A Novel Fucose-binding Lectin from *Photorhabdus luminescens* (PLL) with an Unusual Heptabladed β-Propeller Tetrameric Structure

Received for publication, October 4, 2015, and in revised form, September 18, 2016. Published, JBC Papers in Press, October 7, 2016, DOI 10.1074/jbc.M115.693473

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Edited by Sarkis Mazmanian

*Photorhabdus luminescens* is known for its symbiosis with the entomopathogenic nematode *Heterorhabditis bacteriophora* and its pathogenicity toward insect larvae. A hypothetical protein from *P. luminescens* was identified, purified from the native source, and characterized as an α-fucose-binding lectin, named *P. luminescens* lectin (PLL). Glycan array and biochemical characterization data revealed PLL to be specific toward α-fucose and the disaccharide glycan 3,6-O-Me₂-Glcβ1–4(2,3-O-Me₂)Rhaα-O-(L-FucH₄)-OCH₂CH₂NH₂. PLL was discovered to be a homotetramer with an intersubunit disulfide bridge. The crystal structures of native and recombinant PLL revealed a seven-bladed β-propeller fold creating seven putative fucose-binding sites per monomer. The crystal structure of the recombinant PLL:α-fucose complex confirmed that at least three sites were fucose-binding. Moreover, the crystal structures indicated that some of the other sites are masked either by the tetrameric nature of the lectin or by incorporation of the C terminus of the lectin into one of these sites. PLL exhibited an ability to bind to insect hemocytes and the cuticular surface of a nematode, *H. bacteriophora*.

*Photorhabdus luminescens*, Gram-negative Gammaproteobacteria of the family Enterobacteriaceae, have been a target of research due to their dual behavior: a symbiotic relationship with entomopathogenic nematodes of the family Heterorhabditidae and pathogenicity toward insects (1, 2). A monoculture of *P. luminescens* is carried in the gut of a specialized larval stage of nematodes known as infective juveniles (IJ), which seek out susceptible insect hosts and serve as a vector for bacteria. IJs invade the insect host and release *P. luminescens* into its hemocoel. The bacteria expand in the infected insect and produce a number of proteases, toxins, and other virulence factors (3, 4), which leads to septicemia and host death within 24–48 h. Furthermore, *P. luminescens* serve as the food source for developing nematodes. When the nutrients from the host are depleted, bacteria reside in the intestine of newly developed IJs completing their complex life cycle (5). Although the molecular basis of nematode colonization by bacteria is still not fully understood, *P. luminescens* together with the nematode *Heterorhabditis bacteriophora* create a nematobacterial complex that is pathogenic for a wide range of insect species, especially the larval stages of Lepidoptera, Diptera, and Coleoptera (6, 7).

Various lectins, or carbohydrate-binding proteins, and protein/carbohydrate interactions have been demonstrated to be involved in biological and pathological processes (8). In recent years, structural studies focused on lectins or carbohydrate-interacting proteins from pathogenic bacteria and opportunistic fungi have shown how these proteins interact with the surface glycans present on the host cells and might be involved in initiating the invasion or infection (9, 10). A fungal lectin-mediated binding and its apparent receptors on the nematode surface have been shown to take part in host/microbe interactions (11, 12).

Recently, the set of fungal lectins were demonstrated to inhibit *Hemnomus contortus* development by binding to the structurally conserved glycans found in larval gastrointestinal tissue (13). Moreover, a nematocidal pore-forming crystal protein, Cry5B, from *Bacillus thuringiensis* (14, 15) was reported to target a set of invertebrate-specific glycans present on the surface of intestinal epithelial cells of the nematodes (16–18). Investigating the unique structural aspects of carbohydrate-binding proteins might uncover the possible mechanisms involved in the interrelationships between various microbes and the hosts. Here, we focused on the lectin produced by *P. luminescens*, which is both a nematode symbiont and bacterial pathogen; thereby, this bacterium not only requires a means to be recognized by the nematode host but may also interact with the innate immune system of the insect.

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4 The abbreviations used are: IJs, infective juveniles; PLL, *P. luminescens* lectin; rPLL, recombinant PLL; nPLL, native PLL; SPR, surface plasmon resonance; ITC, isothermal titration calorimetry; BME, β-mercaptoethanol; AAL, A. auran-tia lectin; PVL, *P. velutina* lectin; AUC, analytical ultracentrifugation.
We identified and purified a novel L-fucose-binding lectin, designated PLL, from a native source, the *P. luminescens* bacterial culture, which was subjected to a basic structure-functional characterization. We further cloned the gene, characterized the recombinant form of the lectin (rPLL), and compared in detail its crystal structural features with the native protein (ntPLL). The possible role of PLL in the infection process was investigated on the larvae of the greater wax moth, *Galleria mellonella* (order Lepidoptera, family Pyralidae), which is highly susceptible to *Photorhabdus* infection (19). Furthermore, we investigated the interaction of PLL with a natural symbiont of *P. luminescens*, the nematode *H. bacteriophora*.

**Results**

**Identification and Purification of Native PLL from *P. luminescens***—The dual hosts and the complex life cycle of *P. luminescens* make the bacterium an interesting candidate for studying the host/pathogen interactions. To investigate carbohydrate-binding proteins from *P. luminescens*, the bacterial cell lysate was allowed to bind with Coomassie Brilliant Blue R250 showing purification of ntPLL from *P. luminescens* using L-fucose-Sepharose column. The crude extract was loaded into the column, and the protein was eluted in the presence of L-fucose after extensive washing with Tris-HCl (pH 8.2). The purified protein was excised from the SDS-polyacrylamide gel, trypsin-digested, and analyzed by MS/MS analysis. The five tryptic peptides were identified (Fig. 1B, blue), and the peptide sequences were analyzed further by BLAST at NCBI. Five peptides exhibited 99% identity with the hypothetical protein Plu0732 from *P. luminescens* subsp. *laumondii* TT01 (NCBI accession number WP_011145107.1). The peptide identified by N-terminal sequencing is in red. The possible role of PLL in the infection process was investigated on the larvae of the greater wax moth, *Galleria mellonella* (order Lepidoptera, family Pyralidae), which is highly susceptible to *Photorhabdus* infection (19). Furthermore, we investigated the interaction of PLL with a natural symbiont of *P. luminescens*, the nematode *H. bacteriophora*.
Detailed analysis of the plu0732 gene sequence showed that the gene contained two potential starting methionine (ATG) residues. Subsequent N-terminal amino acid sequencing (PNPDNTE) showed that the final protein starts with proline 9 (Fig. 1B), leading to a hypothetical protein of 368 amino acids, which we named PLL.

Cloning of pll Gene, Sequence Variations, Expression in Escherichia coli, Purification, and Characterization—The coding sequence of PLL was amplified from P. luminescens genomic DNA using PCR with the specific primer set, cloned in the pCR-TOPO vector, and analyzed by sequencing; the nucleotide sequence was 1.1 kbp (Fig. 1C).

The pll gene was further subcloned to introduce a hexahistidine tag on the N terminus of the recombinant protein. Expression of the rPLL protein in E. coli BL21(DE3) was optimized using various growth conditions. rPLL purified using affinity chromatography exhibited a monomeric molecular mass of ~41 kDa on SDS-PAGE (Fig. 1D), which was expected from the deduced amino acid sequence of PLL.

Carbohydrate Specificity of ntPLL—Surface plasmon resonance (SPR) experiments with ntPLL showed that the lectin exhibited an ability to bind to L-fucoside among the 12 tested saccharides (L-fucoside, D-mannoside, D-galactoside, mannan, D-lactoside, D-glucoside, N-acetyl-D-galactosaminide, N-acetyl-D-glucosaminide, Lewis a, Lewis b, Lewis X, and Lewis Y) immobilized onto the chip; slow association and dissociation were observed with the immobilized L-fucoside channel (Fig. 2A). Four monosaccharides (L-fucose, Me-α-L-fucoside, D-mannose, and D-galactose) at different concentrations were tested for competitive inhibition of ntPLL with immobilized saccharides (Fig. 2B). The protein concentration was checked by measuring A_{280} using a theoretical molar extinction coefficient of 145,980 M⁻¹ cm⁻¹. C, hemagglutination of ntPLL with erythrocytes of blood group A. The concentrations of the protein were 19.8–0.019 μM. The last well was a negative control (n.c.: erythrocytes and PBS). The blue squares show the lowest ntPLL concentration at which the agglutination of erythrocytes was visible. D, hemagglutination inhibition assay. 0.02 ml of ntPLL (12 μM) and 0.02 ml of saccharide (geometry dilution from 33 mM) were used, and the minimal inhibitory concentrations of the tested saccharides are shown by blue squares. RU, resonance units.

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l-fucoside. ntPLL binding was inhibited by l-fucose and methyl-α-l-fucoside (IC₅₀ of 5.48 and 1.68 mM, respectively), whereas D-mannose and D-galactose did not show any inhibitory effect (Fig. 2B).

Additionally, ntPLL was tested for hemagglutination activity (Fig. 2C). ntPLL lectin displayed hemagglutination activity toward the human erythrocytes of blood group A. The lowest protein concentration at which the agglutination of erythrocytes was visible was 0.3 μM. The inhibition of hemagglutination activity was evaluated with 10 common monosaccharides, such as D-glucose, D-mannose, D-galactose, D-lactose, D-fructose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, l-fucose, methyl-α-l-fucoside, and D-xylene; however, the inhibition was only observed with l-fucose and methyl-α-l-fucoside (Fig. 2D). The minimal inhibitory concentrations of the tested saccharides were as follows: 0.52 mM for l-fucose and methyl-α-l-fucoside and 33 mM for the weak inhibitor D-galactose. The verification of rPLL binding abilities was carried out using SPR experiments (data not shown).

A microcalorimetry titration was carried out to investigate the interaction of rPLL with 13 sugars (l-fucose, methyl-α-l-fucoside, D-galactose, D-glucose, D-xylene, D-trehalose, D-mannose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, D-mannosyl-α-1-6-D-mannose, N-acetyl-D-mannosamine, Lewis b, and chitin oligosaccharides). The results exhibited a monotonic decrease in the exothermic heat of the binding until saturation was achieved (Fig. 3). Each exothermic heat pulse (top) corresponds to the injection of the sugar into the protein solution. Integrated heat data (bottom) were fitted by a single-site binding model showing low affinity of rPLL toward both monosaccharides. The equilibrium dissociation constants were 1.9 and 5.4 mM with the binding stoichiometry of approximately 2.9 and 3.7 for methyl-α-l-fucoside and l-fucose, respectively (Fig. 3, A and B). Isothermal titration calorimetry (ITC) experiments showed rPLL interaction with chitin oligosaccharides; however, quantification of the interaction was not possible due to the undefined and heterogeneous composition of the commercial sample (Fig. 3C).

A screening of rPLL binding to various glycans was performed using glycan array microchips containing about 600 different glycan and bacterial polysaccharides (supplemental Tables S1 and S2). The lectin was found to be very specific; it only strongly bound to α- and β-fucosides and the unusual disaccharide glycan, 3,6-O-Me-r-Glcβ1-4(2,3-O-Me-β)RhaO-(p-C₆H₄)O-CH₂CH₂NH₂. The other sugars did not exhibit relative fluorescent unit values statistically significantly different from the background. The intensity data for the top 25 glycans are shown in Fig. 4.

Oligomeric State of rPLL and ntPLL in Solution—To further investigate the intact molecular weight of native and recombinant PLL, the purified proteins were subjected to MALDITOF-MS analysis; ntPLL and rPLL were used in solution to examine their molecular masses, which were found to be ~164.5 and 168.3 kDa, respectively, indicating that the protein is in a homotetrameric form in both cases. A determination of the oligomeric state of rPLL by analytical ultracentrifugation revealed that PLL exhibited a homotetramer species with the fractional ratio 1.40 and sedimentation coefficient 7.91 (Fig. 5, A and B).

The presence of the intersubunit disulfide bonds of PLL was confirmed by the protein samples run on SDS-PAGE (14%) treated with β-mercaptoethanol (BME) and heating, with and without reducing agent. rPLL samples treated with BME exhib-
PLL from P. luminescens

itated a molecular mass of ~41 kDa, compared with 160 kDa for the non-treated sample (Fig. 5C).

Crystal Structures of Native and Recombinant PLL—The structure of both native and recombinant forms of PLL were solved. Data collection and refinement statistics are given in Table 1. Both PLL structures form a single domain structure including a seven-bladed β-propeller fold organized around a 7-fold pseudoaxis of symmetry (Fig. 6, A and B). Each repeat (β-propeller) of the PLL (termed here as a W-motif; Fig. 6, A and B) consists of a twisted four-stranded antiparallel β-sheet (A-B-C-D; Fig. 6, A and B) connected by relatively short loops. Additionally, the crystallographic structure of ntPLL revealed the presence of Hg²⁺ and Cl⁻ (heavy atom derivative) and Ca²⁺ ions (crystallization agent) (Fig. 6C). The superposition of the native and the recombinant form of the PLL gives an overall root mean square deviation value of 0.156 Å (Ca, main chain), indicating high similarity. Therefore, the structural features will be derived from the structure of rPLL, unless stated otherwise.

The overall shape of rPLL is a short cylinder, with a diameter of ~45 Å and a height of 30 Å. In the β-propeller fold, the first β-strand (A) defines the inner cavity in the center of the protein (Fig. 6D), and the last β-strand (D) is the most exposed to the solvent at the protein surface (Fig. 6B). The β-strands are mostly hydrophobic except for β-strands A and D, which exhibit an amphiphilic character. The inner cavity has the shape of a tunnel with an approximately constant diameter of 13 Å. The N terminus of rPLL is exposed to the solvent and mainly stabilized by hydrophobic and polar interactions of the loop interconnecting repeats W4 and W5 (behind the repeats, Fig. 6, A and B). The C terminus of the lectin is also exposed to the solvent and is located on the same side of the central cavity as the N terminus. The C terminus is stabilized by hydrophobic and polar interactions mediated by the residues defining the space between repeats W1 and W7. Interestingly, the C terminus contains a short β-strand, which interacts with β-strand B of repeat W1 (V43B-L362Cterm, S44B-T361Cterm, and T45B-I363Cterm). The superposition of the seven β-strand repeats (Ca, main chain) gives an overall root mean square deviation value of <0.63 Å for rPLL, indicating a high similarity of the repeats within the rPLL structure.

Oligomeric State of rPLL in Crystals—The oligomeric state of rPLL in the crystal structure is a tetramer (Figs. 6E and 7A), which is generated by two pseudo-2-fold axes (Fig. 7, A–C). The tetramerization mode creates a “side wall-to-side wall” association in the direction of one of the 2-fold pseudo-axes (Fig. 7B) and a “bottom-to-bottom” association in the direction of the second 2-fold pseudo-axis (Fig. 7C). Therefore, the C and N termini of rPLL are exposed to the solvent region. Two disulfide bridges play a key role in the tetrameric association (clear evidence in X-ray structure; Fig. 6E). One disulfide bridge (Cys-227 in the W5 repeat loop between the A and B β-strands in all monomers) is located in the bottom-to-bottom association (Fig. 7C), and the second disulfide bridge (Cys-260, β-strand D of the W5 repeat in all monomers) is in the side wall-to-side wall association (Fig. 7, D and E). Moreover, the bottom-to-
bottom association is mediated purely by non-polar interactions, which are highlighted by the mutual orientation of the His-298 residues of two monomers, where the distance of the His rings is 4 Å (indicating a stacking interaction).

The topography of the surface of the rPLL differs mainly where the side wall–to-side wall association takes place. Repeats W5 and W6 are directly involved in the association (Fig. 7, D–F). It can be clearly seen that the surface interaction is mediated in a shapewise manner. β-Strand C of the W5 repeat of one monomer interacts with β-strand D of the W5 repeat of the second monomer, and vice versa. Similarly, the interconnecting loop in the W5 repeat of one monomer is in an intermolecular interaction with β-strand D of the W5 repeat and loop of the W6 repeat of the second monomer (Fig. 7D). Additionally, the major stabilization contribution in the side wall-to-side wall association (apart from the disulfide bridge) can be observed to be via polar interactions (Table 2) and stacking interactions (Fig. 7, D–F). The residue that most likely has the greatest effect in oligomeric stabilization via intermolecular polar interactions is Asp-262 in W5 β-strand D, with which four intermolecular interactions were observed (Fig. 7E). The major intramolecular stacking interaction is made by the Trp-255 and Trp-258 residues located in the loop of the W5 repeat, which is mediated by Ile-245 located in β-strand C of repeat W5 (Fig. 7F).

Fucose-binding Sites—In principle, up to seven fucose-binding sites can be located on the upper half of the monomer, where the N and C termini of the protein are found (Fig. 8A). Each of them is localized between two adjacent blades. The residues that define the fucose-binding site come from the same repeat (e.g. W2) and additionally from the loop (interconnecting two repeats) of the previous repeat (W1) (Fig. 8B). As displayed in the sequence alignment of the repeats in Fig. 8B, the amino acids of one blade can participate in site i or site i + 1. The binding pockets are highly conserved or semiconserved, and the architecture of the binding sites has a very similar chemical environment (Fig. 8B). An exception can be seen in repeat W5 in terms of the Trp residues (conserved in other repeats). The binding pocket consists of an Arg instead of a Trp residue at position 243 (Fig. 8B). A detailed analysis of the sites further showed a decrease in the hypothetical number of seven binding sites to four well defined binding pockets. An explanation for this can be found in the tetrameric association of rPLL. First, based on the side wall-to-side wall association, two binding sites between repeats W4 and W5 and repeats W5 and W6 (the W5 repeat is directly involved in the oligomerization) were disabled (Figs. 7D and 8B). Interestingly, because of the surface interactions caused by side wall-to-side wall association, the Trp-291 in repeat W6 is in a shifted orientation (~90°), closing the binding pocket, in contrast to the conserved Trp residues in the other repeats. Moreover, the C terminus of the protein is incorporated between repeats W7 and W1, making a short β-strand, which disables the site between the above mentioned repeats. Additionally, Ile-360 (localized at the C terminus) was observed to form a well defined stacking interaction with the highly conserved Trp residues 50 and 66. The fucose-binding sites are exposed on each side of the tetramer at a distance of 50–55 Å from each other.

The crystal structure of the rPLL·t-Fuc complex revealed three fucose residues bound per monomer. These are located between consecutive blades (W1–W2, W2–W3, and W6–W7) (Fig. 8B). For simplicity, the site located between blades W1 and W2 will be named site II (because site I (W7–W1) is disabled),

### Table 1

|                   | nPLL   | rPLL   | rPLL + t-Fuc |
|-------------------|--------|--------|--------------|
| Protein Data Bank | 5C9L   | 5C9O   | 5C9P         |
| Space group       | I222   | I222   | I222         |
| Wavelength (Å)    | 1.005  | 0.967  | 0.918        |
| a                 | 71.12  | 71.79  | 72.78        |
| b                 | 87.68  | 87.82  | 87.72        |
| c                 | 157.70 | 158.30 | 158.19       |
| Resolution range (Å) | 76.75–1.65 | 79.17–1.50 | 45.71–1.75 |
| Reflections measured | 799,825 (116,183) | 1,076,112 (157,056) | 342,062 (48,905) |
| Unique reflections | 59,360 (8509) | 80,153 (11,577) | 51,368 (7404) |
| Completeness (%)  | 99.7 (99.1) | 100.0 (100.0) | 100.0 (99.9) |
| Reflections used for refinement | 56,352 | 76,120 | 48,756 |
| Reflections used  | 56,352 | 76,120 | 48,756 |
| Reflections used for Rmerge | 3001 | 4023 | 2608 |
| Rmerge (%)        | 15.89 | 15.17 | 18.11 |
| CC½ (%)           | 36.2 (14.4) | 28.2 (6.9) | 14.1 (3.4) |
| Multiplicity      | 13.5 (13.7) | 13.4 (13.6) | 6.7 (6.6) |
| Root mean square deviation | 0.068 | 0.030 | 0.024 |
| Bond angles (degrees) | 2.40 | 2.53 | 2.12 |
| No. of water molecules | 374 | 405 | 384 |
| No. of non-hydrogen atoms (total) | 3244 | 3348 | 3262 |
| Ramachandran plot | 97.8 | 97.6 | 97.5 |
| Residues in allowed regions (%) | 97.2 | 1.9 | 2.5 |

* Rmerge = ΣI -⟨I⟩/ΣI, where ⟨I⟩ is the intensity of observation and ⟨I⟩ is the mean value for that reflection.
* R factor = Σ(|Fo|−|Fc|)/Σ|Fo|, where Fo and Fc are the observed and calculated structure factor amplitudes, respectively.
* The Ser-215(A) in PLL was highlighted by Molprobity as having unusual ϕ/ψ angles for an PLL structure.
PLL from *P. luminescens*
and the following ones will be named consecutively. The interactions observed in sites II, III and VII are listed in Table 3. A detailed structural comparison is presented for binding site II relative to the other binding sites.

The conserved features of binding site II consist of four hydrogen bonds between the fucose and the protein, involving two main chain and two side chain hydrogen bonds of two semiconserved residues, Val-72 and Thr-94 (Fig. 8, C and D).

The residues at the position of Thr-94 were either non-polar or polar in character in all sites. Whereas the non-polar parts of the side chains (Thr-94 in site II, Ile-335 in site VII) point away from the binding pocket, leading to a hydrogen bond between the backbone NH and the O-4 oxygen of L-fucose, the polar functional groups (Thr-94 in site II, Ser-142 in site III) form an additional two hydrogen bonds to stabilize the L-fucose O-3 and O-4 oxygens. At the position of Val-72, the corresponding...
residues in all sites have a side chain orientation that is strictly away from the binding pocket. Therefore, the saccharide stabilization at this position is maintained via backbone hydrogen bonds to the fucose ring oxygen or its O-4 oxygen. The second part of the binding site is characterized by a hydrophobic region. The closer and most conserved hydrophobic interactions involve two Trp residues (Trp-99 and -114). The aromatic rings stack against the non-polar face of fucose, forming interactions with the methine and methyl groups at C-4, C-5, and C-6, respectively. The semiconserved Val-91 residue (other repeats hold the hydrophobic character) establishes an additional hydrophobic interaction with the methyl group at the C-6 of fucose. The hydroxyl groups O-1 and O-2 of the fucose

### TABLE 2

| Monomer A₁ | Monomer A₂ | Distance Å |
|------------|------------|------------|
| Ser-246/O (O) | Ser-256/OG (O) | 3.5 |
| Ser-246/O (O) | Ser-256/N (N) | 2.8 |
| Cys-260/O (O) | Arg-243/NH2 (N) | 3.0 |
| Asp-262/OD1 (O) | Arg-243/NH1 (N) | 3.0 |
| Asp-262/OD2 (O) | Asn-257/N (N) | 2.9 |
| Asp-262/N (N) | Trp-258/O (O) | 3.1 |
| Ser-297/OG (O) | Asn-299/N (N) | 3.4 |
| His-298/O (O) | Asn-299/N (N) | 2.7 |
| Trp-302/O (O) | Asn-257/N (N) | 3.7 |

FIGURE 8. A, surface representation (violet) of rPLL. The fucose-binding site is shown in green with bound L-fucose as cyan sticks. B, sequence alignment of seven repeats (W1–W7) of rPLL lectin with main secondary structure elements indicated (residues color-coded to each β-blade, respectively). The amino acids of seven putative fucose-binding sites are highlighted with a green background. The corresponding binding pockets occupied by L-fucose in the rPLL structure are marked with the star (repeats W1 and W2), sphere (repeats W2 and W3), and triangle (repeats W6 and W7) symbols. C, stick representation of PLL fucose-binding site II (dashed square in A) (residues from repeats W1 and W2 in green) with L-fucose (cyan sticks) showing hydrogen bonds highlighted with dashed lines. D, omit map (2mFo – DFo) at 1.0σ defining the L-fucose in binding site II.
TABLE 3
Polar interactions of rPLL complex with L-fucose (Fig. 8, C and D)
The type of binding site, number, and three-letter code of the residue are listed. The polar interactions at a distance up to 3.5 Å are shown.

| Donor atom | Acceptor atom | Distance Å |
|------------|---------------|------------|
| Site II Val-72/O (O) | Site II FUC/O4 (O) | 2.7 |
| Site II Thr-94/N (N) | Site II FUC/O4 (O) | 2.9 |
| Site II Thr-94/OG (O) | Site II FUC/O4 (O) | 3.5 |
| Site II Thr-94/OG (O) | Site II FUC/O3 (O) | 3.0 |
| Site III Gly-120/O (O) | Site III FUC/O4 (O) | 2.6 |
| Site III Gly-120/N (N) | Site III FUC/O5 (O) | 2.9 |
| Site III Ser-142/OG (O) | Site III FUC/O3 (O) | 2.6 |
| Site III Ser-142/OG (O) | Site III FUC/O4 (O) | 3.3 |
| Site VII Ile-311/O (O) | Site VII FUC/O4 (O) | 3.1 |
| Site VII Leu-333/N (N) | Site VII FUC/O4 (O) | 2.9 |

are exposed to the solvent. Despite the seven β-propeller blades not being identical, fucose is bound in the same configuration (sites II, III, and VII). In all three sites, the sugar adopts the α-configuration (axial O-1, steric hindrance checked for both anomers). The number of hydrogen bonds between fucose and the protein varies from two (site VII) to four (sites II and III). In binding site VII, the saccharide is only stabilized by backbone hydrogen bonds. It is likely that sites II and III have a higher affinity for fucose than site VII.

**Interaction of PLL with Hemocytes of G. mellonella Larvae—**
The interaction of PLL with hemocytes of insect larvae was studied using fluorescence microscopy (Fig. 9). Both nPLL-FITC and rPLL-FITC bound to the surface of G. mellonella hemocytes (Fig. 9A). In addition to binding, PLL was able to agglutinate the cells exhibiting a multivalent interaction with hemocytes. The control protein BSA-FITC did not exhibit any interaction with the hemocyte surface, as expected. The binding of PLL to larval hemocytes was significantly inhibited by L-fucose and chitin oligosaccharide among 10 tested sugars (Fig. 9B).

To investigate the potential role of PLL in pathogenicity toward insect larvae, nPLL and rPLL were mixed with an artificial diet and fed to G. mellonella larvae. No mortality was observed over 7 days of PLL feeding, as well as in the control larvae, fed with the diet mixed with the buffer. PLL was also injected at two different concentrations (0.5 and 1.0 mg ml⁻¹) and volumes (10 and 20 µl) to the seventh instar G. mellonella larvae. In some cases, we observed the death of larvae upon PLL injection, but we were not able to assign it to the effect of the lectin, since it was not observed in all experiments. However, the control larvae injected with buffer or BSA died very rarely, and all larvae injected with wasp venom died within 1 day (data not shown), suggesting a small and inconclusive influence of injected PLL on larval viability.

**Interaction of PLL with Nematodes—**
The role of PLL in its nematode partner was investigated by using rPLL-FITC and fluorescence microscopy. PLL exhibited an ability to bind to the surface of the nematodes (Fig. 10A). In nematodes shedding their surface coat (Fig. 10A, bottom), PLL binding was limited to the shed cuticle (arrow) and was not observed on new surface of the animal (asterisk). Therefore, the binding of rPLL to chitin was tested using a chitin resin (Fig. 10B). The inhibition of PLL-FITC binding to the nematode surface using 10 common sugars did not result in a significant difference in fluorescence intensity (data now shown).

**Discussion**

**Novel Homotetrameric Lectin with Intersubunit Disulphide Bridge—** We have identified a new lectin analogous to the hypothetical protein Plu0732 from the entomopathogenic bacteria P. luminescens, called PLL. Purification of the protein from its native source and characterization with SPR revealed PLL to be a lectin binding L-fucose. PLL was found to exist as a homotrimer of ~164 kDa, with a 41-kDa monomer in both its native and recombinant forms, and the oligomeric state was supported with MALDI-TOF-MS and analytical ultracentrifugation.

The protein was expressed in E. coli BL21 (DE3), which does not support an active process of disulfide bond formation; nevertheless, a specific tetrameric oligomerization of PLL with four disulfide bridges was observed. PLL samples in the presence and absence of reducing agent and with and without heating confirmed the presence of intersubunit disulfide bonds, as seen in the crystal structure.

The screening of more than 600 glycans using glycan array microchips showed significant rPLL binding to few sugars. The highest relative fluorescence was observed with α-L-fucoside. Significant responses compared with the background were also seen with an unusual terminal disaccharide (3,6-O-Me₂-GlcNAcβ(1−4)2,3-O-Me₂-Rhaα(1−2)3-O-Me₂-Rhaα1-phenol) from Mycobacterium leprae glycolipid I (20, 21). O-Methylated glycans are widely distributed among phyla, including bacteria, fungi, amoebae, algae, plants, nematodes, and snails (22), and are conserved targets of animal and fungal innate defense (23). The specificity of PLL toward the above mentioned glycan may indicate its role in interaction with both nematode partner and insect host. Surprisingly, the protein did not exhibit any significant response to short chitooligosaccharides or more complex fucosylated oligosaccharides.

**Overall Structural Fold of PLL and Structural Basis of Fucose-binding Sites—** The monomeric structure of PLL revealed a seven-bladed β-propeller fold. The seven-bladed β-propeller fold of the lectin possibly indicated the presence of seven putative fucose-specific binding sites on each monomer placed between two adjacent blades to potentially facilitate the most important feature of lectins, their multivalency.

However, some of these binding sites are affected or disabled by the tetrameric state of the lectin, resulting in three fucose-binding sites that are accessible to a fucose moiety. The structure of the rPLL-fucose complex revealed the presence of three fucose molecules specifically occupying three fucose-binding sites. The crystallographic observations are in good agreement with microcalorimetric titrations, because the best fitting was done using a model for three binding sites.

The fucose-binding sites have the shape of a deep cavity, which can accommodate L-fucose. The architecture of the fucose-binding site is different and basically novel compared with the closest structural homologs: the 6-bladed β-propeller fucose-specific lectins from Aspergillus fumigatus (10) and Aurelia aurantia (AAL) (24) or the 7-bladed β-propeller GlcNAc-specific lectin from Psathyrella velutina (PVL) (25). The novelty of the PLL-binding sites comes from the different conformational architecture of the highly conserved Trp resi-
dues responsible for purely hydrophobic interaction (with the exception of repeat W5 in the tetrameric association of PLL).

The common feature for the lectins of the AAL family (9, 10, 24, 26) or the PVL lectin (25) is that one of the highly conserved Trp residues present in the binding pocket is fully responsible for saccharide stabilization via a polar interaction (OH group of the saccharide and nitrogen of the Trp heterocycle). The second Trp/Tyr residue present in the binding pocket of the lectins is responsible for saccharide stabilization based on stacking interaction.

The highly conserved Trp residues in PLL create a strong hydrophobic patch, which has the ability to recognize fucosylated saccharides based on the C-6 methyl group of L-fucose. In addition, the binding pockets of the lectins from the AAL family or the PVL lectin contain an effective network of polar interactions that are able to stabilize the saccharide at the C2, C3, and C4 positions. In contrast, the fucose-binding sites of the PLL lectin only contain a limited number of polar interactions stabilizing the fucose, mainly at the C4 position. These observations therefore indicate the presence of a novel fucose-specific binding site in PLL, because its fucose recognition seems to be mainly facilitated by hydrophobic forces rather than hydrophilic forces.

**Biological Role of PLL—P. luminescens** is known for its complex life cycle dependent on the entomopathogenic nematode.
H. bacteriophora (5). Both the nematode and P. luminescens are eminent insect pathogens, which are able to invade, infect, and cause the death of a wide range of soil-inhabiting insect species (7). The high virulence of this nematobacterial complex is mostly attributable to the factors produced by P. luminescens during its growth in the infected host; however, many virulence factors have been described that are produced by nematodes themselves (27). The nematode serves as the vector transmitting P. luminescens into the body cavity of the insect host; therefore, it is thought that the bacterial products participate in the early stages of infection after reaching the hemocoel and subsequent colonization and decomposition of insect tissues. Various factors produced by P. luminescens have been identified, many of them exhibiting immunosuppressive or insecticidal activities (28–30). Carbohydrate-binding proteins such as PLL can participate in immunosuppression by mediating the immobilization of hemocytes, by hemocyte agglutination, or by formation of reaction complexes (31). We confirmed the binding of PLL to the surface of hemocytes and their agglutination, but we did not observe any significant damage in hemocyte microstructure; nor did PLL cause mortality in insect larvae upon feeding or injection. In some cases, we observed the death of larvae upon the injection of PLL in contrast to control larvae injected with the buffer; however, the difference in mortality was insignificant. Notably, all G. mellonella larvae injected with ectoparasitic wasp venom containing multiple potent toxins (32) succumbed to the fatal paralysis (data not shown). Therefore, we suggest that PLL should not be categorized as a lectin with insecticidal activity as reported before (33–35). Although PLL does not exhibit a lethal effect, it can still act in coordination with other virulence factors. Lectins or other oligomeric adhesion molecules have been associated, for instance, with uptake reactions during phagocytosis (36) and receptor internalization reactions (37, 38). Lectins from pathogens also participate in cell recognition and adhesion (39, 40); thus, PLL might act as an adhesion molecule that mediates uptake reactions by rearranging glycoprotein receptors and in the process destabilizing the actin cytoskeleton (31).

PLL-mediated attachment can be utilized not only in interactions with insect hemocytes, but also in interactions with nematode symbionts. We observed PLL binding to nematode cuticles, and supposedly it can also bind to the inner tissues of nematodes (data not shown). Of note is the ability of a specialized larval stage of nematodes, IJs, to retain the cuticle from a previous larval stage; the cuticle is shed during the first steps of infection (41). IJs can use the old cuticle to evade recognition by the host immune system (42). In our experiments, we observed the shedding of some nematodes after treatment with rPLL-FITC; the protein was bound only to the old cuticle, which was shed, and did not penetrate to the new cuticle present underneath. Therefore, the role of PLL might be in mediating the interaction of insect hemocytes with cuticle of nematodes,
PLL from *P. luminescens*

which results in clearance of hemocytes from host circulation and promotion of the infection process. It is noteworthy that the lectin AAL (24) was found to be toxic to nematodes, insects, and amoebozoa (43) and also to the mucoromycete fungus *Mucor racemosus* (44); however, we did not observe PLL to be toxic either to *G. mellonella* larvae or *H. bacteriophora*.

The interaction of *P. luminescens* with *H. bacteriophora* is known for its complexity (5). The process of colonization of newly developed nematodes by their bacterial symbiont is still not fully understood, but it is likely that the lectins and other attachment-mediated factors are necessary for successful transmission (45). During preliminary studies, PLL was observed to be bound to inner tissues exposed after section (data not shown), but further research is needed to confirm and characterize the tissue specificity, which could provide us with a clue to possible multiple functions of PLL.

In summary, we report a new lectin PLL from entomopathogenic bacteria, *P. luminescens*. The lectin was characterized from its native source and subsequently prepared in recombinant form. The crystal structures of ntPLL, rPLL, and the rPLL-fucose complex were compared, which revealed a novel architecture of a seven-bladed β-propeller fucose-specific lectin with intersubunit disulfide bridges. The oligomeric structural complexity of PLL was revealed to contain up to 12 calculated binding sites (for theoretically three accessible sites), which may suggest that multivalency plays a crucial role in the lectin’s binding and explain its very low affinity toward free monosaccharides. PLL displayed binding ability with insect hemocytes and the surface of the nematode.

**Experimental Procedures**

**Materials**—1-L-Fucose was purchased from AppliChem (Darmstadt, Germany); D-glucose, D-galactose, and D-trehalose were from Carbosynth (Compton, UK); isopropyl-β-D-thiogalactoside (IPTG) and D-fructose were from Duchefa Biochemie (BH Haarlem, Netherlands); D-xylose and N-acetylmannosamine were from Dextra Laboratories (Reading, UK); D-lactose was from Lach-Ner (Neratovice, Czech Republic); chitin oligosaccharides were from TCI Europe (Zwijndrecht, Belgium). Chitin resin was from New England Biolabs. N-Acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-mannose, D-mannose-agarose, N-acetyl-D-glucosamine-agarose, and N-acetyl-D-galactosamine-agarose were purchased from Sigma-Aldrich. Imidazole and LB (Lennox) broth were from Molekula (Newcastle Upon Tyne, UK) and ForMedium (Norfolk, UK), respectively. FITC was purchased from Thermo Scientific (Rockford, IL). Protein molecular Marker III was from AppliChem.

**Preparation of 1-Fucose-Sepharose Resin**—1-L-Fucose-Sepharose resin was prepared in our laboratory by coupling epoxy-activated Sepharose 6B (GE Healthcare) and 1-L-fucose according to the instructions and recommendations in the manual with the modifications mentioned below. Seven grams of epoxy-activated Sepharose (GE Healthcare) was resuspended in distilled water. The resin was washed with 400 ml of distilled water and then 700 ml of MilliQ water adjusted to pH 10 (alkaline water). For the coupling of 1-fucose, 20 ml of resin was incubated with 20 ml of 1-fucose (0.25 M) in alkaline water (pH 10.0) at 37°C overnight with stirring. The resin was washed three times with alkaline water, and the coupling was repeated as above. The resin was washed with distilled water three times and incubated with 20 ml of 1.0 M ethanolamine (pH 8.0) (overnight, standing). The resin was washed three times with 500 ml each of 0.5 M NaCl (pH 4.0) followed by 0.5 M NaCl (pH 8.3) and stored in 20% ethanol.

**Preparation of Sensor Chips for SPR Measurements**—The surface of the CM5 sensor chip (GE Healthcare) was coated with streptavidin using a standard amine-coupling method. Twenty-five microliters of each solution used was applied at a flow rate of 5 μl/min. The carboxymethylated surface was activated with N-hydroxysuccinimide/N-(3-dimethylanopropyl)-N’-ethylenediamine hydrochloride solution (GE Healthcare), and streptavidin (100 μg ml⁻¹ in 10 mM sodium acetate, pH 5.0) was injected into the CM5 chip. Unreacted groups were blocked with 1 M ethanolamine-HCl (pH 8.5). Then each saccharide covalently bound to the biotinylated polyacrylamide (biotin-PAAs-monosaccharides, Lectinity, Moscow, Russia) was diluted using the running buffer (10 mM HEPES, 150 mM NaCl, 0.005% Tween 20, pH 7.4) to a concentration of 200 μg ml⁻¹, and 50 μl of the final mixture was injected into the selected channel at a flow rate of 5 μl/min.

**Identification of 1-Fucose-binding Lectins**—Screening for 1-fucose-binding lectins was carried out by incubating *P. luminescens* cell lysate with the 1-fucose-Sepharose resin for 30 min, followed by washing the matrix three times with 0.02 M Tris-HCl (pH 8.2) with 0.1 M NaCl. The matrix-bound proteins were denatured for 5 min at 95°C and analyzed by SDS-PAGE. The bands were cut from the gel, digested by trypsin, and identified by MS/MS analysis (46) performed by the Proteomics Core Facility, Central European Institute for Technology (Masaryk University, Brno, Czech Republic).

**Purification of ntPLL—** *P. luminescens* was obtained from cadavers of *G. mellonella* infected with the nematobacterial complex *H. bacteriophora*.*P. luminescens* subsp. *kayaii* as described before (47).

The bacterial culture was grown either in LB medium (pH 7.4) or in minimal medium (33 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.5 mM NaCl, 48 mM NH₄Cl, 3 mM Fe³⁺ citrate, 2 mM MgCl₂, 0.1 mM CaCl₂, 2.0% d-glucose, pH 7.4). One-liter Erlenmeyer flasks containing 250 ml of medium were inoculated with 2.5 ml of an overnight-grown culture of *P. luminescens* and incubated with shaking at 30°C for 3 days (for LB medium) or 7 days (for minimal medium). Afterward, the cells were harvested by centrifugation at 12,000 × g at 4°C for 10 min. The cell pellet was resuspended in 0.02 M Tris-HCl (pH 8.2) with 0.1 M NaCl and disrupted by sonication. The cytoplasmic extract was separated by centrifugation at 14,000 rpm and 4°C for 1 h. The cytoplasmic extract was further loaded onto 1-fucose-Sepharose resin pre-equilibrated with 0.02 M Tris-HCl (pH 8.2) with 0.1 M NaCl. PLL was allowed to bind to the immobilized 1-fucose, washed with the same buffer, and then eluted with 0.02 M Tris-HCl (pH 8.2) with 0.1 M NaCl and 0.05 M 1-L-fucose. The eluted protein was separated by SDS-PAGE, and the protein band was excised and further analyzed by MALDI-MS and LC-MS/MS. For N-terminal amino acid sequencing using the Edman degradation method, the purified protein was separated by SDS-PAGE and blotted onto a PVDF membrane (48).
Cloning, Expression, and Purification of rPLL—The P. luminescens subsp. laumontii TT01 hypothetical protein plu0732 gene sequence was used to design the primers and amplify the plu coding sequences from genomic DNA obtained from P. luminescens subsp. kayaii. The plu0732 gene fragment was amplified by PCR using PfU Ultra polymerase (Agilent), and the forward (5’-ATGCCAATATTCCAGATAATACCGAAG-3’) and reverse (5’-GATCACAAGGCTGTCATCAAGG-3’) primer set. The resulting plasmid, pCR-TOPO/plu, was transformed into TOPO chemically competent E. coli XL1 cells (Invitrogen). Silent site-directed mutagenesis was carried out on the pCR-TOPO/plu plasmid to alter the restriction site for Ndel present in the gene using the primer set 5’-CCAGATAATACCGAGCAGC-3’ and 5’-CTACCT- CACCCTGCAACATGTGCTTCGGTATTATCTGG-3’, resulting in the pCR-mut/plu plasmid. Finally, the primer set 5’-CCCTCATAATGCCAATATTCCAGATAATACCGCAAGAG-3’ and 5’-ACACTCGAGATCACAGAAGCGTCGATCAAG-3’), containing the Ndel/Xhol restriction sites (underlined) was used for cloning the plu gene in the pET29a vector (Invitrogen), resulting in the pET29aplum/plu plasmid.

E. coli BL21 (DE3) cells containing the pET29aplum plasmid were cultured in LB medium (pH 7.4) with 50 μg ml⁻¹ kanamycin at 37 °C until the A₆₀₀ reached 0.5–0.6. The optimization of the culture growth and protein production was carried out by growing the cells at different conditions, including various concentrations of IPTG for induction (0.1, 0.2, and 0.5 mM), various temperatures (18, 20, 25, 30, and 37 °C), pH of the media (6.5, 7.5, and 8.5), and length of induction (3, 6, 9, 12, and 16 h at 25 °C) (data not shown). The expression of the plu gene was induced by adding IPTG to the optimized final concentration of 0.2 mM, and the cells were grown under optimized cultivation conditions for 9 h at 25 °C. The cells were harvested, washed, and resuspended in 0.02 M potassium phosphate buffer (pH 7.5) with 0.002 M trehalose (buffer A). The cells were disrupted with a sonicator (Sonics, Newtown, CT), and the soluble fraction was eluted with buffer A containing imidazole (0.4 M). The purity of the protein was verified by 12% SDS-PAGE stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich). The fractions containing the purified protein were pooled and dialyzed against buffer A and concentrated with an ultrafiltration unit (Millipore) using a 30-kDa cut-off membrane (Millipore). rPLL tended to precipitate during the dialysis in 0.02 M Tris–HCl (pH 8.2) with 0.1 M NaCl; therefore, an optimization of the buffer for rPLL was carried out. 0.02 M phosphate buffer (pH 7.5) with 0.002 M trehalose was found to be suitable for the stability of rPLL and used in subsequent studies. The concentrated protein was used immediately or lyophilized for long term storage.

SPR—SPR measurements were performed using a Biacore 3000 instrument (GE Healthcare) at 25 °C using a running buffer of 0.01 M Hepes (pH 7.5) with 0.15 M NaCl and 0.005% Tween 20 and 0.02 M phosphate buffer (pH 7.5) with 0.15 M NaCl and 0.002 M trehalose, for ntPLL and rPLL, respectively, and a flow rate of 5 μl/min. The experiments were performed using CM5 and CM4 chips for ntPLL and rPLL, respectively. Sensor chips were immobilized with L-fucose, d-mannose, d-galactoside, mannan, d-lactoside, d-glucose, N-acetyl-d-galactosaminide, N-acetyl-d-glucosaminide, Lewis a, Lewis b, Lewis X, and Lewis Y. Biot-PAAb (biontinated polyacrylamide) probes bearing sugar moieties (Lectinity) were trapped on the sensor chips that were coated with streptavidin using the standard procedure. Each Biot-PAAb-saccharide (50 μl at a concentration of 200 μg ml⁻¹) was injected to the selected channel. The streptavidin-modified channel in CM5 and the carboxymethylated dextran channel in CM4 were used as blanks. Inhibition analyses were carried out after a 1-h preincubation of the lectin with 0–20 mM sugars (L-fucose, methyl-α-L-fucoside, d-mannose, d-galactose) for ntPLL. The chip surface was regenerated using 50 mM EDTA and 50 mM L-fucose. The data were evaluated using BIAevaluation version 4.1 software (GE Healthcare, Little Chalfont, UK).

Hemagglutination Activity—Hemagglutination assays and hemagglutination inhibition assays were performed with ntPLL using human erythrocytes treated with 0.1% papain according to a method described previously (10). Other sugars, such as L-fucose, methyl-α-L-fucoside, d-galactose, d-glucose, d-fructose, d-xyllose, d-lactose, d-mannose, N-acetyl-d-galactosamine, and N-acetyl-d-glucosamine, were used for the inhibition test. Positive hemagglutination led to the formation of a uniform red lattice, whereas non-agglutinated erythrocytes sedimented at the bottom of the well.

ITC—ITC experiments were performed using AutoITC and ITC₂₀₀ calorimeters (MicroCal, Malvern, UK) as described previously (9). In brief, the experiments were carried out at 25 ± 0.1 °C. rPLL was suspended in 0.02 M phosphate buffer (pH 7.5) with 0.05 M NaCl. The same buffer was used to prepare the 60 mM sugar solutions (L-fucose, methyl-α-L-fucoside, d-galactose, d-glucose, d-fructose, d-xyllose, d-lactose, d-mannose, N-acetyl-d-galactosamine, and N-acetyl-d-glucosamine), for which the protein concentration used for ITC was 0.1 mM. Before sample analysis, a control experiment was performed, where the protein sample in the calorimeter cell was replaced with buffer, resulting in an insignificant heat of dilution.

A reverse arrangement of ITC was carried out to measure the interaction between chitin oligosaccharides and rPLL, where 30 mg ml⁻¹ chitin oligosaccharide was titrated with rPLL (0.3 mM). A control experiment, where the protein sample was replaced with buffer, resulted in an insignificant heat of dilution. The heat effect was analyzed by nonlinear regression using a single-site binding model (MicroCal Origin version 7) (49).

Analytical Ultracentrifugation (AUC)—The oligomeric state of PLL was investigated by AUC using a ProteomeLab XL-1 (Beckman Coulter) equipped with an An-60 Ti rotor. Sedimentation velocity experiments were performed at various loading concentrations of rPLL (0.03–0.23 mg ml⁻¹) in 0.02 M phosphate buffer (pH 7.5) with 0.002 M trehalose. Sedimentation
PLL from *P. luminescens*

Velocity experiments were conducted in a standard double-sector centerpiece cell loaded with 420 μl of protein sample and 430 μl of reference solution. Data were collected using absorbance optics at 20 °C at a rotor speed of 42,000 rpm. Scans were performed for 6-min periods at 280 nm and 0.003 cm spatial resolution in continuous scan mode. The partial specific volumes of protein together with the solvent density and viscosity were calculated from the amino acid sequence and buffer composition, respectively, using the software Sednterp. The sedimentation profiles were analyzed with the program Sedfit version 14.1. A continuous size distribution model for non-interacting discrete species was used to determine the distribution of apparent sedimentation coefficients (50).

**Labeling Proteins with FITC**—The conjugation of FITC with nt/rPLL was carried out according to Marchetti *et al.* (51). In brief, lyophilized ntPLL and rPLL were dissolved in 0.05 M borate buffer (pH 8.5) to a final concentration of 2.0 mg ml\(^{-1}\). FITC was dissolved in \(N,N\)-dimethylformamide (10 mg ml\(^{-1}\)) and mixed with the lectin solution in a 20:1 molar ratio (\(n_{\text{FITC}}/n_{\text{lectin}}\)). The mixture was incubated in the dark for 1 h. Excess FITC was removed by dialyzing the labeled ntPLL-FITC and rPLL-FITC against 0.02M Tris-HCl (pH 7.5) with 0.05 M NaCl and 0.02 M phosphate buffer (pH 7.5), respectively, at 4 °C. BSA was used as a control protein for the experiment and labeled with FITC as mentioned above. The FITC-conjugated proteins were used for experiments immediately after the dialysis or stored at 4 °C in the dark for up to 4–5 days. The concentration of the nt/rPLL-FITC and BSA-FITC was determined using a NanoDrop 1000 spectrophotometer and the theoretical molar extinction coefficients of ntPLL, rPLL, and BSA (145,980, 146,105, and 43,824 M\(^{-1}\) cm\(^{-1}\)), respectively.

**Glycan Array**—PPLL-FITC was used for a screening of glycan chips. Briefly, the glycan chips were covered with 1.0 ml of natural and synthetic glycans via a standard manufacturer's drop method. The initial screening found the following conditions: 0.1 M Tris-HCl (pH 8.5) with 10% PEG 4000, 0.2 M CaCl\(_2\) (ntPLL) and 0.1 M Tris-HCl (pH 8.5) with 2.5% ethanol (rPLL). Finally, the optimization of the crystals was established by the hanging drop vapor diffusion method with the selected conditions obtained with screening kits (unchanged conditions). The 4-μl drops containing a 1:3, 2:2, and 3:1 ratio of protein (10 mg ml\(^{-1}\)) to crystallization solution were set against 500 μl of the same equilibration crystallization solution.

The ntPLL crystals were soaked in a 1.0 M solution of HgCl\(_2\) for 10 min before cryoprotection to solve the phase problem and resolve the structure of the protein (no closely homologous structure was found to use the molecular replacement approach). The ntPLL and rPLL crystals obtained from the above conditions were cryoprotected using 40% PEG 400 (ntPLL) and 30% glycerol (rPLL), respectively. To obtain rPLL protein complexed with sugars, such as α-fucose, α-mannose, N-acetyl-d-mannosamine, N-acetyl-d-galactosamine, and chitin oligosaccharides, the crystals of rPLL were soaked with a 50 mM solution of sugars for 10–30 min.

Diffraction data for ntPLL and rPLL were collected on the P13 EMBL beamline of PETRA III (DESY, Hamburg, Germany) with the PILATUS detector (Dectris Ltd., Baden-Dättwil, Switzerland). Image processing was performed using XDS (54) before being scaled and converted to structural factors using SCALA in the CCP4 suite (55).

Diffraction data of rPLL complexed with α-fucose were collected on BL14.1 operated by the Helmholtz-Zentrum Berlin at the BESSY II electron storage ring (Berlin-Adlershof, Germany) (56). Image processing was performed using XDSAPP (57). All further computing was performed using the CCP4 suite. The initial structure of ntPLL was solved using single-wavelength anomalous dispersion data (X-ray wavelength of 1.005 Å) with the help of the program suite hkl2map (determination of heavy atom coordinate sites, phasing, and solvent flattening) (58) together with the automatic model-building module of the ARP/wARP program package (59).

**Structure Solution and Refinement**—A molecular replacement technique was used to solve all three native structures with MOLREP (60) using the monomeric coordinates of the initial ntPLL structure from the single-wavelength anomalous dispersion data collection. Crystallographic refinement was carried out with the program REFMAC (61), and manual model building was achieved using Coot (62). The positions of heavy atoms in ntPLL were checked by overlapping the anomalous map and the \(mFo - DFc\)-weighted maps. The incorporation of α-fucose as a ligand was performed after inspection of the \(mFo - DFc\)-weighted maps. Water molecules were introduced automatically using Coot and inspected manually. Alternative conformations were constructed where necessary (with occupancies estimated from the refined relative B-factors of the conformations). The stereochemical quality of the models was assessed with the program Molprobity (63). Molecular drawings were prepared using PyMOL (Schroedinger, LLC). The final models of ntPLL, rPLL, and rPLL complexed with α-fucose were deposited in the Protein Data Bank database under accession numbers 5C9L, 5C9O, and 5C9P. A summary of the data collection and refinement statistics is given in Table 1.
Interaction of PLL with Insect Hemocytes—Hemolymph was collected from seventh instar G. mellonella larvae by cutting a proleg and pooling the hemolymph into a tube treated with phenylthiourea to prevent melanization and coagulation. Hemolymph was pooled from at least five individuals in each experiment.

The hemocyte agglutination test was performed by mixing ntPLL-FITC, rPLL-FITC, or BSA-FITC (each 100 µg ml⁻¹) with freshly collected hemolymph. The mixture was incubated for 20 min in the dark at 17 °C and then washed three times with PBS (pH 7.4). Hemocytes were examined by fluorescence microscopy (IX81, Olympus, Tokyo, Japan).

To test the binding specificity, rPLL-FITC (0.1 mg ml⁻¹) was incubated for 30 min in a humid chamber with a 1.0 m solution of the saccharide (L-fucose, D-galactose, D-glucose, D-fructose, D-xylene, D-lactose, D-mannose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, and chitin oligosaccharide) prepared in PBS before its application to hemocytes adhered on the glass slide. Hemocytes were washed with PBS three times and examined as mentioned above.

Insect Bioassay—Larvae of G. mellonella were reared on an artificial diet (64) at 30 ± 1 °C in constant darkness. Seventh instar larvae were used in the experiments.

To test its oral toxicity, rPLL, ntPLL, or BSA (control) was added at various concentrations (0.15–2.0 mg ml⁻¹) and in different volumes (15–150 µl) to the insect diet (5 g). The larvae were observed for mortality for 7 days.

Two different concentrations (0.5 and 1.0 mg ml⁻¹) of rPLL, ntPLL, or control protein BSA in two different volumes (10 and 20 µl) were injected into the hemocoel of G. mellonella larvae through the third pair of abdominal prolegs. Larvae were observed for mortality for 7 days. An equal volume (10 and 20 µl) of 0.02 M phosphate buffer (pH 7.5) with 0.01 M trehalose (PB/Treh) or crude venom of ectoparasitic wasp H. hebetor (protein concentration 7 mg ml⁻¹) was injected into the control groups of larvae as negative and positive control, respectively.

Interaction of PLL with Nematodes—Nematodes (H. bacteriophora) were incubated with rPLL-FITC (0.2 mg ml⁻¹) for 30 min at room temperature and washed three times with PBS, followed by examination under fluorescence microscopy (Olympus IX81).

PLL binding specificity to the nematodes was evaluated using various sugars, such as L-fucose, D-galactose, D-glucose, D-fructose, D-xylene, D-lactose, D-mannose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, and chitin oligosaccharides. rPLL-FITC (0.1 mg ml⁻¹) was incubated with and without sugars (1.0 m) at room temperature for 30 min, followed by incubating the nematodes with these preincubated rPLL-FITC samples for 30 min at room temperature. The nematodes were washed with PBS three times and examined under fluorescence microscopy (Olympus IX81).

Author Contributions—A. K., P. S., G. D., P. H., and M. W. have conceived and designed the experiments. A. K., P. S., P. D., and G. D. performed the experiments. A. K., G. D., P. H., and M. W. analyzed the data. A. K., G. D., P. D., and M. W. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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