Evidence for the glycoprotein nature of the crystalline cell wall surface layer of *Bacillus stearothermophilus* strain NRS2004/3a

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The surface layer of *Bacillus stearothermophilus* strain NRS2004/3a was isolated and chemically characterized. The results of these initial studies lead to the conclusion that the cell surface protein is glycosylated.

Bacterial cell wall *Glycoprotein* *Eubacteria* *S-layer* *Self-assembly* *Cell surface*

1. INTRODUCTION

Many Gram-positive and Gram-negative Eubacteria and Archaebacteria are covered by regular arrays of protein [1–3]. Chemical analyses of these crystalline S-layers of a variety of bacteria show that most of them are composed of a single polypeptide species with M_r values ranging from 40 000 to 200 000. Although earlier studies strongly suggest that S-layer subunits of Eubacteria can possess covalently linked carbohydrate residues [4] it was generally assumed that the presence of glycoproteins is restricted to Archaebacteria [5,6]. From our results we propose that the ability for glycosylation of cell surface proteins is also present in Eubacteria.

2. MATERIALS AND METHODS

2.1. Bacteria and growth conditions

Bacillus stearothermophilus NRS2004/3a was grown in batch cultures in S-VIII medium at 60°C as in [7]. For glycosylation inhibition experiments cell suspensions at an absorbance of 0.2 (at 600 nm) were transferred to flasks containing 1–10 μg tunicamycin (Sigma) per ml medium, and incubated for 16 h. For the preparation of cell wall material, the bacteria were grown in continuous culture at 60°C at a dilution rate of 0.25 h^{-1}.

2.2. Preparation of S-layers

By a modification of the procedure in [8], cells harvested by centrifugation were disrupted by ultrasonication. The cell wall fragments were purified by repeated suspension in 50 mM Tris–HCl (pH 7.2) followed by centrifugation at 35 000 × g. The pellets containing cell wall material were extracted with 5 M guanidine hydrochloride (Fluka) in distilled water and the extract was applied to a Sepharose CL-6B column equilibrated with 5 M guanidine hydrochloride in distilled water. Appropriate fractions were pooled, dialyzed against water, lyophilized, and stored at −20°C.

2.3. Electron microscopy

Freeze-etching preparations and thin sections were examined in a Philips EM 301 as in [7].

2.4. Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS–PAGE) was performed as in [9]. The procedures used for staining protein and glycoprotein are described in [7].

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2.5. Carbohydrate and amino acid analyses, protein estimation

Neutral sugar contents are based on the orcinol–H_2SO_4 method [10]. Rhamnose was specifically determined by cysteine–H_2SO_4 assay [11]. After hydrolysis, neutral sugars and hexosamines were analyzed by gas–liquid chromatography (GLC) as the corresponding alditol acetates [12]. Amino acid analyses were performed on a Biotronik LC 5000 amino acid analyzer after hydrolysis of the samples in azeotropic hydrochloric acid for 24 h at 105°C. The protein contents of the eluates were estimated on the basis of the absorbance at 280 nm.

2.6. Glycopeptide preparation

2.6.1. Pronase digestion

Purified S-layers were preincubated (100 mg) with 0.2% SDS in 0.01 M ammonium acetate buffer (pH 5.5) (10 ml) for 1 h at 37°C. Subsequently, pronase (Serva; 2% in relation to protein) and calcium acetate (final concentration 1.5 mM) were added and the pH was adjusted to 7.8 [13]. After incubation periods of 60–80 h at 37°C the digest was centrifuged. The clear supernatant was fractionated over Sephadex G-25 (1.5 × 180 cm), equilibrated with 1% acetic acid, and fractions of 13 ml were collected. The fractions giving positive reaction upon orcinol–H_2SO_4 and rhamnose assay were combined and concentrated to 1 ml, and the digestion was repeated for 48 h under the above conditions. The clear solution was fractionated on a Biogel P-100 (BioRad) column (1 × 120 cm), equilibrated with 1% acetic acid. Fractions of 1.5 ml were collected and analyzed for protein and carbohydrate as described above.

2.6.2. ^3H labeling of glycopeptides

Aliquots of the glycopeptide preparations (up to 1 mg in 100 μl of 0.1 M sodium acetate buffer, pH 6.0) were tritium labelled by sonication in the presence of *N*-succinimidyl[2,3-^3H]propionate (NSP) (Amersham; 30 μl of a toluene solution containing 1 mCi/ml). The samples were evaporated and applied to a Biogel P-100 (100–200 mesh) column as described above.

2.6.3. Attempted β-elimination of the glycan chains

NSP-labelled glycopeptide was treated with 0.15 M sodium borohydride solution (100 μl) at pH 9.0 for 48 h at 4°C [14]. After acidification with acetic acid and removal of boric acid with dry methanol the sample was passed through the Biogel P-100 column. Several attempts to achieve β-elimination of glycan chains from the glycoprotein at higher pH values and/or temperatures apparently resulted in extensive degradation of the peptide moiety. Furthermore, a recent report [14] indicates that the distinction between *N*- and *O*-glycosylation becomes impossible above pH 11.5.

2.6.4. Hydrazinolysis

Thoroughly dried glycopeptide was treated with anhydrous hydrazine (Pierce) for 24 h at 100°C [15]. The samples were evaporated, desalted, re-*N*-acetylated and reduced with sodium borohydride [16]. The product was chromatographed over Biogel P-100.

3. RESULTS

3.1. Electron microscopy

S-layer material covers the bacteria fully and at any time during the cell cycle (fig.1a); it clearly represents the outermost cell wall component (fig.1b).

3.2. Characterization of S-layer protein

Pure S-layer material, obtained from isolated cell walls by extraction and gel-chromatography in 5 M guanidine hydrochloride, produces 4 bands on SDS–PAGE (fig.2, lane b, compared with a). Their apparent M_r values are 93 000, 120 000, 147 000 and 170 000. The 3 higher bands give a positive periodic acid-Schiff reaction (fig.2, lane c). Amino acid (table 1) and carbohydrate analyses (table 2) of undissociated S-layer material reveal a slight preponderance of acidic amino acids, the complete absence of cysteine and a sugar content of approx. 3.6%. Rhamnose is by far the most preponderant monosaccharide (3.0%).

3.3. Effect of tunicamycin

Upon addition of the antibiotic tunicamycin to the growth medium of the bacteria at 5 μg/ml the 4 SDS–PAGE bands characteristic for S-layer material (fig.2, lane e) were no longer detectable. The electrophoretic pattern of the other (cellular) proteins (fig.2, lane d) remained essentially un-

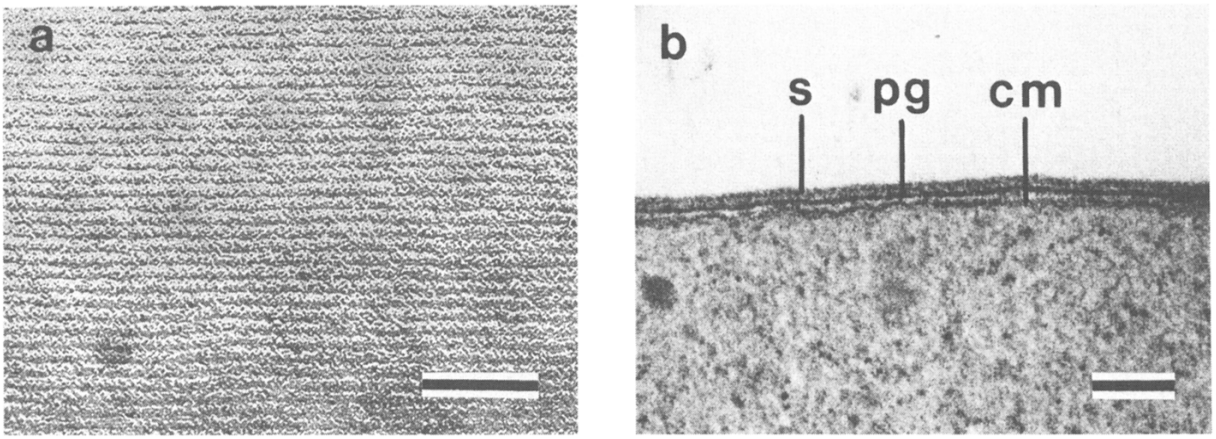

Fig.1. Freeze-fracture replication image (a) and ultrathin section of the cell envelope (b) of *B.stearothermophilus* NRS2004/3a; s, S-layer; pg, peptidoglycan; cm, cytoplasmic membrane. Bars, 100 nm.

changed. Electron microscopical inspection of cells grown in the presence of tunicamycin showed the complete absence of S-layers (not shown).

3.4. *Isolation and characterisation of glycopeptide*

Exhaustive proteoleotic digestion of S-layer proteins yielded a glycopeptide which migrated as a single peak on Biogel P-100 (fig.3, upper panel). Its sugar analysis (table 2) again shows rhamnose as the preponderant monosaccharide (55.0%).

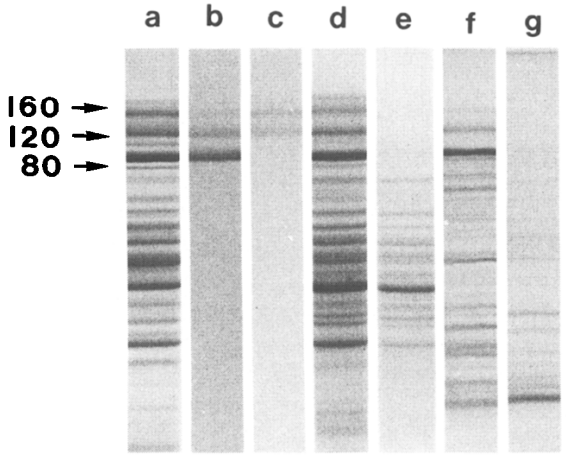

Fig.2. SDS–PAGE of whole cell extracts and S-layer preparations of *B.stearothermophilus* NRS2004/3a. Electrophoreses were performed on slab gels containing 10% acrylamide. Whole cell extracts (lane a), purified S-layers (lane b), and periodic acid-Schiff-staining of purified S-layers (lane c). Tunicamycin treatment of intact cells after 5 min (lane d) and after 16 h incubation at 60°C (lane e). Pronase digestion of S-layers after 6 h (lane f) and 24 h incubation at 37°C (lane g). The arrows denote M_r values of 80 000, 120 000 and 160 000, respectively, derived from calibration experiments with reference proteins.

Table 1

Amino acid composition of S-layer glycoprotein and of the glycopeptide from *Bacillus stearothermophilus* derived from it by treatment with SDS/pronase

Amino acids	Molar ratio		
	S-layer	Glycopeptide	
		Determined	Nearest integer
Asx	1.32	2.28	2.0
Thr[a]	1.00	1.00	1.0
Ser	0.62	1.94	2.0
Glx	0.59		
Pro	0.36		
Gly	0.51	0.86	1.0
Ala	1.26	1.32	1.0
Val	1.18		
Met	0.01		
Ile	0.41		
Leu	0.75		
Tyr	0.24		
Phe	0.21		
His	0.05		
Arg	0.18		
Lys	1.05		
Trp	0.08		

[a] Calculation basis

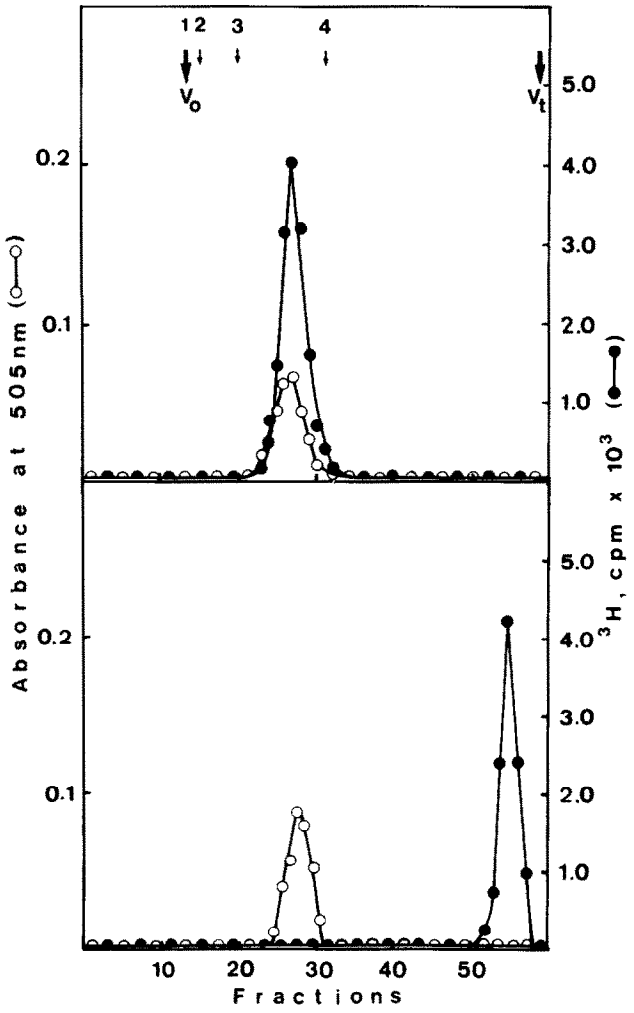

Fig.3. Gel filtrations of the NSP-labelled glycopeptide on a Biogel P-100 column following treatment with alkaline borohydride (upper panel) and following treatment with anhydrous hydrazine (lower panel) as described in section 2. M_r markers (indicated by arrows): Thyroglobule (1), catalase (2), bovine serum albumin (3), carbonic anhydrase (4); V_0, void volume; V_t, total volume.

Other neutral sugars are present only as minor components; no amino sugar was found in this preparation. Amino acid analysis (table 1) of a weighed sample of pure material suggests the presence of 5 amino acids, asparagine, serine, threonine, glycine and alanine in a ratio of approx. 2:2:1:1:1.

3.5. Hydrazinolysis and attempted β-elimination

Treatment of a glycopeptide sample from the Biogel P-100 peak (fig.3, upper panel) with alkaline $NaBH_4$ solution had no effect on its migration on the same column (fig.3, upper panel). In contrast, hydrazinolytic conditions separated the carbohydrate from the radioactivity incorporation in the peptide; the sugar peak appeared in the elution pattern with a slight decrease in molecular mass (fig.3, lower panel).

4. DISCUSSION

The data presented here lead us to postulate that S-layer proteins of *B.stearothermophilus* NRS2004/ 3a contain covalently linked carbohyd-

Table 2

Monosaccharide composition of S-layer glycoprotein and of the glycopeptide from *Bacillus stearothermophilus* derived from it by treatment with SDS/pronase

Monosaccharide	(%)		
	S-layer[a]	Glycopeptide[b]	Product of hydrazinolysis[b]
Rha	3.0	55.0[a]	79.0[d]
Man	0.1		
Gal	0.2	<2.0[a,e]	87.0[c]
Glc	0.2		
GlcNAc	0.1	–	

[a]GC analysis; [b]After Biogel P-100; [c]Orcinol–H_2SO_4 assay [10]; [d]Cystein–H_2SO_4 assay [11]; [e]Individual hexose peaks were too small for exact calculations

rate. This conclusion is based on a number of observations.

Treatment of isolated cell walls with highly dissociative reagents such as guanidine hydrochloride or urea, both in high concentrations, or acid [17], leads to the extraction of S-layer material which, upon restoration of physiological conditions, reaggregates into two-dimensional paracrystalline arrays (self-assemblies) with the same lattice constants as observed on intact cells [18]. SDS–PAGE, after gel chromatography in 5 M guanidine hydrochloride, shows that this self-assembly material is separated into 4 bands of which the 3 corresponding to the highest M_r values are stained with the periodate-Schiff reagent. We assume that these bands correspond to glycoproteins having identical polypeptide moieties but increasing degrees or different patterns of glycosylation, since upon extraction the material of each band can be induced to reaggregate into identical S-layer lattices (unpublished). When cells are grown in the presence of tunicamycin, an antibiotic which inhibits the formation of carbohydrate–polyprenyl conjugates [19], no S-layer material can be detected either on electron micrographs of intact cells or in the electrophoretic pattern of isolated extracted cell walls.

After digestion of isolated S-layers with pronase, a fraction of high carbohydrate content and apparently containing a small peptide moiety was isolated. Sugar and peptide comigrate on SDS–PAGE and on gel permeation chromatography in the presence of high concentrations of chaotropic agents. Amino acid analysis showed that aspartic acid (and/or asparagine) and the hydroxyamino acids serine and threonine represent 5 of the 7 residues forming the peptide. It is well established that these amino acids are, in most glycoproteins so far known, essential structural requirements of protein glycosylation.

Mild conditions which should lead to detectable β-elimination of carbohydrate linked to α-amino-β-hydroxy acids were without effect, suggesting that the carbohydrate is not linked to serine or threonine. Upon hydrazinolysis, however, carbohydrate could be separated from the peptide.

The evidence presented here strongly suggests a glycoprotein nature of the S-layer proteins investigated. Further corroboration of these findings would bear important consequences reaching into the concepts of evolution. The presence of glycoproteins on the outer surface of cell walls of Archaebacteria is held to represent an important biochemical distinction from the broad spectrum of Eubacteria [5,6]. Our experiments indicate that this criterion may require re-evaluation in the near future. Indeed, data from our laboratory show that other Eubacteria *(Clostridium, Desulfotomaculum)* produce glycosylated S-layer proteins as well [20]. Finally, it should be mentioned that recent investigations have demonstrated the presence of mannosylated membrane proteins in *Micrococcus luteus (lysodeikticus)* [21].

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