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Heterologous Expression of 3-O-Deacylase in *Acinetobacter baumannii* Modulates the Endotoxicity of Lipopolysaccharide

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Key Words

Acinetobacter baumannii ATCC 19606 · 3-O-deacylase enzyme · Lipopolysaccharide · Lipid A · Endotoxicity · Outer membrane vesicle · Inactivated whole cell

Abstract

The lipopolysaccharide (LPS) of *Acinetobacter baumannii* is a potent stimulator of proinflammatory cytokines, such as interleukin-6 (IL-6). The 3-O-deacylase (PagL)-modifying enzyme that removes the 3-O-linked acyl chain from the disaccharide backbone of lipid A provides the opportunity to develop a new therapeutic compound that could reduce detrimental inflammatory responses. The plasmid pMMB66EH-PagL obtained by recombinant DNA technology was electroporated into *A. baumannii* ATCC 19606. Compared with wild-type LPS, outer membrane vesicles and inactivated whole cells of engineered bacteria had a statistically significant decreased ability to produce IL-6. Structural analysis of lipid A by negative-ion matrix-assisted laser desorption/ionization time-of-flight mass spectrometry revealed that the profile of lipid A fractions under PagL expression was changed. Taken together, our data showed that recombinant penta-acylated lipid A had less immunoreactivity and that the tetra-acylated version of lipid A with TLR4 antagonist activity decreased the induction of IL-6 production in the murine macrophage cell line J774 A.1.

Introduction

Acinetobacter baumannii has emerged in recent years as a major cause of nosocomial infections associated with significant morbidity and mortality in intensive care units [Falagas and Rafailidis, 2007]. The spread of multidrug-resistant *A. baumannii* in clinical settings all over the world and consequently the failure of antimicrobial chemotherapy have caused a worldwide health care crisis [Dijkshoorn et al., 2007]. Therefore, the development of novel strategies for the treatment and prevention of infections due to this bacterium is essential. In recent years, a few studies have shown that vaccination with inactivated whole cells and outer membrane vesicles (OMVs) elicit

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or genotype	Source or reference
Strains and cell line		
A. baumannii ATCC 19606	Bouvet and Grimont (ATCC® 19606™)	ATCC
E. coli DH5α	F⁻ Δ(*lacZYA-algF*)*U*169 *thi*-1 *hsdR*17 *gyrA*96 *recA*1 *endA*1 *supE*44 *relA*1 *phoA* φ80 Δ*lacZ* Δ*M15*	Invitrogen
E. coli BL21	F⁻ *ompT gal dcmlonhsdS*$_B$(r_B^- m_B^-) λ(DE3 [*lacI lacUV5-T7* gene 1 *ind1sam7 nin*5])	Invitrogen
J774 A.1 murine macrophages	J774 A.1 (ATCC® TIB-67™)	ATCC
Genes and plasmids		
pUC57-PagL$_{(Pa)}$	Plasmid harboring PagL of *Pseudomonas aeruginosa*	GenScript Co.
pMM66EH	Broad-host-range expression vector	Furste et al., 1986
pMM66EH-PagL$_{(Pa)}$	pMM66EH derivative harboring *pagL*$_{(Pa)}$	This study

antibodies against *A. baumannii* antigens and provide protection in animal models [McConnell and Pachon, 2010; McConnell et al., 2011]. A potential drawback is that inactivated whole cells and OMVs contain a wide range of lipopolysaccharides (LPSs) associated with endotoxicity. The lipid A moiety of LPS activates the Toll-like receptor 4 (TLR4)/MD2 complex, resulting in the induction of the expression of proinflammatory cytokines such as interleukin-1, tumor necrosis factor-α and interleukin-6 (IL-6) [Palsson-McDermott and O'Neill, 2004]. The extensive release of bacterial LPS fragments containing lipid A into the blood circulation gives rise to fever, diarrhea and possible fatal septic shock associated with an uncontrolled expression of proinflammatory cytokines [Miller et al., 2005]. Unlike many other nonenterobacterial endotoxins, the LPS of *A. baumannii* is a potent stimulator of inflammatory signaling in human monocytic cells [Erridge et al., 2007]. There is evidence of a strong interaction of the *Acinetobacter* endotoxins with TLR4/MD2/CD14, which contributes to the pathology of this bacterium during systemic infection in mice [Lin et al., 2012].

Several approaches have been used to reduce endotoxicity in vaccine formulations, such as detergent extraction and genetic modifications of the lipid A moiety. Membrane proteins, PagL, 1,3-bisphosphoglyceric acid, LpxR and LpxO have been reported to modify the fatty acyl chain region of lipid A. PagL is a lipase that removes the 3-O-linked acyl chain from lipid A [Wang and Quinn, 2010]. Data have shown that the expression of PagL in bacteria decreases the endotoxicity of LPS as a result of the downregulation of proinflammatory cytokines such as IL-6 [Geurtsen et al., 2006]. The discovery of the enzymes responsible for lipid A modifications provides the opportunity to develop new therapeutic compounds that may reduce detrimental inflammatory responses. The aim of the present study is to compare the endotoxic immunoreactivity of LPS with and without expression of PagL (obtained from *Pseudomonas aeruginosa*) in *A. baumannii* ATCC 19606.

Material and Methods

Bacterial Strains and Growth Conditions

All the bacterial strains used in this study are described in table 1. For selecting the recombinant *Escherichia coli* strains and *A. baumannii* ATCC 19606 on lysogeny broth (LB) agar, ampicillin was used at a concentration of 100 μg/ml. To induce the expression of the pMMB66EH-carrying *pagL* gene in *E. coli* BL21 or *A. baumannii* ATCC 19606, the bacteria were grown in LB supplemented with 0.25 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C with shaking at 150 rpm. *A. baumannii* ATCC 19606 was grown at $OD_{650\ nm}$ = 1 and then incubated on ice for 10 min. The cells were pelleted down by centrifugation at 14,000 *g* for 10 min. Half a volume of the bacterial pellet was inactivated at 56°C for 30 min and stored as whole-cell pellet. The remaining half was used for LPS extraction. The supernatant of the culture was filtered through a 0.22-μm membrane (Millipore Corporation, Bedford, Mass., USA) and used for isolation of OMVs.

Recombinant DNA Techniques

We designed the nucleotide constructs including ribosomal binding site, *pagL* gene (obtained from *P. aeruginosa*), T7 terminator region and digestion sites (*Eco*RI and *Hind*III). The *pagL* and associated constructs were obtained by gene synthesis (GenScript, USA).This sequence was deposited in the GenBank database under the accession number KJ405399. We received a pUC57-PagL plas-

mid and digested it with *Eco*RI and *Hind*III. The PagL fragment was ligated into *Eco*RI- and *Hind*III-digested pMMB66EH as a broad host range expression vector. The ligation mixture was used for the transformation into *E. coli* DH5α. Next, the pMMB66EH-PagL plasmid was electroporated into *E. coli* BL21 as well as *A. baumannii* ATCC 19606. PagL proteins and isolated OMVs were analyzed by SDS-PAGE [Laemmli, 1970] using a Bio-Rad Mini-PROTEAN®3 apparatus (Bio-Rad, USA). Samples were run on a 12% polyacrylamide gel with a 5% stacking gel and subjected to electrophoresis at 150 V. Proteins were stained with Coomassie brilliant blue or with the silver nitrate method.

Electroporation

Electrocompetent strains were produced as follows: overnight cultures of bacteria were grown in LB and subcultured at a dilution of 1:20 in 50 ml of fresh LB medium. Cultures were grown at an $OD_{650\ nm}$ = 0.8 and then incubated on ice for 10 min. Cells were pelleted down by centrifugation and then washed 3 times with 300 mM sucrose and finally resuspended in 300 µl of 300 mM sucrose. An aliquot of 100 µl of the cell suspension was mixed with 3 µg of the recombinant DNA. The mixture was placed in a prechilled sterile electroporation cuvette (1-mm electrode gap, Bio-Rad) and immediately pulsed by means of a Bio-Rad Gene Pulser (2.5 kV, 200 W, and 25 µF). The mixture was incubated at 37°C for 1 h in 1 ml of LB. Cells were spread on LB agar containing ampicillin (100 µg/ml) and incubated at 37°C.

Isolation of OMVs

One liter of LB was inoculated with 10 ml of an overnight culture of *A. baumannii* strain ATCC 19606 and incubated overnight at 37°C and 200 rpm. Bacterial cells were pelleted down by centrifugation at 14,000 *g* for 10 min at 4°C. The supernatant was filtered through a 0.22-µm membrane (Millipore Corporation) and subjected to centrifugation at 60,000 *g* for 4 h at 4°C. The pellet containing vesicles was resuspended in PBS (pH 7.4). The absence of viable bacteria in the OMV preparations was determined by streaking aliquots on agar plates to test for bacterial growth.

Extraction of LPS

The bacteria were grown overnight in LB at 37°C, and the cells were spun down by centrifugation at 6,000 *g* for 10 min. Extraction of LPS was carried out based on the Westphal and Jann [1965] method. The bacterial pellet (1 g wet weight) was mixed with an equal volume of 90% preheated phenol at 65°C for 15 min. The mixture was then placed on ice to facilitate the separation of phases and centrifuged for 20 min at 6,000 *g*. The aqueous phase was collected. A second extraction was performed on a mixture of 90% phenol and the cellular pellet by addition of distilled water at 65°C. Both aqueous phases were combined and dialyzed against distilled water until the phenol odor was completely eliminated. After dialysis, the LPS was lyophilized and stored at room temperature.

Isolation and Characterization of Lipid A

Lipid A isolation was performed based on the Bligh-Dyer method [Delcour, 2013]. Briefly, the bacterial pellet (1 g wet weight) was mixed with single-phase Bligh-Dyer mixture of chloroform, methanol and water (1:2:0.8 v/v) and shaken vigorously. The mixture was left for 20 min and then centrifuged for 20 min at 2,000 *g*. The upper phase was discarded and the lower phase containing insoluble fractions such as LPS was mixed with a hydrolysis buffer (50 mM sodium acetate, pH 4.5, plus 1% SDS) and incubated for 45 min in a boiling water bath. To extract the lipids from the SDS phase, the solution was converted into a 2-phase Bligh-Dyer solution (2:2:1.8 v/v) and shaken vigorously. The mixture was then centrifuged for 10 min at 2,000 *g*. The lower phase was transferred to a clean glass rotary evaporator flask and lyophilized. Finally, the extracted lipid A was subjected to negative-ion matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. Analyses were performed on a Bruker Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Coventry, UK) in negative reflective mode with delayed extraction. Mass spectra were acquired over a range of 800–4,000 m/z. Final mass spectra were externally calibrated against an adjacent spot containing 6 peptides.

Endotoxicity Assay

LPS measurements of samples were done using a quantitative Limulus amebocyte lysate test (Lonza QCL-1000®, Basel, Switzerland). The concentrations of endotoxin were calculated based on a standard curve. The stimulation of IL-6 production was tested with the murine macrophage cell line J774 A.1. The macrophages were seeded in 24-well plates (2.5×10^5 cells/well) in 400 µl of Dulbecco's modified Eagle's medium (Gibco BRL, USA) supplemented with 10% fetal bovine serum (Gibco® cell culture products, Invitrogen Corporation). The cells were stimulated with 100 µl of purified LPS serial dilutions (triplicate method), whole-cell suspensions and OMVs. The stimulation was continued for 18 h at 37°C in a humid atmosphere containing 5% CO_2. Following the stimulation, IL-6 concentrations in the culture supernatants were detected by a mouse IL-6 ELISA Ready-SET-Go kit (eBioscience, San Diego, Calif., USA) and quantified with an enzyme-linked immunosorbent assay.

Statistical Analysis

The means and standard deviations of 3 individual experiments were calculated. A normality test and the paired t test were used. All statistical analyses were performed using SPSS v. 18.0 (IBM Corp., Armonk, N.Y., USA).

Results

DNA Recombination

The *pagL* fragment (676 bp) was ligated into *Eco*RI- and *Hind*III-digested pMMB66EH (8,807 bp). We confirmed the correct DNA recombination with a double digestion of pMMB66EH-PagL plasmid in *E. coli* DH5α (fig. 1). After electroporation, *A. baumannii* ATCC 19606 colonies harboring pMMB66EH-PagL were confirmed by phenotypic and genotypic tests.

Protein Expression and OMV Isolation

The main point of the transformation of pMMB66EH-PagL plasmid into *E. coli* BL21 was to check the functional expression of the PagL construction. The expected molecular mass of PagL protein was ~18 kDa, consistent

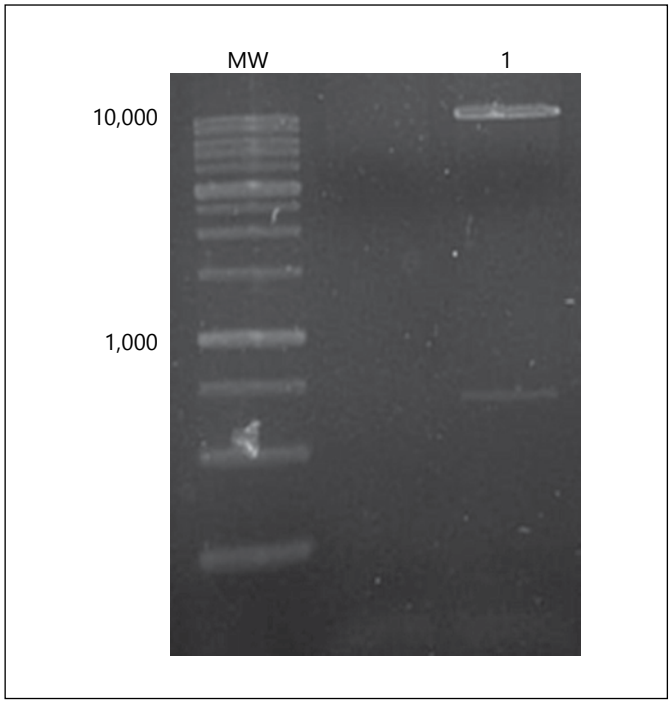

Fig. 1. Electrophoresis of pMMB66EH-PagL that was double-digested by *Eco*RI and *Hind*III in 1% agarose gel. Lane 1 shows 2 fragments including pMMB66EH (8807 bp) and PagL (676 bp). MW = Molecular weight marker.

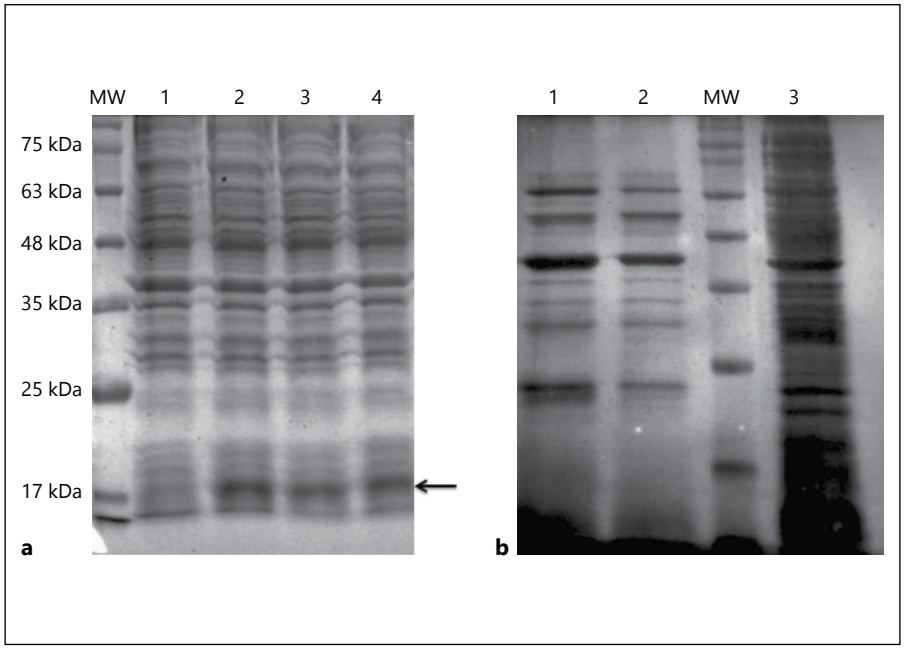

Fig. 2. Polyacrylamide gel electrophoresis of protein samples. MW = Molecular weight marker. **a** Expressed product of *pagL* in 12% SDS-PAGE gel. Lane 1 = Uninduced construct; lane 2 = pMMB66EH-PagL induced by 0.25 mM isopropyl-1-thio-β-D-galactopyranoside after 1 h; lane 3 = induction by 0.5 mM isopropyl-1-thio-β-D-galactopyranoside after 1 h; lane 4 = induction by 1 mM isopropyl-1-thio-β-D-galactopyranoside after 1 h. After induction by isopropyl-1-thio-β-D-galactopyranoside, an ~18 kDa protein of PagL (arrow) appeared in 12% gel. This finding confirmed that our nucleotide construction had a functional expression. **b** OMVs isolated from the *A. baumannii* ATCC 19606 strains with and without PagL expression resolved on a 12% gel and stained using Coomassie blue. Lane 1 = OMVs under expression of PagL; lane 2 = OMVs without PagL expression; lane 2 = total proteins of *A. baumannii* ATCC 19606.

with the protein expression in SDS-PAGE gel (fig. 2a). We separately designed a pET28a-PagL system and detected the protein by Western blot using a His-tag antibody (data not shown). Protein profiles of OMVs in the *A. baumannii* ATCC 19606 strain with and without pMMB66EH-PagL were compared in SDS-PAGE gel (fig. 2b). Data showed that the expression of PagL had no deleterious effect on *A. baumannii* ATCC 19606 because 2 patterns of OMV protein profiles had the same OMV protein pattern.

MALDI-TOF Mass Spectrometry Analysis

We characterized and compared lipid A moieties of 2 strains (with/without expression of PagL) by MALDI-TOF mass spectrometry. The MALDI-TOF mass spectrometry primary data were annotated based on the study by Beceiro et al. [2011]. Analysis showed that the lipid A fraction of *A. baumannii* ATCC 19606 contains 3 major species which correspond to hepta-acylated (m/z 1,910), hexa-acylated (m/z 1,728) and tetra-acylated (m/z 1,404) lipid A (fig. 3a). Figure 3a shows the mass spectrometry of the *A. baumannii* ATCC 19606 strain containing the empty vector pMMB66EH, and figure 3b shows the mass spectrometry of the *A. baumannii* ATCC 19606 strain containing the pMMB66EH-PagL. According to figure 3b (the strain with the expression of PagL), the lipid A underwent some modifications. The new ion peak was shifted to m/z 1,520, which may represent a recombinant penta-acylated lipid A, lacking one 12:0 (3-OH) fatty acid moiety at position 3 of the disaccharide backbone. The m/z 1,728 lost its ion peak, and m/z 1,404 had a higher relative intensity in comparison to lipid A isolated from the *A. baumannii* ATCC 19606 strain containing the empty vector pMMB66EH. This is the first report of a PagL modifying lipid A with a C_{12} fatty acyl chain at position 3.

Endotoxicity Detection Assays

To assess the endotoxic activity of wild-type and PagL-modified *A. baumannii* LPS molecules, their potency to stimulate the production of IL-6 by the murine macrophage cell line J774 A.1 was tested. Compared with wild-type LPS, the purified LPS from the strain expressing PagL had a significant attenuated potency to stimulate the production of IL-6 by the murine macrophages (fig. 4a). Data passed a normality test, and the paired t test showed that endotoxicity changed significantly after PagL expression ($p = 0.017$). As a result of the LPS modification by PagL in whole cells and OMVs, the endotoxicity was reduced (fig. 4b). Data passed a normality test, and the paired t test showed that there was a statistically significant change ($p = 0.011$ and $p = 0.006$ in whole cells and OMVs, respectively).

Discussion

Although lipid A is an essential component of LPS in all Gram-negative bacteria, it is a highly diverse molecule. Gram-negative bacteria have evolved mechanisms to modify the structure of lipid A in different environments to escape recognition by the innate immune system. In *Salmonella enterica* serovar typhimurium and *P. aeruginosa,* PagL acts as an immunomodulator leading to a deacylation of lipid A and a reduction in lipid A recognition when bacteria establish persistent infections. These modifications can alter signaling by the TLR4/MD2/CD14 complex.

The structure-function analysis of lipid A signaling indicates that the length and number of acyl side chains are critical for TLR4 signaling [Hajjar et al., 2002; Kusumoto et al., 2003]. Optimal lipid A potency is achieved by a biphosphorylated, hexa-acylated lipid A species that has acyl chains 12–14 carbons in length [Raetz and Whitfield, 2002]. Lipid A moieties that deviate from this pattern often demonstrate a significant low endotoxic activity [Alexander and Rietschel, 2001]. For example, lipid A molecules with penta- or hepta-acyl chains are ~100 times less active, while lipid A with tetra-acyl chains lacks agonistic activity altogether and acts as an antagonist instead [Hajjar et al., 2002; Kusumoto et al., 2003]. In this study, our data revealed that the whole cells, OMVs and purified LPS under PagL expression lessened toxicity. Previous studies showed that *A. baumannii* normally produced 3 major forms of lipid A including hepta-, hexa- and tetra-acyl chains which correspond to 1,910, 1,728 and 1,404 m/z, respectively [Beceiro et al., 2011]. However, MALDI-TOF mass spectography showed that the heterologous expression of the PagL enzyme changes the spectrum, distribution and structure of lipid A forms. It seems that hexa-acylated lipid A is the substrate target for PagL as a posttranslational modification enzyme which efficiently removes C_{12} fatty acid chains at position 3 of the disaccharide backbone. Therefore, the 1,728 m/z peak was eliminated and the 1,520 m/z peak appeared. Moreover, the hepta-acyl form maintained its position in the spectral profile. This form of lipid A has more anchoring capacity and is probably important for the integrity of bacterial membranes. The higher intensity of the tetra-acylated lipid A form may be due to the bypass effect on LPS biosynthesis under PagL expression. Tetra-acylated lipid A has TLR4 antagonist activity that may have decreased the amount of IL-6 in murine macrophage J774 induction.

Although multiple sequence alignment analysis of PagL homologues in a variety of Gram-negative bacteria showed low sequence similarity, a conserved domain could be detected in the C-terminal region [Geurtsen et al., 2005]. In *P. aeruginosa*, the PagL enzyme consists of an 8-stranded β-barrel with the axis tilted by approximately 30° with respect to the lipid bi-

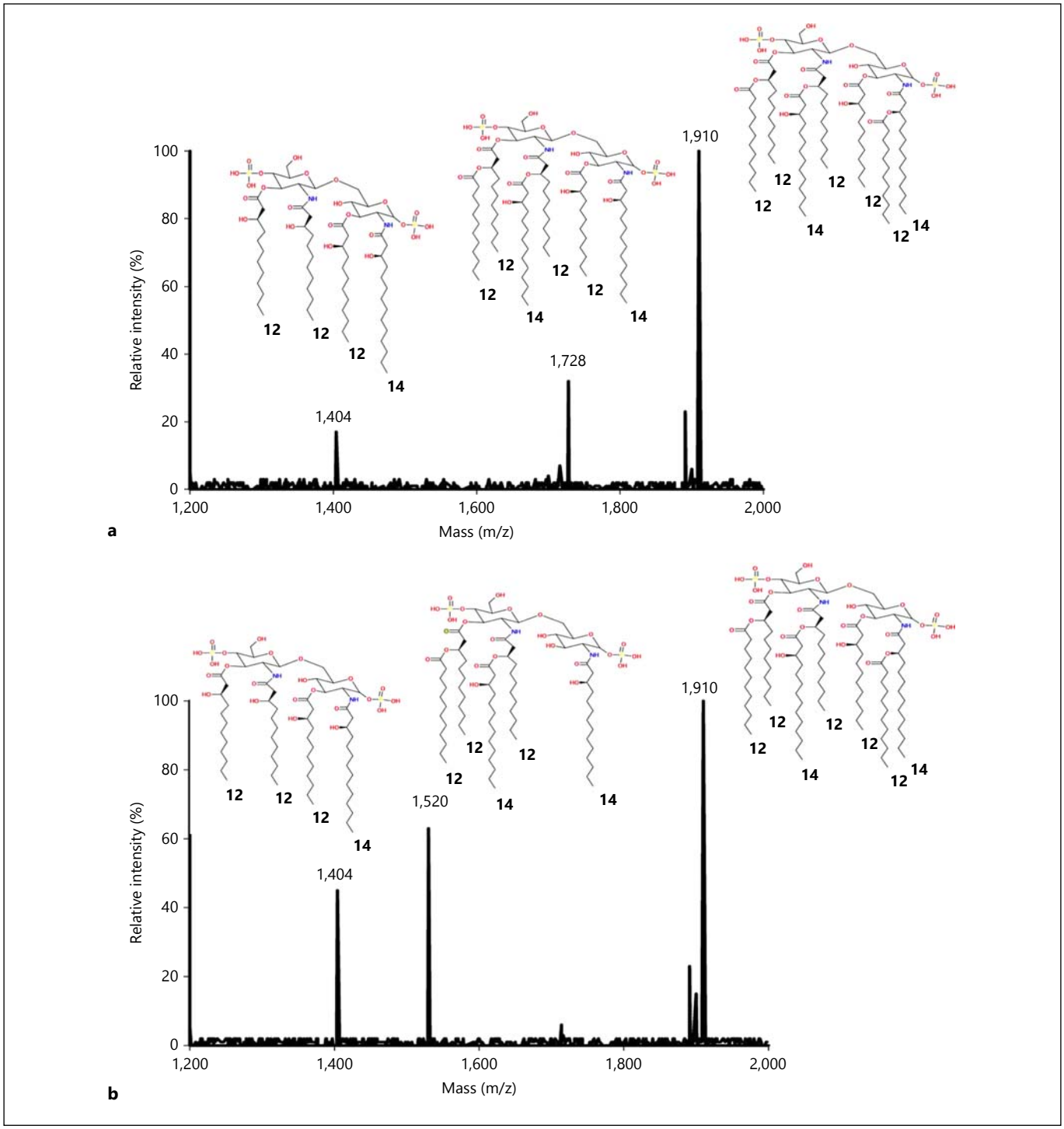

Fig. 3. Structural analysis by negative-ion MALDI-TOF mass spectrometry of lipid A isolated from an *A. baumannii* ATCC 19606 strain containing the empty vector pMMB66EH (**a**) and *A. baumannii* ATCC 19606 under expression of PagL (**b**). **a** Three major peaks, which correspond to hepta-acylated (m/z 1,910), hexa-acylated (m/z 1,728) and tetra-acylated (m/z 1,404) lipid A, were characterized. **b** With the expression of PagL, the new ion peak detected was m/z 1,520, which represents a recombinant penta-acylated lipid A lacking one 12:0 (3-OH) fatty acid moiety at the 3 position of the disaccharide backbone. The m/z 1,728 lost its ion peak, and m/z 1,404 had higher relative intensity.

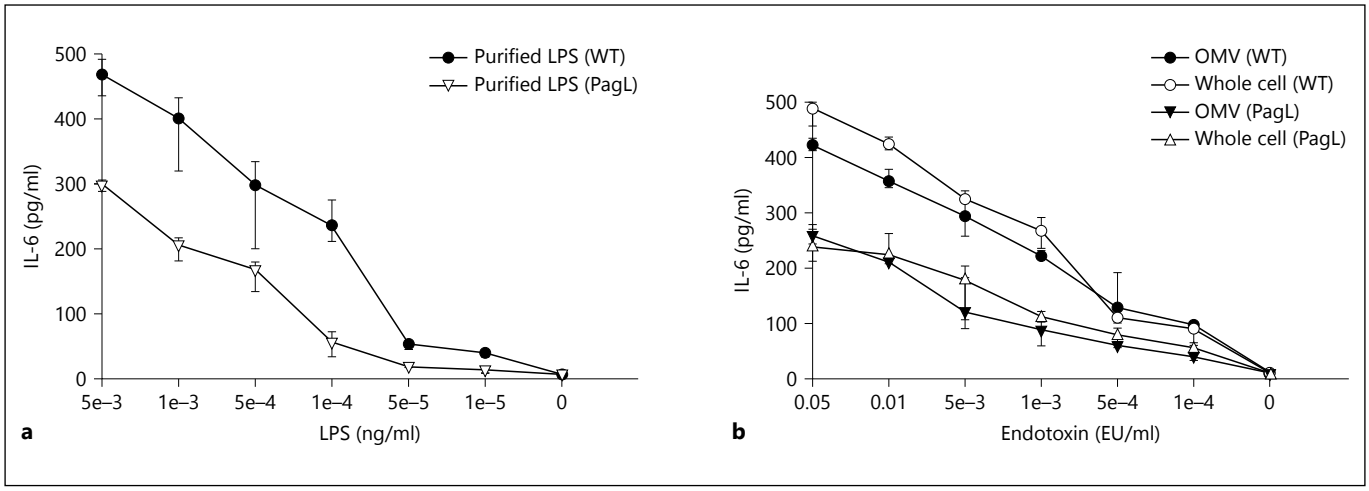

Fig. 4. IL-6 induction by purified LPS, OMVs and whole cells of *A. baumannii* ATCC 19606. The production of IL-6 by the murine macrophage cell line J774 A.1 was stimulated with serial dilutions of purified wild-type (WT) or PagL-modified LPS from *A. baumannii* ATCC 19606 (**a**) or with whole-cell suspensions of *A. baumannii* ATCC 19606 cells expressing PagL or containing the empty vector pMMB66EH (WT) or with OMVs of *A. baumannii* ATCC 19606 isolated from cells expressing PagL or containing the empty pMMB66EH vector (WT) (**b**). EU = Enzyme unit.

layer. It contains an active site with a Ser-His-Glu catalytic triad and an oxyanion hole that comprises the conserved Asn in the C-terminal region [Rutten et al., 2006]. Previous studies revealed that a heterologous expression of *P. aeruginosa* PagL in *S. enterica* serovar typhimurium and *E. coli* resulted in the removal of the 3-OH C_{14} fatty acid from lipid A, indicating that *P. aeruginosa* PagL recognizes either the 3-OH C_{10} or 3-OH C_{14} fatty acid at position 3 of the disaccharide backbone [Ernst et al., 2006]. Our study confirmed that the 3-O-deacylase enzyme of *P. aeruginosa* removes the 3-OH C_{12} fatty acid at position 3 of the disaccharide backbone. In 2011, Beceiro et al. [2011] revealed the structures of the main molecular lipid A in *A. baumannii*. They showed that the recombinant penta-acylated lipid A had 10 units (m/z) less than the same molecule. This may be due to the different position of the fatty acid chains in lipid A and different molecular charges. The asymmetrical structure of recombinant penta-acylated lipid A results in an incomplete interaction with TLR4 and decreases IL-6 secretion. The findings of this study indicated that PagL could change the structure of *A. baumannii* lipid A.

OMVs can be considered as vaccine-adjuvant particles released from Gram-negative bacteria including LPS and outer membrane proteins. These OMVs have been shown to stimulate immune response and have self-adjuvant properties that can be utilized in the development of effective acellular vaccines. OMVs contain toxic components such as LPS associated with unpleasant reactions. Therefore, the reduction of LPS endotoxicity is an important goal for optimized immune responses. Detergent extraction and genetic manipulation are approaches that have been used to reduce endotoxicity in vaccine formulations. The heterologous expression of PagL and LpxR-modifying enzymes or the mutation in enzymes associated with LPS biosynthesis, such as LpxL and LpxM, have been used for the genetic manipulation of LPS in recent years. Recently, isolated OMVs and inactivated whole cells under expression of PagL have been used as a vaccine candidate in *Bordetella pertussis* [Asensio et al., 2011; Geurtsen et al., 2007]. In the future we aim to evaluate OMVs and inactivated whole cells with PagL expression as vaccine candidates.

Acknowledgments

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Disclosure Statement

There are no conflicts of interest declared.

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