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Determination of the cell adhesion specificity of *Streptococcus suis* with the complete set of monodeoxy analogues of globotriose

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Streptococcus suis **causes meningitis and other serious infections in pigs and humans, and binds to host cell globotriosylceramide. In order to determine the essential hydroxyls involved in binding, the complete set of monodeoxy derivatives of the receptor trisaccharide Galα1-Galβ1-4Glc were tested as inhibitors of bacterial hemagglutination. Removal of the 4″-, 6″, 2′ or 3′-hydroxyls abolished inhibitory activity, which indicated that they were critically involved in binding. The same results were obtained using synthetic lipid-linked monodeoxy derivatives of the trisaccharides in a thin-layer overlay assay. The P_N and P_O subtypes of the *S. suis* adhesin showed similar binding patterns. The hydroxyls of the glucose moiety were not critical for binding, although the adhesin binds better to the trisaccharide Galα1-4Galβ1-4Glc than the disaccharide Galα1-4Gal.**

Keywords: **bacterial adhesion, globotriose, monodeoxy analogues, receptor binding, *Streptococcus suis***

Abbreviation: **GbO_3, Galα1-4Galβ1-4Glcβ1-1′Cer (globotriosylceramide)**

Introduction

Streptococcus suis is an important pig pathogen causing severe infections such as septicemia, pneumonia and meningitis in pigs and humans [1]. We have identified two carbohydrate-based cell-binding specificities in *S. suis.* Two strains were found to recognise sialylated polylactosamine chains [2]. Another adhesin, present in all strains so far examined, was found to recognise the disaccharide sequence Galα1-4Gal present in trihexosylceramide, GbO_3 [3]. This adhesin was classified into two subtypes, P_N and P_O, based on differences in their binding specificity. Type P_O was inhibited by galactose only, whereas type P_N was inhibited by both galactose and N-acetylgalactosamine, and bound better P_O to GalNAcβ1-3Galα1-4Gal-containing oligosaccharides present in globoside [4].

For both P_N and P_O types of the *S. suis* adhesin the optimal receptor structure, based on the hemagglutination assays and TLC overlay assays using naturally existing glycolipids, appeared to be the trisaccharide Galα1-4Galβ1-4Glc [4]. The binding pattern of the adhesin to the hydroxyls of the Galα1-4Gal disaccharide has been determined [4], but the mode of binding to the hydroxyls of the Glc moiety has remained unknown. The determination of the hydrogen-bonding specificities in carbohydrate-protein interactions, using well-defined monodeoxysugars as probes, was pioneered by Lemieux, who investigated the binding of antibodies and lectins [5]. Subsequent work along these lines included investigations of the binding of antibodies [6] and bacterial surface lectins [7]. In the present study we have determined the binding pattern of the *S. suis* P_N and P_O adhesins using the complete set of the monodeoxy derivatives of the receptor trisaccharide.

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Materials and methods

Oligosaccharides

The preparation of oligosaccharide derivatives [8–10] and their bis-SO_2-lipid derivatives [11–13] has been reported. The conformations of the saccharides were determined both by NMR and theoretical calculations [14,15].

Sialidase from *Vibrio cholerae* was obtained from Behringwerke AG, Marburg, Germany, Microwell microtiter plates from NUNC, Roskilde, Denmark, high-performance thin-layer chromatography aluminium sheets HPTLC Sil-

ica Gel 60 from Merck, Darmstadt, Germany, polyisobutylmethacrylate (Plexigum P28) from Röhm GmbH, Darmstadt, Germany, Kodak X-Omat AR film from Eastman Kodak, Rochester, NY, and GasPak anaerobic systems from Becton Dickinson and Co., Cockeysville, MD, USA.

Bacterial strains

The origins of the *Streptococcus suis* strains studied have been described before [16]. The bacteria were maintained in 15% glycerol in Todd-Hewitt broth at -80 °C and were grown on sheep blood agar plates overnight at 37 °C under anaerobic conditions. The agglutinating phase of the bacteria was maintained by adsorbing the bacteria to red cells as described before [17].

Erythrocytes

Human erythrocytes were obtained from a young healthy donor (blood group B+). The erythrocytes were purified by centrifugations and treated with sialidase as described before [18]. Sialidase-treated erythrocytes (5% v/v) were used for hemagglutination inhibition assays.

The hemagglutination inhibition assay

The oligosaccharide in P_i/NaCl (10 mM sodium phosphate buffer, 0.15 M NaCl, pH. 7.4) were serially diluted (25 µl) in microtiter plates and 25 µl of bacterial suspension was added. After incubation of the plates at 4 °C for 10 min, 50 µl of a 5% sialidase-treated erythrocyte suspension was added, and the plates were incubated at 4 °C for 2 h. The minimum inhibitory concentration (MIC) was defined as the lowest concentration giving full inhibition of hemagglutination with the accuracy of ±one dilution step.

Thin-layer chromatography overlay assay

For binding assays two micrograms of each glycolipid was eluted on silica gel high-performance thin-layer chromatography plates with the solvent chloroform/methanol/water (60:35:8, by vol.). The plates were coated with 0.3% polyisobutylmethacrylate (Plexigum P28) in ether:hexane (3:1, by vol.), dried at 23 °C, sprayed with P_i/NaCl and blocked with 2% bovine serum albumin in P_i/NaCl at 23 °C for 2 h. The plates were overlaid with radiolabelled bacteria (5×10^8 colony-forming units/ml) in 2% bovine serum albumin in P_i/NaCl and kept at 23 °C for 2 h. The plates were washed 3 times for 5 min with 1% bovine serum albumin in P_i/NaCl and subjected to autoradiography using Kodak X-Omat AR film for 24-70 h. Glycolipids in control lanes were visualised with anisaldehyde reagent [19].

Molecular modelling

Minimum energy calculation of the conformations of Galα1-4Galβ1-4Glc has been reported [20]. The lowest energy conformation of the GbO_3 trisaccharide was used to create the structure shown in Figure 2.

Results

Hemagglutination inhibition by monodeoxy derivatives of disaccharides

In a previous study the inhibitory properties of the monodeoxy derivatives of Galα1-4Gal were studied using a solid phase inhibition assay [4]. Since the presentation of oligosaccharide receptors on solid artificial surfaces may be different from that in natural membranes [21,22], the derivatives were tested with a hemagglutination inhibition assay. The inhibitory concentrations of the disaccharides were found to be one order of magnitude lower in this assay as compared to the solid phase inhibition assay. However, as indicated by the results in Table 1, the same hydroxyls (*i.e.* 4′-, 6′-, 2- and 3-OH) were found to be essential for the binding of the adhesin to the disaccharide for both the P_N and P_O types of adhesins.

Hemagglutination inhibition by monodeoxy derivatives of trisaccharides

Hemagglutination inhibition using the complete set of the monodeoxy derivatives of the receptor trisaccharide (Table 2) reveal a few-fold lower values for the inhibitory concentrations as compared to the disaccharides (Table 1). The same hydroxyls as indicated by the disaccharide inhibitions were indicated to be the essential hydroxyls for binding also in the case of the trisaccharides. Removal of the 2-, 3,- or 6-hydroxyls of the glucose moiety did not abolish binding. Instead, there was a slight increase in the inhibitory strength, which suggests that hydrophobic interactions may improve the binding.

Table 1. Minimum inhibitory concentrations of receptor disaccharide derivatives in the hemagglutination induced by type P_N and P_O *S. suis*

Inhibitor	*S. suis* P_N	*S. suis* P_O
	µM	
Galα1-4Gal	3.1	1.5
Galα1-4Galβ-O-Me	1.5	0.8
Galα1-4Galβ-O-Me; 2-Deoxy-	>100.0	>100.0
Galα1-4Galβ-O-Me; 3-Deoxy-	>100.0	>100.0
Galα1-4Galβ-O-Me; 3-C-Me-	>100.0	>100.0
Galα1-4Galβ-O-Me; 6-Deoxy-	1.5	1.5
Galα1-4Galβ-O-Me; 2′-Deoxy-	1.5	1.5
Galα1-4Galβ-O-Me; 3′-Deoxy-	0.8	0.8
Galα1-4Galβ-O-Me; 3′-O-Me-	12.2	12.2
Galα1-4Galβ-O-Me; 4′-Deoxy-	>100.0	>100.0
Galα1-4Galβ-O-Me; 6′-Deoxy-	>100.0	>100.0

Table 2. Minimum inhibitory concentrations of receptor trisaccharide derivatives in the hemagglutination induced by type P_N and P_O *S. suis*

Inhibitor	*S.suis* P_N	*S.suis* P_O
	μM	
Galα1-4Galβ1-4Glcβ1-O-$(CH_2)_2SiMe_3$	0.76	0.76
Galα1-4Galβ1-4Glcβ1-O-$(CH_2)_2SiMe_3$; 2-Deoxy	0.19	0.76
Galα1-4Galβ1-4Glcβ1-O-$(CH_2)_2SiMe_3$; 3-Deoxy	0.19	0.38
Galα1-4Galβ1-4Glcβ1-O-$(CH_2)_2SiMe_3$; 6-Deoxy	0.19	0.38
Galα1-4Galβ1-4Glcβ1-O-$(CH_2)_2SiMe_3$; 2′-Deoxy	>20.0	>20.0
Galα1-4Galβ1-4Glcβ1-O-$(CH_2)_2SiMe_3$; 3′-Deoxy	>20.0	>20.0
Galα1-4Galβ1-4Glcβ1-O-$(CH_2)_2SiMe_3$; 6′-Deoxy	0.19	0.76
Galα1-4Galβ1-4Glcβ1-O-$(CH_2)_2SiMe_3$; 2″-Deoxy	ND	ND
Galα1-4Galβ1-4Glcβ1-O-$(CH_2)_2SiMe_3$; 3″-Deoxy	0.19	0.095
Galα1-4Galβ1-4Glcβ1-O-$(CH_2)_2SiMe_3$; 4″-Deoxy	>20.0	>20.0
Galα1-4Galβ1-4Glcβ1-O-$(CH_2)_2SiMe_3$; 6″-Deoxy	>20.0	>20.0

Binding to lipid-linked derivatives in thin-layer chromatography overlay assay

In order to study the binding of the adhesin to the receptor disaccharide in the glycolipid form, radioactively labelled bacteria of the P_N and P_O type were incubated with thin-layer chromatograms of the bis-SO_2-glycolipid derivatives [11–13] of the trisaccharides. As shown in Figure 1, bacteria of type P_N bound to the unmodified and the 2″-, 3″, 6′-, 2-, 3-, and 6-monodeoxy derivatives, whereas there was no binding to the 4″-, 6″-, 2′- and 3′-monodeoxy derivatives. Bacteria of type P_O (not shown) gave the same result. Thus, the binding specificity for the bis-SO_2-glycolipid derivatives was the same as for the soluble derivatives in the hemagglutination inhibition assays (Tables 1 and 2).

Discussion

The molecular epitope recognised by the *S. suis* adhesins P_N and P_O using the complete set of monodeoxy derivatives of globotriose oligosaccharides was confirmed to consist of the hydroxyls HO-4″, HO-6″, HO-2′ and HO-3′ (Figure 2). As shown before, the main difference between the two types is the more narrow combining site of P_O around the 3″-hydroxyl [4]. The presence of additional weak hydrogen bonds in P_O at the 3″- and 6′-hydroxyls could not be confirmed in the hemagglutination inhibition assays.

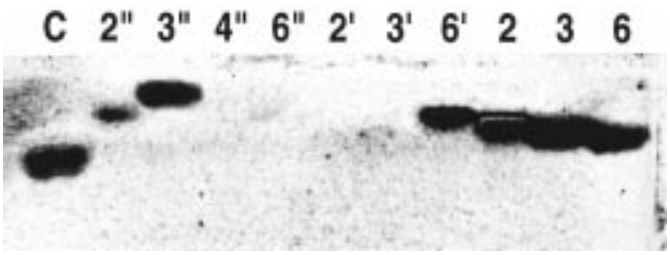

Figure 1. Binding of *S. suis* bacteria to glycolipids on thin-layer chromatography plates. Radioactively labelled bacteria of type P_N (strain628) were incubated with polyisobutylmethacrylate-coated thin-layer chromatography plates containing two μg of each glycolipid derivative of the monodeoxy derivatives of the receptor trisaccharide. C, control unmodified trisaccharide bis-SO_2-glycolipid.

As shown by the previous hemagglutination and TLC overlay assays, the glucose residue improved the binding of the adhesin to its receptor [4]. The role of the hydroxyl groups of the β-glucose residue was studied with both hemagglutination inhibition and TLC overlay assay. Surprisingly, none of the hydroxyls was found to be hydrogen bonded to the adhesin. Slight decreases in the MIC values were found in hemagglutination assays, which suggests that the adhesin might form hydrophobic contacts with the adhesin, and that the combining site could be narrow around the glucose residue. We have recently shown that a derivative containing an aromatic ring in the reducing-end of the receptor disaccharide (2-(5-phenyl-4-thiapentanoylamido)ethyl 4-*O*-α-D- galactopyranosyl-β-D-galactopyranoside) was 6- and 4- fold stronger inhibitor than the trimethylsilylethyl derivative of the two adhesin subtypes [23], which also supports the idea that hydrophobic contacts may be involved.

It has previously been shown that the presentation of the glycolipids on thin-layer chromatograms may be critical for the interpretation of the binding specificity of Galα1–4Gal-binding proteins [21,22,24]. However, the present study shows that in the case of *S. suis* the results of the different assays give comparable results.

A common feature for the known galabiose-recognising adhesins of *E. coli* and *S. suis* is the minimum requirement of Galα1–4Gal. Despite of this similarity, the epitopes that are recognised are different. Both the class I and II ad-

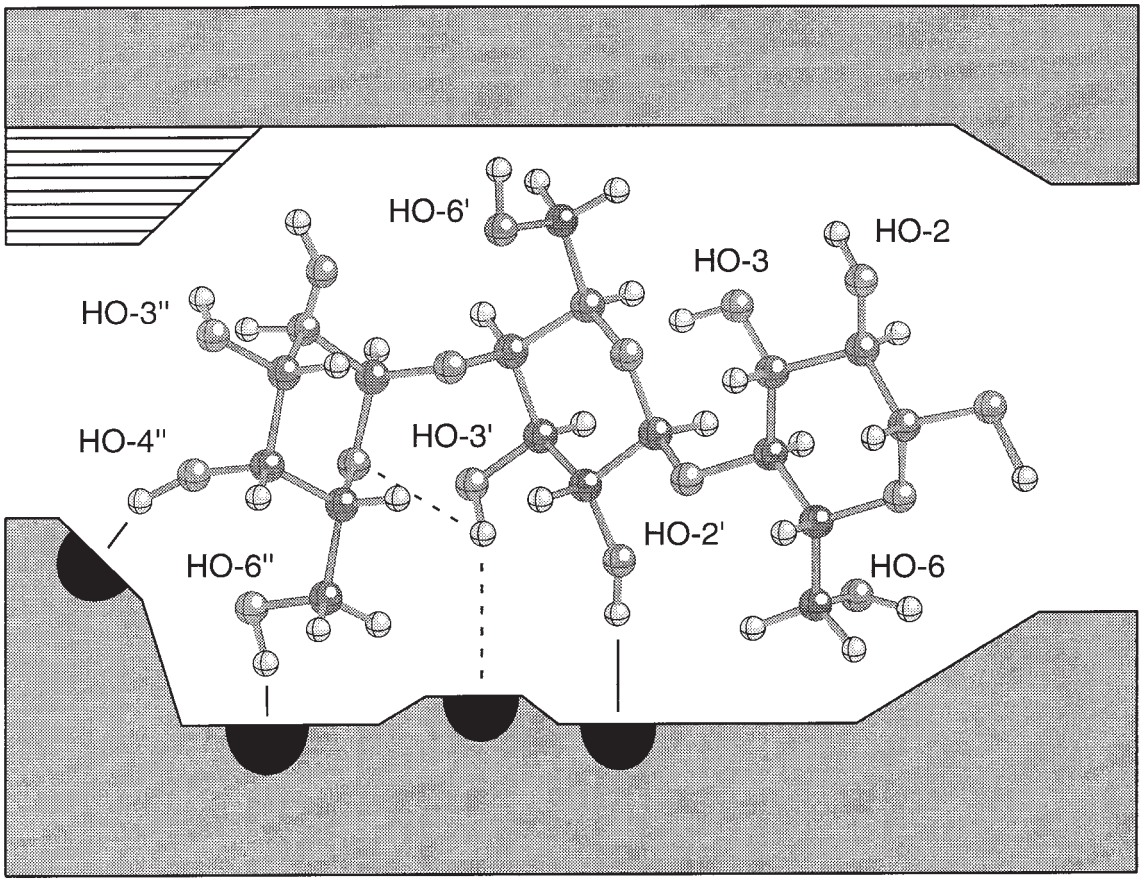

Figure 2. Model of the binding of the receptor trisaccharide Galα1-4Galβ1-4Glc to the *S. suis* adhesins of type P_N and P_O. The lowest energy conformation of the GbO_3 trisaccharide [20] was used to create the structure shown. The essential hydrogen bonds formed between the receptor saccharide and the adhesins are indicated by the solid lines. The broken lines at HO-3′ indicated the potential hydrogen bonds to the adhesin and to O-5″. The hatched area top left indicates the crowded region of close contact in P_O.

hesins of *E. coli* recognise HO-6″, HO-4″, HO-3″, HO-6′, and to a lesser extent HO-2″. Thus it seems that the adhesins of *E. coli* and *S. suis* recognise the receptor saccharide from different sides of the molecule.

The *S. suis* adhesin can be solubilised and purified as a monomer in an active form [25], and the molecular cloning of its gene is underway. The elucidation of the structure of the adhesin by crystallography and NMR, combined with the studies using receptor oligosaccharide derivatives, will be needed for the determination of the complete binding mechanism and the design of anti-adhesive drugs against *S. suis.*

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