

Structural basis of calcium and galactose recognition by the lectin PA-IL of *Pseudomonas aeruginosa*

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Abstract The structure of the tetrameric *Pseudomonas aeruginosa* lectin I (PA-IL) in complex with galactose and calcium was determined at 1.6 Å resolution, and the native protein was solved at 2.4 Å resolution. Each monomer adopts a β -sandwich fold with ligand binding site at the apex. All galactose hydroxyl groups, except O1, are involved in a hydrogen bond network with the protein and O3 and O4 also participate in the co-ordination of the calcium ion. The stereochemistry of calcium galactose binding is reminiscent of that observed in some animal C-type lectins. The structure of the complex provides a framework for future design of anti-bacterial compounds.

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Key words: Lectin; Crystal structure; Galactose; *Pseudomonas aeruginosa*

1. Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium that is found in various environments including soil, water and vegetation. It is also an opportunistic pathogen, responsible for numerous nosocomial infections in immunocompromised patients. The bacteria colonise patients with a number of chronic lung diseases, particularly those on assisted ventilation, and especially cystic fibrosis patients. *P. aeruginosa* virulence is based on several properties, most particularly on the ability of this organism to adhere to surfaces, to form biofilms and to secrete hydrolytic enzymes and toxic compounds.

Since host carbohydrates have been known for many years to constitute specific attachment sites for microbial protein receptors [1,2], the lung mucins, and above all those of cystic fibrosis patients, have been analysed thoroughly [3]. On the other hand, some of the carbohydrate binding proteins of *P. aeruginosa* have been studied and their role in recognition and adhesion is far from being fully elucidated. Several types of receptors have been identified. Flagellin and flagellar cap protein FliD recognise mucin oligosaccharides [4], whereas

type IV pilus adhesins have been shown to recognise glycosphingolipids asialo-GM1 and asialo-GM2 [5]. In addition, two soluble lectins PA-IL (gene *lecA*) and PA-IIL (gene *lecB*), specific for D-galactose and L-fucose, respectively, and containing divalent cations have been characterised [6]. These two lectins are produced at high levels by the bacteria in association with the cytotoxic virulence factors [7] and under quorum-sensing control [8]. The galactophilic lectin PA-IL was the first *P. aeruginosa* lectin to be isolated by affinity chromatography [9]. Its 12.75 kDa subunits consist of 121 amino acids and associate as a tetramer [10]. This lectin has a narrow specificity spectrum for D-galactose-containing molecules, including analogues bearing hydrophobic groups [11] and α -D-galactose-containing disaccharides and other glycoconjugates [12,13], but it also binds adenine with high affinity [14]. The lectin has been demonstrated to bind the most common human antigens, I, B and especially Pk [15], and to be cytotoxic to respiratory epithelial cells in primary cultures [16].

We recently solved the structure of PA-IIL, the fucose binding lectin of *P. aeruginosa*, at very high resolution (1.3 Å) [17], revealing a new mode for carbohydrate binding, based on the interaction with two calcium ions. This was proposed to be the basis for the unusually high affinity of PA-IIL for its ligands. The structural basis of PA-IIL's wide specificity spectrum and its interaction modes with different monosaccharides, such as D-mannose and D-fructopyranose, has been recently elucidated [18]. The successful crystallisation of PA-IL has also been reported recently [19]. The crystal structure in the presence of calcium has been solved at 1.5 Å resolution and deposited in the Protein Data Bank (code 1L7L) although not accompanied by a detailed description [20]. The present communication describes the refined crystal structure of the calcium-free form of PA-IL and its complex with calcium and galactose. This structure unveils a new carbohydrate binding mode and may assist in the development of therapeutic drugs against *Pseudomonas* infections.

2. Materials and methods

PA-IL was isolated and purified by affinity chromatography as described previously [6]. Lyophilised protein was dissolved in water (10 mg/ml) either alone or in the presence of galactose (425 μ g/ml) and salts (1 mM CaCl₂ and MgCl₂). Initial crystallisation conditions were screened using the complete Hampton Screens I and II from Hampton Research (Laguna Niguel, CA, USA) from which thin, needle-shaped

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Abbreviations: PA-IL, *Pseudomonas aeruginosa* lectin I; PA-IIL, *Pseudomonas aeruginosa* lectin II; MBPA, mannose binding protein A

crystals appeared after a few days in the presence of ammonium sulphate and isopropanol. These conditions were then optimised and large needle-shaped crystals were obtained from hanging drops of 2 μ l of PA-IL solution mixed with 2 μ l of reservoir solution (1.5 M ammonium sulphate at pH 4.5, 20% isopropanol for the calcium-free protein and 1.5 M ammonium sulphate at pH 4.7, 5% MPD and 2% glycerol for the complex with galactose). Both crystals belong to space group $P2_12_12_1$ with four molecules per asymmetric unit. The cell dimensions of the calcium-free protein crystal are $a=49.7$ Å, $b=51.5$ Å and $c=166.8$ Å and those of the complex are $a=49.1$ Å, $b=53.3$ Å and $c=160.7$ Å. Crystals were cryo-cooled at 100 K after soaking them for as short a time as possible in either glycerol 30% v/v or MPD 30% v/v in precipitant solution for the calcium-free and complexed form respectively. All data were collected at the ESRF synchrotron (Grenoble, France) at stations ID14-1 and ID14-4 on an ADSC Q4R CCD detector (Quantum) and at station BM30A on a MAR165 CCD detector (Mar Research). Diffraction images were processed using MOSFLM [21] and scaled and converted to structure factors using the CPP4 program suite [22].

The structure of the calcium-free protein was solved by the molecular replacement technique with the MOLREP program [23], using the monomeric structure (PDB code 1L7L) [24] of the calcium-containing PA-IL [20] with calcium and water molecules removed as the search probe. The molecular replacement gave four clear solutions corresponding to the expected tetramer in the $P2_12_12_1$ asymmetric cell. The structure of the complex with galactose was then solved with the same program using one monomer of the refined calcium-free structure, again with calcium and water molecules removed. To retain maximum non-bias, the R_{free} data set of the calcium-free structure was used, extending it to 1.6 Å resolution. After an initial cycle of refinement, the electron density maps of this complex showed clear features corresponding to four galactose molecules and four calcium ions. For the purposes of identification, the ions were modelled as both calcium and magnesium in separate refinements and the B -factors compared after refinement. The B -factors of the magnesium ions were significantly less than those of the surrounding ligating atoms (average of 3.3 Å² against 8.6 Å²) whilst those for calcium were much closer (6.6 Å²), suggesting that the ions were most likely to be calcium. For both structures, automatic placement of water molecules was performed using the ARP/warp program [25]. Crystallographic refinement was carried out with the program REFMAC [26], with an overall B -factor refinement for the lower resolution structure and individual B -factors for the higher resolution structure, and manual model building with O [27]. Co-ordinates have been deposited at the Protein Data Bank [24] under codes 1OKP and 1OKO for the calcium-free and complexed structures, respectively.

3. Results and discussion

The final refined model of calcium-free PA-IL consists of 484 residues, 137 water molecules and two sulphate ions with an R_{crys} of 18.3% and R_{free} of 26.3% to 2.4 Å resolution. The PA-IL/galactose complex contains 484 amino acids, 482 water molecules, three sulphate ions, one MPD molecule, four calcium ions and four galactose residues with an R_{crys} of 15.4% and R_{free} of 18.7% to 1.6 Å resolution (Table 1). In both structures, the asymmetric unit contains four polypeptide chains of 121 amino acids arranged around a pseudo C222 axis (Fig. 1A). The resulting tetramer is very similar to that generated by the I222 space group in the crystal structure of native PA-IL [20]. Each monomer adopts a small jelly-roll type β -sandwich fold, consisting of two curved sheets, each one consisting of four antiparallel β -strands. Tetramerisation occurs by interaction between the largest sheets for one interface and by contacts between C-terminal moieties for the other interface.

The structure obtained from crystals grown in the presence of metal ions and galactose shows each monomer to contain one calcium ion and one galactose ligand in the same binding site (Fig. 1A). There are no major differences between the

Table 1
Data collection and refinement statistics

Crystal	Metal-free	Complex with Ca and galactose
Data collection		
Beamline	ID14-4	ID14-1
Wavelength (Å)	0.932	0.934
Resolution (Å)	2.4	1.6
Highest resolution shell (Å)	2.40–2.48	1.60–1.66
Space group	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions		
a (Å)	49.7	49.1
b (Å)	51.5	53.3
c (Å)	166.8	160.7
α (degrees)	90	90
β (degrees)	90	90
γ (degrees)	90	90
Measured reflections	114 114	241 596
Unique reflections	17 451	53 762
Average multiplicity	6.5 (6.2)	4.5 (4.2)
Completeness (%)	99.8 (99.8)	94.9 (79.1)
Average $I/\sigma(I)$	7.7 (1.9)	6.7 (1.4)
R_{merge} (%)	8.9 (36.0)	7.6 (41.0)
Wilson B -factor (Å ²)	34.0	12.5
Refinement		
Resolution range (Å)	20.37–2.40	18.60–1.60
R_{crys} (observation)	0.183 (16569)	0.154 (51032)
R_{free} (observation)	0.263 (882)	0.187 (2730)
Highest resolution shell		
R_{crys} (observation)	0.200 (1211)	0.197 (3066)
R_{free} (observation)	0.270 (75)	0.256 (144)
Cruickshank's DPI based on maximum likelihood (Å)	0.205	0.062
Average B_{iso} (Å)		
All atoms	30.2	14.0
Protein atoms	30.3	12.5
Solvent atoms	29.2	24.5
RMS deviation from ideality		
Bonds (Å)	0.016	0.017
Angles (degrees)	1.52	1.57
Outliers on Ramachandran plot	0	0
Amino acids	4 × 121	4 × 121
Protein atoms	3604	3604
Sugar atoms	0	4 × 12
Calcium atoms	0	4
Other atoms	2 × 5	3 × 5 + 8
Water molecules	137	482
Residues with alternative conformations	None	S113 _A , Q120 _A , S121 _A , T39 _B , N71 _B , T95 _B , S113 _B , V15 _C , V85 _C , S113 _C , S121 _C
PDB deposition code	1UOJ	1OKO

Values in parentheses refer to the highest resolution shell.

$R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum I$, where I = observed intensity.

Geometric analyses were performed by PROCHECK [37] and WHATIF [38].

calcium-free structure, the calcium-bound one [20] and the complex with calcium and galactose and comparison between the monomer main chain atoms from the different structures yields rms difference values lower than 0.55 Å.

The ligand binding site is located at one apex of the β -sandwich and is directed towards the solvent. The calcium binding site is made up mainly of the region 100–108 of the amino acid sequence which forms a loop and a short one-turn α -helix. The side chains of Asp100, Asn107 and Asn108 participate in the co-ordination of calcium together with the main chain carbonyl of Thr104 (Table 2). A fifth contact is established by the carbonyl main chain group of Tyr36 which is located on a neighbouring loop. When this calcium-bound site is compared to the calcium-free one, in three out of four

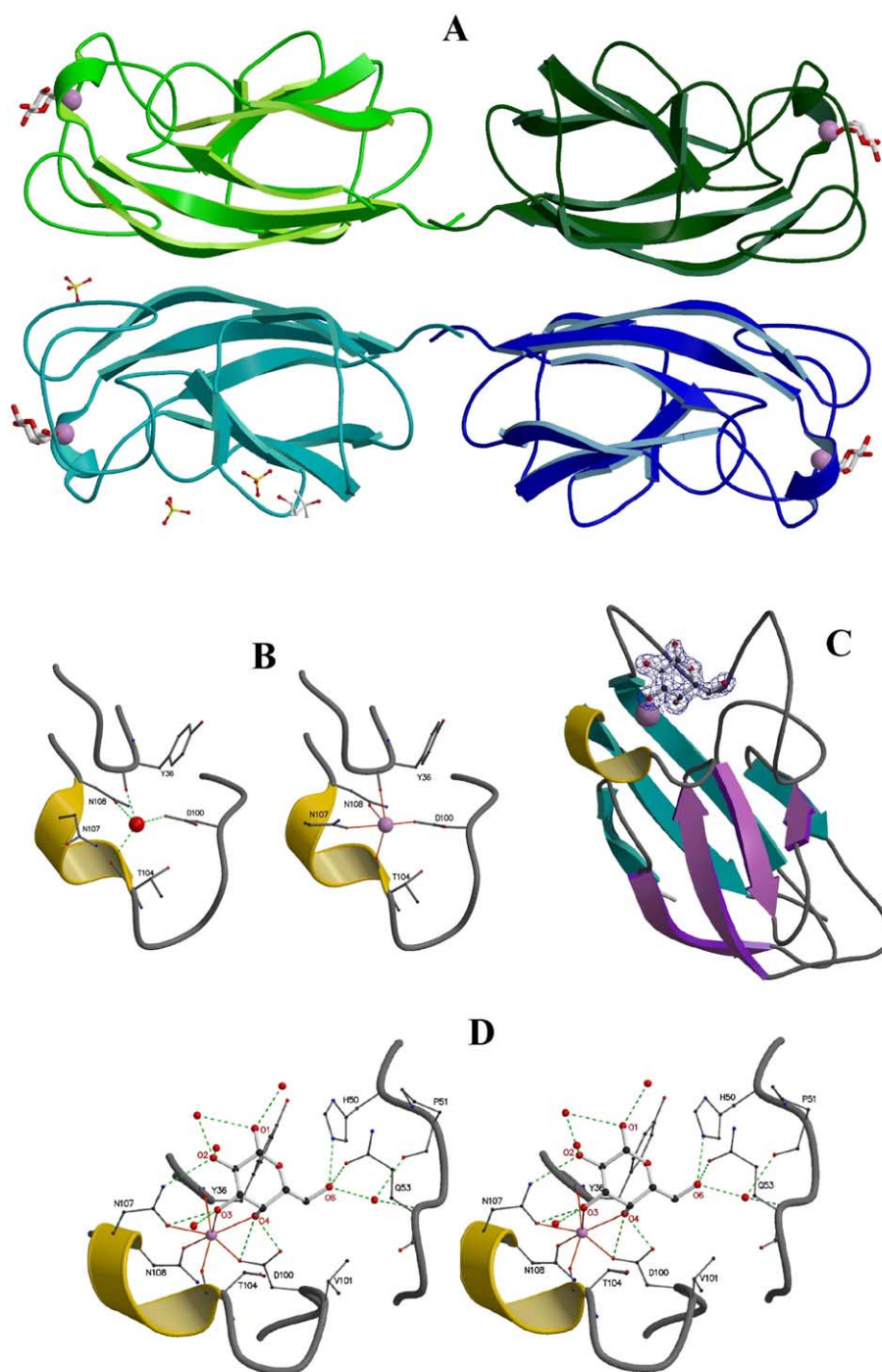


Fig. 1. A: Tetrameric structure of the PA-IL/galactose complex with stick representation of galactose and cpk representation of the calcium ions. Sulphate and MPD molecules are also represented. B: Comparison of the calcium binding site in the calcium-free (left) and in the metal-bound (right) (PDB code 1L7L) structures. Hydrogen bonds are represented as dashed green lines and co-ordination contacts as solid orange lines. Calcium ions and water oxygens are represented by violet and red spheres, respectively. C: Representation of monomer A of the PA-IL/galactose complex showing one sheet in blue, one sheet in pink and the helix in yellow, together with the final sigma weighted $2F_o - F_c$ electron density map (contoured at 1.0σ) around the galactose residue. D: Stereo view of the calcium and galactose binding sites in the crystal structure. Color coding as in B. Molecular drawings in all figures were prepared with MOLSCRIPT [35] and RASTER-3D [36].

monomers of the calcium-free crystal structures a water molecule replaces the calcium ion (Fig. 1B). The oxygen atom of this water is within hydrogen bonding distance of four of the calcium ligands. The only significant difference appears in the Asn107 side chain which is not involved in the hydrogen bond network and adopts a different orientation.

In the structure obtained by co-crystallising PA-IL and ga-

lactose in the presence of calcium and magnesium, electron density is observed at the metal binding site. It has been attributed to calcium, based on the values of B -factors, and also on the fact that calcium was observed at high resolution [20]. Electron density corresponding to monosaccharides is clearly identifiable in contact with each calcium ion (Fig. 1C). The galactose ligand adopts the classical 4C_1 pyranose

Table 2
Characteristics of the calcium and galactose binding sites

Atom 1	Atom 2	Distance (Å) ^a
Co-ordination of calcium ion		
Ca	Tyr36.O	2.37 (9)
Ca	Asp100.OD2	2.46 (6)
Ca	Thr104.O	2.29 (3)
Ca	Asn107.OD1	2.45 (4)
Ca	Asn108.OD1	2.41 (8)
Gal.O3	Ca	2.47 (3)
Gal.O4	Ca	2.50 (2)
Hydrogen bonds between PA-IL and Gal		
Gal.O2	Asn107.ND2	3.07 (6)
Gal.O3	Asn107.OD1	2.98 (4)
Gal.O4	Asp100.OD1	2.60 (3)
Gal.O4	Asp100.OD2	2.97 (1)
Gal.O6	His50.NE2	2.76 (7)
Gal.O6	Gln53.OE1	2.70 (6)
Hydrogen bonds with conserved water molecules		
Gal.O1	Wat1	3.1 (1)
Gal.O2	Wat2	2.7 (1)
Gal.O2	Wat2	2.8 (3)
Gal.O3	Wat3	2.7 (1)
Gal.O6	Wat4	2.79 (3)
Bridging water molecules		
Wat4	Pro51.O	2.73 (7)
Wat4	Gln53.N	2.95 (2)
Hydrophobic contacts		
Gal.C1	Tyr36.CD	4.01 (7)
Gal.C2	Tyr36.CD	4.13 (2)
Gal.C4	Thr104.CB	3.71 (3)
Gal.C6	Val101.CG2	3.78 (3)

^aAverage value of the four monomers with standard deviation of the last digit in parentheses.

ring shape. The β configuration is the only one to be observed in monomer A whereas electron density indicates a significant (c. 40%) proportion of α configuration for galactose in the three other monomers. Interactions between galactose and protein include direct hydrogen bonds and hydrophobic contacts but also interactions bridged by either the calcium ion or one water molecule (Table 2). Two oxygen atoms of galactose, i.e. O3 and O4, are involved directly in the co-ordination sphere of the calcium ion (Fig. 1D). Hydrogen bonds to the sugar residues involve three regions of the protein. From the calcium binding loop (100–108), Asp100 establishes contact

with O4 of galactose and Asn107 forms hydrogen bonds with O2 and O3, while Val101 and Thr104 are involved in hydrophobic contacts. Tyr36, also participating in co-ordination of the calcium ion through its carbonyl backbone oxygen, makes hydrophobic contact with C2 of galactose. Another part of the same long loop (36–64) interacts specifically with the O6 hydroxymethyl group that establishes hydrogen bonds with His50, Gln53 and one water molecule making a bridge to the protein backbone. Several other water molecules are in contact with hydroxyl groups O2, O3 and O4. Altogether, four water molecules are conserved in the four binding sites of the asymmetric unit as listed in Table 2.

The PA-IL sequence does not present any significant similarity to any other protein. The monomer fold is related structurally to a β -sandwich family fold described as galactose binding domain-like in the SCOP database [28], although PA-IL represents a truncated form of this fold lacking one of the N-terminal strands. This family contains, among others, several lectins and carbohydrate binding domains, none sharing similarity with those of PA-IL. This fact is not surprising, since it has already been shown that even carbohydrate binding modules from common ancestors may display different locations of their sugar binding sites [29].

Calcium-mediated recognition of galactose by lectins is well characterised for several members of the C-type lectin family. The carbohydrate binding domain of these animal lectins shows specific calcium-dependent binding of various monosaccharides with dissociation constants in the mM range [30]. The majority of C-type lectins can be divided into D-mannose binding (also recognising D-glucose, L-fucose, etc.) and D-galactose binding ones. The first complex with galactose was obtained with the rat mannose binding protein A (MBPA) that, after appropriate mutations, yielded the galactose binding mutant QPDWG [31]. Recently, TC14, a tunicate galactose-specific C-type lectin from *Polyandrocarpa misakiensis*, has been co-crystallised with D-galactose [32]. Despite the lack of similarity at the sequence or at the fold level, the galactose binding mode of PA-IL shows a striking resemblance to each of these C-type lectins (Fig. 2). In all of them, the galactose O3 and O4 hydroxyl groups participate in the calcium ion co-ordination and O4 establishes a strong contact with one Asp residue that participates in the co-ordination.

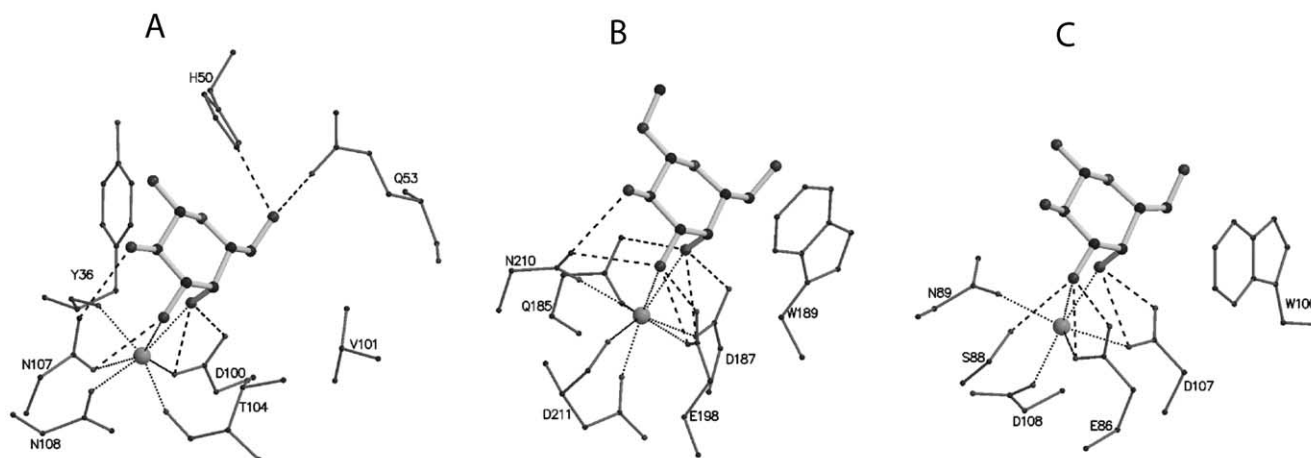


Fig. 2. Comparison of the calcium and galactose binding sites in three different lectin crystal structures. A: PA-IL/ β Gal complex (this work). B: MBPA (QPDWG)/ β Gal-O-Me complex (PDB code 1AFA) [31]. C: TC14/ β Gal complex (PDB code 1TLG) [32]. The galactose ring is shown in the same orientation in the three binding sites.

Since recognition of the axial orientation of O4 is an important point for galactose specificity, this aspartate residue plays a key role in the binding site.

PA-IL displays medium range affinity for D-galactose, with an association constant (K_a) of $3.4 \times 10^4 \text{ M}^{-1}$ as reported from an equilibrium dialysis study [11]. This 10-fold increase in galactose affinity compared to that of C-type lectins could be attributed to the additional strong interaction with O6 of the sugar. PA-IL's limited specificity for galactose does not allow other monosaccharide binding, with the exception of *N*-acetyl-D-galactosamine that can bind, albeit with a lower affinity than galactose. The presence of a hydrophobic group on the sugar anomeric position, in either α or β configuration, enhances the affinity with the tightest binding obtained for phenyl- β -thiogalactoside [11]. This result is in agreement with the observed galactose binding mode, where only hydroxyl O1 and, to a lesser extent, O2 are exposed to the solvent and are therefore free to carry substitution. PA-IL binds to a large number of glycoproteins [13] and glycosphingolipids [12,15]. Amongst them, the glycoconjugates containing terminal non-substituted α Gall-4Gal disaccharide, i.e. the human Pk blood group, present on either red blood cell glycosphingolipids or pigeon egg white glycoproteins [33], and α Gall-3Gal disaccharide, the xeno-antigen present on non-human tissues, are bound most strongly by the lectin. These two disaccharides together with melibiose, α Gall-6Glc, inhibit the binding of PA-IL to glycoconjugates. Further studies are needed to determine if other galactose-containing ligands such as asialo-GM1 and GM2 and Lewis oligosaccharides that were reported to be present in the lungs of cystic fibrosis patients [4,34] could act as high affinity receptors for PA-IL.

The determination of the crystal structures of the two soluble lectins of *P. aeruginosa* in complex with their monosaccharide ligands gives hope for the future design of anti-adhesive carbohydrate-based therapeutics. The two lectins do not seem to be evolutionarily related even though they both adopt a jelly-roll fold. More interestingly, they both use calcium for carbohydrate binding. In PA-IIL the two calcium ions are associated with high affinity (micromolar range) and a wide range of different monosaccharides that are able to bind. In contrast, in PA-IL, calcium together with a unique network of hydrogen bonds generates a binding site endowed with high specificity for galactose. PA-IL's narrow specificity, together with its unique sequence, makes this protein an attractive target for drug design.

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