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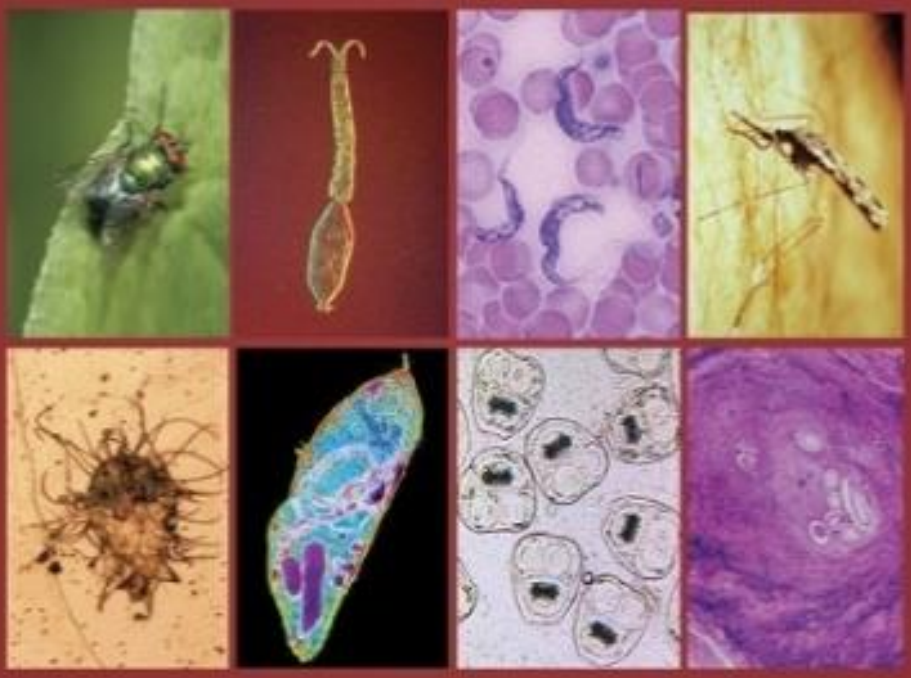

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How Targeting LecB Can Help Treat *Pseudomonas aeruginosa* Infections: The Role of LecB in Pathogenesis and its Therapeutic Potential

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ABSTRACT

Pseudomonas aeruginosa is a major cause of hospital-acquired infections, and LecB, a lectin that mediates bacterial attachment, biofilm formation, and virulence, is a key factor in its pathogenicity. Inhibition of LecB has been proposed as a promising therapeutic approach for *P. aeruginosa* infections, with several potential inhibitors, such as conjugate-antibodies, glycomimetic, and small molecules, being explored. This review summarizes recent research on LecB, including its structural and functional properties, its role in bacterial virulence, and its interactions with host cells. The authors highlight the importance of further understanding LecB's mechanisms of action and regulation, as well as its role in *P. aeruginosa* infections, in order to develop effective treatments. The review concludes by discussing the potential of LecB inhibitors as a novel therapeutic strategy for combating *P. aeruginosa* infections.

INTRODUCTION

Pseudomonas aeruginosa is a highly versatile bacterium and its genetic makeup reflects this versatility. The metabolic flexibility of the bacterium is influenced by genes involved in metabolic pathways, transcriptional regulators, and regulatory mechanisms (Klockgether *et al.*, 2011). In 2000, the completion of the genome sequence for the PAO1 strain of *P. aeruginosa* was reported by Stover and his colleagues. Since then, several strains have had their entire genomes sequenced (Stover *et al.*, 2000). The PAO1 strain of *P. aeruginosa* has a genome size of approximately 6.3 million base pairs, contains 66.6% G + C, and encodes 5,700 genes with 5,584 projected open reading frames (ORFs) (Ozer *et al.*, 2014; Stover *et al.*, 2000). In comparison to other bacterial genomes, the PAO1 strain of *P. aeruginosa* has a significantly larger number of projected outer membrane proteins involved in adhesion, motility, antibiotics, and virulence factor production (Chevalier *et al.*, 2017). Genome comparisons within the species resulted in a three-tiered functional classification of the *P. aeruginosa* genome: the core genome, the accessory genome, and the pan-genome (Freschi *et al.*, 2018, 2019; Kung *et al.*, 2010; Ozer *et al.*, 2014; Valot *et al.*, 2015). The core genome consists of portions of the genome shared by most *P. aeruginosa* strains and is interspersed with accessory genetic components (Klockgether *et al.*, 2011; Ozer *et al.*, 2014). This area of the genome has modest genetic diversity, contains the bulk of genes involved in housekeeping activities, and is not tolerant of significant alterations during short evolutionary periods (Mathee *et al.*, 2008; Spencer *et al.*, 2003; Wolfgang *et al.*, 2003).

The accessory genome of *P. aeruginosa* consists of non-conserved, variable-length segments of DNA often found in extrachromosomal elements and blocks of inserted DNA in specific loci (Dobrindt et al., 2004; Klockgether *et al.*, 2011). These DNA fragments tend to cluster in specific locations rather than being evenly distributed throughout the genome (Mathee *et al.*, 2008). This area of the genome is of significant therapeutic importance as it includes genes encoding virulence factors and genes involved in resistance to multiple antibiotic classes (Freschi *et al.*, 2019; Ho Sui *et al.*, 2009). The presence of resistance genes in the accessory genome contributes to the spread of multidrug-resistant organisms (Kung *et al.*, 2010; L. M. Sommer *et al.*, 2020). The study of *P. aeruginosa*'s evolution, adaptability, and infectious potential is made possible by the identification of these accessory genetic components (Ozer *et al.*, 2014). The pan-genome of *P. aeruginosa* can be thought of as a circular chromosome that includes strain-specific polymorphism portions anchored by conserved genes (Mathee *et al.*, 2008). This chromosome is comprised of genes from both the core genome and accessory genome, with the latter including all dispensable strain-specific genes found in only a subset of strains (Sawa et al., 2020). The current size of the pan-genome is greater than 10 million base pairs, with the core genome comprising approximately 89.7% of the entire genome, or 5.84 million base pairs, and the accessory genome accounting for an average of 11.1% of the overall genome, or 727 thousand base pairs (Ozer e*t al.*, 2014).

P. aeruginosa is a highly adaptable bacterium known for its ability to survive in a variety of ecological niches and challenging environments. This adaptability is in large part due to the bacterium's multiple and redundant secretion and attachment systems (Moradali *et al.*, 2017). *P. aeruginosa*, an opportunistic pathogen, is known to cause severe infections, particularly in immunocompromised individuals such as those suffering from cystic fibrosis, HIV, cancer, and burn wounds. Individuals with weakened immune systems, requiring respiratory assistance or undergoing cancer treatment, are at a higher risk of developing *P. aeruginosa* infections. (Driscoll *et al.*, 2007).

The bacterium's inherent antibiotic resistance and ability to rapidly acquire new drug resistances pose significant clinical challenges, resulting in multi- or extremely drug-resistant strains (Munita & Arias, 2016). *P. aeruginosa* has been put on the World Health Organization's Priority Pathogens List for the Research and Development of New Antibiotics as a priority 1 pathogen. This shows how important it is to find new treatments (Organization, 2017).

One of the ways in which *P. aeruginosa* is able to colonize host tissues and form biofilms is through the surface-exposed protein LecB, which facilitates the bacterium's adhesion to receptors on cells of the same or other species (Tielker *et al.*, 2005). A number of adhesins generated by *P. aeruginosa* have been identified, including pilus and flagellar proteins and the outer membrane protein OprF, which are capable of binding to receptors and initiating the adhesion process (Arora *et al.*, 1998; Azghani *et al.*, 2002; Doig *et al.*, 1990). Additional studies have indicated that LecB exhibits a specific binding pattern to the surface of human nasal polyp explants and fucosylated glycoproteins present in the mucin of cystic fibrosis patients, potentially prolonging infections (Adam *et al.*, 1997). In individuals with cystic fibrosis, it has been observed that respiratory epithelial cells expressing a mutant CFTR gene produce membrane glycoproteins that are more fucosylated than those produced by cells with the wild-type form of the gene (D. *et al.*, 2001; Scanlin & Glick, 2001). This suggests that LecB may play a role in promoting *P. aeruginosa*'s adhesion to the

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epithelium of CF airways (Thomsson et al., 2002). The bacterium has evolved multiple invasion mechanisms, and this review will delve into how LecB assists *P. aeruginosa* in its ability to infiltrate, create biofilms, manipulate host signalling pathways, and explore the potential for developing novel anti-lectin therapies.

Crystal Structure and Sugar Binding Mechanism of LecB:

The structure of LecB and its interaction with fucose and fucose-containing oligosaccharides has been determined through high-resolution protein crystallography (Fig. 1) (E. Mitchell *et al.*, 2002).

Fig.1: Crystal structure of the LecB lectin (PDB DOI: 10.2210/pdb1OUS/pdb).

The crystal structure of LecB reveals a tetramer consisting of four individual subunits, each of which binds a fucose molecule and contains two calcium ions. The overall structure of LecB is a nine-stranded antiparallel beta-sandwich fold (Loris *et al.*, 2003a; E. Mitchell *et al.*, 2002). The five-stranded Greek-key motif is formed by strands 1 to 5 in each subunit and is extended by strands 6 to 8, which connect with strands 1 and 4 to form a curved five-stranded beta-sheet (E. Mitchell *et al.*, 2002). Dimerization occurs through a head-to-tail interaction between two monomers, facilitated by hydrophobic interactions between non-Greek-key strands 6, 7, and 8, as well as hydrogen bonding at their extremities. Tetramerization is primarily achieved by the antiparallel attachment of beta-strands 79 to 85 from one dimer to their counterparts from another dimer (E. Mitchell et al., 2002). The calcium-binding pocket of LecB is made up of the C-terminus of the adjacent monomer and two calcium-binding loops (CBL-1 connecting strands 1 and 2 and CBL-2 connecting strands 7 and 8) (E. Mitchell *et al.*, 2002). The binding of calcium is mediated by six

acidic groups, five of which are essential. Fucose binds to both calcium ions in an unusual manner, with three of its hydroxyl groups coordinating with the calcium ions and forming hydrogen bonds with acidic groups at the calcium-binding site. The main chain nitrogen of Serine 23 in CBL-2 forms a hydrogen bond with the oxygen of the fucose ring, while the hydroxymethyl group of Serine 23 and the methyl group of Threonine 45 interact hydrophobically with the fucose methyl group at position 6 (Loris *et al.,* 2003a; E. Mitchell *et al.,* 2002). LecB has a unique mechanism for binding to fucose and fucosylated oligosaccharides. This mechanism results in a high affinity for fucose, which is significantly greater than what is typically observed in protein-carbohydrate interactions. The high affinity is due to the binding of fucose being facilitated by two calcium ions, and the interaction between these ions, the amino acid side chains, and the sugar. The binding process involves a combination of strong hydrogen bonds and coordination bonds between the sugar, protein, and calcium ions, as well as favourable entropic terms and charges delocalization. Additionally, the binding of fucose causes three strongly bound water molecules from the sugar-free structure to be displaced into the bulk solvent, leading to a favourable entropic term. The high affinity for fucose results from the combination of strong hydrogen bonds, a favourable entropic term, and the charge delocalization that arises from the presence of two nearby calcium ions (Loris *et al.,* 2003a; E. Mitchell *et al.,* 2002).

LecB has a distinctive mechanism of binding to monosaccharides compared to other calcium-containing lectins. Most C-type animal lectins include two calcium ions, but only one calcium ion is involved in sugar binding, resulting in a low association constant (Gilboa-Garber: The Biological Functions of *Pseudomonas.* - Google Scholar, n.d.). On the other hand, LecB's two calcium ions are located close to each other, allowing for high-affinity binding to fucose and other monosaccharides like L-galactose, D-mannose, and D-arabinose, albeit with varying affinities. This sets LecB apart from other calcium-containing lectins, such as the mannose-binding protein and the serum amyloid P component of pentraxin, which bind to carbohydrates with low association constants or only to certain sugar derivatives with negative charges (E. Mitchell *et al.,* 2002).

LecB and Its Role in Biofilm Synthesis:

The role of lectin-like extracellular protein LecB in the synthesis of biofilms is an important aspect of microorganisms' ability to survive in various environments (Funken *et al.,* 2012; Passos da Silva *et al.,* 2019; Tielker *et al.,* 2005). Biofilms are a type of microbial community that is encased in an extracellular polymeric matrix, which is primarily composed of carbohydrates and proteins (Assefa & Amare, 2022). This matrix helps protect the microorganisms from harmful elements and provides a suitable environment for their growth and survival. Biofilms are thought to be the source of most chronic and bacterial infections in humans. This means that patients are more likely to get sick or die, have higher medical costs, and stay in the hospital longer (Elhabibi & Radwan, 2017; Preda & Săndulescu, 2019). The structural and functional integrity of biofilm aggregates is largely dependent on the presence of exopolysaccharides, which serve as intermediaries in facilitating the adherence of biofilms to substrata and promoting cell cohesion (Imberty et al., 2004). *P. aeruginosa* and certain other types of bacteria are inherently resistant to certain antibiotics and other antimicrobial substances. This resistance is believed to result from the formation of biofilms on infected tissues and medical devices (Costerton *et al.,* 1999; Stewart & Costerton, 2001). Multiple studies have shown that the production of polymer networks in biofilms is associated with the presence of lectin-like extracellular proteins, such as LecB (Funken *et al.,* 2012;

Passos da Silva *et al.,* 2019; Tielker *et al.,* 2005). This is because oligosaccharide receptors, which are widely expressed on the surfaces of eukaryotic host cells, are involved in the formation of biofilms (Bogino *et al.,* 2013; Fong & Yildiz, 2015; Viljoen et al., 2022). LecB plays a significant role in the synthesis of biofilms by promoting the formation and stability of the extracellular matrix through its interaction with carbohydrates and sugars (Funken *et al.,* 2012; Passos da Silva *et al.,* 2019; Tielker *et al.,* 2005). This highlights the importance of understanding the role of lectin-like proteins in biofilm formation and the development of effective strategies to control the spread of biofilms and their associated infections. LecB and OprF, the main outer membrane porin, are essential for the adherence of the bacterium to lung epithelial cells (Azghani *et al.,* 2002; Chemani *et al.,* 2009). Another study has uncovered that LecB is found in various subcellular sites within the cell, including the outer membrane, which is presumed to be at the cell surface (Bartels *et al.,* 2011; Tielker *et al.,* 2005). The finding poses queries regarding the involvement of an intracellular lectin in biofilm formation. While the presence of LecB on the surface could elucidate its impact on biofilm development, the mechanism through which the protein traverses the cell envelope remains unidentified, which contradicts the previously held belief. LecB lacks the structural characteristics of outer-membrane proteins, such as a terminal F residue and barrel-forming structures (Loris *et al.,* 2003a; E. Mitchell *et al.,* 2002; E. P. Mitchell *et al.,* 2005), and is lacking in secretory signals known in *P. aeruginosa* (Ma *et al.,* 2003; Tielker *et al.,* 2005). The release of LecB from the outer membrane after treatment with pNPF suggests that it interacts with fucose-containing receptors (Funken *et al.,* 2012; Tielker *et al.,* 2005). Mutations in the part of LecB that binds to sugars prevent it from adhering to the membrane (Funken *et al.,* 2012). This shows that LecB's binding to carbohydrate ligands is an important step in its association with the surface of bacterial cells (Garber *et al.,* 1987). The use of YFP-labelled LecB showed that LecBYFP had adhered to the surface of Red-labelled cells, as indicated by a strong fluorescent signal around their periphery. However, pre-incubation of LecBYFP with L-fucose before labelling the cells effectively prevented the LecB from binding to the cell surface (Tielker *et al.,* 2005). The selectivity of LecB for D-mannose was utilized to isolate potential ligands using affinity chromatography on a mannose agarose matrix. Along with LecB, two other proteins were identified through sequence analysis using MALDI-TOF mass spectrometry, with estimated molecular weights of around 35 kDa. These proteins were identified as the outer membrane porin OprF (PA1777) and probable glutaminase-asparaginase PA1777, with 48% sequence coverage and a mascot score of 328. Notably, when the same experimental conditions were applied to cell lysates from a LecB-negative strain of *P. aeruginosa* PATI2, no proteins could be separated using affinity chromatography. The specificity of the interaction between LecB and OprF was further confirmed by the fucose-induced elution of OprF (Funken *et al.,* 2012). Confirmation of the specific interaction between OprF and LecB was achieved by transferring the eluted OprF onto a blotting membrane and then treating it with peroxidase-labelled LecB. The surface proteins of *P. aeruginosa*, including pili and type A flagella, contain carbohydrate ligands in the form of lipopolysaccharide or glycosylated proteins (Brimer & Montie, 1998; Castric *et al.,* 2001). The presence of the α-l-fucose derivative α-l-N-acetyl fucosamine in the O-antigenic oligosaccharides of several *P. aeruginosa* serotypes and in the trisaccharide found on the pili of strain 1244 has been documented (Glycosylation of *Pseudomonas Aeruginosa* 1244 Pilin: Glycan Substrate Specificity - DiGiandomenico - 2002 - Molecular

Microbiology - Wiley Online Library, n.d.). Mannose, on the other hand, has been found to be a key component of the biofilm extracellular polysaccharide produced by the typically mucoid *P. aeruginosa* PAO1 strain (Wozniak *et al.,* 2003). In addition, glycosylated surface proteins are not limited to *P. aeruginosa* and can also be found in other potentially pathogenic bacteria, such as *Streptococcus sanguinis* and *Mycobacterium tuberculosis* among Gram-positive bacteria, and *Neisseria meningitidis*, *N. gonorrhoeae*, *Campylobacter jejuni*, *Escherichia coli*, and *Helicobacter pylori* among Gram-negative bacteria (Dobos *et al.,* 1995; Erickson & Herzberg, 1993; Power & Jennings, 2003). However, the structures of the glycans and their role in the glycosylation of the proteins are still not fully understood. Furthermore, the discovery of LecB in the periplasmic region of the membrane highlights the importance of glycosylation for the localization of proteins in the outer membrane. Antibodies directed against LecB revealed the presence of a second protein band in the periplasmic fraction, which was significantly heavier than the typically observed LecB. This version was called "high-molecular-weight" (HW) LecB, and it was found to bind mannose at the same level as the "low-molecular-weight" (LW) version (Bartels *et al.,* 2011). Post-translational changes, such as glycosylation, can lead to an increase in the molecular weight of a protein (Swanson & Kuo, 1991; Totten & Lory, 1990). In eukaryotic cells, protein glycosylation, which involves the attachment of sugars to proteins, is a common occurrence and plays a crucial role in protein stability and targeting (Ungar, 2009). In *P. aeruginosa*, the subunits of the type IV pili and flagella are believed to be O-glycosylated (Castric *et al.,* 2001; Schirm et al., 2004; Totten & Lory, 1990), while N glycosylation has yet to be observed. The NetNGlyc 1.0 server found a potential N glycosylation site at position N22 in the LecB sequence. The N-glycosidase F from *Flavobacterium meningosepticum* was employed to investigate its effects on the periplasmic variants of the protein LecB. This enzyme is specifically known for removing N glycosylation from proteins. The results showed that the N-glycosidase was able to completely remove the N glycosylation from the heavy-weight (HW) variant of LecB, but had no effect on the light weight (LW) variant. These observations indicate that the HW LecB is most likely an N-glycosylated protein (Bartels *et al.,* 2011). To gain further insight into the role of N-glycosylation in LecB localization, a mutation was introduced at asparagine N22 to alanine. The strains were collected and analysed for the subcellular localization of LecB after 16 and 48 hours of growth. Results indicated that while both strains produced LecB in the cytoplasm after 16 hours, the mutated LecB accumulated in the cytoplasm while the wild-type LecB was found in both the cytoplasm and periplasmic fraction. This highlights the importance of N-glycosylation at position 22 for proper membrane localization of LecB (Bartels *et al.,* 2011). In addition, the expression of LecB was analysed using Quantitative RT-PCR, which showed consistent expression of the protein during both harvest periods. However, the mutant LecB was quickly cleared from the cells, indicating that *P. aeruginosa* has a mechanism for removing abnormal proteins (Bartels *et al.,* 2011). The importance of N-glycosylation for protein stability has been documented in other studies, including a site-directed mutagenesis analysis of the proteinase cathepsin E, which showed that removing N-glycosylation sites led to significantly reduced protein stability (Yasuda *et al.,* 1999). This highlights the importance of N-glycosylation in maintaining the stability of proteins and its potential impact on their function (Rudd *et al.,* 2001). The role of N-glycosylation in protein stability and function is underscored by the findings that N-glycosylation of LecB in *P. aeruginosa* is vital for its production and transport to the outer

membrane (Bartels *et al.,* 2011). The importance of N-glycosylation in determining protein stability and localization is emphasized, and further investigations in this area could enhance our understanding of cellular processes and disease mechanisms.

The biofilm matrix of non-mucoid *P. aeruginosa* strains contains two extracellular polysaccharides, Pel and Psl, which work together to maintain the biofilm structure (Borlee *et al.,* 2010; Byrd *et al.,* 2009; Colvin *et al.,* 2012). LecB plays a critical role in regulating biofilm formation by binding to mannose residues present in the pentasaccharide component of Psl. The repeating unit of the Psl pentasaccharide is made up of three mannose residues, two of which are connected by β-1,3′ glycosidic linkages, while the other is linked by a α-1,2′ bond. LecB has a binding affinity of 25 M for β-1,3′ mannobiose, which is the same as its affinity for α-1,2′ mannobiose, indicating that the non-reducing mannose interaction with LecB is hindered by the β-linkage rather than the C3 linkage (Fig. 2). Additionally, the interaction of LecB with Psl in the biofilm matrix was confirmed using fluorescent labelling and observed in comparison to negative control strains (Passos da Silva *et al.,* 2019). The COMSTAT image-analysis program was utilized to analyse the surface roughness of biofilms produced by both wild-type and mutant LecB strains. The wild-type cells produced biofilms consisting of multiple cellular aggregates that extended over 50 microns above the attachment surface in a dilute complex growth medium. In contrast, the LecB mutant strain formed a monolayer of cells with small mounds that did not typically extend beyond 25 microns. The wild-type biofilm phenotype was restored through complementing the LecB mutation. However, when the expression of LecB was downregulated, the biofilms lacked a clear Psl localization pattern and were unable to form large aggregates. The role of another matrix protein, CdrA, on biofilm aggregate formation has been shown to be influenced by Psl interactions. Therefore, it is possible that LecB and CdrA serve similar functions (Passos da Silva et al., 2019). When examining the impact of LecB and CdrA on biofilm structure and Psl retention, it was discovered that LecB was primarily responsible for Psl localization and aggregate formation. The expression of LecB in a double mutant background restored wild-type biofilm properties, whereas the expression of CdrA did not. However, another study has shown that CdrA plays a critical role in aggregate formation under different growth conditions. It appears that both LecB and CdrA are partially redundant, providing protection against mutations that may target both gene and potentially hamper aggregate production. Furthermore, the secretion of CdrA from cells can bind to Psl in the matrix, promoting matrix stability. It is possible that other matrix proteins besides LecB and CdrA may also contribute to matrix stability. Despite differences in growth conditions and protein expression levels, both strains produce similar matrix components, including EPS and CdrA, to keep Psl at the base of the biofilm. Overall, the expression of LecB significantly affects both Psl secretion and biomass retention, indicating its importance in biofilm formation (Passos da Silva *et al.,* 2019).

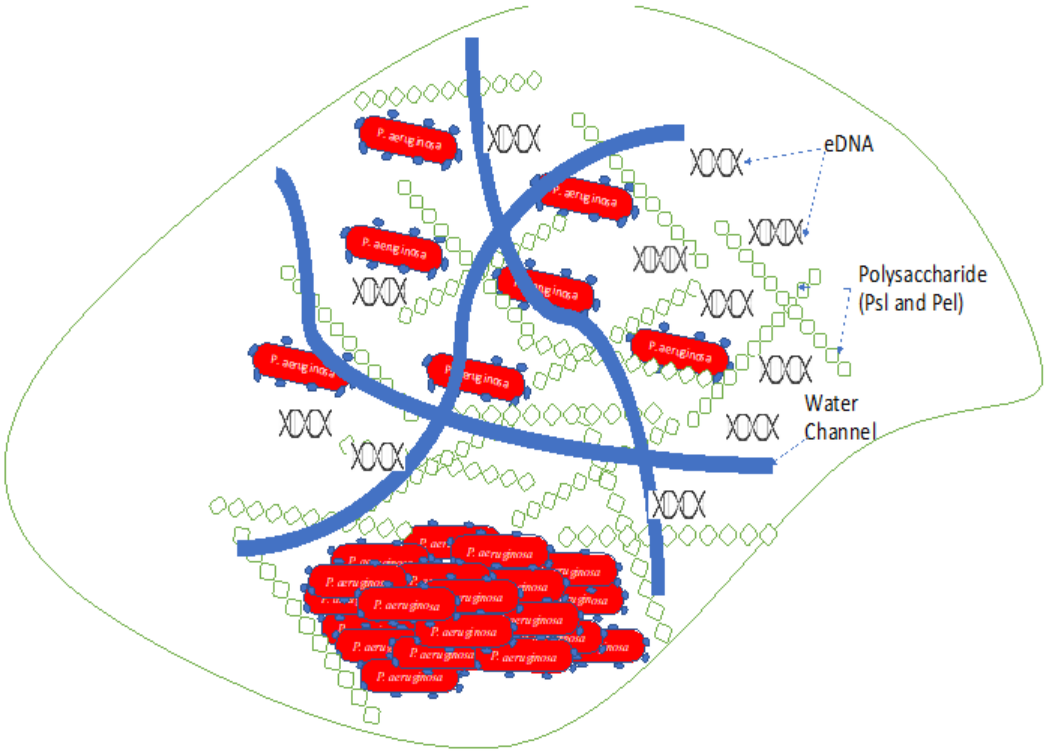

Fig. 2: Role of LecB in *Pseudomonas aeruginosa* biofilm formation. The LecB plays a critical role in polysaccharide formation by binding to the Psl, promoting maintenance of biofilms.

The Role of LecB in the Mechanisms of *P. aeruginosa* Manipulation of Host Cells in Wound Healing:

P. aeruginosa is a bacterium that typically first encounters the human host at the apical pole of epithelial cells (Heiniger *et al.*, 2010). This bacterium can invade these cells in two ways, either through the creation of new cell damage or through pre-existing damage, which enables it to enter the basolateral cell pole (Bucior *et al.*, 2010). One of the ways *P. aeruginosa* manipulates host cells is through a molecule called LecB. This molecule is unique because it specifically targets the carbohydrate moieties of integrins, which are proteins involved in cell adhesion. LecB has four different binding sites that work together to cross-link receptors (Loris *et al.*, 2003a; E. Mitchell *et al.*, 2002). When purified LecB is applied to the apical and basolateral plasma membranes of MDCK cells in a laboratory setting, it elicits very different cellular responses. The binding of LecB to basolateral cell surfaces triggers a series of cellular effects that are directly linked to the internalization of integrins (Thuenauer *et al.*, 2020, 2022). In fully polarized epithelial cells, LecB binding to the basolateral cell surface but not to the apical cell surface (which only contains small amounts of integrins) results in the loss of apicobasal polarity and the subsequent internalization of integrins. This process is necessary for the dissolution of polarity. The loss of polarity can be reversed by washing away the LecB, which coincides with the return of β1-integrin to the basolateral plasma membrane. LecB-mediated internalization of integrins also plays a role in inhibiting cell migration during epithelial wound healing. During this process, integrins are preferentially internalized in cells at the edge of the wound, which have more integrins and therefore more available binding surface for LecB. The apical surfaces of these cells are exposed, unlike those of cells that are deeper in the monolayer (Thuenauer *et al.*, 2020). The treatment of these cells with LecB effectively and quickly blocks their migration, while cells deeper within the

monolayer retain their migratory abilities. Examining the localization of cell adhesion receptors in response to stimulation by LecB on the basolateral side provides insight into the molecular mechanisms underlying the loss of epithelial polarity. This demonstrates the rapid and efficient internalization of β1-integrins (Thuenauer *et al.,* 2020). The timing of β1-integrin internalization and its return to the cell surface corresponds with changes in the transepithelial electrical resistance (TEER), and it is interesting to note that this effect can be reversed after LecB washout. Moreover, *P. aeruginosa* is known to colonize wound environments, and research has shown that wounds infected with this bacterium take longer to heal (Engel & Eran, 2011). To manipulate host cells, *P. aeruginosa* employs a variety of mechanisms, including LecB-mediated epithelial depolarization and inhibition of cell migration. These strategies can vary depending on the type of host tissue involved (Cott *et al.,* 2016)**.** Furthermore**,** Studies showed that LecB can bind to and trigger the endocytosis of functional α3β1-integrin-laminin complexes, which are some of the most abundant ligands of α3β1-integrin expressed by MDCK cells (Myllymäki *et al.,* 2011). Additionally, LecB has been found to internalize both activated and inactivated β1-integrins with similar kinetics, indicating that integrin activation status does not play a major role in LecB-mediated internalization (Greciano *et al.,* 2012). The internalization of β1-integrins was observed in cells treated with LecB, and the presence of β1-integrin was detected on LecB-induced membrane invaginations in cells with low ATP levels. This internalization process may be explained by LecB-mediated lipid-integrin cross-linking, which enables the internalization of fucose-bearing glycosphingolipids. Endogenous lectins, such as galectin-3, also have the ability to mediate integrin internalization (Furtak *et al.,* 2001; Lakshminarayan *et al.,* 2014). LecB has been found to play a role in the migration of MDCK cells by inhibiting the formation of lamellipodia in response to wounding. However, cell migration was re-established after LecB was removed (Cott *et al.,* 2016; Thuenauer *et al.,* 2020). This suggests that LecB is able to control integrin-basement membrane contact, allowing *P. aeruginosa* to move along the basement membrane-epithelial cell interface. LecB appears to be a potent endocytic mechanism that enables *P. aeruginosa* to hijack the host cell's endogenous uptake pathway for its own benefit. This protein is crucial in the regulation of cell migration and integrin internalization, and its binding to host cells is necessary for its migratory defect effects (Thuenauer *et al.,* 2020).

Quorum Sensing and Regulation of LecB:

Gene regulation through quorum sensing is a response to changes in cell population density (Pena *et al.,* 2019). The process is enabled by autoinducers, which are chemical signals produced by bacteria. The levels of autoinducers increase as cell density increases. The expression of genes is altered once the minimum threshold of an autoinducer is detected (Vendeville *et al.,* 2005). *P aeruginosa* uses quorum sensing to control the expression of many genes, most of which are crucial for the production of virulence factors including LecB (Hentzer, 2003; Schuster *et al.,* 2004; Wagner *et al.,* 2003). This bacterium has three distinct quorum sensing systems, named las, rhl, and pqs. The las system activates the rhl and pqs systems (Latifi *et al.,* 1996; Pesci *et al.,* 1997; Wade *et al.,* 2005), but rhl and pqs can also become active without the presence of the las system (Dekimpe & Déziel, 2009; Diggle *et al.,* 2003; Medina *et al.,* 2003). The rhl system also has a negative impact on the pqs system (McGrath *et al.,* 2004; Wade *et al.,* 2005). Each of these systems is composed of a signalling molecule (autoinducer) and a regulatory protein. Many virulence factors, such as elastase, exotoxin A, haemolysing, and others, are regulated by the lasRI and

rhlRI quorum sensing systems of *P. aeruginosa* (Gambello *et al.,* 1993; Hassett *et al.,* 1999; Latifi *et al.,* 1996; Ochsner & Reiser, 1995; Toder *et al.,* 1991). Quorum sensing plays a significant role in the bacteria's regulatory network. It is estimated that up to 4% of *P. aeruginosa* genes are influenced by quorum sensing. However, not all genes are identified as being regulated by quorum sensing. The genes encoding LecA and LecB were not identified as quorum sensing-regulated gene products by Whiteley et al. due to the lack of saturation mutagenesis in their research (Whiteley *et al.,* 1999). Nevertheless, the rhlRI locus, and not the lasRI locus, is believed to directly regulate the expression of both lectins in PANO67. An analysis of lectin production was not able to determine whether RhlR/C4-HSL or LasR/3O-C12-HSL could independently trigger lectin production. Two RpoS elements were identified upstream of the transcription start site, indicating that RpoS is involved in lectin gene expression. RpoS, originally discovered in gram-negative bacteria, was thought to activate survival genes during the stationary phase. However, recent research has shown that RpoS also regulates the general stress response (Muffler *et al.,* 1997). The rpoS mutation increased the sensitivity of *P. aeruginosa* cells to various stressors, but the increased sensitivity was not as pronounced as seen in *E. coli* (Jørgensen *et al.,* 1999; Suh *et al.,* 1999). The rpoS mutant was found to be more virulent than the parent strain in a mouse model of chronic lung infection, demonstrating that RpoS influences the expression of virulence genes in *P.* aeruginosa (Suh *et al.,* 1999). *Latifi et al*. found that RhlR/C4-HSL directly regulates the expression of rpoS in *P. aeruginosa* (Latifi *et al.,* 1996).

Phylogenetic Analysis and Carbohydrate Specificity of the LecB Gene:

The LecA and LecB genes of this bacterium, located in the centre of the genome (chromosome 25), play a crucial role in its virulence and pathogenesis (Muffler *et al.,* 1997; Strateva & Mitov, 2011). The amino acid sequences of the LecB gene from various *P. aeruginosa* strains, including PAO1, PA14, LESB58, PACS2, and PA7, were analysed through multiple sequence alignment. The results of the analysis showed that while the LecA gene is conserved, the LecB gene has undergone significant changes in its amino acid sequence, particularly in the PA14 strain compared to PAO1 (Boukerb *et al.,* 2014). The LecB sequence was used to construct a phylogenetic tree that separated the strains into two distinct branches, one containing the LecBPAO1 sequence and the other containing the LecBPA14 sequence (Roy *et al.,* 2010). The PA7 sequence was observed to be distant from the other two branches. Furthermore, a separate analysis of 200 core genes in these clinical isolates showed a strong correlation with the LecB sequence alignment, grouping the bacteria into two major families: PAO1-like and PA14-like (Dötsch *et al.,* 2015). The LecB sequence was found to be effective in differentiating the clinical isolates into these two groups (R. Sommer *et al.,* 2016). This information can be utilized in selecting an appropriate therapeutic strategy for treating infections caused by these strains. The LecBPA14 and LecBPAO1 sequences were found to diverge in 15 locations, accounting for the observed sequence changes in clinical isolates. In addition to these differences, two extra substitutions (A48G and A105S) were observed in some strains, which were restricted to the PA14-like family only. These changes near the carbohydrate-binding site, including the substitution of S23A, G97S, or P73S, may affect the specificity and affinity of ligand binding (R. Sommer *et al.,* 2016). CFG mammalian glycan array analysed the carbohydrate specificity of LecBPA14 and any possible changes in selectivity between LecBPA14 and LecBPAO1 (Blixt *et al.,* 2004). FITC-labelled lectins showed strong similarities in their glycan binding profiles with only minor differences. Both lectins

displayed a similar binding pattern for fucosylated oligosaccharides, such as fucosylated N-glycans and fucosylated N-acetyl lactosamine repeats, as well as high-mannose-type structures (R. Sommer *et al.*, 2016). The target with the greatest apparent affinity for both LecB variants was the bi-antennae H type II antigen on the di-LacNAc N-linked glycan structure (Loris *et al.*, 2003b). The reason for this high affinity is believed to be due to the simultaneous bivalent binding to two binding sites on the tetramer, as evidenced by the fact that a similar but shorter LacNAc analogue has a reduced apparent affinity (Loris *et al.*, 2003b; E. Mitchell *et al.*, 2002; R. Sommer *et al.*, 2016). The structure of LecBPA14 was determined through X-ray crystallography. Like LecBPAO1, the 1.70 A resolution revealed LecBPA14 to be a homotetramer composed of four carbohydrate recognition domains (CRDs) containing two Ca2+ ions each and located at the vertices of a pseudo tetrahedron. The stability of the lectin's tetrameric structure in solution, as determined by dynamic light scattering tests, is due to the direct binding of G114 to a Ca2+ ion from a neighbouring monomer (R. Sommer *et al.*, 2016). Importantly, the structural analysis of LecBPA14 and LecBPAO1 showed that all amino acid changes in LecBPA14 are confined to the surface of the tetramer, thus preserving its ability to form oligomers, which is essential for its biological activity as a cross-linking agent (R. Sommer *et al.*, 2016). The structures of LecBPA14 complexed with methyl β-L-fucoside, α-1,3-D-mannosyl D-mannose, and the high affinity ligand Lewis^a were determined through X-ray crystallography at resolutions ranging from 1.40 to 1.55 angstroms. The binding of carbohydrates to LecBPA14 is facilitated by the complexation of two Ca2+ ions with three hydroxy groups in the saccharide ligand. Additionally, the hydroxy groups are close to D96, D99, and the C-terminal G114 in the next monomer. The C-6 methyl group of fucose-containing ligands is housed in a lipophilic pocket formed by T45 and A23. The S23A version of the CRD enhances van der Waals interactions between A23 and Ca2+-coordinated carbohydrate aglycones. Unlike LecBPAO1, the G97S version of LecBPA14 has an additional hydroxy group in the CRD that may be used for hydrogen bonding. In all three LecBPA14 complexes, the carbohydrate ligand forms a single hydrogen bond with S97 through a water molecule. The high-affinity ligand Lewis^a creates multiple hydrogen bonds with LecBPA14 through two water molecules that form hydrogen bonds with both the protein and the ligand. Although the reducing end galactose of Lewis^a does not interact with LecBPA14, the remaining three monosaccharides do. Lewis^a has a water-mediated hydrogen bond between its O-6 terminal D-galactose moiety and S97, and the GlcNAc O-6 connects with D96 through a similar mechanism (R. Sommer *et al.*, 2016). The anomeric oxygen of fucose receives a hydrogen from the second water molecule, forming a hydrogen bond between the two water molecules. The binding of Lewis^a, a high affinity ligand, to LecBPA14 is strengthened by a complex network of both hydrophobic and hydrophilic interactions. The anomeric oxygen of fucose is held by a hydrogen bond with a second water molecule, forming a connection between the two water molecules. LecBPA14 has two sequence variants, S23A and G97S, which are located in the carbohydrate binding region. These variants enable robust interactions with all carbohydrate ligands, giving LecBPA14 a greater affinity for its ligands compared to LecBPAO1, which does not possess these polymorphisms (R. Sommer *et al.*, 2016).

LecB as virulence factors in P. aeruginosa-induced injury

The connection between lectins and the pathogenicity of *P. aeruginosa* was explored by Chanez Chemani et al. using a lung epithelial cell line and a murine model of lung injury. Mice infected with a strain lacking LecA or LecB showed less

extravascular lung water, as indicated by lower levels of radioactive tracer leakage, compared to their parental counterparts (Chemani *et al.,* 2009). Re-infection with the complemented strains led to permeability disorders at 16 hours, similar to those seen in the parental strain, revealing that LecA and LecB contribute to the alveolar-capillary barrier injury caused by *P. aeruginosa* (Chemani *et al.,* 2009). Adhesion and recognition through oligosaccharide-mediated mechanisms are crucial in the early stages of *P. aeruginosa* pathogenesis (Kirkeby *et al.,* 2006, 2007), and LecA and LecB play a key role as virulence factors during this phase. This is supported by the significant decrease in lung bacterial load in mice infected with the LecA and LecB mutant strains compared to those infected with the PAO1 group. The bacterial load in all three groups was similar after 6 hours, but the group infected with the parental strain had significantly higher alveolar-capillary barrier permeability compared to the groups infected with the two mutants (Chemani *et al.,* 2009). *Kurahashi et al.* also found a link between permeability increase and bacteremia, supporting these findings (Kurahashi *et al.,* 1999). *Plotkowski et al.* proposed that *P. aeruginosa* could invade intravascular space by adhering to endothelial cells and crossing the endothelial barrier, using glycan structures on the surface of endothelial cells (Plotkowski *et al.,* 1994). Ex vivo studies have shown that LecB can bind to these glycan structures, suggesting that changes in cellular glycan-lectin interactions may play a role in reducing the spread of *P. aeruginosa* and permeability disorders (Kirkeby *et al.,* 2006, 2007). This suggests that LecB are major virulence factor in *P. aeruginosa*-induced lung injury, even though the pathogenesis of *P. aeruginosa* is multifactorial. LecB and LecA significantly affect the severity of lung injury, bacterial load in the lung, and pathogen dissemination, which all impact the likelihood of survival. The use of targeted LecB inhibitors proved to be highly effective. Advances in synthetic chemistry to produce high-affinity carbohydrate-based ligands (Marotte, Préville, *et al.,* 2007; Marotte, Sabin, *et al.,* 2007) and the determination of the crystal structures of *P. aeruginosa* LecB bound to carbohydrate ligands (Cioci *et al.,* 2003; E. Mitchell *et al.,* 2002) have expanded the potential of lectins as therapeutic targets in *P. aeruginosa* infections.

The Role of LecB in Inflammation and Cell Migration:

P. aeruginosa is a ubiquitous bacterium that often causes acute lung injury in patients suffering from ventilator-associated pneumonia (Sawa, 2014). This type of infection results in severe inflammation and degeneration of lung tissue (Johnson & Matthay, 2010). To heal the damaged tissue, it is crucial to suppress inflammation and promote cell proliferation and migration (González-López *et al.,* 2011). Unfortunately, *P. aeruginosa* can interfere with these processes, thereby allowing the bacterium to spread into the bloodstream and potentially causing life-threatening sepsis (Sawa, 2014). LecB was discovered to play a significant role in these mechanisms, found to dramatically decrease host cell migration and proliferation while concurrently activating NF-κB mediated signalling (Cott *et al.,* 2016; Sponsel *et al.,* 2023). *P. aeruginosa*, along with *Salmonella typhimurium*, has been shown to regulate β-catenin and inflammation antagonistically during bacterial infections (Chen *et al.,* 2013; Duan *et al.,* 2007). However, the specific bacterial agents responsible for this regulation remained unidentified. In a mouse model of *P. aeruginosa* keratitis, It was discovered that the bacterium causes β-catenin breakdown and a strong inflammatory response, resulting in persistent tissue damage even after bacterial clearance (Chen *et al.,* 2013). Furthermore, introducing a non-degradable mutant of β-catenin to the system decreased the onset of inflammation by preventing its breakdown. The inflammation caused the

overexpression of genes controlled by NF-κB, including tumour necrosis factor-alpha, interleukin-6, and interleukin-1. Intestinal samples from S. *typhimurium*-infected mice revealed a decrease in β-catenin and an increase in NF-κB activity (Duan *et al.*, 2007). The delayed activation of LecB-mediated effects on NF-κB compared to the quick activation of NF-κB by other molecules, such as TNF, may be due to the activation of NF-κB via β-catenin degradation. LecB is not the only fucose-binding lectin with pro-inflammatory properties, as lectins from *Burkholderia cenocepacia* and *Aspergillus fumigatus* have also been found to have similar effects (Houser *et al.*, 2013; Šulák *et al.*, 2011). The impact of LecB on β-catenin degradation is likely due to the inhibition of cell migration that it induces. Cell migration involves several key processes, including attachment and detachment from the extracellular matrix, adhesion to other cells, maintaining cell polarity, and reorganizing the cytoskeleton (Sheetz *et al.*, 1999). The regulation of cell migration by GSK-3β has been widely studied, but its exact effect can vary depending on the cellular system being used. In some cases, inhibiting GSK-3β has been found to prevent the migration of keratinocytes and reduce the formation of long, thin extensions (lamellipodia) that are necessary for cell movement (Koivisto *et al.*, 2003). This suggests that GSK-3β plays a positive role in cell migration. However, other studies have shown that activating Akt signalling can lead to the inhibitory phosphorylation of GSK-3β, which then boosts the transcriptional activity of β-catenin and promotes the motility of intestinal epithelial cells (Cott *et al.*, 2016). The bacterium *P. aeruginosa*'s LecB may also play a role in this process, as it appears to bypass or overcome the inhibitory effects of Akt on GSK-3β. This indicates that GSK-3β activity is essential for the breakdown of β-catenin, which is induced by LecB. LecB's impact on β-catenin is thought to be the result of proteasomal degradation, rather than lysosomal degradation. The proteasomal degradation of β-catenin is independent of Akt signalling, but requires GSK-3β activity for full degradation to occur. In addition, β-catenin levels in cells are regulated by a feedback loop of ongoing synthesis and proteasomal degradation. LecB had no effect on β-catenin levels that were influenced by protein synthesis, but it did promote the proteolytic destruction of β-catenin (Cott *et al.*, 2016). It is possible for β-catenin to be bound and phosphorylated even in the presence of GSK-3β phosphorylation that inhibits migration (Dajani *et al.*, 2003). The involvement of Axin in the destruction complex may also play a role in preventing GSK-3β phosphorylation (Ding *et al.*, 2000). Confocal fluorescence imaging showed that β-catenin, which was attached to the membrane, was concentrated near the nuclei and around the centrosomes, further indicating that proteasomes were involved in breaking down β-catenin (Wójcik & DeMartino, 2003). Interestingly, after LecB treatment, β1-integrin was also degraded and taken into the same location as β-catenin. Furthermore, it was established that integrins play a critical role in cell motility (Huttenlocher & Horwitz, 2011). β1-integrin has been reported to form a complex with α3-integrin, another subunit of integrin, to promote cell migration. This complex has been shown to be important for regulating the stability of the lamellipodia and promoting cell motility (Cott *et al.*, 2016). In addition, the interaction between β1-integrin and α3-integrin has been implicated in a variety of cellular processes, including cell adhesion, proliferation, and differentiation (Choma *et al.*, 2004). α3β1-integrins can also stimulate and localize to cadherin-catenin complexes, which are typically associated with cell-cell adhesion. The localization of α3β1-integrins to cadherin-catenin complexes has been shown to be mediated by their association with the tetraspanin CD151 (Chattopadhyay

et al., 2003). Tetraspanins are a family of transmembrane proteins that are known to interact with integrins and modulate their function. CD151, in particular, has been shown to associate with α3β1-integrin and promote its localization to cadherin-catenin complexes. This interaction has been implicated in a variety of cellular processes, including cell adhesion, migration, and invasion (Chattopadhyay *et al.,* 2003). The depletion of β1-integrin and its removal from cell-cell and cell-matrix contacts have a negative impact on cell migration, as these contacts are important for regulating cell adhesion and motility. The interaction between CD151 and integrins can also play a role in regulating cell migration, and its release from integrins can lead to suppression of cell migration (Palmer *et al.*, 2014). Immunofluorescence and western blot analysis showed that cells stimulated by Wnt3a had a stronger cytosolic signal of β-catenin and greater nuclear translocation compared to control cells. β-catenin's major effects on migration, proliferation, and inflammation are mediated by its cytosolic and nuclear pools (Perez-Moreno & Fuchs, 2006). The simultaneous treatment of Wnt3a and LecB completely prevented the accumulation and nuclear translocation of β-catenin induced by Wnt3a, indicating a cross-talk between Wnt3a and LecB effects and suggesting that LecB-induced degradation of β-catenin is also impacting the protein's cytosolic pool, which is consistent with the decrease in its transcriptional activity (Cott *et al.,* 2016). Cell division is an important process that plays a crucial role in the maintenance and repair of cells and tissues. The cell cycle is a normal progression of cells through the stages of growth and division, but if the number of cells entering the S-phase of the cycle decreases, then populations of cells in the G1 and SubG1 stages increase (Bertoli *et al.,* 2013; Hunt *et al.,* 2011; Wang, 2021). LecB stimulation was shown to cause this reduction in the number of cells entering the S-phase. This arrest in the cell cycle at the G1-S phase can result in slowed cell growth and some sub-G1 apoptosis. After LecB treatment, cyclin D1, a key regulator of this transition, and other related proteins such as cyclin E2 and Rb were found to be down-regulated. This suggests that LecB causes a decline in the cell cycle progression (Cott *et al.,* 2016). In fact, the levels of cyclin D1 were significantly reduced after 1 and 5 hours of incubation, with a decline to 20% after 16 hours of overnight incubation with LecB. Similarly, c-myc levels were also reduced to the same extent as cyclin D1 levels following an overnight treatment with LecB. Studies have shown that the downregulation of β-catenin by upstream regulators or shRNA can lead to a decrease in cyclin D1 expression (Cott *et al.,* 2016). This downregulation can occur through a variety of mechanisms, including the inhibition of Wnt signaling and the disruption of β-catenin's interaction with other proteins, and the downregulation of cyclin D1 expression is one of the downstream effects of β-catenin downregulation, and it can contribute to a decrease in cell proliferation (Chatterjee *et al.,* 2011; Lau *et al.,* 2011). In the cell cycle, the activity of Rb is regulated by phosphorylation, and in particular, phosphorylation of Rb by the cyclin D1/Cdk4/6 complex at Ser795 has been shown to inhibit its suppressive effect on transcription. This phosphorylation event leads to the dissociation of Rb from E2F transcription factors, allowing for the expression of genes involved in cell cycle progression (Musgrove et al., 2011), and this phosphorylation was also shown to be significantly reduced by LecB treatment (Cott *et al.,* 2016). Additionally, Rb protein was found to have a lower molecular weight, indicating that multiple phosphorylation modifications had been removed from the protein. LecB also prevented the nuclear accumulation and translocation of β-catenin produced by Wnt3a, and activation of LecB for 24 hours decreases the levels of c-myc and cyclin D1, which are classic transcriptional targets of β-catenin. This reduction in cyclin D1

levels also affected the levels of cyclin E2 and Rb phosphorylation (Cott *et al.,* 2016) . When LecB was combined with other cytotoxins produced by *P. aeruginosa*, such as ExoU, ExoT, and LepA, it amplified the inflammatory response and contribute to the development of Acute Lung Injury (ALI) (102–104). However, a study reported a reduction in bacterial load and dissemination in an *in vivo* murine model of ALI when infected with either a LecB-deficient mutant or when treated with specific glycoconjugates that inhibit LecB (Boukerb *et al.,* 2014; Chemani *et al.,* 2009).

LecB-Mediated Signalling and The Host Cell Response:

LecB plays a critical role in the invasion and cellular uptake of host cells (Thuenauer *et al.,* 2020). Studies have shown that LecB can increase the cellular uptake of *P. aeruginosa* by activating a signalling cascade involving Src, PI3K, and Rac proteins. This signalling pathway is regulated by the protein caveolin-1, which is important for the formation of caveolae, small invaginations in the plasma membrane that play a role in various cellular processes. Activation of the Src-PI3K-Rac signalling cascade by LecB leads to changes in the actin cytoskeleton of the host cell, resulting in the formation of protrusions that can facilitate the uptake of the bacterium. Specifically, the activation of Rac leads to the rearrangement of actin filaments, which in turn promotes the formation of these protrusions. PI3K is known to activate both Src kinases and Akt through the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) in response to LecB treatment (Gassama-Diagne *et al.,* 2006). In non-treated LecB cells, PIP3 is mostly localized to the basolateral plasma membrane in polarized epithelial cells, and this helps to maintain the proper cellular polarity and function of these cells (Gassama-Diagne *et al.,* 2006). LecB induces the accumulation of PIP3 on the apical plasma membrane, similar to what is observed when whole *P. aeruginosa* bacteria interact with the apical plasma membrane of MDCK cells (Kierbel *et al.,* 2007). This abnormal localization of PIP3 has consequences for the signalling pathways that normally rely on PIP3 localization at the basolateral membrane, and it contributes to the pathogenic effects of *P. aeruginosa* infection. *P. aeruginosa* uses different invasion mechanisms to infect a wide range of host cells, and one of these mechanisms involves the interaction between the bacterium's lectin A (LecA) and the glycosphingolipid Gb3 on the host cell. This interaction leads to a type of invasion known as the "lipid zipper" (Eierhoff *et al.,* 2014) or the ability of *P. aeruginosa* to exploit CFTR to stimulate caveolin-1-dependent endocytosis (Bajmoczi *et al.,* 2009). However, MDCK cells do not express Gb3, a glycosphingolipid that is known to interact with the *P. aeruginosa* lectin A (LecA) (Müller *et al.,* 2017); as a result, *P. aeruginosa* uses alternative mechanisms to invade MDCK cells such as LecB-mediated signalling (Thuenauer *et al.,* 2022). The deletion of LecB in *P. aeruginosa* has been shown to inhibit invasion efficiency in H1975 cells. These findings illustrate that different invasion processes, such as LecA- and LecB-dependent invasion, are not mutually exclusive, but rather work together to achieve a broader objective (Thuenauer *et al.,* 2022). Applying LecB-biotin to the apical surface of polarized MDCK cells and then lysing the cells to precipitate LecB-receptor complexes using streptavidin beads to identify apical interaction partners of LecB. Mass spectrometry analysis of the precipitated complexes revealed a list of 12 proteins that were significantly enriched, indicating that LecB has the ability to bind to multiple receptors (Thuenauer *et al.,* 2022). However, a specific receptor responsible for LecB-triggered PI3K signalling were unable to pinpoint, since several of the identified proteins had been shown to have the capacity to elicit PI3K signalling, including CEACAM1 (Voges *et al.,* 2012;

Yu *et al.,* 2006), mucin-1 (Raina *et al.,* 2004), ICAM1 (Hamaï *et al.,* 2008), and podocalyxin (Huang *et al.,* 2015; Sizemore *et al.,* 2007). Live-cell microscopy demonstrated the apical uptake of beads by MDCK cells and before the apical uptake of beads, there was a transient event of apical PH-Akt-GFP/PIP3 accumulation. This apical PH-Akt-GFP/PIP3 accumulation was found to occur more frequently and more intensely in response to LecB-coated beads compared to biotin-coated control beads. This suggest that there may be a specific mechanism by which the cells recognize and respond to the LecB-coated beads, potentially involving a receptor or signalling pathway that is not activated by the biotin-coated beads. The apical uptake of *P. aeruginosa* in the polarized MDCK cells was significantly reduced when LecB was abrogated or blocked with L-fucose. This highlighted that LecB is important for the apical uptake of *P. aeruginosa* in MDCK cells and that it may play a role in mediating the interaction between the bacteria and the host cells. Similarly, inhibiting Src kinases with PP2 and inhibiting PI3K with LY294002 both led to a decrease in the uptake of *P. aeruginosa* (Kierbel *et al.,* 2005; Lepanto *et al.,* 2011). However, the adherence of wt and mutant LecB *P. aeruginosa* with the host cells was not significantly different, revealing that the decreased efficiency of invasion observed upon deletion of LecB was not due to reduced host cell binding, but rather to the absence of LecB-mediated signalling (Thuenauer *et al.,* 2022). It is interesting to note that while deletion or blocking of LecB with L-fucose did not decrease the internalization of *P. aeruginosa* bacteria as much as inhibition of Src kinases and PI3K, there may be other bacterial factors that can also cause PI3K-dependent uptake into host cells. One such candidate is type IV pili and deletion of type IV pili led to a small but significant reduction of PI3K/Akt activation upon apical application of *P. aeruginosa* to polarized Calu-3 cells (Bucior *et al.,* 2012). Exotoxins S and T found in *P. aeruginosa* contain N-terminal RhoGTPase activating protein (RhoGAP) domains, which can hydrolyze GTP to GDP in Rho, Rac, and Cdc42. This leads to cytoskeletal depolymerization, which counteracts host cell invasion (Garrity-Ryan *et al.,* 2000; Kroken *et al.,* 2022). Furthermore, treating the apical surface of MDCK and H1975 cells with LecB resulted in a time-dependent activation of Rac. The activation of Rac was preceded by PI3K activity, and blocking PI3K activity with wortmannin inhibited the activation of Rac. LecB also induced ruffle-like structures in sparsely seeded H1975 cells. LecB colocalized with transfected Rac1-wt-GFP and actin in these cells, and the colocalization coefficient between Rac1-wt-GFP and actin significantly increased in LecB-treated cells. This suggest that functional Rac is necessary for the effect of LecB in inducing ruffle-like structures (Thuenauer *et al.,* 2022). Treating the apical cell pole of MDCK cells with LecB caused a significant rearrangement of the actin cytoskeleton in the cell. In untreated cells, the apical cell pole was composed of microvilli, a central actin-free portion of the periciliary membrane, and a primary cilium that appeared as dotted structures (Francis et al., 2011; Stroukov *et al.,* 2019; Thuenauer *et al.,* 2011). After three hours of treatment with LecB, the subapical organization of the actin cytoskeleton was completely disrupted. This actin rearrangement was found to be a result of LecB's stimulation of PI3K signalling, which led to the activation of Rac and subsequent actin reorganization (Thuenauer *et al.,* 2022). These observations were also observed during the internalization of *P. aeruginosa* (Esen *et al.,* 2001; Kierbel *et al.,* 2007; Lepanto *et al.,* 2011), highlighting the crucial role of LecB in this process (Thuenauer *et al.,* 2022). LecB treatment promoted the formation of actin stress fibres that contracted at the base of the primary cilium. After twelve hours of apical LecB administration, the polarized MDCK cells no longer had primary cilia, which is an intriguing observation. If LecB

was removed, the effect would also disappear, showing the substantial impact of LecB-mediated actin rearrangement. However, the potential physiological consequences of the loss of primary cilia during *P. aeruginosa* infection still require further investigation. Additionally, In untreated MDCK cells, caveolin-1 was primarily localized to the basolateral plasma membrane (Verkade *et al.,* 2000). However, treatment with LecB on the apical surface resulted in the abnormal recruitment of caveolin-1 to the apical cell pole. Blocking Src kinases or PI3K inhibited the coprecipitation of caveolin-1 with LecB-biotin complexes. Knocking down caveolin-1 suppressed PI3K activation upon LecB treatment. This suggests that LecB recruits caveolin-1 to the apical surface of cells, and this recruitment is dependent on the activation of Src kinases and PI3K. Additionally, caveolin-1 is essential for LecB-induced PI3K activation, forming a positive feedback loop between caveolin-1 recruitment and PI3K activation (Thuenauer *et al.,* 2022).

LecB and Therapeutics:

Antibiotic resistance is a major public health concern and new treatment approaches are urgently needed (Murray *et al.,* 2022; Serwecińska, 2020). In recent years, antivirulence drugs have emerged as a promising strategy for treating bacterial infections (Fig. 3). Instead of killing the bacteria directly, these drugs target virulence factors that are essential for bacterial pathogenesis, such as bacterial lectins (Dehbanipour & Ghalavand, 2022; Fleitas Martínez *et al.,* 2019; Rezzoagli et al., 2020). Inhibiting bacterial lectins, such as LecB, has emerged as a promising approach for preventing biofilm formation and treating bacterial infections. By inhibiting bacterial lectins, it may be possible to prevent bacteria from adhering to surfaces and forming biofilms, thus making them more vulnerable to antibiotics and other treatments. This approach has the potential to disrupt the formation of bacterial communities and reduce the incidence of chronic and recurring infections (Fig. 4). Clinical trials conducted on adult CF patients have shown a notable decrease in the colonies of sputum *P. aeruginosa* following the administration of fucose/galactose inhalation therapy (Hauber *et al.,* 2008; Kolomiets *et al.,* 2009).

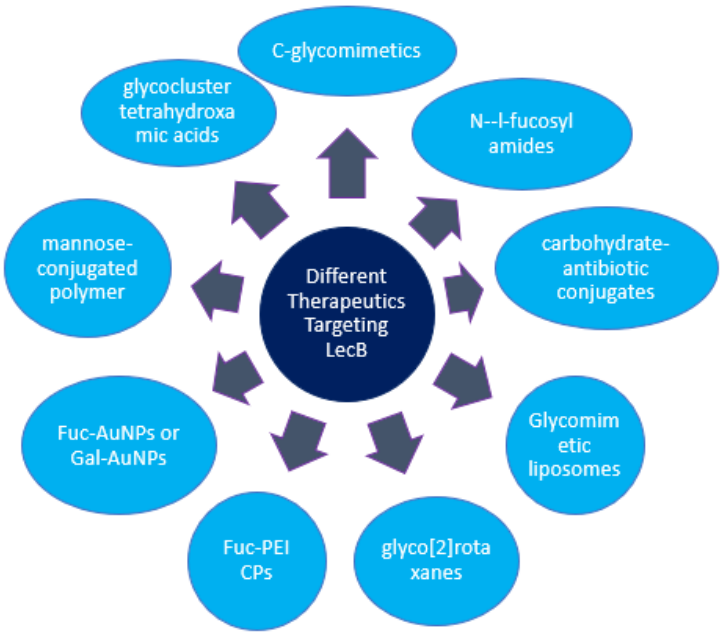

Fig. 3: Current anti-LecB therapeutics for the treatment of Pseudomonas aeruginosa infections. Shown are the various small molecule inhibitors, antibodies-conjugates, glycomimetics and AuNP etc that target the LecB lectin, and inhibits the bacterial adhesion and biofilm formation.

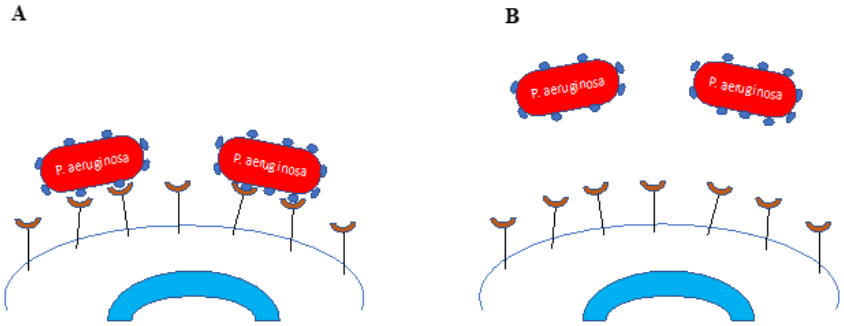

Fig. 4: Mechanism of action of anti-LecB drugs in preventing *P. aeruginosa* adhesion to host membranes. (A) In the absence of anti-LecB drugs, LecB binds to host glycoconjugates on the surface of host cells, leading to bacterial adhesion and colonization. (B) In the presence of anti-LecB drugs, the drugs bind to the LecB lectin, preventing its interaction with host glycoconjugates and inhibiting bacterial adhesion to host membranes. This prevents the formation of bacterial biofilms and reduce the severity of *P. aeruginosa* infections.

Targeting bacterial lectins such as LecB with glycomimetic is a promising approach for preventing biofilm formation and treating bacterial infections. Glycomimetic are small molecule compounds that mimic the structures of carbohydrates and have been designed to target various biological processes that involve carbohydrate-protein interactions (Ernst & Magnani, 2009; Hevey, 2019). In the case of LecB, glycomimetic have been developed based on the structure of the small molecule LecB ligand mannose and optimized for target-binding

Targeting bacterial lectins such as LecB with glycomimetic is a promising approach for preventing biofilm formation and treating bacterial infections. Glycomimetic are small molecule compounds that mimic the structures of carbohydrates and have been designed to target various biological processes that involve carbohydrate-protein interactions (Ernst & Magnani, 2009; Hevey, 2019). In

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the case of LecB, glycomimetic have been developed based on the structure of the small molecule LecB ligand mannose and optimized for target-binding potency and oral bioavailability. The fucose-mannose hybrid glycomimetic that was synthesized combines the properties of d-mannose-derived inhibitors with L-fucose, resulting in sub-micromolar affinities and excellent antibiofilm activity in vitro. The binding of these glycomimetic to LecB was confirmed using techniques such as NMR and isothermal titration calorimetry, which showed that the compounds exhibited nanomolar inhibitory affinities for LecB variants from two representative strains of clinical isolates. One notable aspect of these glycomimetic is their excellent biophysical properties, including high receptor residence times and fully enthalpy-driven binding to the target lectin, which make them suitable for early drug development. Additionally, the choice of amide can affect the inhibitory activity of the compound, with mannose sulphonamides being more effective than the corresponding amides and fucose-mannose hybrid amides being as active as mannose sulphonamides due to hydrophobic interactions. Overall, these findings suggest that glycomimetic, particularly fucose-mannose hybrid glycomimetic, hold promise as potential therapeutics for targeting LecB-mediated biofilm formation in a broad range of clinical isolates (R. Sommer et al., 2018). Moreover, Sommer *et al* modified the weak LecB ligand, methyl α-d-mannoside, into C-6 modified amide and sulphonamide derivatives, which showed an increased potency of up to 20 times. They also developed C-glycosides by combining the functional groups of fucose and mannose, which resulted in glycomimetic 4-7 with improved target binding potency, selectivity, ADME/Tox parameters, and oral bioavailability (R. Sommer et al., 2019). Whereas, N-β-l-fucosyl amides also exhibit high-affinity binding to LecB, with sub-micromolar binding affinities, and good antibiofilm activities (Mała *et al.*, 2022). Another approach could be Carbohydrate-antibiotic conjugates, which are the emerging class of therapeutics that show promise for the treatment of bacterial infections (Cal *et al.*, 2017; Cavaco *et al.*, 2022). These conjugates use the bacterial lectin LecB as a target. By conjugating carbohydrates to antibiotics, these molecules can specifically target LecB and enhance their activity against bacterial biofilms. For instance, mannosylated glycopolymer-b-AMPSm has been used as a carrier to deliver Tobramycin to *P. aeruginosa* biofilms, resulting in a significant reduction in the bacterial load (Boffoli *et al.*, 2022). The conjugation of the antibiotic to the targeting moiety can improve the pharmacokinetic properties of the drug, such as its solubility, stability, and tissue distribution, as well as enhance its efficacy and reduce toxicity. Another carbohydrate conjugate that has demonstrated promising results in targeting LecB is Ciprofloxacin-C-fucosides. This conjugate combines the antibiotic Ciprofloxacin with fucose, a sugar that binds specifically to LecB. In vitro, studies have shown that Ciprofloxacin-C-fucosides effectively inhibit the growth of *P. aeruginosa* biofilms. The conjugate's ability to specifically target LecB is a major advantage, as it minimizes the potential for off-target effects and enhances the therapeutic efficacy of the antibiotic. Ciprofloxacin- C-fucosides conjugates were effective at binding to both LecB variants of PAO1-like or PA14-like structures (Meiers *et al.*, 2020). However, the carbohydrate conjugates had reduced antibiotic activity compared to the parent antibiotic molecules, likely due to their higher molecular weight, which can make it more difficult for them to penetrate the thick outer membrane of Gram-negative bacteria. Comparing the conjugates to N-propargylated ciprofloxacin, which showed that even a small chemical modification to the antibiotic molecule can result in decreased activity (Meiers *et al.*, 2020). Glycomimetic liposomes are a type of

liposome that incorporate glycomimetic. These glycomimetic can bind to specific lectins or carbohydrate-binding proteins, making them a promising strategy for targeting extracellular structures that are involved in bacterial virulence and biofilm formation. Liposomes are spherical vesicles that consist of a lipid bilayer and can encapsulate therapeutic agents like peptides, proteins, or drugs (Akbarzadeh *et al.,* 2013). To enhance their targeting ability, liposomes were modified by attaching multiple copies of targeting ligands to their surfaces, creating a multivalent presentation that increases the liposomes' affinity to their corresponding lectins. The incorporation of sulphonamide derivatives of L-fucosides were utilized as suitable ligands for glycomimetic liposomes since they have previously demonstrated high binding affinity to LecB. The liposomes containing these ligands were evaluated for their ability to inhibit *P. aeruginosa* attachment and biofilm formation in vitro and in an animal model of chronic lung infection. The multivalent presentation of the ligands on the glycomimetic liposomes is believed to improve their binding affinity to the lectins on the surface of *P. aeruginosa* bacteria (Metelkina *et al.,* 2022). Moreover, a type of molecule called glyco[2]rotaxanes, which consist of a macrocycle called a pillar[5]arene that is threaded by two chains, or "axles," each of which has a carbohydrate molecule attached to it has shown the antibiofilm activity. The pillar[5]arene acts as a host for the carbohydrate chains, forming a rotaxane structure that was synthesised to disrupt biofilm formation by targeting LecB. The axles were also decorated with positively charged ammonium groups, which help the molecules to bind to negatively charged bacterial surfaces. Moreover, some of the glyco[2]rotaxanes compounds being more active than the commonly used antibiotic tobramycin. The glyco[2]rotaxanes also demonstrate selectivity towards *P. aeruginosa* over other bacterial species (Mohy El Dine *et al.,* 2021).

Another compound, fucose-modified polyethyleneimine copolymer particles (Fuc-PEI CPs) via nucleophilic addition between primary amine and aldehyde groups was synthesized and characterized, and its antibacterial activity against *P. aeruginosa* was evaluated. The results showed that the PEI-fucose complex had a high antibacterial activity and was found to be non-toxic to human cells, indicating its potential as a safe and effective antibacterial agent (Liu *et al.,* 2021). Furthermore, the use of fucose-functionalized (Fuc-AuNPs) and galactose-functionalized (Gal-AuNPs) gold nanoparticles were used to target LecB to enable specific binding to *P. aeruginosa*. When these nanoparticles were loaded with the antibiotic cefazolin, the Fuc-AuNPs and Gal-AuNPs had improved therapeutic efficacy against *P. aeruginosa* biofilms compared to the antibiotic alone. Moreover, exploiting the phototherapeutic properties of the AuNP core, light irradiation resulted in an enhanced therapeutic effect towards *P. aeruginosa* when combined with cefazolin. The targeted photo/chemotherapeutics Fuc-AuNP@CAZ/Gal-AuNP@CAZ were able to eradicate *P. aeruginosa* biofilm formation on glass slides and clinically relevant steel surfaces with biocompatibility fully confirmed among human cells (Zhang *et al.,* 2020). Another therapeutic application was developed based on the mannose-conjugated polymer. The polymer was designed to target the bacteria's LecB, and the polymer was synthesized using RAFT polymerization and conjugated to mannose using click chemistry. The Man-polymer contains a rhodamine monomer to allow for tracking, mannose ethyl methacrylate or galactose ethyl methacrylate carbohydrate monomers for targeting and biocompatibility, and a cargo of ciprofloxacin prodrug monomers. The fluorescence due to the copolymerized rhodamine monomer was used to measure the interaction of the Man- and Gal-polymer

with the *P. aeruginosa* biofilm. Moreover, the discovery and characterization of a new class of compounds called glycocluster tetrahydroxamic acids. The development of water-soluble calixarene-based iron-chelating glycoclusters that are designed to interfere with the iron uptake process and inhibit bacterial adhesins through glycan mimicry. By incorporating four mannose substituents and four hydroxamic acid groups, the researchers found that one of the glycoclusters, called mannocluster 2, was a strong iron chelator that disrupted the growth of the siderophore-deficient PAD07 strain and mannocluster 2 showed strong biofilm inhibition on its own and that when combined with the antibiotic tobramycin, it achieved complete biofilm eradication without any toxic effects. Furthermore, the glycoclusters were potent inhibitors of LecB. Mannocluster 2 inhibited LecB at a nanomolar IC50, which was 236 times more potent than the traditional Me-α-D-mannoside. The study revealed that the HAG-bearing linkers in the glycoclusters played a crucial role in their activity against biofilms and other bacterial adhesins (Taouai *et al.*, 2019). Overall, the potential advantage of targeting LecB in *P. aeruginosa* is that it may reduce the risk of developing resistance to antimicrobial agents, as LecB is not typically involved in the development of antibiotic resistance. Additionally, targeting LecB may provide an alternative treatment option for infections caused by *P. aeruginosa*, which can be difficult to treat due to its ability to resist many antimicrobial agents. Advances in LecB therapeutics represent an exciting area of research with the potential to improve the treatment of bacterial infections, particularly those caused by *P. aeruginosa*. Ongoing research in this field may lead to the development of effective and safe drugs that can be used to combat a wide range of bacterial infections, including those that are currently difficult to treat.

DISCUSSION

LecB is a virulence factor produced by *P. aeruginosa* that has been implicated in the pathogenesis of *P. aeruginosa* infections. The lectin is capable of binding to glycosylated molecules on host cells, such as mucins and glycolipids, allowing *P. aeruginosa* to adhere to and colonize host tissues. LecB also plays a critical role in biofilm formation, which can confer increased resistance to antibiotics and immune system defences. As such, understanding the structural and functional aspects of LecB is crucial for the development of new strategies for the prevention and treatment of *P. aeruginosa* infections. Recent studies have shed light on the mechanisms of LecB activity and its interactions with host cells. For instance, LecB has been found to bind specifically to the Lewis B blood group antigen and other glycans expressed on the surface of human epithelial cells. Crystallography has revealed the structure of LecB, revealing that the lectin adopts a homotetramer fold. Site-directed mutagenesis studies have shown that specific amino acid residues within the carbohydrate-binding site of LecB are critical for glycan recognition and binding.

The potential of LecB as a target for therapeutics against *P. aeruginosa* infections has been widely recognized, and several approaches have been explored to inhibit LecB activity. One such approach is the use of antibodies-conjugates against LecB, which can neutralize its binding activity and prevent bacterial adhesion. Small molecule inhibitors that disrupt the carbohydrate-binding activity of LecB have also been developed, such as naringenin, a flavonoid found in grapefruit that has been shown to inhibit LecB-mediated biofilm formation in vitro. Synthetic peptides that mimic the LecB-binding site on host cells have also been developed and have demonstrated inhibitory effects on LecB activity.

While these approaches hold great promise, several challenges remain in the development of LecB-targeted therapies. For instance, the potential for off-target effects of LecB inhibitors and the

development of resistance to these inhibitors are important concerns. Furthermore, the efficacy of LecB inhibitors in vivo remains to be demonstrated, particularly in the context of the complex environment of bacterial infections.

In conclusion, LecB is a critical virulence factor of *P. aeruginosa* that plays a key role in bacterial adhesion and biofilm formation. Recent studies have provided insight into the structural and functional aspects of LecB and its interactions with host cells, leading to the development of potential therapeutics targeting this lectin. The development of effective LecB inhibitors holds great promise for the prevention and treatment of *P. aeruginosa* infections, but further research is necessary to fully understand the potential of this approach.

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