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Localization of the attachment site of oligoglucans to *Mesorhizobium loti* HAMBI 1148 murein

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The location and nature of the linkage between peptidoglycan and oligoglucans in the cell wall of *Mesorhizobium loti* HAMBI 1148 have been defined by the analysis of nitrous acid deamination of peptidoglycan glucosaminyl residues. The $MurNH_2$-Glc_n fraction was obtained after converting deaminoacylated and N-deacetylated muramyl residues in the cell wall preparation to lactam forms which were stable during subsequent deamination, followed by reduction and opening of the lactams. GC/MS analysis of this material, subjected to partial hydrolysis and reduction or to methanolysis followed by peracetylation, confirmed the presence of glucosyl residues glycosidically attached to muramic acid. The MALDI-TOF spectroscopic analysis of the deaminated material also revealed the presence of $[M-H]^-$ or $[M+Na-2H]^-$ ions representing fragments containing muramic acid with one to three linked glucose residues. The analysis of fully methylated neutral oligosaccharides released from the peptidoglycan with lysozyme followed by borohydride reduction showed the presence of di- and trisaccharides lacking the reducing end.

Keywords: *Mesorhizobium*, murein, oligoglucan, deamination, MALDI-TOF

INTRODUCTION

Peptidoglycan is a ubiquitous constituent of the bacterial cell wall responsible for the physical integrity of bacteria. It consists of a glycan backbone of β-*N*-acetylglucosaminyl (GlcNAc) (1→4) *N*-acetylmuramyl (MurNAc) units joined by β-1,4 linkages with short peptide chains linked to the lactyl moiety of muramyl residues (Schleifer & Kandler, 1972). In earlier reports (Drożański *et al.* 1981; Karaś *et al.* 2005) it was suggested that *Rhizobium* peptidoglycans were unique as they had an extra glucose constituent besides the usual muramyl glycan components. The liberation of oligoglucans from *Rhizobium* cell wall preparations with lysozyme suggested that they were covalently linked to peptidoglycan sacculi. On the basis of the knowledge of lysozyme specificity, the author hypothesised that the most probable site of attachment was the 'reducing' muramic acid residue. However, till now no direct proof of that hypothesis has been presented.

This paper reports studies on the nitrous acid deamination products of *Mesorhizobium loti* HAMBI 1148 N-deacylated lactamised murein glycan. The results presented here provide evidence that *Mesorhizobium*, in contrast to other bacteria, contains an unusual type of murein to which glucose oligomers are linked covalently. Due to the lack of pure commercial preparations of bacteriolytic enzymes, particularly endo-*N*-acetyl-β-glucosaminidase and *N*-acetylmuramoyl-L-alanyl amidase, chemical methods of murein degradation were applied.

MATERIALS AND METHODS

Strain and growth of bacteria. *Mesorhizobium loti* strain HAMBI 1148 was obtained as a gener-

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Abbreviations: Ac_2O, acetic anhydride; GlcNAc, *N*-acetyl-D-glucosamine; lacMurNH, muramic acid lactam; MurNAc, *N*-acetyl muramic acid; PS, polysaccharide; TFA, trifluoroacetic acid.

ous gift from the Department of Microbiology and Biochemistry, University of Helsinki (Finland). The bacteria were grown to stationary phase in liquid medium 79CA (Vincent, 1970) at 28ºC with vigorous shaking.

Preparation of murein glycans containing linked glucose oligomers. The cells were harvested by centrifugation and washed with 0.5 M NaCl and water. The delipidated (Bligh & Dyer, 1959) cell debris, insoluble in hot phenol/water (Westphal & Jann, 1965) and boiling sodium dodecyl sulfate (Weidel *et al*., 1960), was digested with trypsin (Drożański, 1983) and subjected to the procedure of removing gel-forming PS as described by Zevenhuizen *et al.* (1983). After such treatment, the murein preparation was free from outer rhizobial polysaccharides and other components of the bacterial envelope. The freeze-dried crude murein preparation containing intracellular glycogen was suspended in 30% KOH (100 mg/ml, in a Teflon test tube placed inside a screw-cap vessel) and after flushing with nitrogen it was incubated in a boiling water bath for 3 h to disrupt murein amide bonds (Gerhard *et al*., 1994). To the cooled material, 3 volumes of water were added and the debris insoluble in strong alkali was removed by centrifugation. Glycogen was precipitated from the supernatant with ice-cold ethanol (70%, v/v) and centrifuged. The sediment was dissolved in water and precipitated again with ethanol. The pooled ethanol supernatants were cooled in ice, neutralised with hydrochloric acid, concentrated by rotary evaporation, and freeze-dried. The products of alkaline deaminoacylation and N-deacetylation were fractionated and desalted on a Sephadex G15 column (∅ 2.5 cm×100 cm, Pharmacia) using water as the eluent. The collected fractions (3 ml) were assayed for neutral carbohydrates by the anthrone method (Keleti & Lederer, 1979), and free amino groups were determined with 1-fluoro-2,4-dinitrobenzene (Goodwin, 1968). For sugar analysis, aliquots from appropriate fractions were hydrolysed, N-acetylated, converted to alditol acetates, and analysed by gas chromatography-mass spectrometry (GC/MS). Part of the deacylated muramic glycan (70 mg) was subjected to cation exchange chromatography. The material not bound to the ion exchange resin was eluted with water, whereas the material bound to the column was eluted with 0.5 M NaOH. The pooled alkaline effluent was neutralised with HCl and fractionated on a Sephadex G15 column, giving the glucose-containing material used for establishing the localisation and nature of the linkage between the oligoglucans and the peptidoglycan.

Preparation of the MurNAc-$(Glc)_n$ fraction. Based on the supposition that the MurNAc residue might be linked to oligoglucan, a procedure for special degradation of murein *via* deamination of glucosamine residues was developed as presented in the scheme in Fig. 1. For this procedure, the N-deacylated murein glycan, containing both neutral and aminosugars, was esterified (0.25 M methanolic HCl, 80ºC, 3.5 h; sample concentration 20 mg/ml), rotary evaporated, distilled twice with MeOH, and freeze-dried. The esterified muramic acid residues (in oligoglycan fragments) were subjected to lactamization with hydrazine acetate in dry methanol (Keglević *et al*., 1998) at 65ºC for 36 h in a Pyrex screw-cap test tube with a Teflon-lined cap. After cooling of the material, 5 volumes of acetone were added to precipitate murein glycan and to decompose hydrazine. The resulting pellet was rinsed with another portion of acetone and then dissolved in water and dried on a rotary evaporator to remove traces of acetone. The obtained preparation (84 mg) containing a lactam form of muramic acid residues was subjected to deamination (Caroff *et al*., 1990) in 5 ml of water and equal volumes of 33% CH_3COOH and 5% $NaNO_2$. After 2.5 h of incubation, a fresh portion (0.5 ml) of acetic acid and sodium nitrite was added, and the incubation continued for another hour. The deaminated products, after desalting on Dowex-50W H^+, were freeze-dried, reduced ($NaBH_4$), desalted again on a Dowex-50W H^+ column and co-distilled with MeOH. As the next step, ring-opening of lactams was carried out in 0.5 M NaOH at 100ºC for 3 h, and the products were subjected to column chromatography on Dowex-50W H^+. Unbound products were eluted from the column with water, whereas the adsorbed ones were removed with 2 M NH_4OH. The ammonia-eluted products were evaporated on a rotary evaporator to remove ammonia, triple distilled with water, and lyophilised.

Aliquots of the deaminated material obtained according to the above-described procedures were subjected to partial acid hydrolysis (1 M TFA, 120ºC, 1 h), reduction, and peracetylation, followed by GC/MS analysis.

The remaining material (22 mg) was N-acetylated (Hase & Rietschel, 1976), O-deacetylated at 100ºC for 30 min after lowering the pH to 10.0 with Ac_2O, and fractionated on a Sephadex G15 column using 1% acetic acid as an eluent. The sugar composition of the material from the individual fractions was determined by GC/MS analysis of peracetylated methyl glycosides derived after solvolysis of murein in 2 M methanolic HCl at 85ºC for 18 h, and subsequent peracetylation. Small aliquots of the deaminated material were also subjected to MALDI-TOF spectroscopic analysis.

Determination of glucosyl oligomers. Neutral oligosaccharides were liberated from murein with lysozyme in 20 mM ammonium buffer pH 6.4 at 37ºC for 8 h and then at 45ºC overnight. After centrifugation, the resulting debris was digested again

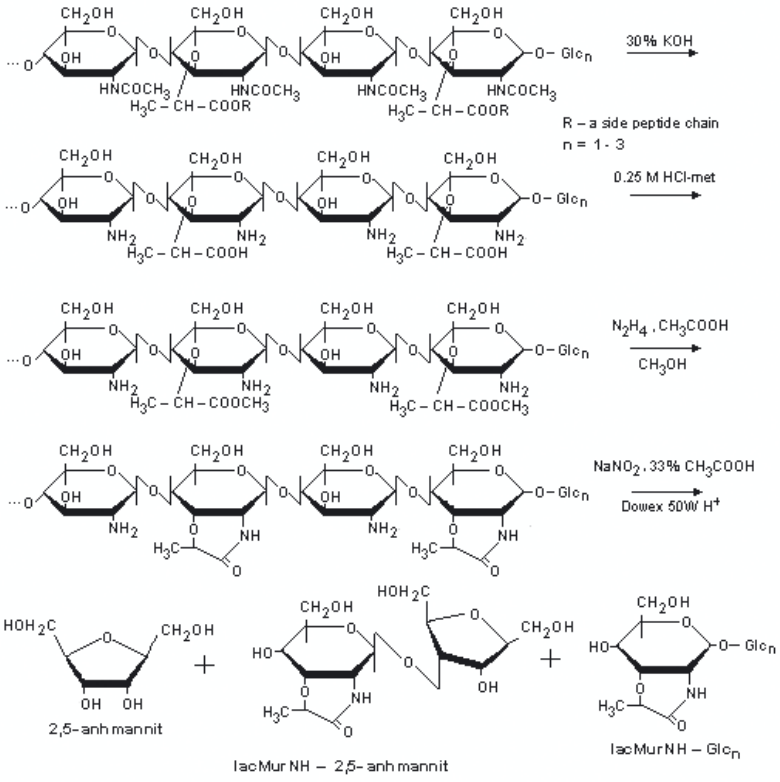

Figure 1. Pathway of the degradation of *M. loti* HAMBI 1148 murein by deaminoacylation and subsequent deamination of glucosaminyl residues.

in the same conditions. The combined supernatants were purified on a Dowex 50W H^+ column and subsequently on a DEAE-Cellulose (OH^-) column to remove the enzyme, salts, and muropeptides. The material from both types of columns was eluted with water. The glycogen present in the fraction was removed as retentate by dialysis against 1% acetic acid. Part of the dialysate after evaporation was reduced with $NaBD_4$, hydrolysed (2 M TFA, 2 h, 120°C), and converted to aldonitrile acetates according to the procedure of Dmitriev *et al.* (1973). Another portion of oligosaccharides (3 mg) was reduced ($NaBD_4$), desalted on a Dowex 50W H^+ column, subjected to the methylation procedure according to Hakomori (1964), derivatized to partially methylated alditol acetates, and analysed by gas-liquid chromatography coupled with mass spectrometry.

GC/MS analysis and analytical equipment. GC/MS determination of peracetylated alditols, aldonitriles, methyl glycosides and partly methylated alditols was performed on an HP 5980 gas chromatograph equipped with an HP-5MS capillary column connected to an HP 5971 mass detector. The GC analysis was performed at 150°C for 5 min, then with a ramp at 5°C/min to 310°C.

MALDI-TOF analysis. MALDI-TOF spectroscopic analysis was performed on a Voyager Elite (PE Biosystems) instrument fitted with nitrogen laser VSL-337 ND (λ=337 nm) and operated in a linear mode with negative ion detection at an accelerating voltage of 20 kV. The spectra obtained are the average of 150 scans. Aliquots of the deaminated material (0.5 µl) were mixed with an equal volume of 50% (v/v) 2,5-dihydrobenzoic acid in acetonitrile as the matrix solution, spotted and dried on a MALDI-TOF sample plate (Zenker *et al.*, 1998).

RESULTS AND DISCUSSION

The hydrolysed aliquots of the deaminoacylated and N-deacetylated murein products after conversion to alditol acetates were analysed in GC/MS. Peaks recorded in the chromatogram at time points 19.39 and 22.27 min corresponded to Glc and GlcNAc, respectively, whereas for MurNAc two main peaks at 24.23 and 25.32 min were observed. The presence of Glc, both in "native" murein and the low molecular deacylated products eluted with alkali from a cation-exchanger, indicated covalent attachment of oligoglucans to the murein of the investigated strain.

It is known that only muramic acid residues with a free amino group can cyclise to form a lactam ring (Gilmore *et al.*, 2004). In nature, certain Gram positive bacteria (*Clostridium* or *Bacillus*) convert around 50% of muramic acid residues to muramic-δ-lactam during spore cortex synthesis (Warth & Strominger, 1969). Removal of the peptide side chain from the muramic acid residue in *B. subtilis* murein is catalysed by muramoyl-L-alanine amidase (CwlD). Peptide cleavage is followed by the N-deacetylation

of muramic acid and the formation of a lactam ring. CwlD and polysaccharide deacetylase PdaA have been demonstrated to act interdependently. Only co-expression of both gene products can lead to the formation of δ-lactam rings in muramyl residues (Gilmore *et al.*, 2004). Due to the lack of appropriate commercial bacteriolytic enzymes (particularly endo-*N*-acetyl-β-glucosaminidase and *N*-acetylmuramoyl-L-alanyl amidase) and problems with the removal of glycogen during the purification of mammalian amidase preparations, the chemical degradation of N-deacetylated and deaminoacylated muramic glycan had to be performed. The major part of the deacylated murein (258 mg) was subjected to the procedure, described in the scheme in Fig. 1, which should give muramyl residues (1→4) linked with 2,5-anhMan or short oligoglucans. To increase the efficiency of the formation of δ-lactam rings between the amino group at C_2 and the lactoyl residue, esterification of the carboxyl group of MurNAc was carried out (Keglević *et al.*, 1998). Nitrous deamination which is known to be capable of breaking down the chitin (Runham, 1961), was used to depolymerise murein glycan chains. In the case of muramic acid residues, lactamization had been carried out to protect them against conversion by nitrous deamination to 3-*O*-lactoyl-2,5-anhydromannose and to preserve the glycosidic linkage of $MurNH_2$ glucosamine residues with unprotected amino groups converted to 2,5-anhydro-D-mannose (Runham, 1961; Sastre *et al.*, 1988). Since this kind of derivative is unstable as a polymer (Runham, 1961), the muramyl glycan chain was degraded due to the cleavage of glucosaminyl glycosidic bonds, releasing the following fragments: a lacMurN-(1→4)-2,5-anhydro-D-mannose disaccharide and a 1,6-anhydro muramic acid lactam from the reducing ends or muramyl lactam with linked glucose oligomers. After borohydride reduction followed by ring-opening of the lactams in alkali (0.5 M NaOH, 100°C, 2 h), ion exchange chromatography was carried out to remove the neutral fraction not bound to Dowex 50W. Finally the deaminated material containing $MurNH_2$ bound with the column was eluted with 2 M ammonia.

To determine the nature of the linkage between glucose residues and the muramic acid residue, partial hydrolysis of the deaminated material sample (1 M TFA, 120°C, 1 h) was carried out. The reaction split the glycosidic linkages of the glucosyl only but not the glycosidic linkages of $MurNH_2$ which resisted hydrolysis due to the presence of the free amino group. The liberated glucose residues, after a reduction with sodium borodeuteride were converted to alditol acetate, whereas the muramic acid residues were converted to acetylated methyl derivatives during the same procedures. The mass spectrum analysis of the peracetylated pseudodisaccharide, giving a peak at t=40.10 min, revealed that the MurNAc residue was linked to hexose. The molecular ion at *m/z* 766 was not observed; however, the secondary ion at *m/z* 605, which could have been derived by the subsequent elimination of acetic acid, acetamide, and ketene was present (Fig. 2A). The secondary ion at *m/z* 561, resulting from the deuterium-reduced disaccharide fragment due to hexitol fragmentation at the C_3–C_4 bond followed by acetic acid elimination from ion 621 *m/z*, appeared the most important in establishing the location of the glycosidic bond between MurNAc and C4 of the Glc residue. The most intense ion, at *m/z* 187, represented the C_2–C_3 fragment of peracetylated muramic acid methyl ester. In the case of the analysis of pseudodisaccharides as acetylated alditol (especially 2-aminoalditol) derivatives, the J_1 ion can often be observed (Gloaguen *et al.*, 1997). Although the corresponding J_1 ion at *m/z* 508 containing a hexitol residue linked by oxygen to the muramyl glycosidic carbon with a transferred lactoyl residue was not observed, an ion at *m/z* 448 which was derived from ion J_1 after the elimination of acetic acid was present (Fig. 2A). To confirm the above data oligosaccharides from deaminated murein (fractions P1 and P2) were methanolysed and peracetylated. GC/MS analysis of the resulting samples indicated the presence of a compound forming a peak at retention time 39.4 min, identified as methyl glucoside substituted by an esterified muramyl residue. Indicative ions (374 and 303 *m/z*) (Fig. 2B) formed as a result of splitting the glycosidic bond between the peracetylated methyl ester of the muramyl residue and the peracetylated methyl glucoside residue during the fragmentation process. The molecular ion at *m/z* 693 was not present; however, the secondary ion at *m/z* 547, obtained by the elimination of methyl lactate and ketene, was observed (Fig. 2B).

MALDI-TOF spectrometry in the range of 200–2000 *m/z* of negative ions for the deaminated material was carried out, and the analysis of the spectrum obtained confirmed the established data that short glucans were linked to *M. loti* HAMBI 1148 murein. The calculation revealed that signals recorded at *m/z* 454 and *m/z* 777 as ions $[M\text{-}H]^-$ could represent fragments containing a MurNAc residue with one and three glucose residues (Table 1), respectively. In addition, the ion $[M+Na^+\text{-}2H]^-$ registered at *m/z* 640 with two glucose residues was also observed. The ion $[M\text{-}H]^-$ with a mass of 394 *m/z* was suggested to represent the fragment with muramic acid in the lactam form substituting the glucosyl residue, whereas the ion $[M+NH_4\text{-}2H]^-$ at *m/z* 573 was proposed to represent the fragment consisting of a lacMurNH residue and two glucose residues. A peak registered at *m/z* 1254 was proposed to represent a longer fragment consisting of two MurNAc and one

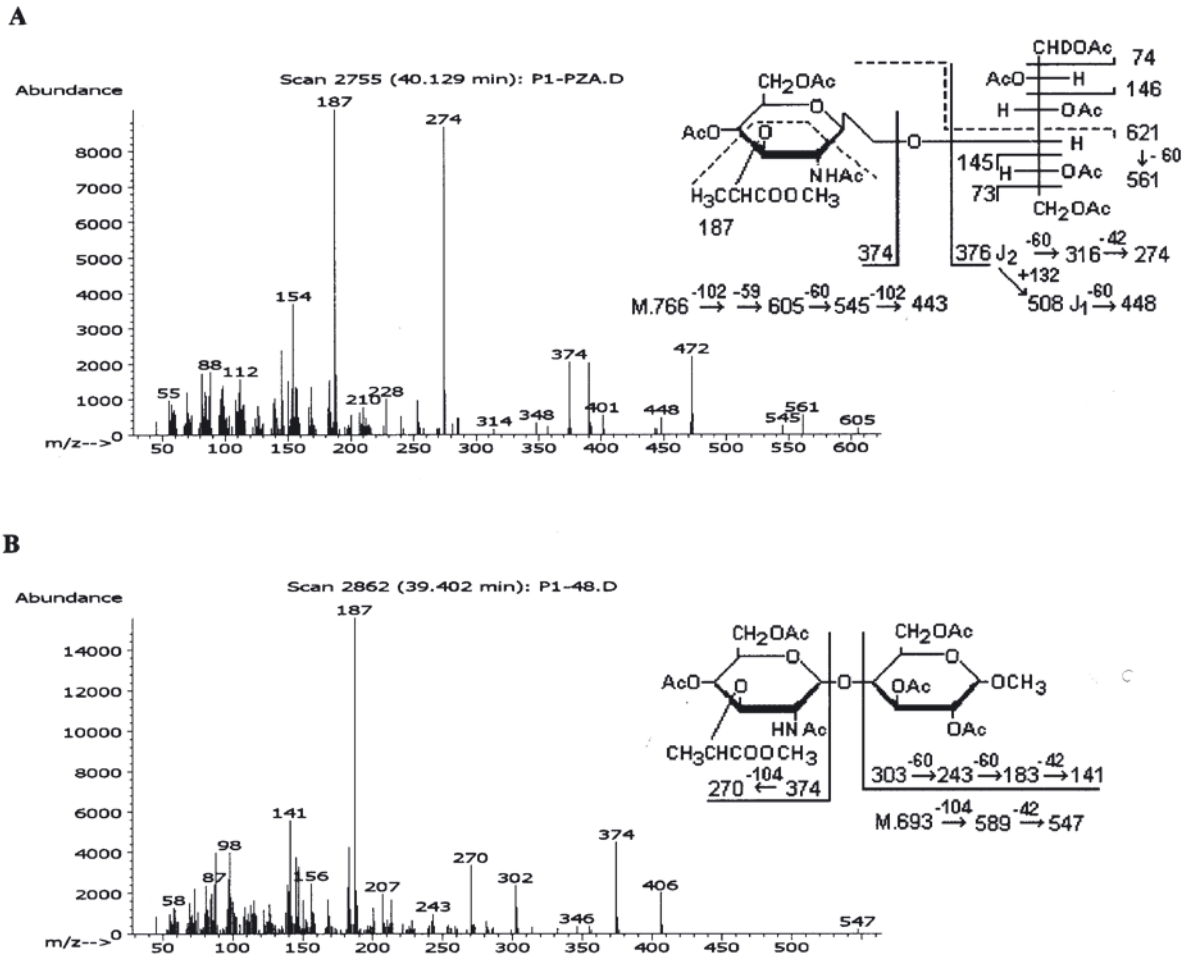

Figure 2. Mass spectrum and the fragmentation pattern of the peracetylated methyl ester of *N*-acetylmuramylo-(1→4)-glucose derivatives in a reduced ($NaBD_4$) (A) or a methanolysed (B) sample.

GlcNAc residue with combined three glucose residues linked to them, probably formed as a result of inefficient alkaline deacetylation (30% KOH) (Gerhardt *et al.*, 1994) or ineffective nitrous deamination of GlcNAc residues. Ions with masses 612, 849, 978 *m/z* were supposed to contain MurNAc residues carrying a part of the side peptide (Ala or Ala-Glu) and one or three glucose residues (Table 1). Amide bonds in murein are very stable, so the action of KOH in removing the oligopeptides may not have been com-

Table 1. Analysis of negative ions derived from *M. loti* HAMBI 1148 murein glycans with deaminated glucosaminyl residues by MALDI-TOF spectroscopy

Peak	*m/z*		Ion	Proposed composition							
	O	C		GlcN	MurNAc	δ-Mur	2,5AnhM	Ala	Glu	A_2pm	Glc
1.	342	341.29	$[M-H]^-$	0	0	0	0	0	0	0	2
2.	394	394.35	$[M-H]^-$	0	0	1	0	0	0	0	1
3.	438	438.416	$[M-H]^-$	0	1	0	1	0	0	0	0
4.	454	454.4	$[M-H]^-$	0	1	0	0	0	0	0	1
5.	459	460.398	$[M+Na^+-2H]^-$	0	1	0	1	0	0	0	0
6.	573	573.52	$[M+NH_4-2H]^-$	0	0	1	0	0	0	0	2
7.	640	638.007	$[M+Na^+-2H]^-$	0	1	0	0	0	0	0	2
8.	777	777.691	$[M-H]^-$	0	1	0	0	0	0	0	3
9.	849	849.767	$[M-H]^-$	0	1	0	0	1	0	0	3
10.	978	978.881	$[M-H]^-$	0	1	0	0	1	1	0	3
11.	1254	1256.151	$[M-H]^-$	1	2	0	0	0	0	0	3

Abbreviations: δ-Mur, muramic acid lactam; 2,5AnhM, 2,5-anhydromannose; O, observed mass of an ion; C, calculated mass of an ion

plete. One of the most intense fragments with a peak recorded at *m*/*z* 342 was calculated to be composed of two glucose residues. If the $MurNH_2$ residue adjacent to glucan was not converted to lactam during lactamization, it could be converted to 3-*O*-lactoyl-2,5-anhydromannose with the release of its aglycon as the result of nitrous acid deamination. This means that after the deamination, part of the glucans could be liberated. The calculation also indicated that the liberated neutral sugar did not possess the reducing end and could, therefore, be a trehalose type.

The GC/MS analysis of the oligosaccharides released from murein with lysozyme after their reduction ($NaBD_4$), hydrolysis (2 M TFA, 120°C, 2 h), conversion to aldonitrile acetate (in hydroxylamine hydrochloride and pyridine), and acetylation revealed that glucose aldonitrile acetate derivatives constituted 98.97% of all glucose derivatives. This confirmed that they did not possess reducing residues of glucose. Also, the analysis of fully methylated neutral oligosaccharides, previously reduced with $NaBD_4$, showed the presence of di- and trisaccharides and the lack of a reducing unit.

In conclusion, our study provided the evidence that some peptidoglycan strands of *M. loti* strain HAMBI 1148 bind glycosidically short oligoglucans. The liberation of muramic acid residues terminated with glucose oligomers was achieved thanks to the degradation of peptidoglycan with an appropriate chemical procedure including esterification, lactamization, and deamination of rhizobial murein instead of unavailable enzymatic digestion. The method used in combination with other spectroscopic analyses simplifies procedures and shortens the time needed to establish some substituents of muramic acid in peptidoglycan.

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