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GLYCOARRAY SECTION

Human tandem-repeat-type galectins bind bacterial non-βGal polysaccharides

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Abstract Galectins are multifunctional effectors, for example acting as regulators of cell growth via protein-glycan interactions. The observation of capacity to kill bacteria for two tandem-repeat-type galectins, which target histo-blood epitopes toward this end (Stowell *et al*. Nat. Med. 16:295–301, 2010), prompted us to establish an array with bacterial polysaccharides. We addressed the question whether sugar determinants other than β-galactosides may be docking sites, using human galectins-4, -8, and -9. Positive controls with histo-blood group ABH-epitopes and the *E. coli* 086 polysaccharide ascertained the suitability of the set-up. Significant signal generation, depending on type of galectin and polysacchride, was obtained. Presence of cognate β-galactoside-related epitopes within a polysaccharide chain or its branch will not automatically establish binding properties, and structural constellations lacking galactosides, like rhamnan, were found to be active. These data establish the array as valuable screening tool, giving direction to further functional and structural studies.

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Abbreviations

BSA	Bovine serum albumin
CRD	Carbohydrate recognition domain
PS	Polysaccharide
RFU	Relative Fluorescence Units

Introduction

Epitopes of glycan chains of natural glycoconjugates are increasingly delineated as biochemical signals, their information turned into cellular effects by lectins [1, 2]. In addition to endogenous glycan determinants, tissue lectins, *e.g*. C-type lectins and galectins, have the ability to target pathogens, as part of defence mechanisms or exploited as sites for bacterial/viral/yeast attachment [3–6]. From the family of galectins, which share the β-sandwich fold, a sequence signature and specificity to β-galactosides, special attention has so far been given to galectin-3 with respect to bacterial recognition [4, 7]. The binding of this chimera-type protein (with its tridomain structure constituted by an N-terminal section, collagen-like repeats and the carbohydrate recognition domain (CRD)) involves carbohydrate-dependent/independent mechanisms. Interest in studying galectins was recently further stimulated by the observation that the tandem-repeat-type galectins-4 and -8 (proteins with two CRDs covalently connected by a linker [8]) can abolish bacterial viability after targeting histo-blood group B antigen on *E.coli* 086 surface [9]. In fact, the repeating unit of its O-polysaccharide (PS) [10] mimics the B (type 4) antigen. Overall, rather strong interaction with histo-blood group epitopes had been reported for the tandem-repeat-type galectins-4, -8, and -9 by different methods [9, 11–15]. Using a cell-based assay, which measures the ligand selection

of cell-bound galectins for cross-linking, a clear preference for such epitopes had even been revealed (Fig. 1) [16, 17].

This preference is phylogenetically conserved, pointing to a physiological role [18] and hereby directing interest to characterizing the range of potential binders among bacterial PSs. The occurrence of such epitopes in PSs of infectious bacteria is very low. Inspection of the relevant public database at http://csdb.glycoscience.ru, which covers about 10,000 structures from around 5,000 organisms, demonstrates that only few of them contain ABH-like motifs, including the polysaccharide from *E. coli* O86 mentioned above:

$$[-2)\mathbf{Gal\alpha1-3(Fuc\alpha1-2)Gal\beta1-3GalNAc\beta}1-3\mathrm{GalNAc}\beta1-]_{n,}$$

and an *oligo*saccharide from *Helicobacter mustelae* strain ATCC43772 terminating with the A (type 1) tetrasaccharide:

$$\mathbf{GalNAc\alpha1-3(Fuc\alpha1-2)Gal\beta1-3GlcNAc\beta}1-\text{(lipopolysaccharide core)}.$$

Since the mode of epitope presentation is known to affect galectin reactivity [19, 20], the first aim of our study is to figure out whether epitope presence will translate into binding activity. Also, molecular mimicry may endow PSs without β-galactosides, the canonical ligands, with binding capacity, warranting a robust screening. Experimentally, we established an array of PSs from bacteria of the genera *Escherichia, Shigella, Salmonella, Cronobacter, Proteus,* and *Pseudomonas,* putting together a total of nearly 150 structurally defined polysaccharides (Table 1). The array also contained a set of non-bacterial glycans as controls, including ABH epitopes [21].

Material and methods

Reagents

Carbohydrate-free bovine serum albumin (BSA), Na_2HPO_4, NaH_2PO_4, NaCl and KCl were purchased from Sigma (USA), Tween-20 was from ICN (USA). Alexa-555-labeled streptavidin was obtained from Invitrogen (USA).

Galectins

Recombinant human galectins -4, -8 and -9 were produced using suited vector/bacteria systems as described and then purified from extracts by affinity chromatography on lactosylated Sepharose 4B [22, 23]. Homogeneity was ascertained by gel filtration, also removing any cleaved material such as free CRDs, and by one/two-dimensional gel electrophoreses, biotinylation performed as described in detail previously [24] and carbohydrate-dependent activity tested by solid-phase/cell assays [25, 26].

Glycans

Polysaccharides were obtained by mild acid degradation (2 % HOAc, 100 °C, 1–4 h) of the corresponding lipopolysaccharides isolated from bacterial cells by the Westphal procedure [27] modified as described [28]. The polysaccharides were purified by gel-permeation chromatography on Sephadex G-50 (Superfine) in pyridinium acetate buffer (4 mL pyridine and 10 mL HOAc in 1 L water). Oligosaccharides in form of aminopropyl-, aminoethyl- or glycylamido-glycosides are described in [21].

Glycan array

Microarrays on glass microscope slides presenting the oligosaccharides and polysaccharides were printed as described

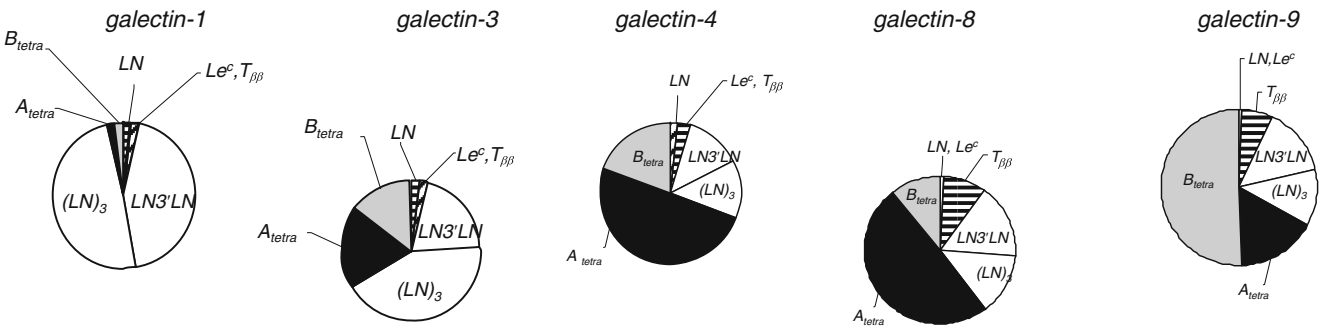

Fig. 1 Elevated affinity of galectins -4, -8 and -9 towards blood group A and B (types 1 and 2) glycans, data taken from [3] and [4] are shown as partial 'weight' of the corresponding glycan. *LN* Galβ1-4GlcNAc, Le^C Galβ1-3GlcNAc, $T_{\beta\beta}$ Galβ1-3GalNAcβ, A_{tetra} GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAc, B_{tetra} Galα1-3(Fucα1-2)Galβ1-4GlcNAc

Table 1 List of bacterial strains used for isolation of the polysaccharides included into the array

№	Name
1	*Salmonella enterica O28deAc*
2	*Salmonella enterica O47deAc*
3	*Salmonella enterica O16deAc*
4	*Salmonella enterica O13*
5	*Salmonella enterica O28*
6	*Salmonella enterica O16*
7	*Salmonella enterica O17*
8	*Salmonella enterica O67*
9	*Salmonella enterica O58*
10	*Salmonella enterica O41*
11	*Salmonella enterica O62*
12	*Salmonella enterica O60*
13	*Salmonella enterica O18*
14	*Salmonella enterica O59*
15	*Salmonella enterica O42*
16	*Salmonella enterica O52*
17	*Salmonella enterica O11*
18	*Salmonella enterica O51*
19	*Salmonella enterica O44*
20	*Salmonella enterica O21*
21	*Salmonella enterica O57*
22	*Salmonella enterica O56*
23	*Salmonella enterica 038*
24	*Cronobacter sakazaki G2356 O2*
25	*Cronobacter sakazaki G2592 O7*
26	*Cronobacter sakazaki G2594 O4*
27	*Cronobacter sakazaki G2726 O3*
28	*Escherichia coli O11*
29	*Escherichia coli O15*
30	*Escherichia coli O44*
31	*Escherichia coli O49*
32	*Escherichia coli O51*
33	*Escherichia coli O52*
34	*Escherichia coli O57*
35	*Escherichia coli O58*
36	*Escherichia coli O71*
37	*Escherichia coli O73*
38	*Escherichia coli O85*
39	*Escherichia coli O86*
40	*Escherichia coli O95*
41	*Escherichia coli O99*
42	*Escherichia coli O108*
43	*Escherichia coli O112ab*
44	*Escherichia coli O118*
45	*Escherichia coli O119*
46	*Escherichia coli O123*
47	*Escherichia coli O125*
48	*Escherichia coli O127*

Table 1 (continued)

№	Name
49	*Escherichia coli O130*
50	*Escherichia coli O148*
51	*Escherichia coli O150*
52	*Escherichia coli O151*
53	*Escherichia coli O161*
54	*Escherichia coli O168*
55	*Escherichia coli 010a,010b*
56	*Escherichia coli O12*
57	*Escherichia coli O14*
58	*Escherichia coli O19ab*
59	*Escherichia coli O27*
60	*Escherichia coli O36*
61	*Escherichia coli O37*
62	*Escherichia coli O41*
63	*Escherichia coli O54*
64	*Escherichia coli O62*
65	*Escherichia coli O81*
66	*Pseudomonas aeruginosa O1(F4)*
67	*Pseudomonas aeruginosa O10ac(F5)*
68	*Pseudomonas aeruginosa O11ab*
69	*Pseudomonas aeruginosa O13ab*
70	*Pseudomonas aeruginosa O13ac*
71	*Pseudomonas aeruginosa O14*
72	*Pseudomonas aeruginosa O15*
73	*Pseudomonas aeruginosa O2abc*
74	*Pseudomonas aeruginosa O2ac*
75	*Pseudomonas aeruginosa O2ac(F3)*
76	*Pseudomonas aeruginosa O2adf*
77	*Pseudomonas aeruginosa O3(Habs 3)*
78	*Pseudomonas aeruginosa O4ac*
79	*Pseudomonas aeruginosa O9ad*
80	*Proteus genomospecies 11B-r*
81	*Proteus mirabilis 12B-r*
82	*Proteus mirabilis 1B-m*
83	*Proteus mirabilis 3B-m*
84	*Proteus mirabilis O11*
85	*Proteus mirabilis O13*
86	*Proteus mirabilis O16*
87	*Proteus mirabilis O23*
88	*Proteus mirabilis O28*
89	*Proteus mirabilis O31*
90	*Proteus mirabilis O33*
91	*Proteus mirabilis O38*
92	*Proteus mirabilis O3ac*
93	*Proteus mirabilis O58*
94	*Proteus mirabilis O6*
95	*Proteus mirabilis O60*
96	*Proteus mirabilis OE*
97	*Proteus pinneri 107*

Table 1 (continued)

№	Name
98	*Proteus pinneri 113*
99	*Proteus pinneri 28*
100	*Proteus pinneri 31*
101	*Proteus pinneri 40*
102	*Proteus pinneri 75*
103	*Proteus vulgaris 32/57 O17*
104	*Proteus vulgaris O19ab*
105	*Proteus vulgaris O22*
106	*Proteus vulgaris O4*
107	*Proteus vulgaris O46*
108	*Proteus vulgaris O65*
109	*Proteus vulgaris OX19*
110	*Proteus vulgaris TG251*
111	*Shigella boydii type 10*
112	*Shigella boydii type 12*
113	*Shigella boydii type 14*
114	*Shigella boydii type 15*
115	*Shigella boydii type 16*
116	*Shigella boydii type 17*
117	*Shigella boydii type 18*
118	*Shigella boydii type 2*
119	*Shigella boydii type 6*
120	*Shigella boydii type 7*
121	*Shigella boydii type 8*
122	*Shigella boydii type 9*
123	*Shigella boydii type X*
124	*Shigella dysenteriae type 1*
125	*Shigella dysenteriae type 11*
126	*Shigella dysenteriae type 3*
127	*Shigella dysenteriae type 4*
128	*Shigella dysenteriae type 7*
129	*Shigella dysenteriae type 8*
130	*Shigella dysenteriae type 8*
131	*Shigella dysenteriae type 9*
132	*Shigella flexneri type 2a*
133	*Shigella flexneri type 2b*
134	*Shigella flexneri type 3a*
135	*Shigella flexneri type 3b*
136	*Shigella flexneri type 4a*
137	*Shigella flexneri type 4b*
138	*Shigella flexneri type 5a*
139	*Shigella flexneri type 5b*
140	*Shigella flexneri type 6*
141	*Shigella flexneri type 6b*
142	*Shigella flexneri type X*
143	*Shigella flexneri type Y*
144	*Streptococcus equi sp.*
145	*Acetobacter methanolicus LPS*
146	*Saccharomyces cerevisiae (Zymozan A)*

Table 1 (continued)

№	Name
147	*Laminarin from L. hyperborea*
148	*Barley β-glucan*
149	*Scleroglucan*
150	*Curdlan*
151	*high MW chitosan*

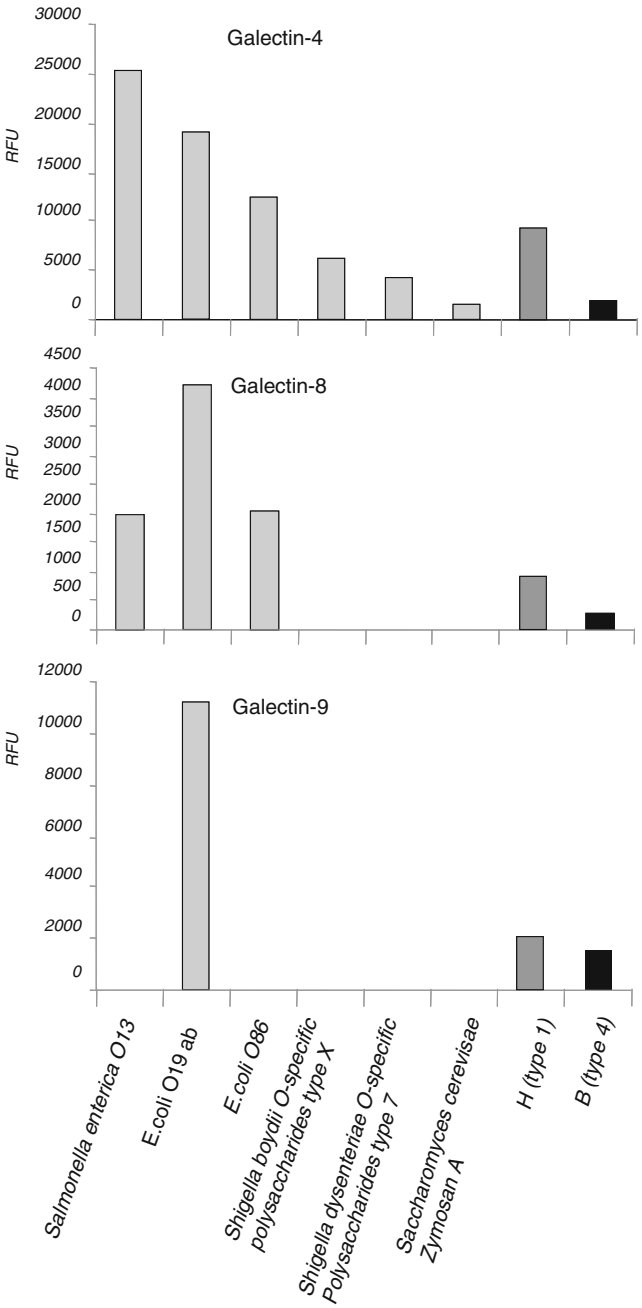

Fig. 2 Binding of galectins to bacterial polysaccharides immobilized on microchip compared to trisaccharide H (type 1) and tetrasaccharide B (type 4). Other PSs listed in Table 1 were also assayed, no binding was observed. Background of the assay is considered as ~500 RFU

Table 2 Structures of the repeating units of bacterial PSs reactive with tandem-repeat-type galectins (structural information from http://csdb.glycoscience.ru). Histo-blood group H- and B-motifs are shown in bold

Bacterium	Structure of the repeating unit
Salmonella enterica O13	[-2**Fucα1-2Galβ1-3GalNAcβ1**-3GlcNAcβ1-]$_n$
E. coli O19ab	[-2Rhaα1-2Rhaα1-2Rhaα1-2Glcβ1-3GlcNAc6Acβ1-]$_n$
E. coli O86	[-2**Galα1-3(Fucα1-2)Galβ1-3GalNAcα**1-3GalNAcβ1-]$_n$
Shigella flexneri type X	[-2(Glcβ1-3)Rhaα1-2Rhaα1-3Rhaα1-3GlcpNAcβ1-]$_n$
Shigella dysenteriae type 7	[-3Qui4NGlyAcβ1-4GalNAcA6NH$_2$ 3Acα1-4GalNAcAα1-3GlcNAcα1-]$_n$

[29]. Aliquots of solution of biotinylated galectins (75 μg/ml) in PBS (0.01 M Na_2HPO_4, 0.01 M NaH_2PO_4, 0.138 M NaCl, and 0.0027 M KCl, pH 7.4) containing 1 % BSA were applied to slides (pretreated with PBS containing 0.1 % Tween-20 for 15 min) and incubated in a humidified chamber at 20 °C for 1 h. Therefore, the slides were washed with PBS containing 0.05 % Tween-20 and incubated with a streptavidin-Alexa-555 conjugate (diluted 1:1,000 with PBS containing 0.1 % Tween-20) under the same conditions. After washing with PBS containing 0.05 % Tween-20 and deionized water, slides were scanned on a ProScanArray HT scanner (PerkinElmer, USA) using the excitation wavelength 543 nm. The obtained data were processed using ScanArray Express 3.0 software and the fixed 70 μm-diameter rings method as well as Microsoft Excel. Twelve spots represent each glycan on the array and data are reported as median RFU of replicates. A signal, which fluorescence intensity exceeded the background value by a factor of five, was considered as significant. Controls using non-spacered glycans ensured carbohydrate dependence of binding.

Results and discussion

A wide panel of PSs was used for probing, among them the one from *E. coli* 086 as control (Table 1) and also synthetic histo-blood ABH-epitopes. Illustrating the highly selective recognition by galectins, only few cases of significant reactivity were detected, and the extent of reactivity between the galectins was found to differ markedly (Fig. 2). All three galectins bound to α(1–2)-rhamnan from *E. coli* with the extent exceeding that obtaining for all ABH glycans.

Despite shared binding properties to the β-galactoside core owing to the homology the galectins can non-uniformly react with glycan epitopes presented in PSs. Also of note, eight PSs present β-galactosides, which would be expected to be galectin binders, but failed to be reactive; particularly, side β-Gal 1-3-connected to core GalNAc in *E. coli* 0125, side β-Gal 1-4-connected to core Glc in *E. coli* 014, inner Galβ1-3(4)Glc(NAc) or Galβ1-3GalNAc in *Proteus mirabilis* O33 and *Proteus mirabilis* OE, in *Proteus penneri* 75, and in *Salmonella enterica* 013,—in all cases Gal moiety has no "forbidden" for galectin binding substituent at O–4 position. Obviously, the spatial presentation and/or structural context can matter, as also valid in the interpretation of cyto- and histochemical results after galectin staining [30, 31].

Equally important, the compilation of the structures of the repeating units of reactive PSs, given in Table 2, disclosed binding properties to non-canonical determinants. As to *L*-rhamnose, a molecular mimicry with *D*-galactose has been suggested, rendering a recognition process possible [32]; however, L-Rha monosaccharide in the array (data not shown) was found to be non-binder. Taking into account topological aspects, these studies should not only be performed on free sugars in solution but also on clustered arrangements, which may be required to generate adequate avidity. Methodologically, the array testing has proven to be a convenient way to select candidates for further measuring effects on cell viability when bacteria are exposed to galectins, giving our study model character.

Conclusions

Our results document the ability of array-presented bacterial PSs to detect galectin reactivity. Binding will not automatically occur if a canonical ligand is part of the PS's repeating unit. Obviously, the mode of presentation plays into this process. Intriguingly, reactivity to PSs without presence of a β-galactoside is detected, stimulating interaction analysis of core-unit sugars due to the potential biomedical relevance of this recognition process, in infection as are effector branch of host defense.

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