*Pseudomonas aeruginosa* LecB is involved in pilus biogenesis and protease IV activity but not in adhesion to respiratory mucins

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ABSTRACT

Pseudomonas aeruginosa expresses two lectins which are implicated in adhesion and biofilm formation. In this study, we demonstrate that P. aeruginosa lecB is involved in pilus biogenesis and proteolytic activity. Moreover, neither lectin was involved in adhesion to human tracheobronchial mucin. We infer that some of the ascribed functions are secondary effects on other systems rather than effects of the lectins themselves.
Lectins are sugar-binding proteins that presumably bind to carbohydrates located on cell surfaces and possibly to secreted glycoproteins. They are commonly found in plants, animals, and bacteria, and are thought to play roles in bacterial infections. For example, some bacterial lectins have been suggested to mediate bacterial adhesion to mammalian cells, respiratory mucins in cystic fibrosis (CF), and in cell-cell interactions (5, 9, 10, 29).

The opportunistic pathogen *Pseudomonas aeruginosa* synthesizes two lectins, LecA and LecB (also known as PA-IL and PA-IIL), having specificity for galactose and fucose, respectively. Many roles have been suggested for these lectins, including adhesion of the bacterium to airway epithelia cells and injury to cells (1, 15). Furthermore, LecB was recently reported to be involved in biofilm formation (27). This latter phenomenon has been ascribed to surface exposed LecB protein. These observations, combined with the properties of lectins, led us to hypothesize that the lectins in *P. aeruginosa* may exert some effect on surface exposed proteins. Although both *P. aeruginosa* lectins were initially thought to be located in the cytoplasm (11), later results suggested that LecB may be present on the surface of sessile *Pseudomonas* cells (20, 27).

The data on the role of these lectins in bacterial adhesion is, however, not entirely clear. To date, there have not been any studies with mutants of these lectins that demonstrate a role for these proteins in bacterial adhesion. Given that LecA is intracellular and there is some question concerning the amount of LecB on the surface of the cell, we initially approached the issue of the functions of these lectins with the hypothesis that they may function in posttranslational modification of bacterial proteins, perhaps by acting as sugar
carriers. However, our investigations have led us to some striking new conclusion on the function of LecB, the most well-studied of these two lectins.

Comparative membrane proteome analysis

LecA and lecB mutants were constructed by insertional inactivation of the P. aeruginosa PAK lecA and lecB genes with gentamicin resistance gene cassettes. The insertions were verified by sequencing the genes and Southern blotting. The bacterial strains and plasmids used in this study are listed in Table 1. This study was initially designed to ascertain whether there were any differences in the expression of any of the membrane proteins of the mutants as a result of the mutations. Membrane proteins were chosen because the implied actions of the lectins were all surface related phenomena e.g. adhesion and biofilm formation. Membrane proteins were isolated from the wild-type strain PAK and its isogenic lec mutants as described previously (12) and were subjected to two-dimensional differential fluorescence gel electrophoresis (2D-DIGE). The preparations were labeled with fluorescent Cy3 and Cy5 dyes according to the supplier’s instructions (Amersham Biosciences). Labeled samples were mixed together and run on the same isoelectric focusing (IEF) and SDS-PAGE gels so that the same protein labeled with a CyDye migrates to the same position on the 2-D gel. Image analysis of 2-D gels by Phoretix™ software (Nonlinear Dynamics, UK) revealed that one protein spot identified as PilJ by QSTAR-mass spectrometry was 6-fold less in the lecB mutant (Fig. 1), however, in the lecA mutant no difference in the protein expression profile was observed. PilJ is a member of an operon containing pilG-L. To determine if the observed differential expression of PilJ is regulated at the transcriptional level, we generated a
transcriptional \( \text{pilG::lacZ} \) promoter fusion construct and inserted it into \( P. \ aeruginosa \) strain PAK and its \( \text{lecB} \) mutant. \( \beta \)-galactosidase assays were performed as described by Miller (17). No significant difference in the transcriptional activity was observed (data not shown) suggesting that the effect seen on the expression of PilJ in the 2-D studies takes place at the post-transcriptional level. Neither lectin was detected in the wild-type membrane preparations used for proteomic studies, suggesting that they may be expressed at very low levels or are not found in significant amounts in the outer membranes. Since there was a difference in the expression of the PilJ protein in the \( \text{lecB} \) mutant, the wild-type protein was subjected to gas chromatography-mass spectrometry and liquid-chromatography-mass spectrometry at the Complex Carbohydrate Research Center, (Athens, Georgia) to examine whether it was glycosylated. No carbohydrates were found attached to this protein.

**LecB mutant was defective in twitching motility**

In \( P. \ aeruginosa \), twitching motility is mediated by type IV pili and is controlled by a complex chemosensory pathway comprised of the proteins PilG, PilH, PilI, PilJ, PilK, ChpA, ChpB and ChpC (30). Kearns et. al. (13) had observed that a \( P. \ aeruginosa \) \( \text{pilJ} \) mutant was deficient in twitching motility and expressed no type IV pili. Therefore, the twitching motility phenotype of the mutants was examined via the subsurface stab assay. Twitching assays demonstrated that the wild-type strain PAK and the \( \text{lecA} \) mutant twitched normally, whereas the \( \text{lecB} \) mutant was non-twitching. This defect could be complemented by providing an intact copy of the \( \text{lecB} \) gene cloned into the low-copy
broad host range plasmid pMMB67EH (8), indicating that inactivation of lecB was responsible for the loss of twitching motility.

To further characterize the defect in twitching motility, Western blot analysis was performed to determine the levels of PilA, the major pilus subunit, in the lecB mutant. The mutant produced wild-type levels of intracellular PilA (Fig. 2A). However, transmission electron microscopic examination of the lecB mutant and the complemented lecB mutant demonstrated that lecB was involved in pilus biogenesis (Fig. 2B) in the mutant lacked pili. Thus, the twitching defect in the lecB mutant is not due to a transcriptional change in pilJ or pilA synthesis but most likely due to an inability to assemble surface pili.

Impairment in biofilm formation is due to lack of pilus synthesis

LecB, flagellar, and type IV pilus mutants of P. aeruginosa have been shown to be impaired in biofilm formation (14, 21, 27). We, therefore, compared the ability of the P. aeruginosa wild-type strain, lecA and lecB mutants and a non-piliated PAK strain (PAK-NP, pilA-) to form biofilms (22). The behavior of the lecB mutant was identical to that of the pilA mutant. As shown in Fig. 3, more than 50% defect in biofilm formation was observed compared to the wild-type strain. The complemented lecB mutant formed a biofilm comparable to the wild-type strain. The results are expressed as the means ± standard deviations (SD) of three independent experiments. Student’s t test was used to compare mean values. Differences were considered significant when P values were <0.05. It was proposed that LecB may facilitate cell to cell interaction since purified
LecB binds to the surface of *P. aeruginosa* (27). The LecB protein was recently reported to be present in the outer membrane fraction (27), which would support such a role, but whether the protein is localized on the periplasmic face or the external surface of the outer membrane is unclear. With this new information that pili are not assembled in the *lecB* mutant, the most likely explanation for the defect in biofilm formation may be the absence of pili in this mutant.

**Role of LecB in protease(s) activity**

*P. aeruginosa* is a prolific exporter of virulence factors. Its genome harbors genes for the six known protein secretion systems that have been described in Gram-negative bacteria (26). Since the *lecB* mutant was defective in type IV secretion, we examined whether the mutants also had defects in other secretion systems. In *P. aeruginosa*, the type II secretory pathway is used to export the largest number of proteins, including phospholipase C, alkaline protease, exotoxin A, LasA and LasB (elastase) (3, 4, 16, 19, 23). We examined the proteolytic activities of *lecA* and *lecB* mutants by growing them on Casein-Milk agar plates. In *P. aeruginosa*, caseinolytic activity is due mainly to the actions of LasB, alkaline protease and protease IV (6). After 36 h, wild-type strain, the *lecA* mutant and the complemented *lecB* mutant produced a proteolytic zone, while a very weak zone was observed in the *lecB* mutant (Fig. 4A). We therefore examined the levels of LasB in the LecB mutant by immunoblot using antibody against LasB protein. Both the intracellular pool as well as secreted LasB were unaltered in the mutant (Fig. 4B), suggesting that the reduced caseinolytic activity is not due to a LasB defect, but either to alkaline protease or protease IV. Alkaline protease is a metallo-protease whose activity is
inhibited by EDTA (6). Therefore, to eliminate alkaline protease, we took advantage of
the specific property of protease IV to cleave lactoferrin in the presence of EDTA (31).
Supernatants from overnight cultures of the wild-type, lecB mutant and complemented
lecB mutant were concentrated 125 fold and lactoferrin degradation by these supernatants
was examined as described by Wilderman et. al. (31) in the presence of EDTA.
Degradation products were then analyzed by SDS-PAGE. As shown in Fig. 4C, the lecB
mutant did not exhibit any lactoferrin degrading activity, whereas hydrolysis of the
substrate was observed with the wild-type and the complemented lecB mutant. Since
neither the secretion nor the size of LasB is affected in the lecB mutant we presume that
the defect in caseinolytic activity is probably due to modulation of protease IV activity.
Notably, SDS-PAGE analysis of the secreted proteins showed that in lecB mutant many
extracellular proteins were absent.

In addition, the mutants were assayed for the phospholipase and exotoxinA secretion but
found to be unaffected. Furthermore, no effect on the secretion of ExoS and ExoT, which
are secreted through type III secretion system (7, 32), was observed in the lectin mutants.

\textit{P. aeruginosa} lectins are not involved in adhesion to respiratory mucins.

\textit{Pseudomonas} lectins have been suggested but not demonstrated to be involved in
adhesion to respiratory mucins in cystic fibrosis (18). In order to examine this, we
performed mucin binding assays to CF mucins as described previously (24). A \textit{P. aeruginosa}
mutant lacking pili (PAK-NP) attaches to mucins as efficiently as wild-type
strain (24), whereas a PAK-NP mutant lacking functional \textit{fliD} (PAK-NPD), which
encodes the flagellar cap protein, was severely impaired in mucin adhesion. (2). These
strains were used as controls. As shown in Fig. 5, both lectin mutants were unimpaired in
their ability to bind to CF mucin when compared to wild-type strain, whereas the PAK-
NPD mutant showed a marked defect. Respiratory mucins contain galactose and fucose
among a number of sugars, therefore, one would predict that if there is surface expression
of these lectins, then mucin binding may be a prominent function. This was not
demonstrable in these studies.

In summary, our results indicate that *P. aeruginosa* LecB is involved in multiple
functions. It affects the expression of PilJ, a member of the pilus biogenesis pathway,
which in turn affects pilus formation. Its role in pilus biogenesis would explain its role in
biofilm formation in vitro. The studies also demonstrate a role for LecB in the the
proteolytic activity of *P. aeruginosa*. Given that this protein affects both type II and type
IV secretion processes, one can assume that the action may be at the level where these
two secretion pathways intersect. In addition, we have shown that neither lectin is
involved in mucin adhesion. Thus, the exact place and role of these interesting lectins in
cell physiology remains unsolved.

**ACKNOWLEDGMENTS**

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REFERENCES


FIGURE LEGENDS

Figure 1. Proteomic profile of the outer membranes of *P. aeruginosa* PAK and its lecB mutant using a 2-D differential fluorescence gel electrophoresis system. Membrane extracts from the wild-type PAK and the lecB mutant were subjected to two-dimensional gel electrophoresis. The normal protein spot was denoted by closed circle, whereas the corresponding down-regulated protein in the lecB mutant is denoted by open circle.

Figure 2. A, Western blot analysis of PilA production by PAK, lecA and lecB mutants. PilA was detected with anti-PilA antibody and is indicative of intracellular pilin in these strains. B, Transmission electron microscopy of a, PAK; b, lecB mutant; and c, the complemented lecB mutant (lecB\(^+\)). Cells were coated on carbon grids, stained with 1% phosphotungstic acid, washed and dried. The mounted bacteria were viewed with a Hitachi H-7000 transmission electron microscope.

Figure 3. Quantification of biofilm formation by the PAK, lecA mutant, lecB mutant, complemented lecB mutant (lecB\(^+\)) and PAK-NP strains in a crystal violet microtiter plate assay after 24 h. Absorbance was measured at 600 nm. Error bars denote standard deviations. The means ± SD were determined from at least three independent experiments. The difference between the parent strain and lecB mutant was statistically significant (p<0.05).

Figure 4. A, Caseinolytic activity of PAK, lecA mutant, lecB mutant, and the complemented lecB mutant (lecB\(^+\)). The bacterial strains were grown on Milk Casein
agar plates. Appearance of clear zone was observed after 36 h. B, Western blot analysis of elastase (LasB) production by wild-type PAK, lecA and lecB mutants. LasB was detected with anti-LasB antibody and is indicative of levels of elastase in these strains. C, Lactoferrin degradation by wild-type PAK, lecB mutant and the complemented lecB mutant. Lactoferrin (1 μg) was incubated with concentrated supernatants of the bacteria at 37 °C for 1h. Metallo-proteases were inhibited by adding EDTA to the supernatants. Digestion products were analyzed by SDS-PAGE and gels stained with Coomassie blue. Molecular weight markers are shown with numbers. Lane 1, lactoferrin (~90 kDa); lane 2, wild-type PAK; lane 3, lecB mutant; lane 4, lecB complemented.

Figure 5. Adhesion of PAK, lecA mutant, lecB mutant, PAK-NP (PAK non-piliated mutant) and PAK-NPD (PAK-NP fliD mutant) to human tracheobroncial mucin. Error bars denote standard deviations. The means ± SD were determined from at least three independent experiments. Only the NPD mutant was significantly different from the wild type strain (p<0.001)
Table 1. Bacterial strains, plasmids, and primers used in this study

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<th>Strains, plasmids or primers</th>
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<td>Promoterless lacZ oriV oriT TeR StrR Ω</td>
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a GmR, gentamicin resistance; AmpR, ampicillin resistance; CarbR, carbenicillin resistance; TeR, tetracycline resistance; StrR, streptomycin resistance. In primer sequences, lower case denotes
nucleotides added to facilitate restriction digestion at the marked sites in bold. Nucleotides added
to create a restriction site are highlighted and underlined.
Figure 1
Figure 2
Figure 3
Figure 4.
Figure 5