The Fucose-binding Lectin from *Ralstonia solanacearum*

A NEW TYPE OF $\beta$-PROPELLER ARCHITECTURE FORMED BY OLIGOMERIZATION AND INTERACTING WITH FUCOSIDE, FUCOSYLLACTOSE, AND PLANT XYLOGLUCAN$^*$

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Plant pathogens, like animal ones, use protein-carbohydrate interactions in their strategy for host recognition, attachment, and invasion. The bacterium *Ralstonia solanacearum*, which is distributed worldwide and causes lethal wilt in many agricultural crops, was shown to produce a potent L-fucose-binding lectin, *R. solanacearum* lectin, a small protein of 90 amino acids with a tandem repeat in its amino acid sequence. In the present study, surface plasmon resonance experiments conducted on a series of oligosaccharides show a preference for binding to αFuc1–2Gal and αFuc1–6Gal epitopes. Titration microcalorimetry demonstrates the presence of two binding sites per monomer and an unusually high affinity of the lectin for αFuc1–2Gal-containing oligosaccharides ($K_D = 2.5 \times 10^{-7}$ M for 2-fucosyllactose). *R. solanacearum* lectin has been crystallized with a methyl derivative of fucose and with the highest affinity ligand, 2-fucosyllactose. X-ray crystal structures, the one with α-methyl-fucoside being at ultrahigh resolution, reveal that each monomer consists of two small four-stranded anti-parallel $\beta$-sheets. Trimerization through a 3-fold or pseudo-3-fold axis generates a six-bladed $\beta$-propeller architecture, very similar to that previously described for the fungal lectin of *Aleuria aurantia*. This is the first report of a $\beta$-propeller formed by oligomerization and not by sequential domains. Each monomer presents two fucose binding sites, resulting in six symmetrically arranged sugar binding sites for the $\beta$-propeller. Crystals were also obtained for a mutated lectin complexed with a fragment of xyloglucan, a fucosylated polysaccharide from the primary cell wall of plants, which may be the biological target of the lectin.

*Ralstonia solanacearum* is a Gram-negative $\beta$-proteobacterium, inhabiting water and soil and causing lethal wilt in more than 200 plants worldwide (1). From the soil, it can enter plant roots via wounds or secondary roots, invade the xylem vessels, and spread in the plant (2). This bacterium causes major agronomic and economic losses in tropical climates and threatens potatoes in temperate climates, especially with the recent extension of strains adapted to cooler environmental conditions in Europe and North America. The expression of genes encoding the machinery for adhesion to plant cells (*hrp*) is enhanced by plant cell wall fragments (3). Although the specific signal molecule has not yet been identified, it appears to be a component of the cell wall polysaccharide matrix, since removal of lipids and proteins does not affect the activity of cell wall fragments. In addition to linear insoluble cellulose, complex branched polysaccharides such as pectins and xyloglucans, also called hemicelluloses, are found in the primary cell walls of higher plants (4). Fucose-containing xyloglucan oligomers have been shown to exert signaling effects on plant tissues (5).

Since host carbohydrates have been known for many years to constitute specific attachment sites for pathogen protein receptors (6, 7), there is great interest in structure-function studies of bacterial proteins enabling the pathogen attachment to host glycans. However, only a limited number of their complexes with receptors have been characterized by crystallography. Most of the structural studies dealt with toxins that bind specifically to intestine (8) or to neural glycans (9). Recently, adhesins located on the tip of pili from uropathogenic or enterotoxigenic *Escherichia coli* strains were structurally characterized (10), as well as *Pseudomonas aeruginosa* soluble lectins, PA-IL and PA-III, that are specific for galactose and fucose, respectively (11). These latter structures provide clues for the binding of the bacteria to the overfucosylated mucins present in the lungs of cystic fibrosis patients (12).

Studies of lectins involved in interactions of phytopathogens with plant glycans are in their very early stage. The search for a fucose-binding lectin in *R. solanacearum* led to the characterization of two soluble lectins. The first, RS-III, binds to fucose but has a strong preference for mannose despite its strong sequence and structural similarity to PA-III (13). The second lectin, RSL,$^1$ is slightly smaller than PA-III (90 amino

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$^1$ The abbreviations used are: RSL, *R. solanacearum* lectin; AAL, *A. aurantia* lectin; SPR, surface plasmon resonance; Fuc, L-fucose; r.m.s., root mean square; ITc, isothermal titration microcalorimetry.

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acids) and has strong affinity for fucose and fucose-containing oligosaccharides such as human ABO blood group determinants (14). This lectin displays sequence similarity to the fucose-binding lectin of another soil inhabitant, the orange peel mushroom *Aleuria aurantia*. Structural determination of *A. aurantia* lectin (AAL) showed a six-bladed β-propeller with five fucose binding sites (15). Intriguingly, the RSL monomer sequence is three times shorter than that of AAL, corresponding to only two repeats, and therefore cannot adopt the same fold.

The present paper describes the production, binding properties, and structure of RSL. The specificity of recombinant RSL toward a large number of human and plant oligosaccharides was screened by surface plasmon resonance (SPR). The affinity toward the best ligands was further characterized by titration microcalorimetry, confirming a very high affinity toward the αFuc1–2Gal disaccharide. Crystal structures of three different RSL-sugar complexes revealed the molecular basis of both the affinity and specificity of this lectin.

### EXPERIMENTAL PROCEDURES

**Materials**—Lewis a (α-l-Fuc(1→4)[β-D-Gal(1→3)]→O-GlcNAc), Lewis X (α-l-Fuc(1→3)[β-D-Gal(1→4)]→O-GlcNAc), blood group A (α-l-Fuc(1→2)[β-D-Gal(1→3)]→O-Gal), and group H II (α-l-Fuc(1→2)-β-D-Gal(1→4)→O-GlcNAc) trisaccharides were purchased from Dextra Laboratories (Reading, UK). α-L-fucose, p-nitrophenyl-α-L-fucoside, p-nitrophenyl-β-L-fucose, α-l-Fuc(1→2)-β-D-Gal(1→4)→O-Glc (Prolac) and other monosaccharides were purchased from Sigma. α-Methyl-l-fucopyranoside (Methyl) was purchased from Interchim.

**Synthesis of Methyl 1-Seleno-α-l-fucopyranoside**—Organic reagents were used as purchased except CH₂Cl₂, which was distilled from CaH₂ and stored over molecular sieves (4 Å), and MeCN, which was dried and stored over molecular sieves (3 Å). Organic solutions were concentrated and stored over molecular sieves (4 Å), and MeCN, which was dried and stored over molecular sieves (2 Å). NMR spectrum were recorded on a 300-MHz (Varian) and a 400-MHz (Jeol) spectrometer in CDCl₃ or D₂O. TMS was used as the internal standard (δ = 0) for 1H spectra in chloroform, and 13C spectra were referred to the chloroform signal (δ = 77.17). Silica gel (40–63 μm, Prolabo; VWR International) was used for flash chromatography. TLC was performed on silica gel 60 F₂₅₄ (Merck) glass plates with detection by charring with anisaldehyde sulfuric acid reagent.

**Preparation of Xyloglucan Polysaccharides and Oligosaccharides**—Fucosylated xyloglucans were extracted from cell walls of *Rubus fruticosus* suspension cells. Pectic polysaccharides were solubilized by sequential extraction (16), the main hemicellulose fraction was obtained with 2.5 N NaOH solution and xyloglucan was purified as previously described (17). Xyloglucan from a 20-day-old cell suspension was used for the addition of 0.5 mM isopropyl-β-D-thiogalactoside (IPTG). After induction using an Amicon cell fitted with M₅₀, 10 kDa membrane (Amicon, Beverly, MA) (18). The fucosylated nonasaccharide (XXFG) was prepared from xyloglucan from 24-day-old cell suspension by enzymatic digestion using xyloglucanase Cell26B produced by *Aspergillus aculeatus* and kindly supplied by Novozymes. The resulting oligomers were separated and characterized as described previously (17).

**Production of Recombinant RSL and Mutagenesis**—Native RSL has been purified from *R. solanacearum* ATCC 11696 purchased from the American Type Culture Collection as previously described (14). Purified DNA from the same bacterial strain has been used for the construction of recombinant RSL. The initial primers to amplify the genomic fragment around the gene of hypothetical protein RSc2107 (TREMBL access code Q6X8X6), which was previously assigned to be RSL, were designed based on the sequenced genome of *R. solanacearum* GMI1000 (19). To solve strain-dependent sequence variations, initial primers complementary to about 10–20 bp upstream (5′-GATGATCAACAATGTCCATTAATTATTCC-3′) and downstream (5′-GATTCACACCTTGGCCAG-3′) of the rsc2107 gene were used, and the amplified PCR product from the ATCC 11696 strain was sequenced. New gene-specific primers (5′-GAG ACG ACA TAT GTC GAC GTG CAG GAC CG-3′ and 5′-CAA AGG TTC ATG TCG TGG CTT AGG CGC-3′) were designed and HindIII restriction sites to edge the gene were used for preparation of the recombinant protein. After digestion with HindIII and HindIII, the amplified fragment was introduced into the cloning site of pET-25(b +) vector (Novagen, Madison, WI), resulting in plasmid pET25sr. The expression vector pET25sr was transformed into *E. coli* BL21(DE3) (Novagen). Cells harboring pET25sr plasmid were grown in LB broth containing 100 μg ml⁻¹ ampicillin at 37 °C until the A₆₀₀ reached ~0.6. The cells were then cultured for an additional 3 h with the addition of 0.5 mM isopropyl-β-D-thiogalactoside. After induction by 0.5 mM isopropyl-β-D-thiogalactoside, bacterial cells were harvested by centrifugation at 8,000 × g at 4 °C for 20 min and washed with an equal volume of buffer containing 20 mM Tris/HCl, 100 mM NaCl, (pH 7.4). The cell suspension was disrupted by ultrasonic vibration, and cell-free extract was collected by centrifugation at 20,000 × g at 4 °C for 50 min.

Recombinant RSL was purified by affinity chromatography on a mouse-mucin-galactose column. Protein extract was loaded on a column pre-equilibrated by 20 mM Tris/HCl, 100 mM NaCl buffer at pH 7.4, and unbound proteins were washed out with the same buffer. RSL was eluted with 0.1 M NaOH in the same buffer, dialyzed against deionized water at 4 °C for 5 days, and then concentrated by lyophilization. Protein purity was assessed by SDS-PAGE and mass spectrometry.

Recombinant RSL mutants were generated by amplification of the *rsl* gene by DNAzyme™ II recombinant DNA polymerase (Finzymes, Finland). A selected double mutant (D77G/G84S) was cloned, expressed, and purified as described for the wild-type recombinant RSL.

**Crystallization and Data Collection**—Crystallization trials were performed with Hampton crystallization screens I and II (Hampton Research, Laguna Niguel, CA). Crystals of the RSL-fucose complex were obtained using the following conditions: 2 μl of precipitant (0.1 M so-
dium cacodylate buffer, pH 6.5, 0.2 mM magnesium acetate tetrahydrate, 20% polyethylene glycol 8000) mixed with 2 μl of RSL solution at a concentration of 10 mg/ml and t-fucose at a concentration of 137 μM/ml. The drops were allowed to equilibrate over a reservoir of 1 ml of the reservoir solution (30% polyethylene glycol 6000 in 0.1 M Tris/HCl, pH 6.5, 0.2 M magnesium acetate tetrahydrate, 20% polyethylene glycol 8000) mixed with 2 μl of solution of 50 mM L-fucose. They belonged to space group P31 with unit cell dimensions of a = 44.13 Å and c = 103.66 Å at 100 K. The asymmetric unit corresponding to a cell of 2.0 Å³ Da⁻¹ and 38% solvent content.

Crystals of the RSL-Me-selenofucoside and RSL-FucLac complexes were obtained by co-crystallization of RSL with sugars in the presence of 3.0 Å³ Da⁻¹ and 58% solvent content. Since trials of RSL crystallization in the presence of xyloglucan were not successful, a mutant (RSIm) in which half of the binding sites are altered (D77G/G84S) was also screened. Co-crystals of RSIm and the nonasaccharide fragment XXFG were obtained by soaking protein/fucose crystals prepared in 1.5 M (NH₄)₂SO₄ in 2.7 mg/ml XXFG solution. Crystals were obtained in space group P4₁2₁2 with unit cell dimensions of a = 64.1 Å, c = 128.1 Å at 100 K with three monomers in the asymmetric unit corresponding to a Vₐ₀ of 2.3 Å³ Da⁻¹ and 45% solvent content.

Crystals were cryo-cooled at 100 K, after soaking them for as the shortest possible time in 30% (v/v) glycerol in precipitant solution. Data images were recorded on an ADSC Q4R CCD detector (Quantum Corp.) or a MAR 225 detector (MAR USA Inc.) at beamlines ID14-2 and ID23-1, respectively, at the ESRF (Grenoble, France). Highly redundant single wavelength (0.9793 Å) anomalous diffraction data to 1.7 Å resolution were collected using ID23-1 from a single crystal containing the seleno-ligand. The three native forms were measured at a 0.93 Å wavelength using ID14-2. Diffraction images were processed using MOSFLM (20) and scaled and truncated to structure factors using the CCP4 (21) programs SCALA and TRUNCATE. Data processing statistics are presented in Table I.

Structure Determination—The crystal structure was solved using the single wavelength anomalous diffraction technique with data from the selenofucoside complex. Harker sections of the anomalous difference Patterson map showed peaks corresponding to one selenium per monomer in the asymmetric unit. Selenium site coordinates, phasing, and analysis were determined by PROCHECK (23).

### Table I

| Data collection and phasing statistics | RSL/SeMeFuc | RSL/MeFuc | RSL/FucLac | RSL/XXFG |
|---|---|---|---|---|
| Beamsline | ID23–1: ESRF | ID14–2: ESRF | ID14–2: ESRF | ID14–2: ESRF |
| Space group | P3₁ | P3₁ | H32 | P4,22 |
| Resolution (Å) | 25.92 to 1.70 | 30.67 to 0.94 | 55.6 to 2.10 | 64.15 to 1.80 |
| Highest resolution shell (Å) | 1.74 to 1.70 | 0.96 to 0.94 | 2.15 to 2.10 | 1.85 to 1.80 |
| Wavelength (Å) | 0.9793 | 0.923 | 0.923 | 0.933 |
| Cell dimensions (Å) | a = 44.1 | a = 43.985 | a = 76.8 | a = 64.1 |
| Total number of hkl | 687,564 | 825,992 | 35,012 | 213,335 |
| Average completeness (%) | 99.9 (98.3) | 98.3 (98.3) | 97.1 (96.5) | 99.7 (96.7) |
| Completeness for anomalous data (%) | 99.8 (97.1) | 98.3 (98.3) | 97.1 (96.5) | 99.7 (96.7) |
| Wilson B factor (Å²) | 1.17 (47.3) | 1.37 (37.7) | 1.13 (45.7) | 1.07 (47.2) |
| Values in parentheses refer to the highest resolution shell. |
| RA (observation) | 0.120 (7,126) | 0.253 (317) | 0.188 (1,299) |
| Refinement statistics | | | | |
| Resolution limits (Å) | 30.67 to 0.94 | 55.6 to 2.1 | 64.15 to 1.80 |
| Working set R | 0.089 (135,348) | 0.183 (6,435) | 0.146 (24,221) |
| Test set R | 0.120 (7,126) | 0.253 (317) | 0.188 (1,299) |
| Average completeness (%) | 99.9 (98.3) | 98.3 (98.3) | 97.1 (96.5) | 99.7 (96.7) |
| Overall completeness (%) | 99.8 (97.1) | 98.3 (98.3) | 97.1 (96.5) | 99.7 (96.7) |
| Wilson B factor (Å²) | 1.17 (47.3) | 1.37 (37.7) | 1.13 (45.7) | 1.07 (47.2) |

### Table II

| Refinement statistics | RSL/MeFuc | RSL/FucLac | RSL/XXFG |
|---|---|---|---|
| Resolution limits (Å) | 30.67 to 0.94 | 55.6 to 2.1 | 64.15 to 1.80 |
| Working set R | 0.089 (135,348) | 0.183 (6,435) | 0.146 (24,221) |
| Test set R | 0.120 (7,126) | 0.253 (317) | 0.188 (1,299) |
|Overall G factor | 0.05 | 0.188 | 0.106 |
| Distance deviations | 0.055 | 0.188 | 0.106 |
| Bond distances (Å) | 0.04 | 0.007 | 0.009 |

a For RSL/MeFuc refinements, the value was estimated from a Luzzati plot.

b Analysis was determined by PROCHECK (23).

c For RSL/MeFuc refinements, values refer to root mean square deviations (in Å) for angle and planar restraint distances.
2-fucosyllactose with sugar concentrations from $0.097 \, \mu M$ (lowest curve) to $100 \, \mu M$ (highest curve). Inset, fitting of steady state 1:1 model. B, plant xyloglucan polysaccharide at concentrations from $0.16 \, \mu M$ (lowest curve) to $20 \, \mu M$ (top curve). C, fucosylated XXFG (black) and nonfucosylated XXLG (gray) xyloglucan fragments at concentrations of $7.8 \, \mu M$ (lowest curve) to $500 \, \mu M$ (top curve).

**FIG. 1.** SPR sensograms representing binding of RSL to different oligosaccharides at 25°C and with a flow rate of 5 μl/min. A, 2-fucosyllactose with sugar concentrations from 0.097 μM (lowest curve) to 100 μM (highest curve). Inset, fitting of steady state 1:1 model. B, plant xyloglucan polysaccharide at concentrations from 0.16 μM (lowest curve) to 20 μM (top curve). C, fucosylated XXFG (black) and nonfucosylated XXLG (gray) xyloglucan fragments at concentrations of 7.8 μM (lowest curve) to 500 μM (top curve).

**TABLE III**

Equilibrium dissociation and association constants for the interaction between fucosylated oligosaccharides and RSL measured by SPR experiments

| Ligand                          | $K_D$ (μM) | $K_A$ ($10^9$ M$^{-1}$) | Potency |
|--------------------------------|------------|-------------------------|---------|
| Blood group B trisaccharide    | 0.21       | 484.8                   | 9.9     |
| α-Fuc1→2[β GalNAc(1→3)]Gal    | 0.25       | 400.1                   | 8.2     |
| Blood group A trisaccharide    | 6-Fucosyl-GlcNAc | 333.3                  | 6.8     |
| α-Fuc1→6β GlcNAc-O-Me          | 0.30       | 255.8                   | 5.2     |
| 2-Fucosyllactose (FucLac)      | 0.39       | 168.6                   | 3.4     |
| α-Fuc1→2βGal                   | 2-Fucosylgalactose (FucGal) | 156     | 3.2     |
| α-Fuc1→2βGal                   | 0.64       | 121.8                   | 2.5     |
| α-1-Me-fucoside (MeFuc)        | 0.82       | 117                     | 2.4     |
| H type II trisaccharide        | p-Nitrophenyl-α-l-fucoside | 117     | 2.4     |
| α-Fuc1→2βGal                   | 2.04       | 49.0                    | 1.0     |
| XXFG                           | 2.76       | 36.3                    | 0.74    |
| Lewis α trisaccharide          | 3.71       | 27.0                    | 0.55    |
| α-Fuc1→4[β Gal(1→3)]GlcNAc    | 8.47       | 11.8                    | 0.24    |
| p-Nitrophenyl-β-l-fucoside     | 9.01       | 11.1                    | 0.23    |
| α-Fuc1→4βGal                   | 12.5       | 8.0                     | 0.16    |
| Lewis X trisaccharide          | 14.4       | 7.0                     | 0.14    |
| 2-Fucosyllactose (3FucLac)     | 15.0       | 6.7                     | 0.14    |
| α-Fuc1→3βGal                   | 18.7       | 5.3                     | 0.11    |
| Sialyl Lewis X tetrasaccharide | α-Fuc1→3βGal(2→3)β Gal(1→4)GlcNAc | 118.7    | 0.22    |

solvent flattening were carried out with autoSHARP (22). An initial structure was built automatically using ARP/wARP (24), and side chains were docked to give 245 residues of a total of 270 for the asymmetric unit. Manual building using O (25) and several cycles of refinement with Refmac (26) gave a more complete model consisting of a tightly associated trimer forming a 6-bladed β-propeller.

This model was used as the search model for molecular replacement for both the P3, and P4,22 native data sets using Molrep (21), whereas a monomer was used for the H32 space group. This was followed by a complete automatic construction, side chain docking, and an initial water molecule construction with ARP/wARP. In each complex, the few missing amino acids and all sugar residues clearly defined in density were positioned manually using O. Refinement cycles with Refmac, including further automatic water molecule placement using ARP/wARP, manual rebuilding with O, and construction of alternative conformations where necessary (with occupancies estimated from the refined relative B-factors of the conformations), resulted in final models with refinement statistics listed in Table II.

**SPR Measurements**—All SPR experiments were performed with a Biacore 3000 (Biacore AB, Uppsal, Sweden) at 25°C using HBS buffer (10 mM Hepes, 150 mM NaCl, pH 7.4) and a flow rate of 5 μl/min. Measurements were carried out simultaneously on all four measuring channels using three different concentrations of immobilized RSL, whereas the fourth channel was used as the control flow cell. A research grade CM5 sensor chip was activated with 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide, N-hydroxysuccinimide) for 10 min, and 50 μl of RSL in 5 mM maleate buffer, pH 6.0, at concentrations of 300, 100, and 50 μg/ml, respectively, was injected into a particular flow cell. The unreacted species on the sensor surface were

C. Vonrhein, E. Blanc, P. Roversi, and G. Bricogne, manuscript in preparation.
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**RESULTS**

**Cloning and Production of Recombinant RSL**—Sequencing of the amplified fragment from *R. solanacearum* ATCC 11696 strain harboring the gene for RSL protein revealed nine differences in the nucleotide sequence compared with the GM11000 strain. Two result in amino acids Thr and Ala instead of two initial methionine. Spectroscopy analysis confirmed the molecular mass of 9.9 kDa, which moved as a sharp band of about 10 kDa. Mass spectrometry analysis determined from the standard equation,

\[
Q_r = \frac{n_P A_r}{G - K_P n_P} = \frac{1 + \frac{A_r}{n_P} + \frac{1}{nK_P} + \sqrt{\left(\frac{1 + \frac{A_r}{n_P} + \frac{1}{nK_P}\right)^2 - 4 \frac{A_r}{n_P}}}{2}
\]  

(Eq. 1)

where \( P \) represents the total protein concentration, \( A_r \) is the total concentration of the ligand, \( V_r \) is the volume of the cell, and \( Q_r \) is the total released heat for the injection of ligand. \( \Delta G \) values and entropy contributions were determined from the standard equation,

\[
\Delta G = -RT \ln K = \Delta H - T \Delta S
\]

where \( \Delta G \), \( \Delta H \), and \( \Delta S \) are the change in free energy, enthalpy, and entropy of binding; \( T \) is the absolute temperature; \( r = 8.32 \text{J mol}^{-1} \text{K}^{-1} \); and \( K \) is the association constant. All experiments were performed with c values 100 < c < 200 (c = KM, where M is the initial concentration of the macromolecule).

**Specificity Studies of RSL by Surface Plasmon Resonance**—A surface plasmon resonance binding assay was used to determine equilibrium dissociation constants \( K_D \) for RSL binding to a series of fucose-containing saccharides. Fig. 1 shows typical sensorgrams obtained after injection of analyte over the lectin-covered surface. Because association and dissociation phases were rapid, binding curves for all substrates were carried out using the steady-state parts of experimental curves. Dissociation constants were investigated using Scatchard plot analysis and by fitting the data to saturation curves (Fig. 1A). Final values were obtained by curve-fitting using Origin Software version 6.1 that enabled simultaneous evaluation of the curves obtained for each substrate at three different concentrations of immobilized RSL. The calculated constants were in agreement with values obtained by linearization methods. The results are summarized in Table III.

Among monosaccharides, the highest affinity is for l-fucose with a \( K_D \) value of \( 2 \times 10^{-6} \text{ M} \). The specificity for precise carbohydrate stereochemistry is high, since monosaccharides with different hydroxyl group orientations such as d-mannose, d-fructose, d-galactose, and l-rhamnose were also tested but did not show significant binding. Only l-galactose and d-arabinose, differing only by the group at C6, displayed some binding albeit with affinity 4–5 times lower than that of fucose. Among monosaccharide derivatives, the addition of a hydrophobic group on the anemic position increased the binding for the \( \alpha \)-anomer with a 3 times higher affinity for \( \alpha \)-methyl-fucoside.

Fucosylated oligosaccharides of biological interest were also tested. A comparison of their \( K_D \) values derived from SPR experiments revealed that RSL shows a high preference for terminal fucose bound by \( \alpha \)-1,2 or \( \alpha \)-1,6 linkages and a lower preference for \( \alpha \)-1,4 and \( \alpha \)-1,3 linkages. All \( \alpha \)-Fuc(1→2)-Gal-containing oligosaccharides have a \( K_D \) in the \( 10^{-7} \text{ M} \) range, with slightly higher affinity for the human blood group A and B determinants, which are branched trisaccharides. On the other hand, fucosylated saccharides with different hydroxyl group orientations such as D-mannose, D-fructose, D-galactose, and L-rhamnose were also tested but did not show significant binding.

**TABLE IV**

| Ligand          | \( K_D \) \( 10^6 \text{ M}^{-1} \text{ M} \) | \( K_D \) \( \mu \text{M} \) | \( n \) | \( \Delta G \) \( \text{kJ mol}^{-1} \) | \( \Delta H \) \( \text{kJ mol}^{-1} \) | \( T \Delta S \) \( \text{kJ mol}^{-1} \) |
|-----------------|--------------------------------|----------------|-----|----------------|----------------|----------------|
| \( \alpha \)-Me-fucoside | 1.37 ± 0.06 | 0.73 ± 0.03 | 1.98 ± 0.13 | 35.0 ± 0.11 | 42.1 ± 1.3 | -7.1 ± 1.4 |
| 2-Fucosyllactose | 3.99 ± 0.07 | 0.25 ± 0.005 | 2.00 ± 0.05 | 37.7 ± 0.05 | 39.3 ± 0.1 | -1.6 ± 0.07 |

S.D. values were calculated from three independent measurements for both ligands.

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**FIG. 2.** Titration calorimetry results of \( \alpha \)-methyl-fucoside (0.325 mM) binding to RSL (18.3 \( \mu \text{M} \)) in 100 mM Tris buffer, pH 7.5 at 25 °C. Top, data from 30 automatic injections of 10 \( \mu \text{L} \) of MeFuc each into the RSL-containing cell. Lower, plot of the total heat released as a function of ligand concentration for the titration shown above (squares). The solid line represents the best least square fit for the obtained data.
hand, the human histo-blood Lewis oligosaccharides that con- 
tain αFuc(1→3)GlcNAc or αFuc(1→4)GlcNAc motifs have 
lower affinity than fucose itself. 

Xyloglucans are plant polysaccharides with αFuc(1→2)Gal-

exposed extremities carried by xylose branching on a cellulose 
backbone (27). In order to assay the binding ability of RSL to 
xyloglucan, a high molecular weight polysaccharide (>10 kDa) 
and two oligosaccharides were tested (Fig. 1C). The first, a

Fig. 3. RSL trimer complexed with fucose. A, ribbon diagram of the complex with fucose (shown as sticks). B, same representation for AAL monomer complexed with fucose. C, fucose in binding site 1 with hydrogen bonds represented by dashed lines. D, same representation for binding site 2. E, final 2Fo − Fc electron density map (contoured at 1.0 σ) around the fucose molecule. All molecular drawings were prepared with MOLSCRIPT (46) and RASTER-3D (47).
nonasaccharide containing one terminal fucose residue, and the second, an octasaccharide lacking this particular fucose, were designated as XXFG and XXLG, respectively, according to the unified nomenclature of xyloglucans (28). When the polysaccharide was injected into the RSL-loaded chips, fast association was observed in a dose-dependent fashion (Fig. 1 B). In contrast to the fucosylated oligosaccharides, dissociation did not occur, and almost all polysaccharide remained bound to the protein surface upon washing with buffer. Such a behavior could be attributed to the large number of fucose residues on each polysaccharide molecule responsible for an “avidity” or “rebinding” effect that can be observed with multivalent ligands. The XXFG nonasaccharide has fast association and fast dissociation behavior (Fig. 1 C), and $K_D$ values, calculated from the sensorgrams, are of the same order as that of fucose. The binding of RSL to xyloglucans is directed only toward the fucose residue, since XXLG, an octasaccharide missing the terminal fucose, did not bind at all to the protein surface (Fig. 1 C).

**Affinity Studies by ITC Microcalorimetry**—In order to further characterize the interactions between RSL and fucosylated compounds, the $K_a$ values and the thermodynamic binding parameters were analyzed using titration microcalorimetry, a method that is well suited for the characterization of protein-carbohydrate interactions (29). Both α-methyl-fucoside, a substituted monosaccharide, and 2-fucosyllactose (FucLac), a trisaccharide, were used for the study. A typical titration curve for RSL binding to MeFuc is shown in Fig. 2. The titration curve has its inflection point for a ligand/protein molar ratio of 2, indicating clearly that each RSL monomer binds two fucose residues. The data were therefore analyzed using equations for either two identical sites or two different ones. Both mathematical treatments gave an excellent fit to the experimental curve. Fig. 2 B and Table IV display the data obtained using the two identical site hypothesis. For both the MeFuc and 2FucLac ligands, when two independent sites were considered, the sets of data for each site were almost identical, and therefore this model was not further considered.

Analysis of the data confirms the very high affinity of RSL for MeFuc ($K_D = 7.3 \times 10^{-7}$ M) and FucLac ($K_D = 2.5 \times 10^{-7}$ M). When looking at the details of the thermodynamic contribution, the binding of MeFuc presents the highest enthalpy contribution ($\Delta H = 42$ kJ/mol) that is partially counterbalanced by an unfavorable entropy term ($\Delta S = -7.1$ kJ/mol). The entropy loss corresponds to 20% of the free energy. The higher affinity binding of FucLac is characterized by a not so favorable enthalpy term ($\Delta H = 39$ kJ/mol), but the entropy barrier is much smaller than for MeFuc, which results in a lower value of free energy. The binding of both compounds can
be described as enthalpy-driven but with different contributions from the entropy term.

Crystal Structure of RSL with the Use of Selenofucoside Ligand—The use of a selenium-labeled sugar for phasing lectin crystal structures was pioneered by Loris and co-workers (30) using seleno-GlcNAc for phasing the E. coli adhesin G17. This method is particularly elegant, since the selenium-derivatized sugars generally bind very well, and therefore a short soak in

**FIG. 5.** A, ribbon diagram of RSL trimer complexed with fucosyllactose (shown as sticks). B, fucosyllactose in binding site 1 with hydrogen bonds represented by dashed lines. C, ribbon diagram of mutated RSL trimer complexed with XXFG shown as sticks. D, XXFG in binding site 1 with hydrogen bonds represented by dashed lines. E, Connolly surface of RSL color-coded according to the hydrophobicity potential (from brown for hydrophobic to blue for hydrophilic) displayed with the MOLCAD program (48). F, superimposition of fucosyllactose and XXFG trisaccharide in binding site 1 represented with its Connolly surface.
solution containing the selenated molecule gives high phasing power with a strong isomorphicity to the native structure.

Solving the RSL structure demonstrated that the 90 amino acids are arranged in two consecutive and very similar four-stranded antiparallel \( \beta \)-sheets, lying side by side in parallel and connected by one long loop. They assemble in a unique trimer that reproduces the fold previously described as a six-bladed \( \beta \)-propeller (31, 32) (Fig. 3A). The contacts between blades from different monomers are almost similar to those between the two blades of the same monomer, which mainly involve hydrophobic amino acids. The global shape of the trimer is a deep torus with an approximate diameter of 45 Å and a height of 35 Å. Superposition of the main chain atoms of the three monomers gives r.m.s. deviation values of between 0.2 and 0.3 Å. The cavity has a strong hydrophobic character, being formed mostly by the conserved alanine residues of the first strands of each propeller blade. The core is filled with well-ordered water molecules. Our assays using dynamic light scattering (data not shown) indicate that the protein is a trimer in solution, independent of the presence of fucose.

The six-bladed \( \beta \)-propeller formed by the RSL trimer has strong similarity with that formed by one monomer of AAL, the \( A. aurantia \) fucose-binding lectin (15) (Fig. 3B). Analysis of the sequences indicates the presence of two repeats in RSL and six repeats in AAL. The sequence identity score varies between 20 and 30\% (14) (Fig. 4), but the three-dimensional similarity is very clear; superimposition of the two propellers gives an r.m.s. deviation value of 2.8 Å.

**The Fucose Binding Site and Crystal Structure of RSL/MeFuc at a Very High Resolution**—The fucose binding mode can be described in detail by analyzing the very high atomic resolution crystal structure (0.94 Å) of the complex with methyl-fucoside. Two fucose binding sites are observed for each RSL monomer. One is located between the two \( \beta \)-sheets of a monomer (site 1), whereas the second is located at the interface between the neighboring monomers in the \( \beta \)-propeller formed by trimerization (site 2). Due to the high sequence and structure similarity between the two \( \beta \)-sheets, there are actually very few differences between the two sites. They are made up in the crevasses between two adjacent blades, and it is mostly amino acids of the four strands (rather than loops) in each blade that participate in the binding (Fig. 3, C and D).

The conserved features of the RSL binding sites are very close to the previously described five fucose binding sites of AAL (15). The hydrogen bonding network consists of six hydrogen bonds between fucose and protein (Table V). The fucose hydroxyl O-2 is bridged to the main chain by the amide group of Ala\(^{40} \) in site 1 (Ala\(^{85} \) in site 2). Hydroxyl O-3 receives hydrogen from NE1 of Trp\(^{81} \) (Trp\(^{36} \) of neighboring monomer in site 2) and donates to the carboxyl group of Glu\(^{28} \) (Glu\(^{73} \) in site 2). The other acidic oxygen of this carboxyl receives a hydrogen bond from O-4 of fucose that is also hydrogen-bonded to NE of Arg\(^{17} \) (Arg\(^{62} \) in site 2). Finally, the ring oxygen O-5 receives a hydrogen bond from the terminal NH\(_2\) of the same arginine. In the high resolution structure of RSL complexed with MeFuc, water molecules are observed bound to the most accessible oxygen of fucose, O-1 and O-2, but they are not present in all sites and do not bridge to the protein. Hydrophobic contacts play an important role in the binding of fucose; Trp\(^{76} \) (Trp\(^{31} \) of the neighboring monomer in site 2) stacks to the fucose hydrophobic face (C-3, C-5, and C-6) with distances smaller than 4 Å. On the other face of fucose, the sulfur atom of Cys\(^{30} \) (Cys\(^{75} \) in site 2) establishes a hydrophobic contact with the methine carbon C-2. The methyl group at C-6 is inserted in a hydrophobic pocket made by isoleucine residues 59 and 61 (monomer Pro\(^{14} \) and Ile\(^{16} \) in site 2) and tryptophan residues 10 and 81 (Trp\(^{53} \) of the same monomer and Trp\(^{96} \) of the neighboring one in site 2).

The same fucose-binding mode is observed in all of the complexes described here, with a very limited variation in the fucose orientation. The only difference between site 1 and site 2 is the Ile\(^{30}/\text{Pro}^{14} \) substitution in the hydrophobic pocket. In all binding sites, the hydrogen bond network is fully conserved, except in the mutated protein (i.e., site 2 of the RSLm-XXFG complex, where the S84N mutation induces a slight conformational change in the corresponding loop). Fucose binding to the mutated site does not involve a hydrogen bond between O-2 and Ala\(^{46} \), and a water molecule is bridged instead.

**Crystal Structure of the Complex between RSL and Fucosylated Oligosaccharides**—The RSL-FucLac complex crystallized in the H32 space group with a 3-fold axis of symmetry generating the six-bladed \( \beta \)-propeller (Fig. 5A). The trisaccharides in the two sites (intramonomer (site 1) and intermonomer (site 2)) adopt very similar conformations and establish the same contacts with the protein. The interactions involving the fucose residue have been described above. The galactose does not interact with the protein surface but makes an interresidue GalO2–Fuc.O3 hydrogen bond with the fucose residue (Fig. 5B). The glucose residue is folded back on the protein surface with its hydrophobic face, made by the CH of C-1, C-3, and C-5, establishing van der Waals interactions with the plane surface created by a side chain of Asp\(^{77} \) that creates a salt bridge with Arg\(^{17} \) (Asp\(^{77} \) and Arg\(^{62} \) in site 2). In site 2, there is an additional stabilization by O6 interacting with the side chain of Trp\(^{52} \) through a bridging water molecule.

Co-crystallization or soaking trials of XXFG with RSL were not successful. With the hypothesis that the nonasaccharide may be too bulky to be accommodated in the six sites of the trimer, we used the double mutant (N77G/6B4S) in which binding site 2 is altered and co-crystallized this with fucose. Soaking with XXFG yielded a structure with one fucose in mutated site 2 (with similar contacts to the protein as described above) and one XXF nonasaccharide in site 1 (Fig. 5C). The electron density map clearly showed the nonreducing trisaccharide \( \alpha \)Fuc1–2 \( \beta \)Gal–1–2Xyl. Since the xylene is linked to a glucose residue by a highly flexible 1–6 linkage (three bonds in length) and since there is a large void in the crystal packing near the binding site in this space group, it is likely that the remaining six residues were flexible, and therefore no electron density could be observed for them. The xylose itself is distorted to a half boat conformation and settles on the flat area described above. The O2–O3 hydrogen bond between galactose and fucose is also observed (Fig. 5D).

When comparing the crystal structures of the \( 2 \)FucLac and XXFG RSL complexes, there is almost no variation in the conformation of the \( \alpha \)Fuc1–2Gal linkage (Table VI). The crystal structure of the disaccharide itself has been solved (33) as well as those of two \( \alpha \)Fuc1–2Gal-containing trisaccharides (i.e., blood group B (34) and the xyloglucan fragment (35)). In these three crystal structures, the \( \alpha \)Fuc1–2Gal linkage adopts rather different conformations, illustrating the potential flexibility of this linkage that was previously characterized by a molecular mechanics calculation (36). The conformation observed in the \( \alpha \)Fuc1–2\( \beta \)Gal1–2Xyl is quite similar to that observed in complex with RSL (\( \mu = -134^\circ, \phi = -138^\circ \)), and the same interglycosidic bond hydrogen bond is observed, indicating that the lectin binds to a low energy conformation of the oligosaccharide.

**DISCUSSION**

In the SCOP structural data base (37), \( \beta \)-propeller folds are classified according to the number of blades, which varies from four to eight. Almost 30 \( \beta \)-propeller families have been described, displaying very diverse functions but no sequence sim-
### Table VI

| Linkage       | XxLac | Site1 | Site2 | Site3 |
|---------------|-------|-------|-------|-------|
| αFuc1-2Gal    |       | A     | B     | C     |
| \(\Psi\)      | 124.5 | 124.1 | 124.6 | 125.4 | 123.1 |
| \(\Psi\)      | 139.7 | 142.4 | 132.9 | 139.2 | 131.8 |
| βGal1-4Glc    |       |       |       |       |
| \(\Psi\)      | 70.7  | 48    |       |       |
| \(\Psi\)      | 96.2  | 118.5 |       |       |
| βGal1-2Xyl    |       |       |       |       |
| \(\Phi\)      | 82.4  | 90    | 91.9  |       |
| \(\Psi\)      | 85.9  | 96.3  | 100.8 |       |

The remarkable structural repeats that generate the pseudoaxial symmetry of the propellers are not always visible in the peptide sequences. In many cases, the propeller structure is rigidified by a Velcro closure that brings together the C- and N-terminal moiety as part of the same blade. To our knowledge, RSL is the only example where the propeller is generated by oligomerization and not by peptide repeats in the same continuous protein chain. Prior to the RSL structure, only two lectins have been demonstrated to adopt a \(\beta\)-propeller fold and to make use of symmetrically arranged blades for multivalency. Tachylectin-2 consists of five highly similar blades with five GlcNAc binding sites (38). At the present time, the biological role of microbial lectins is not fully elucidated. The strong preference of RSL for fucose is striking, since, in plants, this residue is present in well defined glycoconjugates and polysaccharides. Plant \(\alpha\)-glycoproteins often bear fucose residues on position 3 of the core GlcNAc or as part of the terminal Lewis a determinant (\(\beta\)Gal1-3[aFuc1-4]GlcNAc) (42). The present specificity study established that none of these fucosylated epitopes is a good ligand for RSL. In contrast, xylaglucan polysaccharide, which is part of the hemi-cellulose fraction in primary cell walls, presents terminal fucose residues on position 2 of a \(\beta\)-galactose. Our SPR study indicated that the affinity of RSL for such linkages is strong and, furthermore, that fucosylated xylaglucan is tightly bound by RSL. Therefore, it would be tempting to consider the possibility that the lectin can bind to hemicellulose in the primary cell walls during infection. Interestingly, all of the solanaceous xyl glucans that have been characterized from tobacco (43), tomato (44), and potato (45) were reported to lack the terminal fucose residues and are built from XxGG type subunits with eventual replacement of the \(d\)-Galp units by \(L\)-Araf. Further studies are required to explore the correlation between the absence or presence of fucose on xylaglucan and the infectivity by \(R\). solanacearum.

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