Pseudomonas aeruginosa lectin LecB is located in the outer membrane and is involved in biofilm formation

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Pseudomonas aeruginosa is an opportunistic pathogen which causes a variety of diseases, including respiratory tract infections in patients suffering from cystic fibrosis. Therapeutic treatment of P. aeruginosa infections is still very difficult because the bacteria exhibit high intrinsic resistance against a variety of different antibiotics and, in addition, form stable biofilms, e.g. in the human lung. Several virulence factors are produced by P. aeruginosa, among them the two lectins LecA and LecB, which exert different cytotoxic effects on respiratory epithelial cells and presumably facilitate bacterial adhesion to the airway mucosa. Here, the physiology has been studied of the lectin LecB, which binds specifically to L-fucose. A LecB-deficient P. aeruginosa mutant was shown to be impaired in biofilm formation when compared with the wild-type strain, suggesting an important role for LecB in this process. This result prompted an investigation of the subcellular localization of LecB by cell fractionation and subsequent immunoblotting. The results show that LecB is abundantly present in the bacterial outer-membrane fraction. It is further demonstrated that LecB could be released specifically by treatment of the outer-membrane fraction with p-nitrophenyl α-L-fucose, whereas treatment with D-galactose had no effect. In contrast, a LecB protein carrying the mutation D104A, which results in a defective sugar-binding site, was no longer detectable in the membrane fraction, suggesting that LecB binds to specific carbohydrate ligands located at the bacterial cell surface. Staining of biofilm cells using fluorescently labelled LecB confirmed the presence of these ligands.

INTRODUCTION

Lectins represent a specific class of carbohydrate-binding proteins different from enzymes or antibodies (Barondes et al., 1988). They are found in a wide range of organisms including viruses, bacteria, plants and animals, and are believed to play an important role in cell–cell interactions (Gabius et al., 2002). Specific recognition of or attachment to target cells was demonstrated for the mannose-specific lectin FimH from Escherichia coli, which mediates adhesion between the bacterium and the urothelium (Beachey, 1981). Furthermore, lectins may have significant biotechnological and medical potential, as exemplified by the galactoside-specific mistletoe lectin, which is used on a large scale to support anti-cancer therapy (Beuth et al., 1995).

Pseudomonas aeruginosa, an important opportunistic pathogen associated with chronic airway infections, synthesizes two lectins, LecA and LecB (formerly respectively named PA-IL and PA-IIL) (Gilboa-Garber, 1982). Strains of P. aeruginosa that produce high levels of these virulence factors exhibit an increased virulence potential (Gilboa-Garber & Garber, 1992). The lectins play a prominent role in human infections, as it was demonstrated that a P.
P. aeruginosa-induced otitis externa diffusa (Steuer et al., 1993) as well as respiratory tract infections (von Bismarck et al., 2001) could successfully be treated by application of a solution containing LecA- and LecB-specific sugars. The sugar solutions presumably prevented lectin-mediated bacterial adhesion to the corresponding host tissue. The expression of lectin genes in P. aeruginosa is coordinately regulated with certain other virulence factors and controlled via quorum sensing and by the alternative sigma factor RpoS (Winzer et al., 2000).

The galactophilic LecA has been characterized in great detail over the last 30 years. It consists of four 12-75 kDa subunits (Gilboa-Garber, 1972; Avichezer et al., 1992) and has been shown to cause cytotoxic effects on respiratory epithelial cells by decreasing their growth rate, thus contributing to respiratory epithelial injury during P. aeruginosa infection (Bajolet-Laudinat et al., 1994). In addition, it was demonstrated that LecA induces a permeability defect in the intestinal epithelium, resulting in increased absorption of exotoxin A, an important extracellular virulence factor of P. aeruginosa (Laughlin et al., 2000).

LecB consists of four 11-73 kDa subunits, each exhibiting a high specificity for L-fucose and its derivatives (Gilboa-Garber et al., 2000; Garber et al., 1987) and also for D-mannose, with lower affinity. Its crystal structure was determined recently (Mitchell et al., 2002, 2005; Loris et al., 2003). In cystic fibrosis (CF), increased terminal fucosylation of airway epithelial glycoproteins is found, as well as a higher percentage of sialylated and sulfated oligosaccharides in Lewis A oligosaccharide side chains, which presumably represent preferential ligands for LecB (Mitchell et al., 2002), which thereby contributes significantly to chronic respiratory Pseudomonas infections (Scanlin & Glick, 2001). In addition, LecB decreases in vitro the ciliary beat frequency of the airway epithelium, hence inhibiting an important defence mechanism of the human lung (Adam et al., 1997a, b).

Both P. aeruginosa lectins were shown to be located mainly in the cytoplasm of planktonic cells (Glick & Garber, 1983). These findings make it difficult to explain the lectin-mediated cytotoxic and adhesive properties. Recently, it was suggested that LecB is exposed on the surface of sessile Pseudomonas cells, since the addition of L-fucose-branched chitosan leads to specific cell aggregation (Morimoto et al., 2001).

Biofilms are accumulations of micro-organisms at solid-liquid or solid-air interfaces, where the cells are embedded in a matrix of self-produced extracellular polymeric substances, with polysaccharides and proteins as characteristic components (Wingender et al., 1999). Exopolysaccharides are considered as key components which determine the structural and functional integrity of microbial biofilm aggregates by the formation of a three-dimensional, gel-like, highly hydrated and locally charged biofilm matrix, in which the micro-organisms are immobilized. Also, binding of biofilm cells (cohesion) and anchoring of biofilms to the substratum (adhesion) are mediated by exopolysaccharides. Consequently, extracellular proteins with lectin-like functions may contribute to polymer network formation in biofilms (Higgins & Novak, 1997; Imberty et al., 2004). The formation of P. aeruginosa biofilms on tissues of infected patients as well as on medical devices was recently shown to be responsible for the inherent resistance of the bacterium to certain antibiotics and other various antimicrobial agents (Costerton et al., 1999; Stewart & Costerton, 2001). Since cell-surface-exposed oligosaccharide receptors on eukaryotic host cells are ubiquitous in nature, it is reasonable to assume that both lectins are involved in the attachment of cells to biotic surfaces. Moreover, lectin-mediated cell–cell interactions within a given bacterial population may be involved in the development and maintenance of biofilms, thereby determining the ability of P. aeruginosa to colonize biotic and abiotic surfaces and to persist in the biofilm mode of growth (Parsek & Greenberg, 2000; Imberty et al., 2004).

In this paper, we report on the role of LecB in biofilm formation and its cellular localization in planktonic and sessile P. aeruginosa cells. Confocal laser scanning microscopy (CLSM) was used to monitor biofilm formation of a LecB-deficient mutant strain and the localization of LecB in sessile cells was investigated by fractionation of bacterial cells grown as a biofilm and determination of LecB localization in the different cellular compartments using a LecB-specific polyclonal antiserum. The interaction of LecB with the outer membrane of P. aeruginosa was investigated by constructing a LecB-mutant defective in sugar binding as suggested by analysis of the LecB crystal structure we have recently solved (Loris et al., 2003). The presence of putative ligands on the surface of biofilm cells was visualized by cell staining using fluorescently labelled LecB.

**METHODS**

**Bacterial strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. E. coli DH5α was used for cloning experiments and E. coli BL21(DE3) as a heterologous expression host for plasmid-encoded LecB. E. coli S17-1 was used for conjugation transfer.

**Media and growth conditions.** Precultures for all experiments were prepared overnight in 5 ml Luria–Bertani (LB) medium in glass tubes at 37 °C. Plasmid-carrying E. coli cells were selected with 50 μg chloramphenicol ml⁻¹, 100 μg ampicillin ml⁻¹, 10 μg tetracycline ml⁻¹ and/or 20 μg gentamicin ml⁻¹. In the case of plasmid- or cassette-carrying P. aeruginosa strains, 300 μg chloramphenicol ml⁻¹, 100 μg tetracycline ml⁻¹ and/or 50 μg gentamicin ml⁻¹ were added.

**Cultivation of biofilms.** Biofilms were grown under static conditions on glass slides. Fifty millilitres nutrient broth (NB) [8 g nutrient broth (Oxoid); 4 g NaCl l⁻¹] in a sterile Petri dish was inoculated to an OD₅₈₀ of 0.05 from overnight cultures grown in NB, glass slides were submerged and the cultures were incubated for 48 or 72 h at 30 °C. Prior to CLSM analysis, glass slides were rinsed with 2 ml 0-14 M NaCl to remove unadsorbed cells. For staining of P. aeruginosa PA01 cells with fluorescently labelled LecB, biofilms were grown on membrane filters (Strathmann et al., 2002). Bacteria...
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/phenotype</th>
<th>Reference/source</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>P. aeruginosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Wild-type</td>
<td>Holloway et al. (1979)</td>
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<tr>
<td>SG81</td>
<td>Mucoid biofilm isolate from technical water system</td>
<td>Grobe et al. (1995)</td>
</tr>
<tr>
<td>PAT2</td>
<td>LecB mutant strain derived from PAO1. lecB::Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>PAT4</td>
<td>LecB mutant strain carrying a chromosomal deletion derived from PAT2</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
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<tr>
<td>DH5α</td>
<td>supE44 ΔlaczZYA-argF)U196 (p80MacZM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>Woodcock et al. (1989)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; ompT hsdS&lt;sub&gt;B&lt;/sub&gt;(r&lt;sub&gt;B&lt;/sub&gt; m&lt;sub&gt;B&lt;/sub&gt;) gal dcm (lecT857 ind1 Sam7 nin5 lacUV5-T7 gene1)</td>
<td>Studier &amp; Moffat (1986)</td>
</tr>
<tr>
<td>S17-1</td>
<td>Ec294::(RP4-2 (Tc::Mu) (Km::Tn7)), pro, res, repA, tra&lt;sup&gt;+&lt;/sup&gt;, Tp&lt;sup&gt;+&lt;/sup&gt;, Sm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Simon et al. (1986)</td>
</tr>
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<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pET22b</td>
<td>T7 expression vector for E. coli, PeIB signal sequence, Ap&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Novagen</td>
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<td>pBCSK</td>
<td>P&lt;sub&gt;73&lt;/sub&gt;, P&lt;sub&gt;T7&lt;/sub&gt;, lacz&lt;sup&gt;+&lt;/sup&gt;, ColE1</td>
<td>Stratagene</td>
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<tr>
<td>pBSL141</td>
<td>ColE1 Ap&lt;sup&gt;+&lt;/sup&gt; Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Alexeyev et al. (1995)</td>
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<tr>
<td>pSUP202</td>
<td>pBR325, Ap&lt;sup&gt;+&lt;/sup&gt; Gm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; mob</td>
<td>Simon et al. (1983)</td>
</tr>
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<td>pEC2</td>
<td>pET22b containing the 345 bp Nd/el/BamHI PCR product bearing the lecB gene</td>
<td>Loris et al. (2003)</td>
</tr>
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<td>pELY2</td>
<td>pET22b containing yfp::lecB fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pEXCH2</td>
<td>pET22b containing the 345 bp Nd/el/BamHI PCR product bearing the lecB&lt;sup&gt;+&lt;/sup&gt; gene</td>
<td>This study</td>
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<tr>
<td>pBRR1MCS</td>
<td>lacZs CM&lt;sup&gt;+&lt;/sup&gt; rep mob</td>
<td>Kovach et al. (1994)</td>
</tr>
<tr>
<td>pBBC2</td>
<td>pBRR1MCS containing a 398 bp Xbal/SacI fragment with lecB derived from pEC2</td>
<td>This study</td>
</tr>
<tr>
<td>pBBXCH2</td>
<td>pBRR1MCS containing a 345 bp Xbal/SacI fragment encoding lecB&lt;sup&gt;+&lt;/sup&gt; derived from pEXCH2</td>
<td>This study</td>
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<td>pL2US</td>
<td>515 bp Xbal/BamHI fragment upstream of lecB in pBCSK</td>
<td>This study</td>
</tr>
<tr>
<td>pL2DS</td>
<td>616 bp BamHI/HindIII fragment downstream of lecB in pBCSK</td>
<td>This study</td>
</tr>
<tr>
<td>pL2UG</td>
<td>1–6 kb MluI fragment derived from pBSL141 (Ω-Gm&lt;sup&gt;+&lt;/sup&gt; cassette) in pL2US</td>
<td>This study</td>
</tr>
<tr>
<td>pUGD2</td>
<td>2·1 kb Xbal/BamHI fragment derived from pL2UG in pL2DS</td>
<td>This study</td>
</tr>
<tr>
<td>pSUGD2</td>
<td>2·6 kb Xbal/HindIII fragment derived from pUGD2 (ΔlecB::Ω-Gm&lt;sup&gt;+&lt;/sup&gt; cassette) in pSUP202</td>
<td>This study</td>
</tr>
<tr>
<td>pSUD2</td>
<td>pSUGD2 without Ω-Gm&lt;sup&gt;+&lt;/sup&gt; cassette</td>
<td>This study</td>
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<tr>
<td>pFF19-EYFP</td>
<td>Plant expression vector carrying yfp</td>
<td>Timmermans et al. (1990)</td>
</tr>
</tbody>
</table>

were grown for 24 h at 37 °C on Pseudomonas isolation agar (PIA; Difco) containing 2% glycerol. Single colonies were suspended in 0·14 M NaCl to a concentration of approximately 10<sup>8</sup> cells ml<sup>-1</sup>. Ten millilitres of this suspension was vacuum-filtered onto 25 mm black polycarbonate filters (0·4 μm pore size; Millipore), which were then placed on the surface of PIA plates and cultivated for 24 h at 37 °C.

**Lectin staining of biofilm cells.** The method was modified from Strathmann et al. (2002). Fifty microlitres of a solution containing 10 μg LechB<sub>FP</sub> ml<sup>-1</sup> and 75 μM of the red fluorescent nucleic-acid-binding dye SYTO 9 (Molecular Probes) as described by the manufacturer. Images of biofilms were obtained with a confocal laser scanning microscope (LSM 510; Zeiss) using an LD Achromplan 40x/0·60 NA (corr.) lens. Three-dimensional image stacks were recorded at 488 nm excitation wavelength using an LP 505 nm long-pass detection filter. Confocal images were recorded from at least three fields of view for each sample. The extent of the analysis was done in order to fulfill the minimum sampling area requirement as suggested by Korber et al. (1993) for biofilm samples. The pinhole size was adjusted to equal 1·0 Airy unit. Image recording of CLSM optical thin sections was performed with the Zeiss LSM software (version 2·8). Biofilm mean thickness, surface coverage, roughness and biomass were determined by digital image processing using COMSTAT image-analysis software (Heydorn et al., 2000). Images of the biofilms on polycarbonate filters were recorded by CLSM as described above at 488 nm excitation wavelength using a BP 505–550 nm band-pass detection filter and at 633 nm excitation wavelength using an LP 650 nm long-pass detection filter for LechB<sub>FP</sub> and SYTO 62, respectively.

**DNA manipulation and PCR.** Recombinant DNA techniques were performed as described by Sambrook et al. (1989). DNA fragments were amplified by standard PCR methods. DNA-modifying enzymes (Fermentas) were used according to the manufacturer’s instructions. Plasmid DNA was prepared as described by Birnboim & Doly (1979) and by using the HiSpeed plasmid purification midi kit or, for genomic DNA from P. aeruginosa, the DNeasy Tissue kit (Qiagen).

**Construction of a P. aeruginosa PAO1 lecB-deletion mutant.** A P. aeruginosa lecB mutant was constructed by PCR amplification
of the regions located upstream of the lecb gene were amplified with primers L2USA (5'-GGGATCACGCGTGATTCTGTCAGTTCGTTTCGAGGAGGGATT) and L2USB (5'-GGGATCACGCGTGATTCTGTCAGTTCGTTTCGAGGAGGGATT). The amplified DNA fragment was cloned into the SacI site (underlined). This fragment was digested with XbaI and ligated into pET22b, resulting in plasmid pEC2, which was transformed into E. coli DH5α. The overexpression of lecb was studied using the lecb-negative strain P. aeruginosa PATI2, which respectively represented the inner and outer membrane fractions. For immunodetection of LecB, 100 μl of the outer-membrane fractions was diluted to 2 ml with 10 mM Tris/HCl, pH 8. Proteins were collected by precipitation with trichloroacetic acid (Sivaraman et al., 1997).

Isolation of inner and outer membranes. This was done as described by Wilhelm et al. (1999). P. aeruginosa PATI2 cells containing plasmid pBBC2 (50 mg dry weight) were isolated after growth for 48 h from NB agar plates by resuspension in 20 ml 0.14 M NaCl. Cells were incubated for 30 min at 37 °C with 1 mg lysozyme, disrupted using a glass bead mill followed by three freezing and thawing cycles and intact cells were separated from the cell extract by centrifugation at 5000 g for 10 min. The supernatant was centrifuged at 13 000 g for 1 h. The pellet, consisting of the total membrane fraction, was resuspended in 1 ml 10 mM Tris/HCl, pH 8, containing 1 mM EDTA and 30 % (w/v) sucrose, and layered on the top of a discontinuous gradient prepared by combining sucrose solutions of the following concentrations: 0-5 ml 60 %, 2-1 ml 55 %, 2-1 ml 50 %, 2-1 ml 45 %, 2-1 ml 40 % and 2-1 ml 35 % (w/v) sucrose in 10 mM Tris/HCl, pH 8, 1 mM EDTA. The gradient was centrifuged using an SW41 rotor in an L8-70 ultracentrifuge (Beckman) at 36 000 r.p.m. and 4 °C for 36 h, and 0.5 ml fractions were subsequently removed from the centrifugation tube.

Enzyme assays. These were described by Wilhelm et al. (1999). NADH oxidase and esterase activities were determined as marker enzymes for the inner and outer membrane, respectively, by spectrophotometric determination of the absorbance decrease at 340 nm for NADH oxidase and the absorbance increase at 410 nm for esterase. For NADH oxidase, 900 μl reaction mixture (50 mM Tris/HCl, pH 7.5, 0-2 mM DTT, 0-12 mM NADH) was incubated for 5 min at 25 °C with 100 μl of each fraction. For esterase activity, 23-7 mg p-nitrophenyl caproate (pNPC; Sigma) was dissolved in 5 ml ethanol and added to 95 ml 100 mM potassium phosphate buffer, pH 7, supplemented with 10 mM MgSO₄, to yield a final pNPC concentration of 1 mM. Aliquots (20 μl) of each fraction were added to 180 μl substrate solution in a microtitre plate, the samples were incubated for 10 min at 25 °C and the A₅₀₄ was recorded.

The fractions with the highest NADH oxidase and esterase activities, which respectively represented the inner and outer membrane fractions, were pooled. For immunodetection of LecB, 100 μl of these fractions was diluted to 2 ml with 10 mM Tris/HCl, pH 8. Proteins were collected by precipitation with trichloroacetic acid (Sivaraman et al., 1997).

Washing the outer membrane with solutions of different carbohydrates. Proteins from 100 μl of the outer-membrane fraction were collected by centrifugation at 13 000 g for 1 h. The pellet was suspended in 100 μl 100 mM Tris/HCl, pH 8, containing 20 mM p-nitrophenyl α-L-fucose (pNPC; Sigma) or 20 mM D-galactose (Sigma) as a negative control, and shaken gently for 1 h at 37 °C. After centrifugation of the samples, the proteins in pellets and supernatants were analysed by immunoblotting using LecB-specific antibodies.
Western blotting. Prior to electrophoresis, samples were suspended in SDS-PAGE sample buffer, boiled for 5 min at 95 °C and loaded onto an SDS-16% polyacrylamide gel followed by electrophoretic protein transfer at 0.3 A for 30 min to PVDF membranes. LecB was detected using the polyclonal antibody at a dilution of 1:20000 in TBST (25 mM Tris/HCl, pH 8, 150 mM NaCl, 3 mM KCl, 0.2% v/v Tween 20) followed by an anti-rabbit immunoglobulin G–horseradish peroxidase conjugate (Bio-Rad) and developed with the ECL kit (Pharmacia).

RESULTS

A LecB-negative mutant is impaired in biofilm formation

The biofilm-forming capacities of the P. aeruginosa wild-type strain PAO1 and the LecB-deficient mutant PATI2 were investigated by growing static biofilms in NB medium on glass slides over growth periods of 48 and 72 h at 30 and 37 °C. Regular examination of the glass slides by CLSM revealed that the LecB-mutant P. aeruginosa PATI2 was affected in biofilm formation at both temperatures. The biofilms of the mutant strain were found to be thinner than those formed by the wild-type (Fig. 1; Table 2). Furthermore, the surface coverage of the mutant strain was lower than that of the wild-type. This effect was not caused by a general growth defect, as planktonic populations of the wild-type and the LecB-negative mutant exhibited the same growth rates and reached the same maximal cell densities (data not shown).

LecB is associated with the outer membrane

The finding that the LecB-deficient P. aeruginosa strain was impaired in biofilm formation prompted us to determine the subcellular localization of LecB. For this, planktonic cells were compared with sessile P. aeruginosa PATI2 cells, which harboured the plasmid pBBC2 containing the wild-type lecb gene under transcriptional control of the constitutive lac promoter and had been grown as an unsaturated biofilm on the surface of NB agar. This method has been shown to result in the formation of unsaturated biofilms (Steinberger et al., 2002) of the type that is also found in the lungs of CF patients suffering from a P. aeruginosa infection (Lyczak et al., 2002). Cellular compartments were isolated using differential cell fractionation and proteins were separated in SDS-PAGE sample buffer and analysed for the presence of mutant LecB in comparison with the wild-type. Both strains produced LecB in the cytoplasm as well as in the total membrane fraction in both sessile and planktonic cells (Fig. 2).

The precise localization of LecB was investigated by separating the inner and the outer bacterial membrane of sessile P. aeruginosa cells by discontinuous sucrose-density centrifugation. Activities of the marker enzymes NADH oxidase and esterase were determined to identify inner- and outer-membrane fractions, respectively. The fractions showing the highest enzyme activities were pooled and analysed by SDS-PAGE and Western blotting, revealing that LecB was located exclusively in the bacterial outer membrane (Fig. 3).

LecB binds to carbohydrate ligands in the outer membrane

The localization of LecB in the outer membrane of P. aeruginosa and its high affinity for L-fucose and its derivatives, like pNPF (Garber et al., 1987), suggested that this lectin might be bound to the outer membrane via fucose-containing structures. This assumption was tested by incubating an isolated outer-membrane fraction with 20 mM of either pNPF or D-galactose, which served as a negative control because the affinity of LecB for D-galactose is extremely low (Garber et al., 1987). The outer membrane was isolated by centrifugation and the proteins of the membrane-containing pellet and the supernatant were subjected to SDS-PAGE and Western blotting. LecB was detected in the supernatant obtained from the pNPF-treated outer-membrane fraction, whereas it was missing in the corresponding pellet (Fig. 4). In contrast, treatment with D-galactose did not result in any detectable release of LecB from the outer membrane. These findings strongly suggest that LecB is bound to the outer membrane by interaction with fucose-containing ligands.

The outer-membrane localization of LecB depends on an intact sugar-binding site

LecB was located in the outer membrane at an advanced stage of biofilm formation (Fig. 3). Treatment of the outer-membrane fraction with pNPF resulted in the release of LecB, suggesting that this lectin interacts with fucose-containing receptors at the cell surface (Fig. 4). Analysis of the LecB crystal structure we have recently solved (Loris et al., 2003) allowed us to identify those amino acid residues which are involved in carbohydrate binding. Among them, D104 plays a crucial role by coordinating two calcium atoms which themselves are directly involved in carbohydrate binding. Therefore, we have constructed plasmid pBBXCH2, which expresses a mutated LecB carrying the substitution D104A, which renders this protein unable to bind carbohydrates (Fig. 5a). This plasmid was introduced into the LecB-deficient P. aeruginosa strain PATI4. After growth for 48 h on NB agar plates, cells were fractionated and analysed for the presence of mutant LecB in comparison with the wild-type. Both strains produced LecB in the cytoplasm, but, in contrast to the wild-type lectin, the mutated LecB was not detected in the membrane fraction (Fig. 5b), indicating that the sugar-binding capacity of LecB may be essential for its outer-membrane localization.

LecB specifically binds to the surface of biofilm cells

Our results clearly indicated that LecB interacted with the outer membrane via binding to carbohydrate ligands. The presence of these ligands on the surface of living biofilm cells was investigated by growing P. aeruginosa PAO1 on
membrane filters for 24 h at 37 °C on PIA plates. After treatment for 1 h with a staining solution containing a fluorescently labelled LecB (green) and the DNA-binding dye SYTO 62 (red), biofilms were washed and analysed by CLSM. The intense fluorescent signal covering the cell periphery shown in Fig. 6(a, c) demonstrates the binding of LecBYFP to the surface of the red-stained cells. This binding was even more pronounced when the mucoid *P. aeruginosa* strain SG81 was used (Fig. 6b, d). Preincubation of LecBYFP with L-fucose prior to cell staining inhibited this interaction of the lectin with the cell surface (Fig. 6e, f). These results clearly showed that carbohydrate receptors on the surface of *P. aeruginosa* biofilm cells were accessible to LecB.

**DISCUSSION**

*P. aeruginosa* is the major pathogen in the respiratory tract of patients suffering from CF. The treatment of these chronic *P. aeruginosa* airway infections is thwarted by its innate antibiotic resistance, which is aggravated by

<table>
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<tr>
<th>Strain</th>
<th>Growth period (h)</th>
<th>Biomass (μm³ μm⁻²)</th>
<th>Mean thickness (μm)</th>
<th>Surface coverage (%)</th>
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<tr>
<td>Wild-type</td>
<td>48</td>
<td>4.14</td>
<td>8.19</td>
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<td></td>
<td>72</td>
<td>18.35</td>
<td>27.38</td>
<td>55.5</td>
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<tr>
<td>LecB⁻</td>
<td>48</td>
<td>0.02</td>
<td>0.06</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.12</td>
<td>0.33</td>
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the formation of biofilms on the respiratory epithelium (Costerton et al., 1999; Singh et al., 2000). The application of drugs that inhibit or even prevent biofilm formation seems to be a promising approach (Stewart & Costerton, 2001). Therefore, the mechanisms responsible for biofilm formation have been the subject of numerous recent studies and several proteins involved in biofilm formation and development have been identified (O’Toole & Kolter, 1998; Vallet et al., 2001; Klausen et al., 2003).

Fig. 2. Subcellular localization of LecB in biofilm and planktonic cells of P. aeruginosa grown for 48 h at 37°C. Equivalent amounts of fractions obtained from the extracellular space, the periplasm, cytoplasm and the total membrane were subjected to SDS-PAGE analysis followed by immunoblotting using a LecB-specific antibody. The LecB-negative strain P. aeruginosa PATI2 and 50 ng purified LecB respectively served as negative and positive controls.

Fig. 3. Subcellular localization of LecB in biofilm cells of P. aeruginosa. A crude membrane preparation was subjected to sucrose-density-gradient centrifugation and fractions were tested for NADH oxidase and esterase activities indicating inner and outer membrane. Fractions showing the highest activities of the respective marker enzyme were pooled and used for immunodetection of LecB. LecB was detected in the inner- and outer-membrane fractions after immunoblotting using a LecB-specific antiserum. Purified LecB (50 ng) was used as a positive control.

Fig. 4. Fucose treatment specifically removes LecB from P. aeruginosa outer membranes. Outer membranes isolated from P. aeruginosa biofilm cells were incubated with 20 mM pNPF (lanes labelled fucose) or D-galactose for 1 h at 37°C. Cells were separated from supernatants by centrifugation and LecB was detected in both fractions by immunoblotting. The LecB-deficient strain P. aeruginosa PATI2 and purified LecB respectively served as negative and positive controls.

Fig. 5. The sugar-binding ability of P. aeruginosa LecB is essential for outer-membrane localization. (a) D104 was replaced by an alanine and the resulting lecB mutant gene was expressed in the LecB-deficient strain P. aeruginosa PATI4. (b) Subcellular localization of wild-type and mutant LecB in P. aeruginosa biofilm cells was performed by isolation of cellular compartments after growth for 48 h at 37°C on NB agar plates. LecB was detected by Western immunoblotting.
This study clearly demonstrates that the lectin LecB is an important factor in the development of biofilms by \textit{P. aeruginosa} for the following reasons: (i) a LecB-deficient \textit{P. aeruginosa} mutant strain was clearly impaired in the formation of biofilms and (ii) LecB has been shown to be localized mainly in the cytoplasm of planktonic cells (Glick & Garber, 1983). This finding raises the question of how an internally localized lectin can contribute to the formation of a biofilm. Biofilm cells differ fundamentally from their planktonic counterparts in a variety of physiological aspects (Costerton \textit{et al}., 1999). Therefore, we decided to investigate the subcellular localization of LecB in planktonic and sessile cells. In contrast to previous observations (Glick & Garber, 1983), our results showed that, in both cases, LecB was present in the outer membrane of \textit{P. aeruginosa}, even though the protein lacks structural characteristics of outer-membrane proteins, e.g. a terminal F residue and typical \(\beta\)-barrel-forming structures (Mitchell \textit{et al}., 2002, 2005; Loris \textit{et al}., 2003). The binding constants of LecB for L-fucose and D-mannose were determined to be \(1.5 \times 10^6 \text{M}^{-1}\) and \(3.1 \times 10^2 \text{M}^{-1}\), respectively (Garber \textit{et al}., 1987). Treatment of the outer-membrane fraction with pNPF, which has been shown to be bound by LecB with an even higher affinity than L-fucose itself (Garber \textit{et al}., 1987), caused the dissociation of LecB from the outer membrane. A mutation in the sugar-binding site of LecB resulted in a mutant LecB which no longer appeared in the membrane fraction. These results indicated that LecB is associated with the bacterial cell surface via binding to carbohydrate ligands. The presence of such putative LecB receptors on the surface of \textit{P. aeruginosa} cells was demonstrated by specific cell staining using YFP-labelled LecB. As expected, this process could be inhibited by preincubation of the lectin with L-fucose. The carbohydrate ligands may reside on either lipopolysaccharide or glycosylated cell-surface proteins, which include \textit{P. aeruginosa} pili and type A flagella (Brimer & Montie, 1998; Castric \textit{et al}., 2001). Interestingly, O-antigenic oligosaccharides of different \textit{P. aeruginosa} serotypes contain the \(\alpha\)-1-fucose derivative \(\alpha\)-1-N-acetyl fucosamine, which is also part of the trisaccharide which decorates the pili of the clinical \textit{P. aeruginosa} isolate 1244 (DiGiandomenico \textit{et al}., 2002). Additionally, it was recently demonstrated that mannos is one of the primary constituents of the extracellular polysaccharide of biofilms formed by the typically non-mucoid strain \textit{P. aeruginosa} PAO1 (Wozniak \textit{et al}., 2003). Furthermore, several other pathogenic bacteria are known to contain glycosylated cell-surface proteins, among them the Gram-positive organisms \textit{Streptococcus sanguinis} (Erickson & Herzberg, 1993) and \textit{Mycobacterium tuberculosis} (Dobos \textit{et al}., 1995) and the Gram-negative \textit{Neisseria meningitidis}, \textit{N. gonorrhoeae}, \textit{Campylobacter jejuni}, \textit{E. coli} and \textit{Helicobacter pylori} (Power & Jennings, 2003). However, in most of these cases, the structures of the glycans are unknown, as are the physiological roles of the glycosylation of the proteins.

Surface-exposed LecB may mediate the adhesion of \textit{P. aeruginosa} to receptors located on cells of either the same or different species, thus enabling the colonization of host tissues or the formation of mono- or multispecies biofilms. Several \textit{P. aeruginosa} proteins, including pilus and flagellar proteins as well as the outer-membrane protein OprF, have...
previously been identified as adhesins, which may bind to receptors, e.g. those present on the respiratory epithelium, thus initiating bacterial adherence (Doig et al., 1990; Arora et al., 1998; Scharfman et al., 2001; Azghani et al., 2002). In an earlier report, it was shown that biotinylated LecB specifically binds to the surface of human nasal polyp explants (Adam et al., 1997a). Interestingly, it was demonstrated that mucin from patients suffering from CF contains increased amounts of fucosylated glycoproteins (Shori et al., 2001; Scanlin & Glick, 2001), suggesting that surface-exposed LecB may bind to these glycoproteins and promote persistent infections with P. aeruginosa. This hypothesis is further substantiated by the recent finding that the up-regulation of a Fuc(x1-2) fucosyltransferase was responsible for increased fucosylation of intestinal mucins from CF mice (Thomsson et al., 2002). For CF, the membrane glycoproteins from respiratory epithelial cells expressing a mutated cftr gene were shown to be more fucosylated than those from cells expressing the wild-type gene (Rhim et al., 2001). Therefore, LecB in particular may facilitate the attachment of P. aeruginosa to the CF airway epithelium. The successful treatment of a patient suffering from a Pseudomonas-induced respiratory tract infection with a solution containing LecA- and LecB-specific sugars (von Bismarck et al., 2001) supports the hypothesis that LecB contributes to the development of chronic airway infections via binding to fucosylated glycoproteins of the mucin and/or the respiratory epithelium.

Interestingly, P. aeruginosa LecB does not contain any of the presently known secretion signals (Ma et al., 2003). Thus, it is unlikely that it is secreted via the type I pathway or via the Sec or Tat pathway. In a previous report, it was suggested that cell lysis was responsible for release of LecB into the extracellular medium (Wentworth et al., 1991). This hypothesis seems unlikely, as we have found that mutant LecB was produced in the cytoplasm in the same amount as the wild-type protein (see Fig. 5b), but it was neither translocated into the outer membrane nor released into the extracellular space.

In conclusion, our findings support the notion that LecB may contribute to the pathogenicity of P. aeruginosa in CF in several different ways and therefore represents an interesting target for the development of anti-P. aeruginosa drugs.

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REFERENCES


