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Detailed Structural Characterization of Succinoglycan, the Major Exopolysaccharide of *Rhizobium meliloti* Rm1021

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The detailed structure of the symbiotically important exopolysaccharide succinoglycan from *Rhizobium meliloti* Rm1021 was determined by mass spectrometry with electrospray ionization and collision-induced dissociation of the octameric oligosaccharide repeating unit. Previously undetermined locations of the succinyl and acetyl modifications were determined, in respect to both the residue locations within the octamer and the carbon positions within the pyranose ring. Glycosidic linkages determined previously by methylation analysis were also verified.

Nitrogen-fixing soil bacterial species of the genus *Rhizobium* colonize specific host plants through the induction and invasion of root hair nodules. Successful colonization involves a number of chemical messages that control this complex, multistep process. In many *Rhizobium*-legume symbioses, extracellular polysaccharides are required for nodule invasion (15). Mutants of *Rhizobium meliloti* Rm1021 that fail to produce the major extracellular polysaccharide of this strain, known as succinoglycan, induce the formation of nodules on alfalfa but are unable to invade these nodules (17). Subsequently, it was demonstrated that specific structural features of this succinoglycan are necessary for nodule invasion. The functional significance of the extracellular polysaccharide structures in nodule invasion motivated an investigation of the details of succinyl and acetyl substitution in *R. meliloti* succinoglycan (9, 10, 16, 20).

The earliest structural studies on *R. meliloti* reported that the succinoglycan contained glucose, galactose, pyruvic acid, and *O*-acetyl groups in the proportion of 7:1:1:1 (3). The glycosyl linkage composition was determined by methylation analysis, and the *O*-acetyl group was reported to be located at carbon 6 (referred to herein as C_6) of 3- and 4-O-linked glucose residues (3). At this time the succinate modification was not recognized, and the assignment of the *O*-acetyl group to a 3-O-linked glucose was done in error. Evidence for an octamer repeating unit of the succinoglycan was reported, and the linkage sequence in the main chain was determined by a modified Smith degradation method (13). The hexosyl residues were determined to be β-pyranosidic from the low optical rotation and the ^{1}H nuclear magnetic resonance spectrum of the polysaccharide (13). Most of these structural details for *R. meliloti* Rm1021 were subsequently confirmed as glycosyl-sequencing methodology developed (1). Polymer degradation with β-D-glycanases and methylation analysis demonstrated that the exopolysaccharides from *R. meliloti*, *Alcaligenes faecalis* var. myxogenes, *Agrobacterium radiobacter*, *Agrobacterium rhizogenes*, and *Agrobacterium tumefaciens* were identical except for the acetyl or succinyl substituents (11).

This report describes the structural characterization of the *R. meliloti* succinoglycan by electrospray ionization (ES) (7) and collision-induced dissociation and tandem mass spectrometry (CID). The specific residue locations in the octamer repeating unit of the succinyl and acetyl modifications are identified, interior glycosyl linkages are confirmed, and the ring positions for the acetyl and succinyl modifications are determined. Approximately 10 μg of starting material was used for methylation and to obtain both an ES mass spectrum and a collision-induced fragmentation spectrum.

MATERIALS AND METHODS

Succinoglycan. Octasaccharide repeating units were prepared by digesting the succinoglycan with partially purified succinoglycan depolymerase from *Cytophaga arvensicola* (2) and then purified by gel filtration chromatography on Bio-Gel P4 (20).

Methylation. Vacuum-desiccated samples were dissolved in 200 μl of an NaOH-dimethyl sulfoxide (DMSO) suspension, which was prepared by vortexing DMSO and powdered sodium hydroxide. After 1 h at room temperature, 50 μl of methyl iodide was added, and the solution was kept for 1 h at room temperature with occasional vortexing (5). The sample was partitioned by adding 1 ml of chloroform, the suspension was extracted four times with 2 to 3 ml of 30% acetic acid, and the chloroform layer was concentrated to dryness. The sample was stored at −20°C prior to analysis. In experiments using deuteromethylation, CD_3I was used. Neutral permethylation was carried out as previously described (19) but modified for smaller amounts of sample and lower temperatures. The samples were analyzed directly after extraction and washed with aqueous dilute bicarbonate solutions.

Glycosyl linkage analysis. Glycosyl linkage analysis was carried out essentially as described previously (4), with modifications to methylation as described above. The partially methylated alditol acetates were analyzed by gas chromatography-mass spectrometry (MS) with a capillary column (8).

ES-MS. ES-MS was performed with a TSQ-700 (Finnigan-MAT Corp., San Jose, Calif.) equipped with an electrospray ion source (Analytica, Inc., Branford, Conn.). The methylated sample was dissolved in a 3:7 water-methanol solution containing 0.5 mM sodium acetate and infused at a rate of 0.75 μl/min

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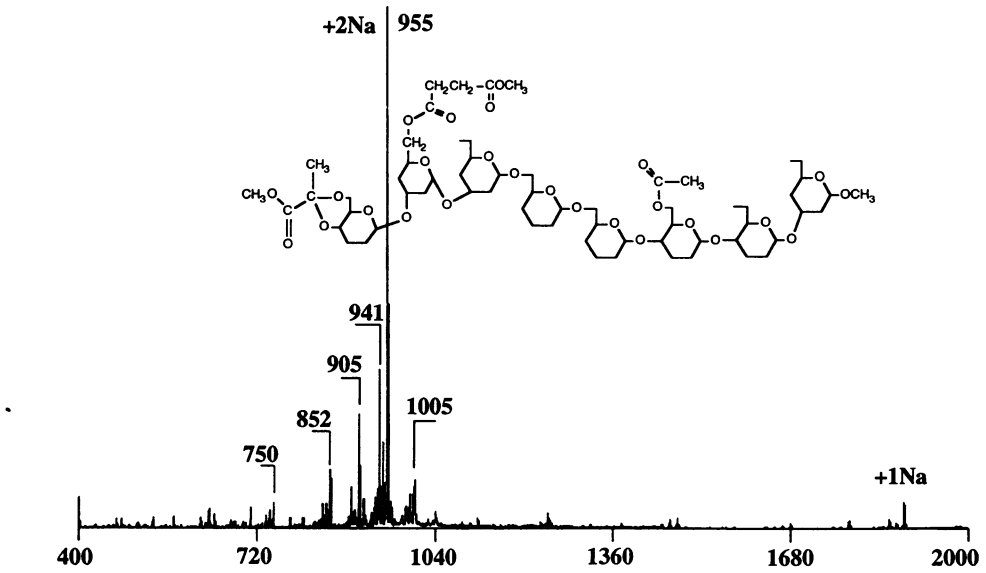

FIG. 1. ES mass spectrum of the neutral methylated octasaccharide derived from succinoglycan. Spectra are labeled with mass-to-charge ratios reported by the peak centroid algorithm rounded to the nearest integer.

directly into the ES chamber through a stainless steel needle. The voltage difference between the needle tip and the source electrode was −4.0 kV. For CID studies, parent ions were selectively transmitted by the first mass analyzer and directed into the collision cell containing argon gas. The cell was held at a potential of −37 V relative to the instrument ground. Mass-to-charge ratios discussed below are the isotopic average masses rounded to the nearest integer. The spectra in the figures are labeled with the mass-to-charge ratios reported by the peak centroiding algorithm, rounded to the nearest integer. Because of a partial resolution of the isotope peaks, mass-to-charge ratios reported by the data system may be shifted to a Thompson (Th [*m/z*]) value that is lower than the isotopic average.

RESULTS

Succinoglycan from Rm1021. The ES mass spectrum of the alkylated octasaccharide generated by neutral methylation (Fig. 1) shows a major ion at 955 Th. This ion is doubly charged and therefore corresponds to a molecular mass of 1,864 Da adducted with two sodium ions [955 = (1,864 + 23 + 23)/2]. This molecular mass is consistent with an alkylated oligosaccharide composed of eight methylated hexosyl residues with three of the residues modified by a pyruvyl, an acetyl, and a succinyl group. Methylation under neutral conditions, as opposed to methylation under basic conditions, was used to retain the succinyl and acetyl moieties. However, this procedure often results in incomplete methylation which gives a series of ions decreasing in mass by 14 Da (e.g., 948 and 941 Th [Fig. 1]). The ions at 905 and 1,005 Th suggest succinyl heterogeneity with small amounts of the octasaccharide containing no or two succinyl groups. The absence of an acetyl group would correspond to a 28-Da decrement in the mass of the methylated product (14-Th loss in the double-charge state), which would be isobaric with the mass of the twice-undermethylated product (941 Th). The fact that the 941-Th ion is more abundant than the singly undermethylated product (948 Th) strongly suggests that a portion of the sample is lacking one acetyl residue.

Collision-induced fragmentation of methylated and alkali metal-cationized oligosaccharides yields a combination of glycosidic and cross-ring cleavages. Glycosidic cleavages involve breaking the carbon-oxygen bond on either side of the glycosidic oxygen. Following the notation of Domon and Costello (6), glycosidic ions containing the nonreducing end are labeled B_1 and B_2, etc., where B_n indicates cleavage of the glycosidic bond on the nonreducing side of the glycosidic oxygen between the *n*th and (*n* + 1)th residue (from the nonreducing end). Fragments resulting from cleavage of the same glycosidic bond but containing the reducing end are denoted Y_n ions (*n* is counted from the reducing end). Cleavages of the carbon-oxygen bond on the other side of the glycosidic oxygen produce C_n ions, containing the nonreducing end, and Z_n ions, containing the reducing end (with analogous counting rules for *n*) (Fig. 2A).

Ring-opening fragments containing the nonreducing end are denoted by ${}^{i,k}A_n$, where *n* indicates the fragment cleaved at the *n*th residue (from the nonreducing end) and *i,k* specifies that the carbon-carbon bonds in the saccharide ring between carbons C_i and C_{i+1} and between C_k and C_{k+1} were cleaved. The reducing-end fragments are similarly labeled ${}^{i,k}X_n$, but *n* is counted from the reducing end.

The fragments generated by cleavage of the glycosidic bonds allow the determination of the residue positions of the acetyl, succinyl, and pyruval modifications within the octamer repeat unit while the ions originating from fragmentation of the pyranose ring provide information on the interresidue linkage and on the site of modification within the pyranose ring.

Residue position of substituent groups. Collision-induced fragmentation of the doubly charged parent ion $(M \cdot 2Na)^{2+}$ at 955 Th (Fig. 3) yields a prominent singly natriated molecular ion at 1,887 Th and a series of singly charged B_n and Y_n ions. In principle, the residue location of the acetyl and succinyl groups could be determined from either the B_n or Y_n sequence of glycosidic fragments (Fig. 2b). The entire sequence of Y_n reducing-end fragment ions (at 259, 463, 696, 900, 1,104, 1,308, and 1,613 Th) was observed, and these ions clearly position the acetyl group on the third residue from the reducing terminus

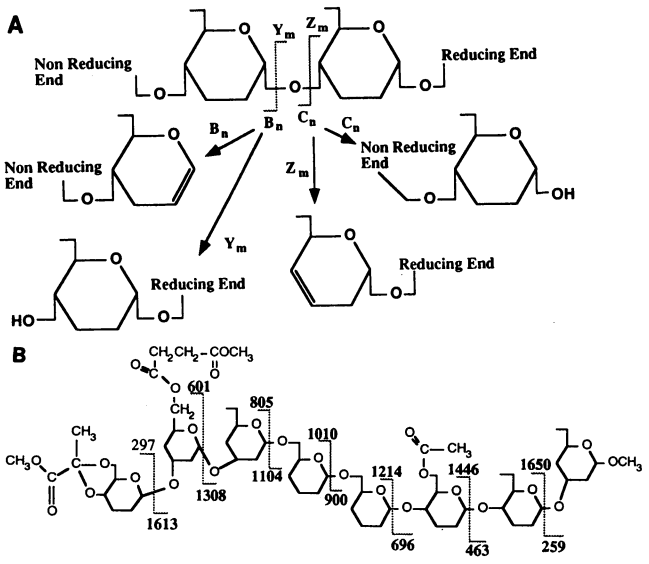

FIG. 2. (A) Glycosidic cleavage nomenclature for collision-induced fragmentation of oligosaccharides. (B) Scheme illustrating fragment masses of the glycosidic cleavages observed in the CID spectrum of the neutral methylated octasaccharide derived from the succinoglycan.

and place the succinyl and pyruvyl moieties on the penultimate and terminal residues, respectively, at the nonreducing terminus.

A somewhat unusual feature of the CID spectrum was the lack of the B_1 (297-Th) and C_1 (313-Th) fragments (although a Y_7 ion at 1,613 Th was observed). It has been postulated that the C_6 oxygen plays a role in stabilizing the sodium cation coordinated to the pyranose ring oxygen (14). For the succinoglycan, the pyruvate group would block the rotation required for the participation of the C_6 oxygen and hence prevent stable adduction of the sodium cation on the nonreducing terminal residue. The other ions in the B_n glycosidic sequence were observed at 601, 805, 1,010, 1,214, 1,446 and 1,650 Th, and the ions in the corresponding C_n sequence were observed at 619, 824, 1,029, 1,233, 1,465 and 1,669 Th.

Linkage position of substituent groups. Bjorndal et al. (3) have reported that acetyl esters were located at C_6 of 3-O- and 4-O-linked glucosyl residues of the octasaccharide. Succinylation of extracellular polysaccharides was unknown at that time, and it took several years to establish the complete repeating-unit composition: glucose-galactose-pyruvyl-acetyl-succinyl (7:1:1:1:1) (1, 12).

Glycosidic linkages can be determined by tandem MS from the A and X fragments arising from collision-induced opening of the pyranose ring (18). Since ring-opening fragments from CID are generally less abundant than glycosidic cleavage fragments, a high signal-to-noise ratio is crucial for their identification. The fragments that convey linkage information in our (low-energy) collision experiments are the A ions, i.e., ions containing the nonreducing terminus. In particular, (1,4)- and (1,6)-O linkages are identified by the presence of $^{3,5}A$ fragments and both $^{3,5}A$ and $^{0,4}A$ fragments, respectively (Fig. 4A). (1,2)-O linkages are not present in this molecule, but when they are present they can be identified by $^{1,3}A$ fragments. The (1,3)-O linkages do not produce ring-opening fragments with the same intensity as do (1,4)- and (1,6)-O linkages in the low-energy collisions of the triple quadrupole MS.

The CID spectrum of the alkylated octamer obtained by neutral methylation was complex because of the labile esters which provided both a large increase in the total number of fragments and new fragmentation pathways. This complexity did not obscure those ions identifying ring-opening fragments at the residues that were not modified by the acetyl or succinyl esters. However, the pyranose rings containing these esters did not cleave in the expected way. Thus, to study the glycosidic linkages at these residues and also to determine the substitution positions, the acetyl and succinyl esters were replaced with trideuteriomethyl ethers by base-catalyzed CD_3 methylation. The combination of neutral methylation followed by base methylation results in a linear permethylated oligosaccharide containing the pyruvate group at the nonreducing-end termi-

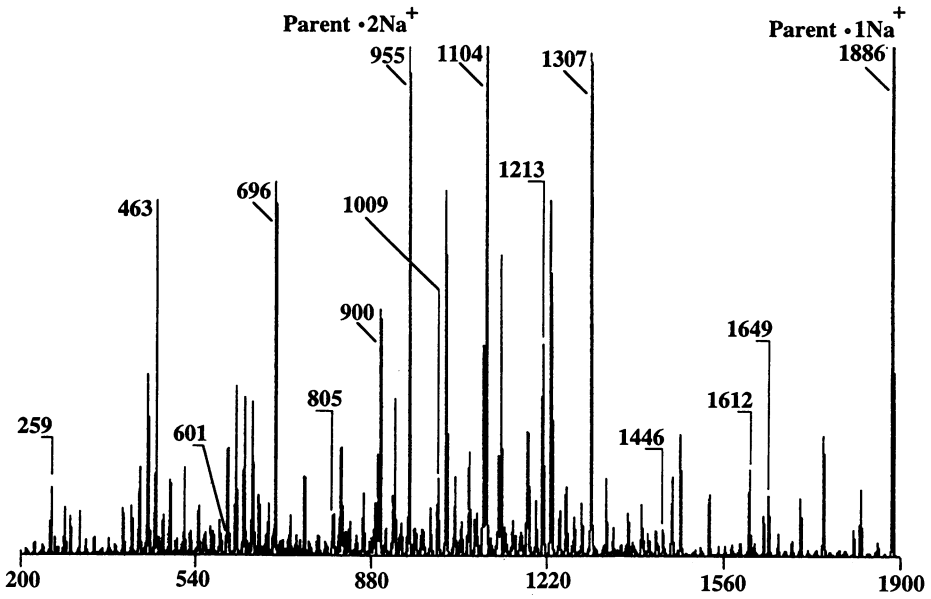

FIG. 3. ES-CID of the 955-Th molecular ion, with glycosidic cleavage ions labeled as in the legend to Fig. 1.

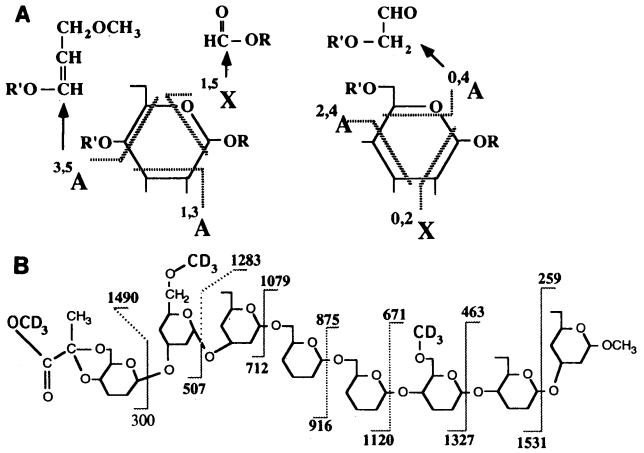

FIG. 4. (A) Ring-opening fragments illustrating nomenclature and ion structure in the collision-induced fragmentation of methylated oligosaccharides. (B) Scheme illustrating fragment masses of the glycosidic cleavages observed in the CID spectrum of the methylated succinoglycan with the succinyl and acetyl esters replaced by trideuteriomethyl ethers.

nus. This derivative gives a much simpler CID spectrum from which both the linkages and substitution positions can be determined.

The ES mass spectrum of the methylated and deuteriomethylated octasaccharide contained the doubly charged 895.4-Th ion. This corresponds to a molecular mass of 1,744.8 Da and shows that the succinyl and acetyl esters have been replaced with a deuterated methoxy group and that the pyruvate is trideuteriomethyl esterified (Fig. 4b).

Collision-induced fragmentation of the doubly charged 895.4-Th ion gave a spectrum in which the reducing-end glycosidic fragments dominate (Fig. 5). Both glycosidic sequences, i.e., sequences containing the nonreducing terminus and the reducing terminus, readily identify the residues that were deuteriomethylated, confirming the residue positions of the esters determined from CID of the neutral methylated succinoglycan. The B_1 and C_1 fragment pair was again not observed, consistent with the interpretation that the pyruvyl group blocks sodium adduction at the nonreducing terminal residue.

The structural information provided by cross-ring cleavages is illustrated by a detailed investigation of the mass region above the glycosidic fragment B_5 at 1,120 Th for the doubly methylated molecule (Fig. 6). In this region the $^{i,j}A_6$ fragments originate from the opening of a pyranose ring that possesses a deuterated methyl group at the 6 carbon. The ion, Th 1211, suggests an $^{3,5}A_6$ fragment that would indicate both linkage at the 4 position and substitution at the 6 position. A reciprocal structure, involving linkage at the 6 position and 4 substitution, would also produce a 1,211-Th fragment; however, 6-linked residues also produce $^{0,4}A$ ions that are 60 Da larger than the glycosidic B_5 fragment. In this case, no $^{0,4}A_6$ ions were observed at 1,180 Th. In contrast, the $^{0,4}A_4$ and $^{0,4}A_5$ fragments were detected at 772 and 976 Th, indicating that these two residues are linked at position 6.

All of the internal glycosidic linkages could be determined from the CID fragments of the twice-methylated molecule. The third residue was linked at position 3 since there were no $^{0,4}A_3$ (567-Th), $^{3,5}A_3$ (595-Th), or $^{1,3}A_3$ (581-Th) ions. The fourth and fifth residues were linked at position 6 as $^{0,4}A_4$ (772-Th) and $^{0,4}A_5$ (976-Th) ions were present along with $^{3,5}A_4$ (800-Th) and $^{3,5}A_5$ (1,004-Th) ions. The sixth residue was 4-O linked, with substitution at the sixth position due to the 3-Da shift in the $^{3,5}A_6$ (1,211-Th) ion as discussed above. The seventh residue gave a $^{3,5}A_7$ (1,415-Th) ion with no $^{0,4}A_7$ ion; hence, it was also 4-O linked.

The CID experiments with the neutral methylated and the twice-methylated succinoglycan failed to identify the linkage at the reducing end; in general, low-energy CID fails to fragment the ring at the reducing terminus, which would be required to identify this linkage. At the nonreducing terminus, neither the

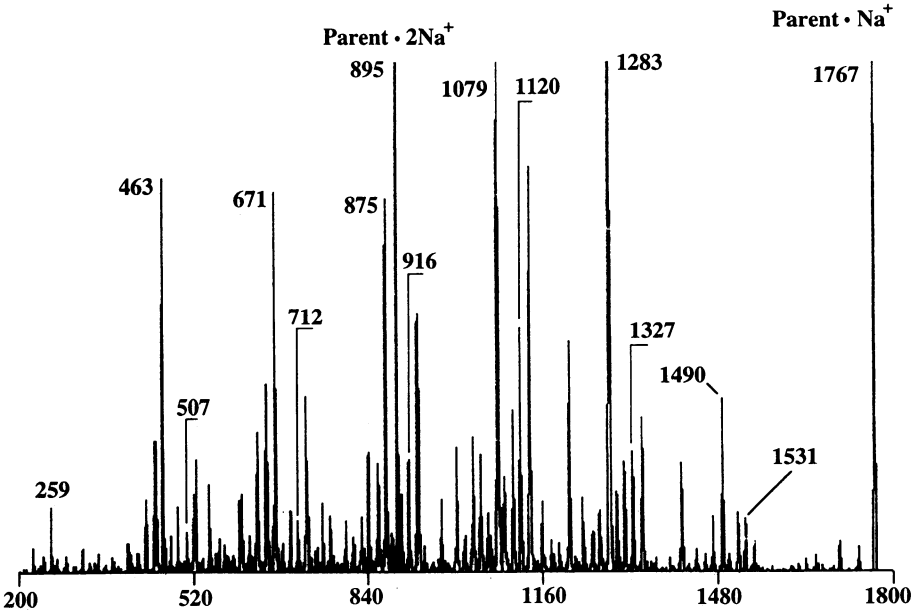

FIG. 5. ES-CID of the 85-Th molecular ion derived from the methylated succinoglycan by replacing the succinyl and acetyl esters with trideuteriomethyl ethers. Labeling was done as described in the legend to Fig. 1.

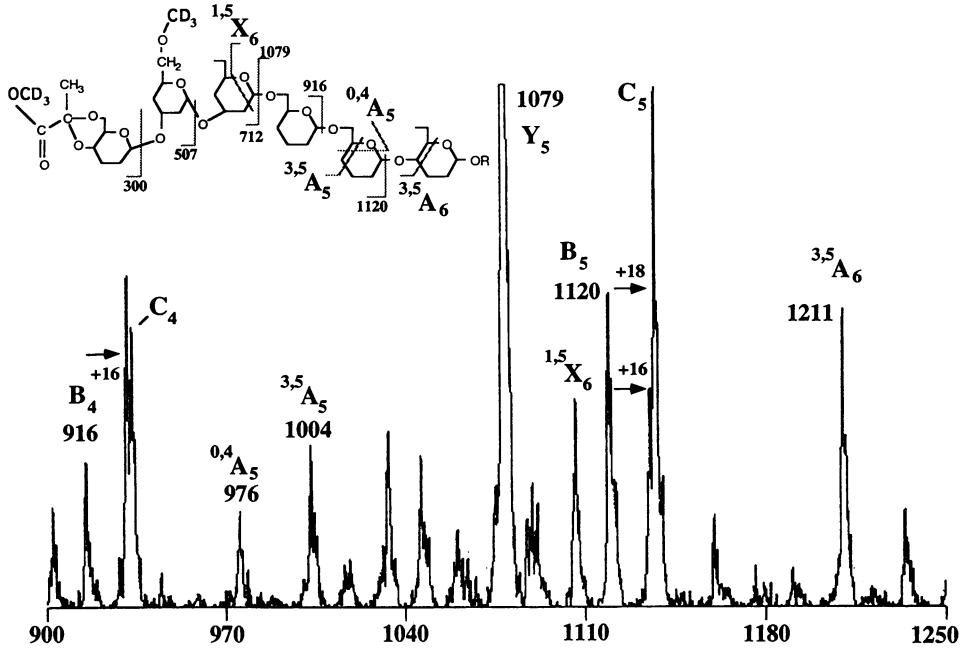

FIG. 6. Expanded view of the 900- to 1,250-Th region of Fig. 5, illustrating some of the ring-opening fragments used in identifying the glycosidic linkages (labeled as in the legend to Fig. 1).

glycosidic B_1 fragment nor ring-opening fragments from the penultimate (succinylated) residue (A_2 fragments) were observed in the singly methylated or the doubly methylated molecule.

Methylation analysis. The failure of the nonreducing terminal residue to adduct a sodium ion precluded the observation of any A_2 fragments. Thus, to determine the ring location of the replaced succinyl group at the second residue and to confirm the structural assignments determined by the collision spectrum, the CH_3- and CD_3-derivatized octamer was hydrolyzed, reduced, and acetylated. The resulting methylated alditol acetates were analyzed by gas chromatography-MS, and their retention times and electron impact mass spectra (EI-MS) were compared with those of standards. The linear octamer yielded approximately equimolar ratios (2 mol each) of 3-O-, 4-O-, and 6-O-linked glucosyl residues.

EI-MS of methylated alditol acetates contain fragments originating from the cleavage of carbon-carbon bonds, which are used to locate the methylated or acetylated hydroxyl groups. The first component in the total ionization plot eluted with a retention time and mass spectrum consistent with those of a methylated alditol acetate derived from a 3-O-linked glucosyl residue. The presence of two paired ions (161/164 and 277/280 Th) of approximately equal abundance indicate that half of the residues were deuterated. In methylated alditol acetates, the fragment bearing the methoxyl group adjacent to the C-C bond ruptured carries the major charge. Rupture of the C_3-C_4 bond is the most diagnostic cleavage and, in this case, identified that C_4 or C_6 was deuteriomethylated. This follows from the fact that positions 1, 3, and 5 must be acetylated, excluding a C_1, C_3, or C_5 origin. The 161/164-Th pair and the absence of an ion pair at 233/236 Th strongly suggest that the CD_3-methyl group is located at C_6. Thus, the EI-MS data identify a 3-O-linked glucopyranosyl moiety deuterated at position 6 (Fig. 7).

The partially methylated alditol acetate derived from the 4-O-linked glucosyl residues was likewise analyzed, and fragmentation by EI-MS indicated that the glucosyl group contained a deuterated methyl group at position 6, a result which is consistent with the CID data discussed previously.

DISCUSSION

The locations within the octamer repeating unit of the acetylated and succinylated hexose residues of the succinoglycan from *R. meliloti* Rm1021 were determined by MS with EI and CID of the intact octamer. Interior glycosidic linkages and the acetylation at position 6 were also determined by this technique after a deuteriomethylation to replace the succinyl and acetyl esters with deuteriomethyl ethers. The pyruvate group at the nonreducing terminus prevented the stable adduction of a sodium cation on the nonreducing terminal glycosyl residue, and this precluded the observation of $^{i,j}A_2$ fragments that would identify the position of the succinyl modification. Thus the location of the succinyl group was determined by glycosyl-linkage composition analysis and

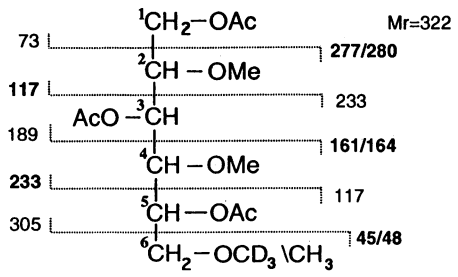

FIG. 7. Scheme illustrating EI-MS fragments of the methylated alditol acetates derived from the methylated succinoglycan with the succinyl and acetyl esters replaced by trideuteriomethyl ethers.

EI-MS on the penultimate nonreducing terminal glucosyl residue. The full structure of the methylated succinoglycan is shown in Fig. 1.

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