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Inhibition and Dispersion of *Pseudomonas aeruginosa* Biofilms by Glycopeptide Dendrimers Targeting the Fucose-Specific Lectin LecB

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SUMMARY

The human pathogenic bacterium *Pseudomonas aeruginosa* produces a fucose-specific lectin, LecB, implicated in tissue attachment and the formation of biofilms. To investigate if LecB inhibition disrupts these processes, high-affinity ligands were obtained by screening two 15,536-member combinatorial libraries of multivalent fucosyl-peptide dendrimers. The most potent LecB-ligands identified were dendrimers FD2 (C-Fuc-LysProLeu)$_4$(*Lys*PheLysIle)$_2$ *Lys*HisIleNH$_2$ (IC_{50} = 0.14 μM by ELLA) and PA8 (OFuc-LysAlaAsp)$_4$(*Lys*SerGlyAla)$_2$ *Lys*HisIleNH$_2$ (IC_{50} = 0.11 μM by ELLA). Dendrimer FD2 led to complete inhibition of *P. aeruginosa* biofilm formation (IC_{50} ~ 10 μM) and induced complete dispersion of established biofilms in the wild-type strain and in several clinical *P. aeruginosa* isolates. These experiments suggest that LecB inhibition by high-affinity multivalent ligands could represent a therapeutic approach against *P. aeruginosa* infections by inhibition of biofilm formation and dispersion of established biofilms.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic human pathogen causing a variety of infections and is regarded as a primary cause of death in immunocompromised patients and those with cystic fibrosis or cancer. There is an urgent need to develop novel therapeutic agents effective against *P. aeruginosa* because this bacterium exhibits multiantibiotic resistance. This resistance is mediated in part by the formation of surface attached biofilms, which form a physical barrier to antibiotic penetration and provide an altered microenvironment (Wagner and Iglewski, 2008). Targeting the mechanisms of tissue adhesion and biofilm development should increase antimicrobial tolerance and facilitate clearance by the host immune system (Drenkard, 2003), in particular by targeting key mediators such as the galactose-specific lectin LecA (PA-IL) and the fucose-specific lectin LecB (PA-IIL) (Loris et al., 2003; Mitchell et al., 2002; Tielker et al., 2005; Diggle et al., 2006).

One might expect that multivalent ligands against the galactoside or fucoside-binding lectins, LecA and LecB, respectively, might exert useful biofilm blocking activities. Indeed mutants lacking either of these lectins are impaired in biofilm formation (Mewe et al., 2005; Tielker et al., 2005), whereas isopropyl-β-thiogalactoside (IPTG) promotes LecA-dependent biofilm dispersal (Mewe et al., 2005). LecA and LecB cause inhibition of human airway cilia beating, which could be abolished by preincubation with galactose and fucose (Mewe et al., 2005). Furthermore, an induced otitis externa diffusa (Steuer et al., 1993) and respiratory tract infections (von Bismarck et al., 2001) due to *P. aeruginosa* have been successfully treated by concentrated solutions of these carbohydrates. Nevertheless, these effects were observed using high concentrations of relatively weak lectin ligands, and the link between the antibacterial effects and lectin binding was not formally established. To test if a therapeutic strategy against *P. aeruginosa* would indeed be possible based on lectin inhibitors, it would be necessary to identify high-affinity ligands to investigate whether such ligands can perturb biofilm processes in the live bacterium.

Lectins such as LecA and LecB are ubiquitous carbohydrate-binding proteins that occur in all known life forms. These proteins bind to multivalent carbohydrates on cell surfaces such as glycoproteins and glycolipids, and mediate a broad range of cellular and tissue adhesion processes. The carbohydrate-binding sites of these lectins are generally specific but rather weak, yet efficient ligand recognition is achieved by a multivalency effect upon binding, called the cluster effect (Lee and Lee, 1995; Lundquist and Toone, 2002). It has been shown in a number of systems that this multivalency effect can be exploited to obtain high-affinity synthetic ligands against various types of lectins in the form of glycoclusters (Kiessling et al., 2006), glycopolymers (Mammen et al., 1998), and glycodendrimers (Newkome et al., 2001; Tomalia and Dvornic, 2002; Lee et al., 2005; Roy, 2003; Deguise et al., 2007). Octavalent dendrimers with galabiose

were recently shown to inhibit hemagglutination induced by the bacterium *Streptococcus suis*, revealing the potential of glycodendrimers as anti-adhesins (Joosten et al., 2004). These precedent results suggested that multivalent fucose ligands should be a suitable compound class to inhibit *P. aeruginosa* lectins.

We focused our search for high-affinity multivalent ligands toward LecB because fucose binds more strongly to LecB than galactose to LecA (Imberty et al., 2004), implying that high-affinity ligands might be more easily identified for LecB. Thus, we hypothesized that useful ligands tailored to these lectins might be accessible based on our recently reported combinatorial synthesis of multivalent peptide dendrimers (Clouet et al., 2004), which follows the principle of split-and-mix synthesis (Furka et al., 1991) and was originally developed in the context of enzyme model studies (Darbre and Reymond, 2006). These compounds belong to the general class of amide-bond-containing dendrimers (Crespo et al., 2005), and the related lysine-branched multivalent peptides have been previously used in various biomedical applications such as synthetic vaccines (Sadler and Tam, 2002). Glycodendrimers assembled on a peptide backbone are more easily accessible than other synthetic dendrimers because they are prepared from standard amino acid building blocks by the same solid-phase synthesis methods used for linear peptides. Peptide dendrimers can furthermore be tuned by variation of amino acid side chains for specific affinity toward the target mediated via secondary interactions outside the carbohydrate binding site, as well as for other properties such as solubility. Preliminary studies in the context of drug delivery had shown that glycosylated peptide dendrimers are not intrinsically cytotoxic (Lagnoux et al., 2005).

RESULTS

Identification of High-Affinity Ligands toward LecB

Two 15,625-member combinatorial libraries of peptide dendrimers were prepared and functionalized with α-L-fucoside end groups as primary recognition elements for LecB on non-cleavable Tentagel beads as the solid support. Various cationic, anionic, polar, hydrophobic, or aromatic residues were placed at the variable positions X^1–X^6 to provide a diverse set of dendrimers, in an arrangement allowing bead decoding by amino acid analysis as described previously (Clouet et al., 2004). The terminal amino groups were acylated either with the protected α-C-fucoside **1** or with the protected α-carboxyphenyl fucoside **2**, followed by acidic removal of the side-chain-protecting groups and deacetylation, to provide solid-supported fucosylated dendrimer libraries **L1** and **L2**, respectively (Figure 1).

Preliminary screening of library **L1** in an enzyme-coupled on-bead binding assay with a biotinylated fucose-specific lectin from the plant *Ulex europaeus* showed mostly cationic amino acids in the dendrimer sequences of stained beads (e.g., **FD1**–**FD4**, Table 1),(Kolomiets et al., 2007), an effect also seen in divalent glycopeptide dendrimers (Johansson et al., 2007). Screening of **L1** and **L2** with rhodamine-B labeled LecB similarly gave sequences containing cationic residues, although neutral and anionic amino acids were also present (e.g., **PA5**–**PA9**, Table 1). The selected hits were resynthesized by solid phase peptide synthesis (SPPS), cleaved from the resin, and purified as soluble dendrimers. When tested in an enzyme-linked lectin assay

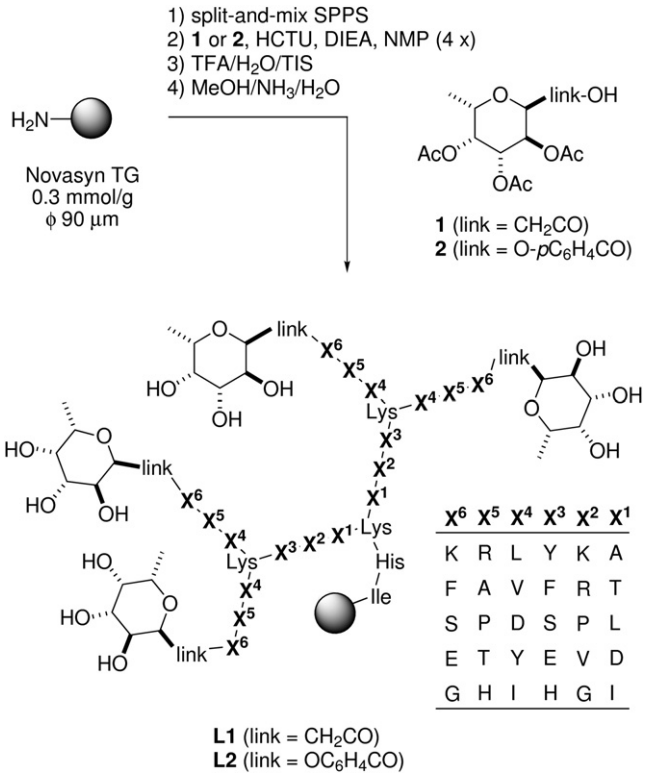

Figure 1. Synthesis and Structure of the C-Fucosyl Peptide Dendrimer Libraries, Displayed on Tentagel Resin as Solid Support

The "link" between amino acid X^6 and the fucose is either an acetyl spacer in the C-fucosyl library **L1** or phenoxyacyl in the O-fucoside library **L2**, obtained by coupling the corresponding building blocks **1** and **2**. The positions X^1–X^6 display various amino acids according to the table at right (one-letter codes of L-amino acids) as a "one-bead-one-compound" library obtained by the split-and-mix SPPS protocol.

(ELLA), the dendrimers inhibited LecB with IC_{50} in the range of 0.11 to 0.75 μM, with up to a 100-fold affinity enhancement relative to fucose (Table 1; see Figure S1 available online).

The diversity of amino acid sequences compatible with good binding to LecB suggested that the affinity enhancement was primarily due to multivalency (Kitov and Bundle, 2003). The multivalency effect on binding was further evidenced in the case of **FD2** by investigating lower-valency analogs. Thus, tripeptide **2G0** featuring the terminal branch of **FD2** exhibited an affinity similar to that of the reference monovalent ligand α-NPF (*p*-nitrophenyl-α-L-fucoside). Similarly, the first-generation dendrimer **2G1** featuring the first- and second-generation branches with two fucoses exhibited the same relative potency per fucose as the monovalent ligands.

Inhibition and Dispersion of *P. aeruginosa* Biofilms

Dendrimers, **FD2**, **PA5**, **PA6**, **PA8**, and **PA9** were selected for a study of *P. aeruginosa* biofilm inhibition. Biofilm inhibition was investigated using the steel coupon assay in which the formation of biofilms in culture suspensions is revealed by staining cells attached to the steel surface (Figure S2) (Diggle et al., 2006). Addition of α-NPF as reference LecB ligand or IPTG as reference LecA ligand to the cultures reduced biofilm formation by 30%

Table 1. Structure and ELLA Data with *P. aeruginosa* LecB for Reference Ligands and Glycopeptide Dendrimers from Library L1 or L2 Identified by On-Bead Affinity Assay

Number	n[a]	Structure[b]	Yield[c] (% mg)		IC_{50} (μM) ELLA[d]	Rel. Pot.[e] Tot. /Fucose	
L-Fuc	1	α-L-Fucose	—	—	11 ± 1.5	1.0	1.0
α-NPF	1	4-Nitrophenyl-α-L-fucoside	—	—	5.27 ± 0.55	2.1	2.1
2G0	1	cFuc-LysProLeuNH_2	68	140	5.94 ± 1.24	1.9	1.9
2G1	2	(cFuc-LysProLeu)$_2$*Lys*PheLysIleNH_2	33	82.3	2.7 ± 0.56	4.1	2.0
FD1	4	(cFuc-LysHisVal)$_4$(*Lys*HisGlyAla)$_2$*Lys*HisIleNH_2	6.7	18	0.43 ± 0.11	25.6	6.4
FD2	4	(cFuc-LysProLeu)$_4$(*Lys*PheLysIle)$_2$ *Lys*HisIleNH_2	14	38.5	0.14 ± 0.035	78.6	19.7
FD3	4	(cFuc-LysHisLeu)$_4$(*Lys*GluLysIle)$_2$ *Lys*HisIleNH_2	4.4	12.4	0.19 ± 0.05	57.9	14.5
FD4	4	(cFuc-LysArgAsp)$_4$(*Lys*SerArgAla)$_2$*Lys*HisIleNH_2	4.7	13.2	0.54 ± 0.14	20.4	5.1
PA5	4	(cFuc-GlyArgVal)$_4$(*Lys*GluGlyLeu)$_2$ *Lys*HisIleNH_2	10	26	0.75 ± 0.08	14.7	3.7
PA6	4	(cFuc-GlyThrVal)$_4$(*Lys*HisProThr)$_2$*Lys*HisIleNH_2	9	22	0.30 ± 0.09	36.7	9.2
PA7	4	(cFuc-GluHisTyr)$_4$(*Lys*TyrGlyAsp)$_2$ *Lys*HisIleNH_2	6	22	0.28 ± 0.02	39.3	9.8
PA8	4	(OFuc-LysAlaAsp)$_4$(*Lys*SerGlyAla)$_2$ *Lys*HisIleNH_2	12	21	0.11 ± 0.01	100	25
PA9	4	(OFuc-GluHisAsp)$_4$(*Lys*GluValAla)$_2$ *Lys*HisIleNH_2	9	17	0.35 ± 0.12	31.4	7.8

[a] Number of fucosyl groups in the ligand.
[b] Linear peptide notation from N to C terminus using three-letter codes for amino acids. *Lys* denotes a branching lysine with both amino groups acylated; cFuc, c-fucoside from building block **1**; OFuc, O-fucoside from building block **2**.
[c] Yield after purification by preparative HPLC.
[d] Average of three independent IC_{50} determinations with standard deviation.
[e] Rel. Pot., relative potency = IC_{50}(fucose)/IC_{50}(ligand); rel. pot. per fucose, rel. pot. / n.

and 40%, respectively, and by 50% when applied simultaneously. Although the effect of monovalent control ligands was only weak, addition of the dendrimers to the culture caused a strong reduction of biofilm formation in the assay (Figure 2, black bars, and Figure S2). The most potent inhibition was observed with ligand **FD2**, which showed a complete inhibition of biofilm formation at 50 μM (see below). A 55% inhibition was observed with **FD2** at 10 μM, and the effect was increased to 70% in the presence of IPTG (Figure S3). The dendrimers were also remarkably active when tested in a biofilm dispersion assay, which revealed the ability of the ligand to disrupt already established biofilms, a closer resemblance to chronic infections (Figure 2, gray bars, and Figure S5). In both biofilm inhibition and dispersion assays, the effect observed with the dendrimers was much stronger than the partial effect observed with the monovalent α-NPF. Quite importantly, bacterial growth was not affected by any of the ligands, indicating that the observed effect was not due to the toxicity of the dendrimers (Figure S4b).

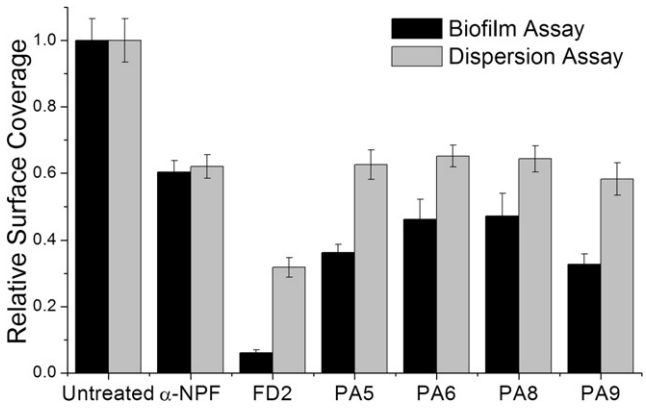

Figure 2. Biofilm Assays with *P. aeruginosa* and Dendrimers
Biofilm inhibition and dispersion were tested with α-NPF (0.5 mM), dendrimers (0.05 mM), and wild-type strain PAO1 (Figure S3). The values are normalized so that growth without any inhibitor is set to 1. A background control (no bacteria) gave reading values similar to the ones shown in the graph for **FD2** biofilm assay (0.05 mM). Mean values of three independent readings ± standard deviation are plotted.

The best biofilm formation inhibitor **FD2** was also the most potent in the dispersion assay and in fact caused complete clearance of *P. aeruginosa* colonies from the steel coupons. Analysis of the steel coupons after biofilm dispersal using confocal imaging clearly indicated that any residual staining after treatment with **FD2** was not caused by attached *P. aeruginosa* cells but probably by residues from formerly attached *P. aeruginosa* cells or by impurities (Figure 3). Most interestingly, **FD2** also inhibited biofilm formation by several *P. aeruginosa* clinical isolates derived from the cystic fibrosis lung, suggesting that the inhibitory effect is general and supporting the potential of these dendrimers in a clinical context (Figure 4).

LecB Ligands Do Not Block Biofilms in *ΔlecB* Strains

Evidence that biofilm inhibition was mediated by LecB-binding was provided by investigating biofilm inhibition in deletion mutants lacking either the *lecA* (PAO1Δ*lecA*) or *lecB* (PAO1Δ*lecB*) genes. The chromosomal *lecA* and *lecB* mutants were constructed by standard in-frame deletion and exhibited normal growth. The monovalent control ligand α-NPF inhibited biofilm formation in wild-type *P. aeruginosa* PAO1 and in PAO1Δ*lecA* ($p = 0.0001$ and $p = 0.0011$, respectively) but showed no reduction in biofilm formation for PAO1Δ*lecB*. However, the galactose ligand, IPTG, inhibited *P. aeruginosa* PAO1 wild-type ($p < 0.0001$) and PAO1Δ*lecB* biofilms ($p < 0.0001$), but not those of PAO1Δ*lecA* ($p = 0.20$) (Figure 5). Similar to α-NPF, dendrimer **FD2** had no effect on PAO1Δ*lecB* biofilm formation (Figure 4). Together with the fact that the dendrimers are not directly toxic

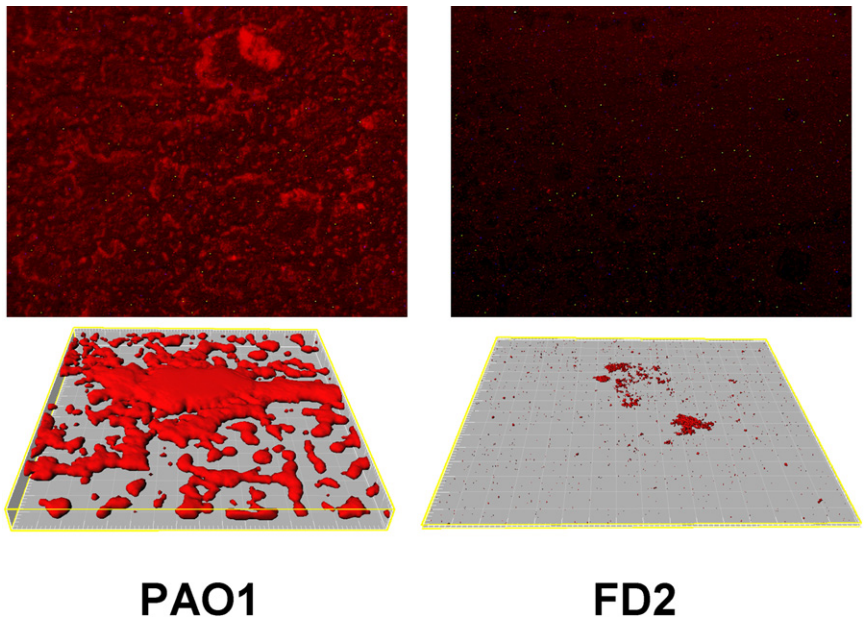

Figure 3. Representative Fluorescent and Confocal Laser Scanning Micrographic Images of Acridine-Orange-Stained Biofilms before and after Dispersion by Dendrimer FD2

to the bacterium as evidenced by the unaltered growth curves in suspension cultures in the presence of the ligands (Figure S4b), these experiments support the hypothesis that the effect of **FD2** on biofilm formation is mediated by specific binding to LecB.

Crystal Structure of the LecB-2G0 Complex

The dependence of LecB inhibition potency by the glycopeptide dendrimers on their amino acid composition suggests that part of their affinity toward LecB might be caused by secondary interactions with the lectin outside the fucose binding site. A crystallographic study was undertaken to determine the nature of the glycopeptide dendrimer-lectin interactions.

Crystallization attempts with LecB and **FD2** or **2G1** at various stoichiometries formed precipitates, and no crystals of these complexes could be obtained. However, a crystal structure of a 1:4 complex of LecB with tripeptide **2G0** featuring the terminal arm of **FD2** could be obtained. In this complex, the fucose residues in each of the four fucose binding sites are anchored to the binding site via two Ca^{2+} ions also coordinated by acidic side chains from the local chain (Glu95, Asp96, Asp99, Asp101, and Asp104), and by the C-terminal carboxyl group of the adjacent chain (Gly114). This binding mode is identical to that reported for free fucose in LecB (Imberty et al., 2004). Furthermore, the tripeptide arms protrude in solution with only few contacts with the protein and only partially observable electron density. For the best-resolved ligand, the conformation of the tripeptide linker (Lys-Pro-Leu) is well-defined by the experimentally observed electron density, except for the flexible side chain of the lysine residue (Figure 6A). The side chain of Asn70 makes an extended contact with the tripeptide linker, and might help to stabilize this particular conformation. The occurrence of different ligand conformations in each of the binding sites is most likely due to local differences in the crystal packing environment, which select different conformations from the intrinsic variability in the peptide moiety (Figure 6B). These various poses indicate that the tripeptide ligand **G0** is conformationally labile in its bound state, in agreement with the relatively low affinity of **2G0** to LecB.

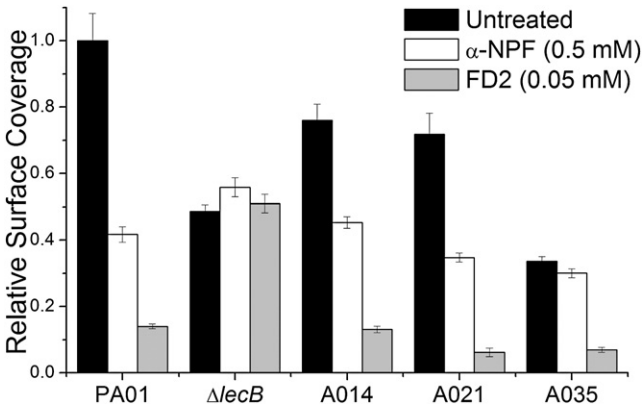

Figure 4. Biofilm Inhibition with α-NPF (0.5 mM) and FD2 (0.05 mM) of Wild-Type PAO1, *ΔlecB* Mutant (PAO1 *ΔlecB*), and Three Clinical Strains (A014, A021, A035)

The values are normalized so that growth of PAO1 without any inhibitor is set to 1. Mean values of three independent readings ± standard deviation are plotted.

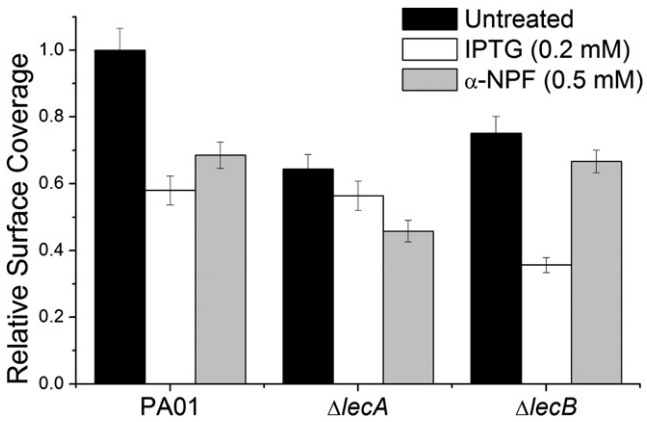

Figure 5. Biofilm Inhibition with Deletion Mutants Showing the Effect of IPTG (0.2 mM) and α-NPF(0.5 mM)

The values are normalized so that growth of PAO1 without any inhibitor is set to 1. Mean values of three independent readings ± standard deviation are plotted.

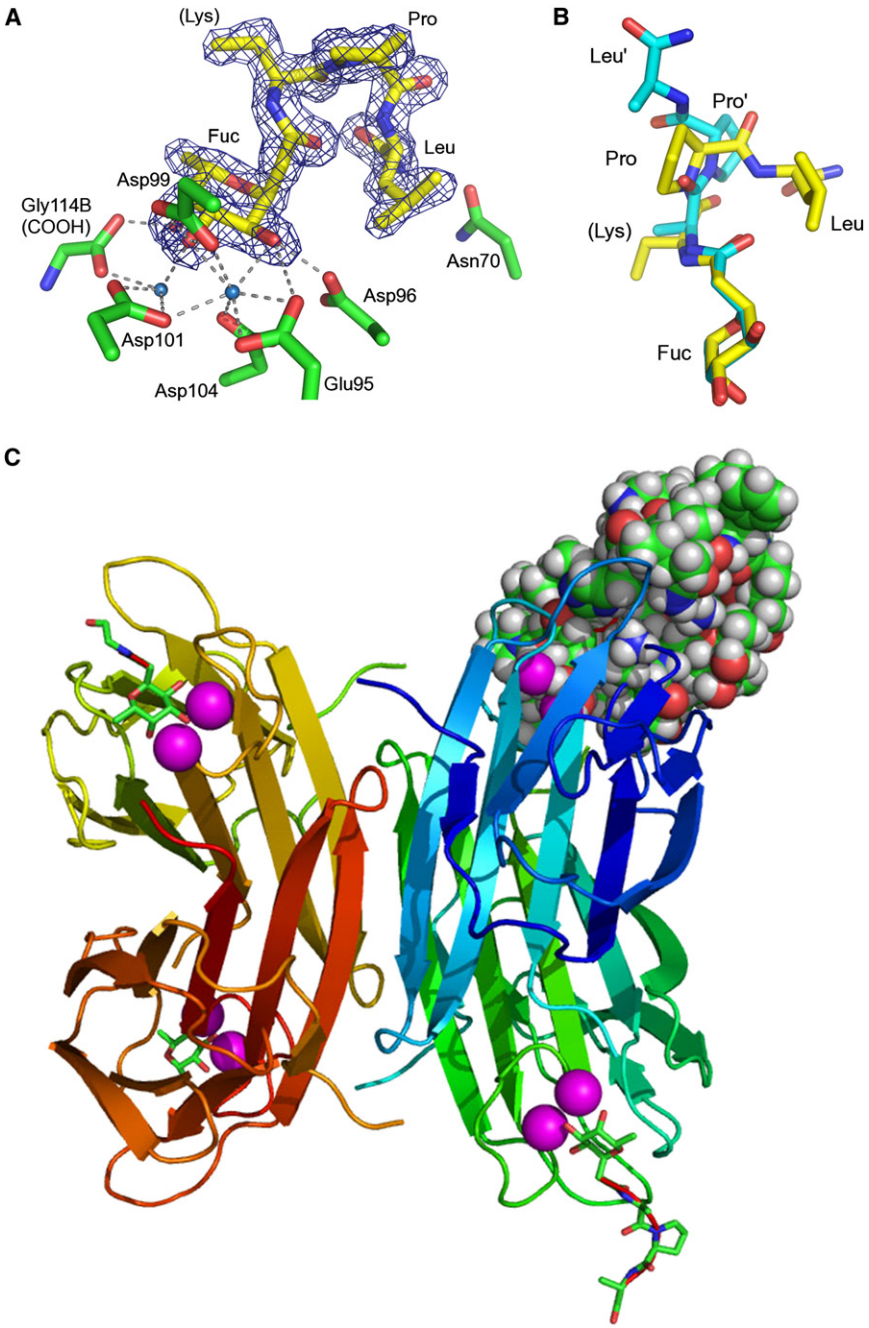

Figure 6. Structural Model of the LecB-FD2 Complex

(A) Crystal structure of the LecB-**2G0** complex, detailed view of a single LecB binding site (green) with the bound **2G0** ligand (yellow). The experimentally observed electron density is shown as a blue mesh, contoured at a density level of 1 sigma.

(B) Superposition of two distinct conformations of the **2G0** ligand, observed in LecB subunits A and B in the crystal structure. The fucose and lysine residues are in identical positions, but the proline and leucine residues adopt clearly distinct conformations.

(C) Model of the dendrimer **FD2**-LecB complex obtained by molecular dynamics and energy minimization. The Ca^{2+} ions are shown in magenta and indicate the location of the fucose binding pocket. Three binding sites are shown with the resolved fucosyl residues of **2G0** and the fourth site is modeled with dendrimer **FD2**.

Molecular Modeling of the LecB-FD2 Complex

The multivalency effects observed upon **FD2** binding to LecB, evidenced by the lower affinity of the monovalent and divalent analogs **2G0** and **2G1**, respectively (Table 1), might be caused by simultaneous coordination of **FD2** with two fucose binding sites, or by lectin-dendrimer interactions involving dendritic arms not directly involved in presenting the fucose residue occupying the fucose binding site. To investigate this point, a molecular model of the LecB-**FD2** complex was constructed. First the **FD2** ligand was assembled, energy minimized, and subjected to a molecular dynamics simulation over 10 ns in implicit solvent. The resulting model of the **FD2** dendrimer was then combined with LecB by removing one of the terminal tripeptide arms and fusing it with that observed in the crystal structure. The resulting model of the **FD2**-LecB complex was then energy-minimized and subjected to molecular dynamics simulation using implicit solvent over 100 ps (see supporting information for details).

The **FD2**-LecB model thus obtained showed that the dendrimer molecule is too small to bridge two distinct fucose binding sites within the same lectin tetramer (Figure 6C). However, the simulation suggests that additional protein-dendrimer contacts outside of the fucose binding site are possible using the fucosyl-tripeptide arm adjacent to the one directly bound to the fucose binding site. These additional contacts are predicted to involve a hydrogen bond between Arg13 on LecB and the proline carbonyl group of this dendritic arm as well as several hydrophobic interactions. Such interactions could explain the observed sequence dependence of LecB affinity in the different dendrimers investigated.

DISCUSSION

The fucose-specific lectin LecB from *P. aeruginosa* has been previously shown to be implicated in biofilm formation, although its exact mechanism of action remains poorly understood. In the present study, we asked whether specific LecB ligands might efficiently interfere with biofilm formation. To this end, we have used multivalent fucosyl-peptide dendrimers as ligands, and the results of our experiments clearly suggest that they indeed

prevent biofilm formation, and, furthermore, disrupt existing biofilms. The absence of biofilm inhibition by the most potent ligand **FD2** in the *lecB* deletion strain provides strong evidence that biofilm inhibition is mediated by direct interaction with LecB. Biofilm inhibition was observed not only in the laboratory wild-type *P. aeruginosa* strain, but also in various clinical isolates derived from the cystic fibrosis lung, which is an encouraging finding in the perspective of therapeutic applications of dendrimers.

The choice of carbohydrate multivalency as a strategy toward high-affinity lectin ligands is well reported in the literature for other lectins, as discussed in the introduction. The crystal structure of LecB furthermore showed that productive binding interactions with the lectin outside of the fucose binding pocket might be difficult to achieve due to the absence of other binding pockets on the protein. Interestingly, the reference ligand α-NPF bearing a hydrophobic phenyl fucoside group next to the carbohydrate shows only marginal enhancement of affinity to the lectin. A survey of several related monovalent aryl fucosides failed to uncover significant affinity gains to LecB (data not shown). This is in contrast to other lectins such as concanavalin A, for which the attachment of an aryl group next to the carbohydrate provides quite strong affinity gains (Kanellopoulos et al., 1996; Loganathan et al., 1992). Although such effects provided the motivation to investigate library **L2** displaying phenyl fucosides as recognition group, the resulting dendrimers exhibit similar potency toward LecB compared with the simple C-fucosyl ligands from library **L1**.

Our multivalent fucosyl dendrimers exhibit an up to 100-fold higher affinity toward LecB compared with fucose as estimated by ELLA. The affinity increase of **FD2** over fucose is 46-fold when estimated by isothermal titration calorimetry (data not shown). Thus, these glycopeptide dendrimers represent the strongest multivalent LecB ligands identified to date. Our structural investigations and the dependence of affinity on the amino acid sequence of the dendrimers suggest that auxiliary dendrimer-LecB interactions outside of the fucose binding pocket are likely to occur. Furthermore, molecular modeling clearly excludes the possibility of multivalent coordination of a LecB dimer or tetramer by the same dendrimer. We believe that the dendrimer leads to the formation of multiprotein aggregates that effectively remove the soluble LecB from the system. The formation of such aggregates was clearly indicated by precipitates observed upon attempted crystallization of **FD2**-LecB complexes.

The IC_{50} of the strongest ligand **FD2** is approximately 10 μM for biofilm inhibition, which represents a 50-fold affinity gain over the monovalent ligand α-NPF. Although the potency gain is significant, further improvements will be necessary. In the course of our investigations, synthetic di- and trisaccharide analogs of the natural LecB ligand Le^a were reported to bind LecB approximately 3- to 10-fold stronger than fucose due to additional contacts outside the fucose binding pocket (Marotte et al., 2007a), although there was no multivalency effect when these ligands were displayed as dendrimers (Marotte et al., 2007b). Synthetic fucosylated polymers were also recently shown to bind LecB (Morvan et al., 2007). Although none of these ligands were investigated for their biofilm inhibition potential, these reports provide clear indications that improvements in binding should be possible by ligand tuning, as also suggested by the modulation of affinities resulting from variations in amino acids in the dendrimers.

The investigated peptide dendrimers did not show any detectable toxicity to the bacterium aside from the biofilm inhibition and dispersion effect. Dendrimer **FD2** was additionally tested for cytotoxicity against a human cell line using an LDH cytotoxicity assay and did not show any significant increase in LDH release up to concentrations of 0.1 mM over the untreated controls (Table S1 and Figure S6). The fact that potent lectin recognition was obtained with dendrimers bearing cationic but also anionic and neutral amino acids suggests that fine-tuning of the structure should be possible by amino acid modification.

SIGNIFICANCE

The widespread occurrence and significant impact of *P. aeruginosa* biofilms in a variety of clinical settings offers promising opportunities to exploit the actions of anti-lectin agents. Beyond cystic fibrosis, *P. aeruginosa* is an important respiratory pathogen in pneumonia and chronic obstructive airways disease. It is also a major colonizer of chronic wounds such as burns and diabetic foot ulcers. Beyond the clinical setting, *P. aeruginosa* biofilm formation leads to contamination of industrial and commercial waste pipes. The economic implications of these effects are sizeable in terms of costs to patients and their caregivers, health care providers, industrial and commercial sectors, and in general the global economy.

Inhibition and dispersion of biofilms provides an attractive antibacterial strategy against *P. aeruginosa*, which can be resistant to multiple antibiotic agents. Interfering with biofilms is expected to escape resistance because the effect is not lethal to the bacterium and would only restore sensitivity to antibiotics or allow clearance by the host immune system. In the present experiments, we have shown that interference with LecB can lead to complete inhibition and dispersion of biofilms, which clearly marks this lectin as a valuable target for developing biofilm inhibitors. The glycopeptide dendrimer ligands are readily amenable to structural modifications and large-scale synthesis by standard SPPS for further development as selective biofilm-dispersion agents. Such agents would have great clinical significance given the difficulties of treating *P. aeruginosa* biofilm-centered human infections.

EXPERIMENTAL PROCEDURES

Library Synthesis

The peptide-dendrimer library was prepared from a 500 mg resin batch of NovaSyn® TG (0.30 mmol/g) divided equally in five reactors. In each of the reactors, the resin was acylated with one of the five amino acids (3 Eq) in the presence of BOP (3 Eq) and DIEA (5 Eq). Amino acids were acylated for 1.5 hr. After each coupling, the five resin batches were then mixed together and split into five parts, equally introduced in the five reactors. These split-and-mix steps were repeated after each amino acid coupling. After each coupling the resin was successively washed with NMP, MeOH, and CH_2Cl_2 (three times with each solvent), then checked for free amino groups with the TNBS test. Proline coupling was checked with the chloranil test. If the test indicated the presence of free amino groups, the coupling was repeated. The Fmoc-protecting groups were removed with a solution of 20%

piperidine in DMF (2 × 10 min) and the solvent was removed by filtration. At the end of the synthesis, the Fmoc-protected resin was dried and stored at −4°C.

Just before screening, the Fmoc-protecting groups were removed and the library was capped with a C-fucoside residue **1** or **2** (5 Eq) in the presence of DIEA (5 Eq) and HCTU (3 Eq) in NMP overnight. The side-chain-protecting groups were removed with TFA/TIS/H_2O (95:2.5:2.5) for 4 hr. Then the carbohydrate was deacetylated with a solution of MeOH/NH_3/H_2O (v/v 8:1:1) for 24 hr, resulting in the glycopeptide dendrimer library on beads **L1** or **L2**.

In the N-acetylated control library, Fmoc-protecting groups were removed and the library was capped with Ac_2O/CH_2Cl_2 1:1 for 30 min. The side-chain-protecting groups were removed with TFA/TIS/H_2O (95:2.5:2.5) for 4 hr to give a library with acetylated N-termini and free ε-lysine side chains.

Expression and Purification of LecB

The *lecB* gene of *P. aeruginosa* on plasmid pEC2 was expressed in *Escherichia coli* BL21(DE3) (Loris et al., 2003; Tielker et al., 2005 ; Tielker et al., 2006). Bacterial cells were grown at 37°C in Luria-Bertani medium containing 0.4% (w/v) glucose to an absorbance at 580 nm of 0.6 and then induced with 0.5 mM isopropyl-β-D-thiogalactoside. After an additional 16 hr of growth, cells were harvested by centrifugation at 8000 *g* for 10 min and suspended in 20 ml 100 mM Tris-HCl buffer (pH 8.0).

LecB was purified by affinity chromatography after lysis of the bacterial cells by sonication (Sonopuls HD60, Bandelin, Berlin, 3 × 4 min at 30 W). The cell lysate was centrifuged at 10,000 *g* for 30 min, and the supernatant was loaded onto a mannose agarose column (Sigma, volume 20 ml) previously equilibrated by washing with 100 ml 100 mM Tris-HCl (pH 8.0). The column was washed with 200 ml saline to remove unspecifically bound proteins. LecB was eluted with 50 ml 20 mM D-mannose in 100 mM Tris-HCl buffer (pH 8.0). LecB-containing fractions were identified by SDS-PAGE and subsequent gel staining with Coomassie-Brilliant Blue R-250 and concentrated by ultrafiltration using Vivaspin 20 devices (Virafines, 5 kDa cut-off) and the buffer subsequently replaced by ultrapure water. The LecB yield usually was 60 mg per liter of culture, and the pure lectin protein was stored in aliquots at a concentration of 1 mg/ml at −20°C.

Labeling of LecB with Rhodamine B isothiocyanate

The LecB lectin (0.5 mg in 400 μl PBS buffer, pH 8.3) was labeled with Rhodamine B isothiocyanate (0.18 mg in 400 μl of same buffer) giving a lectin/dye = 1:4 (per lysine), for 21 hr at room temperature. Purification was performed using a HiTrap desalting column (eluent PBS buffer, pH 7.2). The fractions were checked using SDS-PAGE 20% standard and labeled lectin was observed at a wavelength of 473 nm.

On-Bead Immunosorbent Lectin Binding Assay

Fifty milligrams of the glycodendrimer library on TG resin was washed three times with 1 ml PBST buffer (2 mM Na_2HPO_4, 10 mM NaH_2PO_4, 150 mM NaCl, 0.05% Tween-20, pH 6.8) and then suspended in 2 ml PBST buffer containing 3% bovine serum albumin (BSA). After having been shaken for 30 min, the beads were washed three times with 1 ml PBST buffer containing 1% BSA and subsequently incubated for 1 hr at room temperature with 1 ml of a solution of Rhodamine-B-labeled LecB (6 μg/ml). After removal of lectin solution, the beads were incubated with 0.45 M L(-)-fucose in PBST buffer for another 1 hr. The beads were washed three times with 1 ml PBST buffer and twice with 1 ml water and monitored under a fluorescence microscope (Axiovert 35). Fluorescent beads were manually selected and washed with 90% TFA aqueous solution (10 × 1 ml), water (ten times), MeOH (two times), CH_2Cl_2 (five times), MeOH (two times), and water (five times) in order to remove bound lectin. The screening was repeated with 3 M L(-)-fucose solution and without fucose competition. Negative control was done with acetylated library and no lectin binding was observed in this case.

Sequence Determination

Single dendrimer-containing resin beads were hydrolyzed with aqueous HCl (6M) at 110°C for 22 hr. The amino acids were derivatized with phenylisothiocyanate and the phenylthiocarbamoyl derivatives were analyzed on a reverse-phase C18 Novapack column.

Procedure for Dendrimer Synthesis

Peptide syntheses were performed manually in a glass reactor or plastic syringes (5 or 10 ml). The resin NovaSyn® TGR (loading: 0.18–0.29 mmol/g) was acylated with each amino acid or diamino acid (3 Eq) in the presence of BOP or PyBOP (3 Eq) and DIEA (5 Eq) for 1.5 hr (3 hr after the first generation). After each coupling, the resin was successively washed with NMP, MeOH, and CH_2Cl_2 (three times with each solvent), then checked for free amino groups with the TNBS test. If the TNBS test indicated the presence of free amino groups, the coupling was repeated. After each coupling, the potential remaining free amino groups were capped with acetic anhydride/CH_2Cl_2 for 10 min. The Fmoc-protecting groups were removed with a solution of 20% piperidine in DMF (2 × 10 min) and the solvent was removed by filtration. In the end of the sequence the resin was capped with C-fucosyl acid **1** (5 Eq) in the presence of DIC (5 Eq) and HOBt (5 Eq) or DIEA (5 Eq) and HCTU (3 Eq) in NMP overnight. The carbohydrate was deprotected with a solution of MeOH/NH_3/H_2O (v/v 8:1:1) for 24 hr. The resin was dried and the cleavage was carried out with TFA/TIS/H_2O (95:2.5:2.5) for 4 hr. The peptide was precipitated with methyl *tert*-butyl ether then dissolved in a water/acetonitrile mixture. All dendrimers were purified by preparative HPLC with detection at λ = 214 nm. Eluent A contained water and TFA (0.1%); eluent B contained acetonitrile, water, and TFA (3/2/0.1%).

Enzyme-Linked Lectin Assays

Enzyme-linked lectin essays were conducted using 96-well microtiter plates (Nunc Maxisorb) coated with LecB lectin (5 μg/ml) diluted in carbonate buffer (pH 9.6, 100 μl) for 1 hr at 37°C. After removal of lectin, the wells were blocked with 100 μl per well of 3% (w/v) BSA in PBS at 37°C for 1 hr. BSA solution was removed and each inhibitor was added in serial 2-fold dilutions (54 μl/well) in PBS to lectin-coated microplates and incubated at 37°C for 1 hr. Then 54 μl of biotinylated polymeric fucose (Lectinity Holding, Inc.) at 5 μg/ml was added to the above solutions of inhibitors and the plates were incubated for another hour at 37°C. After washing (three times with 150 μl/well) with T-PBS (PBS containing 0.05% Tween), 100 μl streptavidin-peroxidase conjugate (dilution 1:5000 in PBS) was added and left for 1 hr at 37°C. The wells were then washed three times with 150 μl/well with T-PBS and once with water, and 50 μl/well of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (1 mg per 4 ml) in citrate phosphate buffer (0.2 M, pH 4.0 with 0.015% [v/v] H_2O_2) was added. The reaction was stopped after 20 min by adding 50 μl/well 1 M H_2SO_4. Absorbance was read at 415 nm using a microtiter plate reader (spectra MAX 250). Every experiment was made in triplicate.

Determination of IC_{50} Values

The logarithm of the concentration of the dendrimer was plotted versus the percentage of inhibition. The sigmoidal curve was fitted and the concentration at 50% of inhibition was determined. The percent of inhibition was calculated as follows: Inhibition (%) = $[(A_{max}\text{-}A)/A_{max}] \times 100$. A_{max} corresponds to the mean value of the higher absorption limit reached in the dilution series and not to an external reference. Percent inhibition was plotted versus log C (inhibitor).

Steel Coupon Assay

Biofilms were grown on stainless steel coupons (grade 316L) under conditions similar to those described previously (Dhir and Dodd, 1995). Sterile steel coupons were placed at the edges of Petri dishes with 10 ml 0.05% (v/v) nutrient broth no. 2 (Oxoid) containing the appropriate concentration of the test compound. Inocula of *P. aeruginosa* strains PAO1, PAO1Δ*lecA*, PAO1Δ*lecB*, and PAO1Δ*lecA*Δ*lecB* were prepared from 5 ml overnight cultures grown in LB broth. The optical density (600 nm) was corrected to 1 and aliquots of 100 μl were used to inoculate the medium contained within the Petri dishes. The Petri dishes were incubated at 37°C on a rotary shaker (60 rpm) for 24 hr. An additional 100 μl LB broth was added and the Petri dishes were incubated for a further 24 hr. The test was performed in triplicate for each strain.

Acridine Orange Staining of Stainless Steel Coupon Biofilms

The inoculated medium was removed from the Petri dish with a transfer pipette without disturbing the steel coupons. Fifteen milliliters sterile PBS was then added and the dish agitated gently for 5 min to rinse the coupons. The PBS was removed and this rinse step was repeated. Following this, the coupons were air-dried and then heat-fixed by passing them through a Bunsen flame

three times. The surface attached biofilms were then stained by applying 200 μl 0.1% (w/v) acridine orange to the upper surface of each coupon for 2.5 min. To remove unbound acridine orange, 20 ml sterile PBS was then added to the Petri dish and the dish gently agitated as before for 5 min. The PBS was removed and this wash step was repeated two more times. The coupons were then air-dried and fixed with tape along their edges to a glass microscope slide.

Visualization of Biofilms

The coupons were examined for bacterial attachment with an inverted fluorescent microscope (Nikon Eclipse TE200) using the ×10 objective lens and green filter. Ten images were collected per coupon using a JVC KY-F58 video camera. Sampling was conducted at random from each coupon, avoiding areas at the edges. With the "red" and "low" options set to the maximum level of 255, the area fraction for each image was calculated using the Lucia G/Comet software (Nikon UK) with a threshold set at between 175 and 185.

Capturing of Confocal Images

Coupons were placed in the universal slide holder of an inverted Zeiss Axiovert100M microscope and confocal images and Z-stacks were captured with a Zeiss LSM510uv META Combi confocal system. The objective used was a Zeiss Plan-NeoFluar 40x/1.3 oil immersion and the acridine orange was excited with an argon laser at 488nm and emission collected with a LP560 filter.

Tecan Scan (Growth Curve)

An overnight culture of *P. aeruginosa* strain PAO1, grown in LB, was standardized to an OD_{600} of 0.1 and aliquots were inoculated in test wells of a 96-well plate. The appropriate volume of the test compounds was added to give the final test concentrations in a 300 μl volume. Growth (optical density at 600 nm) was measured over 24 hr at 37°C in Tecan, a combined photometer/luminometer.

Dispersion Test

Biofilms were grown on stainless steel coupons (grade 316L) under conditions similar to those described elsewhere (Dhir and Dodd, 1995). Sterile steel coupons were placed at the edges of Petri dishes with 10 ml 0.05% (v/v) nutrient broth. Inocula of *P. aeruginosa,* strain PAO1were prepared from 5 ml overnight cultures grown in LB broth. The optical density (600 nm) was corrected to 1 and aliquots of 100 μl were used to inoculate the medium contained within the Petri dishes. The Petri dishes were incubated at 37°C on a rotary shaker (60 rpm) for 24 hr. An additional 100 μl LB broth was added and the Petri dishes were incubated for a further 24 hr. The inoculated medium was removed from the Petri dish with a transfer pipette without disturbing the steel coupons and the coupons were rinsed with 10 ml 0.05% nutrient broth. Then, 10 ml 0.05% (v/v) nutrient broth containing the appropriate concentration of the test compound was added to each Petri dish. To the control dish, 0.05% nutrient broth alone was added. The dishes were incubated at 37°C on a rotary shaker (60 rpm) for 2 hr. The media was collected, vortexed, and serially diluted to a final dilution of 1:10,000. Aliquots of 100 μl were spread onto LB agar plates in triplicate and incubated at 37°C overnight. The resulting colonies were counted and a calculation of the viable bacterial count made.

ACCESSION NUMBERS

Coordinates have been deposited in the Protein Data Bank under the accession code 3DCQ.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, two tables, eight figures, and Supplemental References and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(08)00412-2.

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