglycan complex. More severe alkaline treatment solubilized a fraction composed of both arabinogalactan and some peptidoglycan. This fraction appeared to be arabinogalactan linked to defective peptidoglycan judging from its composition (Table 1). It is noteworthy, however, that some peptidoglycan was still linked to arabinogalactan after such severe alkaline treatment. Peptide bonds in the peptidoglycan moiety were also hydrolysed by this treatment and we believe that all the phosphodiester linkages present between arabinogalactan and peptidoglycan would have been hydrolysed. On the other hand, the arabinogalactan itself did not contain any peptidoglycan components except for a very small amount of GlcN (Table 1). These results suggest that another type of linkage besides the phosphodiester linkage was present. Kanetsuna & San Blas (1970) had reported that it might be a glycosidic linkage between a GlcN residue in

Table 1. *Chemical composition of N-CWS and the three wall polymers*

For N-CWS the mean values of five lots of samples are given. At least three determinations were made for each component tested in each sample. The values in parentheses are molar ratios. ND, No data; —, not detected.

Component	Percentage dry weight of fraction			
	N-CWS	Arabinogalactan-peptidoglycan complex	Arabinogalactan containing defective peptidoglycan	Arabinogalactan
Lipids	27.6	1.2	ND	ND
Sugars				
Ara	15.2 (2.05)	27.9 (2.22)	41.4 (2.60)	67.9 (2.54)
Gal	8.9 (1.00)	15.1 (1.00)	19.1 (1.00)	32.0 (1.00)
Rha	0.9 (0.1)	1.3 (0.09)	1.7 (0.10)	2.4 (0.08)
Amino sugars				
GlcN	4.8 (0.54)	8.0 (0.53)	4.4 (0.23)	0.6 (0.02)
Mur	6.2 (0.50)	11.3 (0.54)	5.2 (0.20)	0.08 (0.002)
Amino acids				
Ala	8.5 (1.94)	8.6 (1.15)	2.7 (0.29)	0.03 (0.002)
Glu	5.8 (0.79)	7.3 (0.59)	2.2 (0.14)	0.03 (0.001)
Dap	7.2 (0.77)	10.4 (0.65)	3.3 (0.16)	0.15 (0.004)
Gly	1.2	0.2	0.03	—
Ser	1.7	0.1	0.02	—
Thr	1.4	0.1	0.01	—
Leu	1.2	0.2	0.01	—
Phe	1.5	0.3	—	—
Phosphorus	0.09 (0.06)	0.1 (0.04)	ND	ND
Loss on drying	7.2	11.9	ND	ND

the peptidoglycan and the arabinogalactan. To investigate this possibility arabinogalactan containing defective peptidoglycan was partially hydrolysed and the occurrence of oligosaccharides containing both GlcN and constituents of arabinogalactan was studied by GC-MS.

Fig. 1 shows a total ion current chromatogram of the permethylated alditol derivatives of the oligosaccharides mixture obtained from the partial acid hydrolysis of arabinogalactan containing defective peptidoglycan. The oligosaccharide structures corresponding to each peak in Fig. 1 were identified by mass fragmentation analysis (Kärkkäinen, 1970, 1971; Lönnngren & Svensson, 1974; Lundblad *et al.*, 1975; Mononen *et al.*, 1978).

EI mass spectra for peaks E and I are shown in Figs 2 and 3 respectively.

The A-series fragments (Lönnngren & Svensson, 1974; Lundblad *et al.*, 1975) A_1 (m/z 189), A_2 (m/z 157) and A_3 (m/z 125) and the fragment Ald (m/z 277) show the sequence Rha-GlcNAc (Fig. 2). The presence of additional fragments such as $M - 45$ (m/z 437), $M - 46$ (m/z 436), $M - 89$ (m/z 393), $M - 133$ (m/z 349) and the fragment m/z 131 indicates that the linkage position between Rha and GlcNAc is 1→3.

In Fig. 3, the A-series fragments aA_1 (m/z 219), aA_2 (m/z 187), aA_3 (m/z 155), baA_1 (m/z 393) and the J-series fragment (Lönnngren & Svensson, 1974; Lundblad *et al.*, 1975) $abAldJ_1$ (m/z 511) and the fragments Ald (m/z 277) and $bAld$ (m/z 451) show the sequence Gal-Rha-GlcNAc. The presence of additional fragments such as $M - 45$ (m/z 641), $M - 46$ (m/z 640), $M - 89$ (m/z 597) and $M - 133$ (m/z 553), and the fragment m/z 131 indicated that the linkage position between Rha and GlcNAc was also 1→3, but the linkage position between Gal and Rha has not been determined.

In the case of EI mass studies, neither molecular ions of the permethylated alditol derivatives of Rha-(1→3)-GlcNAc nor Gal-Rha-(1→3)-GlcNAc were identified, but CI mass spectra obtained using methane as a reagent gas clearly showed $M + H$, $M + C_2H_5$ and $M + C_3H_5$ ions of each oligosaccharide. The m/z values and relative intensities (%) of the CI mass spectra of peaks E and I were: $M + H$, 483 (2.1%); $M + C_2H_5$, 511 (13.7%); $M + C_3H_5$, 523 (4.6%) for peak E and $M + H$, 687 (4.9%); $M + C_2H_5$, 715 (12.5%); $M + C_3H_5$, 727 (4.8%) for peak I.

Fig. 1. Total ion current chromatogram of the permethylated alditol derivatives obtained from partial acid hydrolysis of arabinogalactan containing defective peptidoglycan. The conditions used were 2% OV-1 on Gaschrom Q (3 mm $\times$ 100 cm), injection temperature 285 $^{\circ}$ C, column temperature 180–280 $^{\circ}$ C, ionizing voltage 20 eV, total emission current 100 μ A. A and B, permethylated alditol derivatives of arabinose dimers; C and D, galactose dimers; H, galactose trimer; J and K, arabinose tetramers.

Fig. 2. Mass spectrum of peak E.

The GC–MS conditions were 1.5% OV-1 on Chromosorb W HP (2 mm $\times$ 70 cm); injection temperature, 230 $^{\circ}$ C; column temperature, 180–280 $^{\circ}$ C; ionizing voltage, 70 eV; total emission current, 400 μ A; reagent gas, methane 0.3 Torr (40 Pa).

As shown in Table 1, both Gal and Rha were constituents of arabinogalactan whereas GlcNAc was a constituent of peptidoglycan. Thus, the presence of Rha-(1 $\rightarrow$ 3)-GlcNAc and Gal-Rha-(1 $\rightarrow$ 3)-GlcNAc strongly suggested that a novel glycosidic linkage existed between arabinogalactan and peptidoglycan in N-CWS. The proposed structure of the arabinogalactan–peptidoglycan moiety of N-CWS is illustrated in Fig. 4 together with the phosphodiester linkage between arabinogalactan and peptidoglycan. However, we could not identify any oligosaccharides containing a GlcNAc-(1 $\rightarrow$ 4)-MurNGl sequence in our experiments. Consequently, some ambiguity still remains concerning the origin of GlcNAc in Rha-(1 $\rightarrow$ 3)-GlcNAc

Fig. 3. Mass spectrum of peak I.

Fig. 4. Tentative structure of the arabinogalactan-peptidoglycan moiety in walls of *Nocardia rubra* AN-115.

and Gal-Rha-(1→3)-GlcNAc. A structure, such as arabinogalactan-Gal-Rha-GlcNAc-(?)-peptidoglycan is possible whereby GlcNAc is part of the arabinogalactan rather than the peptidoglycan. However, it is more likely that the GlcNAc is derived from peptidoglycan for the following reasons. The linkage between GlcNAc and peptidoglycan seems to be glycosidic rather than a phosphodiester. If it were a phosphodiester linkage, it should have been hydrolysed along with most of the phosphodiester linkages during alkaline hydrolysis, and as a consequence, the molar ratio of Glc:Mur in the arabinogalactan containing defective peptidoglycan would be > 1 not 1 (see Table 1). A ratio greater than 1.0 would be obtained if the GlcNAc was derived from the arabinogalactan.

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