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Similarity of "Core" Structures in Two Different Glycans of Tyrosine-Linked Eubacterial S-Layer Glycoproteins

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Previously, the repeating-unit structure of the S-layer glycoprotein from the eubacterium *Bacillus alvei* CCM 2051 has been determined to be [→3)-β-D-Gal*p*-(1→4)-[α-D-Glc*p*-(1→6)-]-β-D-Man*p*NAc-(1→]$_n$ (E. Altman, J.-R. Brisson, P. Messner, and U. B. Sleytr, Biochem. Cell Biol. 69:72–78, 1991). Nuclear magnetic resonance spectroscopic reexamination of this glycan reveals that the O-antigen-like domain of the polysaccharide is

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connected with the S-layer polypeptide through the "core" structure →3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→O)-Tyr. Except for the substitution in position 4 of the nonreducing rhamnose with the modified glyceric acid phosphate residue GroA-2→OPO_2→4-β-D-Man*p*NAc-(1→, this core is identical to the core of the tyrosine-linked glycan from the S-layer glycoprotein of *Thermoanaerobacter thermohydrosulfuricus* L111-69 (K. Bock, J. Schuster-Kolbe, E. Altman, G. Allmaier, B. Stahl, R. Christian, U. B. Sleytr, and P. Messner, J. Biol. Chem. 269:7137–7144, 1994).

Recent investigations have shown that a number of crystalline surface layers (S-layers) (for reviews, see references 4 and 19) of members of the family *Bacillaceae* are composed of glycosylated S-layer protein protomers (18, 19). Most of the eubacterial S-layer glycoprotein glycans investigated contain polysaccharide regions composed of oligosaccharide repeating units, similar to the O antigens of gram-negative bacteria (20). Considerable diversity exists among the repeating-unit structures, even among closely related strains within a species (17, 22).

The O-antigen-like repeats of the S-layer glycan from *Thermoanaerobacter* (*Clostridium*) *thermohydrosulfuricus* L111-69 have the structure [→3)-α-L-Rha*p*-(1→4)-α-D-Man*p*-(1→]$_n$ (6). They are attached to a "core" structure, →3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→, which in turn is linked to the S-layer polypeptide by an O-glycosidic linkage to tyrosine (5).

Tyrosine was found not only in all pronase-derived glycopeptides from the investigated *T. thermohydrosulfuricus* strains (17, 22) but, unexpectedly, also in the glycopeptides analogously obtained from S-layer glycoproteins of *Bacillus alvei* CCM 2051. In a previous work, the repeating-unit structure of the respective glycan has been identified as [→3)-β-D-Gal*p*-(1→4)-[α-D-Glc*p*-(1→6)-]-β-D-Man*p*NAc-(1→]$_n$ (1).

To compare the tyrosine-linked cores of the S-layer glycoprotein glycans from *T. thermohydrosulfuricus* and *B. alvei*, a suitably large amount of a pronase-derived glycopeptide from the *B. alvei* S-layer glycoprotein was prepared. The low-intensity nuclear magnetic resonance (NMR) signals corresponding to the core sugars were analyzed and compared with the results of Bock et al. (5). Here, we report on the complete S-layer glycan structure of *B. alvei* CCM 2051 and draw conclusions about similar core structures in tyrosine-linked eubacterial S-layer glycoproteins.

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MATERIALS AND METHODS

Growth of bacteria. *B. alvei* CCM 2051 was obtained from the Czechoslovakian Collection of Microorganisms, Brno, Czech Republic, and was grown at 33°C as described previously (1, 21).

Analytical methods and electron microscopy. Carbohydrate and amino acid analyses, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electron microscopy, and sequence analysis were performed according to published methods (5). The phosphate contents of the glycopeptide preparations were determined as described elsewhere (15).

Isolation of S-layer glycoprotein and S-layer glycopeptides. Following isolation of intact S-layer glycoprotein, glycopeptides were prepared by exhaustive pronase digestion (1). Final purification of the glycopeptide fractions of interest included gel filtration over Bio-Gel P-4 and P-100 columns and cation-exchange chromatography over Dowex 50W-X8 resin, chromatofocusing between pH 9.0 and 5.6, and reversed-phase high-performance liquid chromatography (RP-HPLC) as described previously (5). The required glycopeptides were lyophilized and stored at −18°C. Sequence analysis of the glycopeptides was performed as described previously (5).

NMR measurements. Solutions of ~40 mg of glycopeptide in 0.5 ml of D_2O were used. Spectra at 320 K in 5-mm-diameter tubes at 500.13 MHz for ^1H, at 200.46 MHz for ^{31}P, and at 125.77 MHz for ^{13}C were recorded with a Bruker AMX-500 spectrometer. The carbon and proton signals of the polysaccharide were referenced via the acetyl CH_3 group of *N*-acetylmannosamine to 23.00 and 2.08 ppm, respectively, in order to enable the comparison with NMR data given in the literature (1). 2-Phosphoglyceric acid (Sigma) was referenced via internal dioxane at 67.40 and 3.76 ppm. Phosphorus NMR spectra were referenced relative to external phosphoric acid at 0.00 ppm. Shift values δ (in parts per million) and coupling constants *J* (in hertz) were determined on a first-order basis.

Nuclear Overhauser experiments, phosphorus-decoupled proton spectroscopy, total correlated spectroscopy, phase-sensitive correlated spectroscopy, and proton-carbon correlated spectroscopy were done by using Bruker software and a reversed probe head, where appropriate.

RESULTS

Characterization of the S-layer glycopeptide. After pronase digestion, a mixture of high-molecular-weight glycopeptides which eluted in the void volume of a Bio-Gel P-100 column was obtained. The combined fractions of interest were subjected to chromatofocusing and resulted in two major glycopeptide frac-

TABLE 1. NMR data for fraction F-II and part of F-IV of *B. alvei* CCM 2051 and reference compounds

Fraction and unit[a]	Residue and parameter[b]	Value for proton or carbon[c]: 1	2	3a/b	4	5	6a/b	7	8	Reference
F-II										
A	α-Glc									
	δ_C	99.55	72.10	74.04	70.38	72.83	61.54			
		(99.6)	(72.1)	(74.0)	(70.3)	(72.8)	(61.4)			1
	δ_H	5.005	3.61	3.72	3.46	3.74	3.87/3.75			
		(5.001)	(3.605)	(3.724)	(3.456)	(3.729)	(3.871/3.768)			1
	$J_{H,H}$	3.7	9.9	9.3	9.3					
		(3.8)	(9.5)	(9.5)	(9.5)	(2.2/5.4)	(−12.3)			1
B	β-Gal									
	δ_C	NR	71.55	72.65	69.10	75.91	NR			
C	β-ManNAc									
	δ_C	101.46	53.41	71.45	77.15	74.82	66.91	176.35	23.0	
		(101.6)	(53.5)	(71.5)	(77.0)	(74.8)	(66.8)	(176.5)	(23.0)	1
	δ_H	4.985	4.640	3.94	3.84	3.71	4.05/3.88		2.08	
		(4.981)	(4.634)	(3.951)	(3.829)	(3.713)	(4.046/3.892)		(2.080)	1
	$J_{H,H}$	1.0	5.3	9.0	NR	NR	−10.6			
		(1.0)	(4.5)	(9.5)	(10.0)	(5.0/1.0)	(−11.0)			1
D	β-Gal									
	δ_C	103.54	70.64	82.80	69.08	76.01	61.89			
		(103.6)	(70.6)	(82.9)	(69.1)	(76.1)	(62.0)			1
	δ_H	4.49	3.63	3.79	4.12	3.715	3.80/3.75			
		(4.488)	(3.638)	(3.783)	(4.116)	(3.711)	(3.797/3.764)			1
	$J_{H,H}$	7.7	10.0	4.0	1.0					
		(8.0)	(10.0)	(3.8)	(1.0)	(7.6/4.4)	(−12.3)			1
E	β-ManNAc									
	δ_C	100.73	53.67	71.45	NR	74.94	67.08			
	δ_H	5.03	4.61	3.96	NR	NR	4.03/3.87			
	$J_{H,H}$	1.7	4.6	9.5						
F	GroA[d]									
	δ_C	NO	77.27	63.37						
		(178.95)	(76.98)	(65.64)						
	$J_{C,P}$		6.0	4.4						
		(6.1)	(5.0)	(1.5)						
	δ_H		4.67	3.940/3.925						
			(4.466)	(3.902/3.879)						
	$J_{H,H}$		3.5/3.5							
			(3.9/5.6)	(−11.7)						
	$J_{H,P}$		9.2							
			(8.5)							
G	PO_3									
	δ_P	2.034								
	$J_{H,P}$	9.2/9.3								
H	β-ManNAc									
	δ_C	99.09	53.89	72.25	73.6	76.93	60.78	176.48	22.84	
	$J_{C,P}$			2.0	6.0					
	δ_H	4.995	4.615	4.00	4.17	3.725	3.99/3.90			
	$J_{H,H}$	1.7	4.6	9.4	9.3					
	$J_{H,P}$			4.4	9.3					
I	α-Rha									
	δ_C	102.84	71.19	79.00	80.76	68.51	17.58			
		(102.96)	(67.05)							5
	δ_H	5.055	4.080	3.940	3.705	3.885	1.335			
		(5.102)	(4.300)	(3.945)	(3.528)	(3.901)	(1.31)			5
	$J_{H,H}$	1.8	3.4	10.0	10.0	6.2				
		(1.0)	(4.0)	(10.0)						5
J	α-Rha									
	δ_C	102.92	70.82	79.01	72.20	70.08	17.43			
		(103.05)	(70.72)	(79.25)	(72.08)					5
	δ_H	5.060	4.140	3.910	3.565	3.90	1.315			
		(5.050)	(4.173)	(3.926)	(3.560)	(3.902)	(1.31)			5
	$J_{H,H}$	1.7	3.4	9.7	9.7	6.2				
		(1.0)	(4.0)	(10.0)						5
K	α-Rha									
	δ_C	102.90	70.78	79.00	72.20	70.19	17.48			
		(103.05)	(70.61)	(79.12)	(72.08)					5
	δ_H	5.082	4.195	3.940	3.595	3.90	1.315			
		(5.064)	(4.201)	(3.939)	(3.580)	(3.896)	(1.31)			5

Continued on following page

TABLE 1—*Continued*

Fraction and unit[a]	Residue and parameter[b]	Value for proton or carbon[c]: 1	2	3a/b	4	5	6a/b	7	8	Reference
	$J_{H,H}$	1.9	3.4	9.0	9.0	6.2				
		(1.0)	(4.0)	(10.0)						5
L	β-Gal									
	δ_C	101.36	70.69	80.91	69.1	76.12	61.45			
		(101.30)	(70.79)	(80.96)	(69.04)	(76.16)	(61.44)			5
	δ_H	5.110	3.945	3.830	4.10	3.87	3.77/3.77			
		(5.119)	(3.932)	(3.834)	(4.097)	(3.88)	(3.77/3.76)			5
	$J_{H,H}$	7.9	10.0	4.4						
		(7.5)	(10.0)	(3.0)	(1.0)					5
M	Tyr									
	δ_C	NO	56.90	36.70	NO	131.50	117.90	156.75		
		NO	(56.84)	(36.37)	(131.39)	(131.54)	(117.82)	(156.62)		5
	δ_H		3.905	3.220/3.06	7.148	7.296				
			(3.939)	(3.232/3.082)	(7.136)	(7.284)				5
	$J_{H,H}$		4.5/8.5	−14.5	8.4					
			(NO/8.5)	(−14.5)						5
F-IV (peptide portion)										
M	Tyr, δ_C	NO	55.10	36.72	129.03	131.65	117.96	157.10		
N	Glu, δ_C	NO	53.04	26.64	30.58	NO				
O	Gly, δ_C	NO	45.66							

[a] Units in the NMR experiments.
[b] Glc, glucose; Gal, galactose; ManNAc, *N*-acetylmannosamine; Rha, rhamnose; GroA, glyceric acid; PO_3, phosphate; Tyr, tyrosine; Glu, glutamic acid; Gly, glycine; δ_c, carbon chemical shift (in parts per million); δ_H, proton chemical shift (in parts per million); δ_P, phosphorus chemical shift (in parts per million); $J_{H,H}$, proton-proton coupling constant (in hertz); $J_{C,P}$, carbon-phosphorus coupling constant (in hertz); $J_{H,P}$, proton-phosphorus coupling constant (in hertz).
[c] NMR reference data are given in parentheses. NO, not observed; NR, not resolved. If more than one nonequivalent proton is linked to a given carbon, both the corresponding shift values and coupling constants are separated by shills.
[d] Reference data for GroA (given in parentheses) were obtained from analysis of authentic 2-phosphoglyceric acid.

tions (F-II and F-IV) and two minor glycopeptide fractions (F-I and F-III) (not shown). Chemical analysis of the two minor fractions showed carbohydrate and tyrosine contents similar to those in fractions F-II and F-IV. After final purification by RP-HPLC, both major fractions yielded carbohydrate contents of approximately 95% (wt/wt) of the dry weight. The colorimetric phosphate determination indicated a phosphate content of <0.2%. Amino acid analysis revealed tyrosine as the only amino acid in F-II, whereas F-IV additionally contained glutamic acid and glycine. These findings were confirmed by NMR (Table 1) and by N-terminal sequencing (not shown).

NMR analyses. The structures of glycopeptide fractions F-II and F-IV of *B. alvei* were derived by interpretation of their NMR data. Since the ^{13}C chemical shifts of the glycan chain in the two fractions showed no differences, the detailed structure was determined with fraction F-II, containing only tyrosine at the reducing end (Fig. 1). The proton signals of the individual subunits A, C and D, and F to M (Fig. 2) were fully assigned by using phase-sensitive correlated spectroscopy and total correlated spectroscopy techniques. The protons of unit E could be traced only from protons 1 to 3; the remaining signals are presumably hidden by the signals of unit C. The stereochemistry of the individual subunits was established by using proton-proton coupling constants as obtained from either one-dimensional spectra, phase-sensitive correlated spectroscopy, or proton-detected heteronuclear shift correlation experiments (Table 1). All carbon signals connected to already-assigned protons were deduced by using proton-detected heteronuclear shift correlation. Possible ambiguities in the correlation as they occur at carbons 4 and 5 of unit I are mentioned in the following section. Unit B, which represents the terminal unit, showed no signals separated from the repeating-unit signals A, C, and D in the proton dimension, and the remaining minor carbon signals not assigned by the above procedure are therefore empirically associated with unit B. The Gal→Tyr linkage was verified by using nuclear Overhauser spectroscopy in the difference mode, and connectivities from phosphorus were established by using coupled and decoupled ^{31}P NMR spectroscopy (Fig. 3) in combination with ^{31}P-decoupled proton spectra recorded in the difference mode.

The structure of the O-antigen-like portion of the S-layer glycoprotein of *B. alvei* CCM 2051 determined by means of ^1H and ^{13}C NMR spectroscopy was already established in a previous analysis (1). Small deviations of shift values in the current work are attributable to different probe temperatures used.

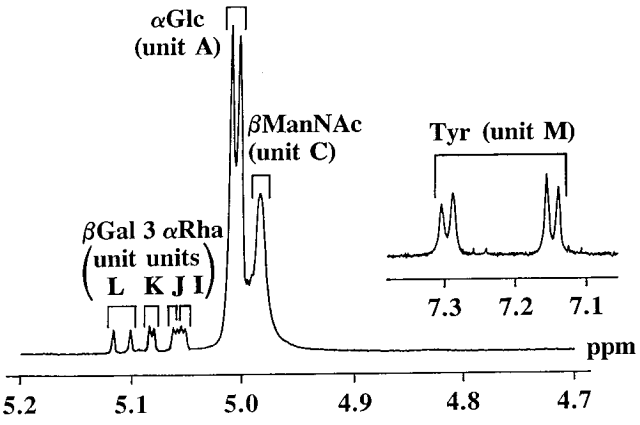

FIG. 1. Partial 500-MHz ^1H NMR spectrum of the anomeric region of glycopeptide fraction F-II of *B. alvei* CCM 2051. Units with distinct signals in the glycopeptide are indicated. For details, see text and Table 1.

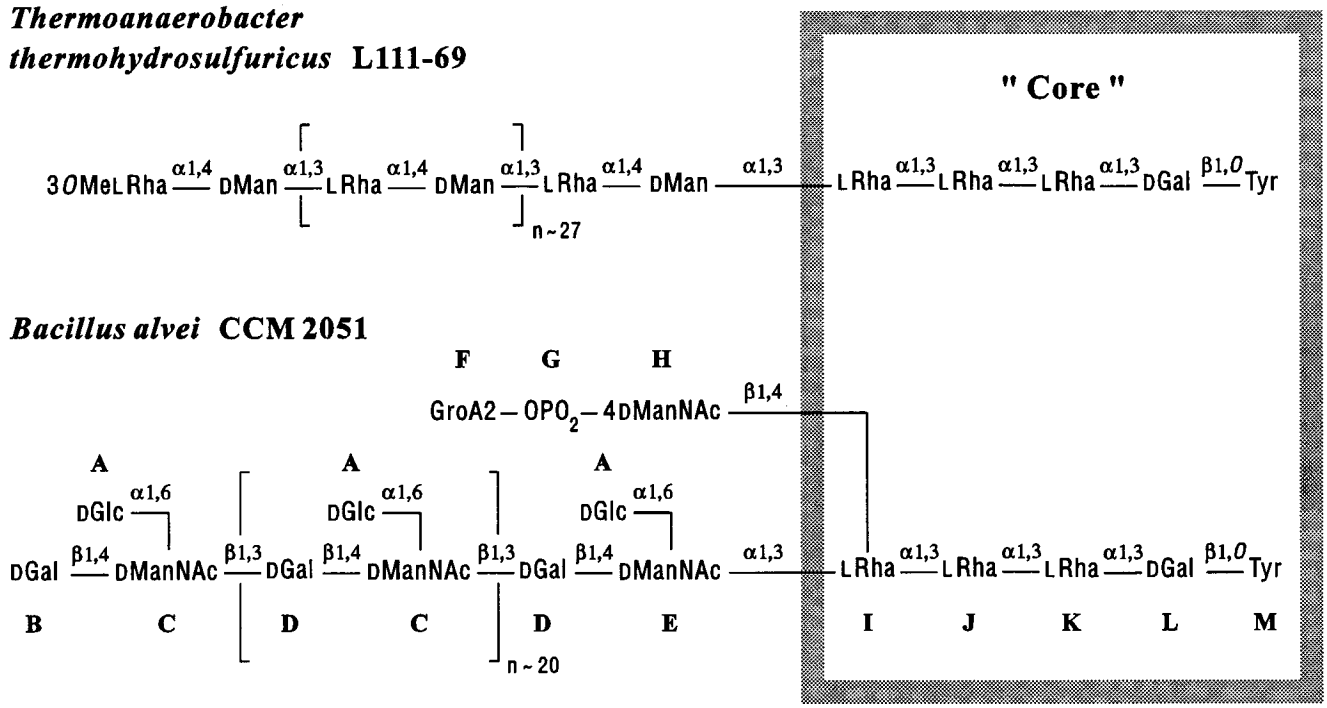

FIG. 2. Comparison of the glycopeptide structures of *B. alvei* CCM 2051 and *T. thermohydrosulfuricus* L111-69 (5). This schematic representation demonstrates both the most probable structure for the glycan chain of the S-layer glycoprotein of *B. alvei* CCM 2051 and the similarity of core structures in different tyrosine-linked S-layer glycans. The units (A through M) in the NMR experiments (Table 1) are indicated.

The present study thus confirms the previously reported structure of the glycan chain to be [→3-β-D-Gal*p*-(1→4)-[α-D-Glc*p*-(1→6)-]-β-D-Man*p*NAc-(1→]$_n$ (Fig. 2, units A, C, and D). In addition to the large signals attributable to the glycosyl units A, C, and D, several small signals corresponding to α-rhamnose, β-galactose, tyrosine, and β-*N*-acetylmannosamine of the core and the nonreducing terminal units of the O-antigen-like chains were observed. Thus, from the information obtained from the proton integrals (α-Rha:β-Gal:Tyr = 3:1:1) and the closely similar spectroscopic properties (shift values, coupling constants, and nuclear Overhauser experiment values; Table 1) of the comparable core structure of *T. thermohydrosulfuricus* (5), the reducing end of the glycan was identified as α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→O)-Tyr (units I to M).

A significant difference is observed with respect to the linkage pattern of the terminal rhamnose of the core (unit I), which is substituted in oxygen 3 by the α-mannose of the O-antigen-like repeats in *T. thermohydrosulfuricus* (5). The available NMR data are best interpreted by assuming that in *B. alvei* this rhamnose is not only substituted in position 3 by the ManNAc residue E of the O-antigen-like repeats, but also carries in position 4 a branching ManNAc residue H which is connected in position 4 by a phosphodiester linkage to oxygen 2 of glyceric acid, for the following reasons.

There is a conspicuous single-proton signal (at 4.67 ppm) which shows proton coupling to a CH_2 group and an additional, large coupling to a heteroatom. By means of phosphorus-decoupled proton NMR measurements, this heteroatom was shown to be part of a phosphate residue (unit G), which itself, as shown by proton-phosphorus coupling, is linked to a sugar unit. The additional constituent was identified as 2-phosphoglyceric acid on the basis of the observations that (i) no coupling was observed except from CH to CH_2 and to PO_3 and (ii) the observed spectral lines are closely similar to those of authentic 2-phosphoglyceric acid (Table 1). By proton-decoupled phosphorus NMR, the phosphate group itself was shown to be disubstituted (Fig. 3). The attached sugar unit was identified as the β-ManNAc residue H by tracing the signals obtained by phosphorus-decoupled proton NMR in the total and phase-sensitive correlated spectra, by means of proton-proton couplings, and from the corresponding carbon shifts.

The proposed 3,4-di-O-substitution pattern of rhamnose unit I is deduced from the spectral data as follows. As can be seen from shift values of carbons 2 and 3 of rhamnose unit I, oxygen 2 is unsubstituted (71.2 ppm) and oxygen 3 is substituted (79.0 ppm) (14). However, signals for carbon 4 (80.76 ppm, dd 2 × 10 Hz, as determined from proton-detected CH correlation) and carbon 5 (68.51 ppm) cannot be unequivocally identified because of heavily overlapping proton signals at 3.7 ppm and low signal intensities. Therefore, the possibility of

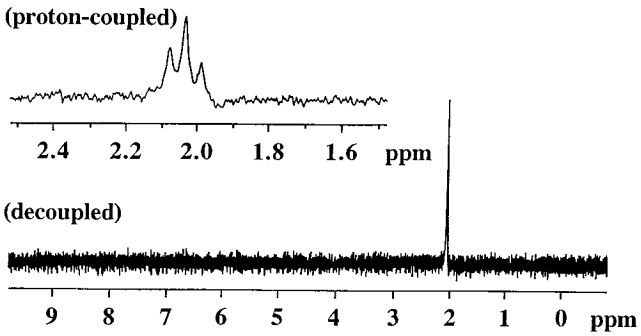

FIG. 3. A 200-MHz ^{31}P NMR spectrum of glycopeptide fraction F-II of *B. alvei* CCM 2051. For details, see the text.

rhamnose I being monosubstituted in position 3 needs to be excluded.

Despite the carbon 1 signals of the core sugars, there are only two shifts of anomeric carbons separated from the intense signals of the repeating units. Both of them belong to β-ManNAc residues (99.09 ppm [unit H] and 100.73 ppm [unit E]). Since a 3-substituted D-galactose is stereochemically related to a 3-substituted L-rhamnose (23), for a β-ManNAc linked to position 3 of L-rhamnose one would expect a shift value of 101.5 ppm, as observed for unit C. Thus, if unit I was monosubstituted in position 3, units H and E would have to be linked to some other sites. The shift value of 79 ppm observed for carbon 3 of rhamnose I is, however, in agreement for L-rhamnose substituted in position 3 by β-D-ManNAc (10).

On the other hand, the shift values assigned to the protons and carbons 4 and 5 of rhamnose I are in good agreement with values reported for α-rhamnose substituted in position 4 by β-ManNAc (7, 9). The carbon 1 signal of β-ManNAc, however, would be expected at 100.4 ppm. If one assumes an upfield shift of approximately 1 ppm for both β-ManNAc carbon 1's, if linked to positions 3 and 4 of the same rhamnose unit (3), one would expect shift values of 100.5 ppm for the mannose linked to positions 3 and 99.4 ppm for the mannose linked to position 4. This would indicate unit H (C-1 at 99.1 ppm) linked to position 4 and unit E (C-1 at 100.7 ppm) linked to position 3 of rhamnose I. This assumption is supported by the fact that only one extra signal at 80.9 ppm is observed in the range where glycosidated carbons reside, whereas at least two signals would be expected if the H and E units of the β-ManNAc are linked to the repeating-unit sugars A, C, and D instead of unit I.

The remaining few small carbon signals not assigned are best interpreted as signals of a terminal β-galactose unit B (Table 1). Carbons 1 and 6 should be indistinguishable from the corresponding carbons in unit D. From the combined NMR evidences, we propose the S-layer glycan structure shown in Fig. 2, which would also be in agreement with a blockwise biosynthesis of the glycan chain of this glycopeptide.

DISCUSSION

Comparison of the NMR spectra of the glycoprotein glycan from *B. alvei* CCM 2051 with those of *T. thermohydrosulfuricus* L111-69 (5) indicates that each O-antigen-like region is linked to the respective polypeptide by means of a tetrasaccharide core and that the two cores are very similar (Fig. 2). Their basic structure is →3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→ (5). However, the nonreducing rhamnose (unit I) is additionally substituted at oxygen 4 in *B. alvei*. The reducing β-D-Gal*p* residues of both cores are linked to tyrosine in an O-glycosidic linkage (5).

Unexpectedly, the β-D-galactose→tyrosine linkage unit was found to be present among phylogenetically rather distant strains: although both investigated organisms belong to the family *Bacillaceae*, *B. alvei* is positioned in rRNA group 3 of aerobic bacilli (2), whereas *T. thermohydrosulfuricus* belongs to the anaerobic thermophilic bacteria (13). Tyrosine was also found in the glycopeptides of several other organisms of the species *T. thermohydrosulfuricus*, but their linkage regions have not yet been characterized (17). However, there is evidence for the occurrence of linkages other than the described β-D-galactose→tyrosine unit. For example, the complex glycan chain of *Bacillus* sp. strain L420-91 definitely has no tyrosine in its glycopeptide (11). Furthermore, in some short glycan chains without repeats, either β-D-glucose→tyrosine linkages (16) or, as in a few archaeal S-layer glycoproteins, predominantly N-linked oligosaccharides are present (8, 12, 24).

The two glycans show in some parts a common architecture, i.e., a core structure by which the glycan chain, consisting of a number of identical repeats, is linked to the S-layer polypeptide. However, we have not observed any modification of the repeating-unit structure at the nonreducing end of the glycan chain of *B. alvei* as was shown for *T. thermohydrosulfuricus* L111-69 (5, 22). This modification prevented degradation of the polysaccharide chain by different exoglycosidases (5).

The architecture of the *B. alvei* and the *T. thermohydrosulfuricus* S-layer glycans resembles that of the lipopolysaccharides of gram-negative eubacteria (20). Rather than being attached to a lipid moiety, the glycans are covalently linked to the S-layer proteins. Therefore, the S-layer glycans create a polysaccharide coat for the bacteria similar to that in the lipopolysaccharides of gram-negative eubacteria. Support for this notion comes from the observation that several of the recently identified unusual sugar constituents of S-layer glycans, such as 3-*N*-acetylquinovosamine (1a), and D-rhamnose and 3-*N*-acetyl-D-fucosamine (11) have not been found in glycoconjugates other than lipopolysaccharides (for a review, see reference 14). Further investigations of the biosynthesis of the polysaccharide domains of S-layer glycoproteins will be required to justify this assumption.

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