Chapter 2

Dissemination of lipid A deacylases (PagL) among Gram-negative bacteria

IDENTIFICATION OF ACTIVE-SITE HISTIDINE AND SERINE RESIDUES

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Abstract

Lipopolysaccharide (LPS) is one of the main constituents of the Gram-negative bacterial outer membrane. It usually consists of a highly variable O-antigen, a less variable core oligosaccharide, and a highly conserved lipid moiety, designated lipid A. Several bacteria are capable of modifying their lipid A architecture in response to external stimuli. The outer membrane-localised lipid A 3-O-deacylase, encoded by the pagL gene of Salmonella enterica serovar Typhimurium, removes the fatty acyl chain from the 3 position of lipid A. Although a similar activity was reported in some other Gram-negative bacteria, the corresponding genes could not be identified. Here, we describe the presence of pagL homologs in a variety of Gram-negative bacteria. Although the overall sequence similarity is rather low, a conserved domain could be distinguished in the C-terminal region. The activity of the Pseudomonas aeruginosa and Bordetella bronchiseptica pagL homologs was confirmed upon expression in Escherichia coli, which resulted in the removal of an R-3-hydroxymyristoyl group from lipid A. Upon deacylation by PagL, E. coli lipid A underwent another modification, which was the result of the activity of the endogenous palmitoyl transferase PagP. Furthermore, we identified a conserved histidine-serine couple as -residues, suggesting a catalytic mechanism similar to serine hydrolases. The biological function of PagL remains unclear. However, because PagL homologs were found in both pathogenic and non-pathogenic species, PagL-mediated deacylation of lipid A probably does not have a dedicated role in pathogenicity.
Introduction

Lipopolysaccharide (LPS), a major component of the Gram-negative bacterial outer membrane, is known to be important for the functioning of this membrane as a permeability barrier and for the resistance against complement-mediated cell lysis (for review, see Raetz and Whitfield, 2002). It consists of three covalently linked domains: lipid A, the core, and the O-antigen. Lipid A forms the hydrophobic membrane anchor and is responsible for the endotoxic activity of LPS. In *Escherichia coli*, it consists of a 1, 4'-bisphosphorylated β-1,6-linked glucosamine disaccharide, which is replaced by R-3-hydroxymyristic acid residues at positions 2, 3, 2', and 3' via ester or amide linkage. Secondary lauroyl and myristoyl groups replace the hydroxyl group of R-3-hydroxymyristoyl at the 2' and 3' positions, respectively (Fig. 1A). Previous studies have shown that the phosphate groups, the glucosamine disaccharide, and the correct number and length of the acyl chains are important for the biological activity of lipid A (Raetz and Whitfield, 2002; Loppnow et al., 1989; Steeghs et al., 2002). The basic structure of lipid A is reasonably well conserved among Gram-negative bacteria, although slight variations in the pattern of the substitutions of the two phosphates and the acyl chain number and length are observed (Nikaido and Vaara, 1987; Caroff et al., 2002). Additional modifications of lipid A (Fig. 1B) are regulated in *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) by the two-component regulatory system PhoP/PhoQ (Guo et al., 1997; Guo et al., 1998). In response to low Mg$^{2+}$ levels, the sensor kinase PhoQ phosphorylates and thereby activates the transcriptional activator PhoP, which leads to the activation or repression of 40 different genes (Guo et al., 1997; Gunn et al., 1998a). A second regulatory system involved in lipid A modification is the PmrA/PmrB two-component system, which itself is PhoP/PhoQ-regulated (Gunn et al., 1998b; Gunn et al., 2000). Mutants with alterations in the PhoP/PhoQ system exhibit reduced virulence and an increased susceptibility to anti-microbial peptides (Miller et al., 1989; Gunn and Miller, 1996). Homologs of the PhoP/PhoQ and PmrA/PmrB systems have been identified in other Gram-negative bacteria, including *E. coli*, *Yersinia pestis*, and *Pseudomonas aeruginosa* (Ernst et al., 1999a; Ernst et al., 1999b).

Until now, several lipid A-modifying enzymes have been identified (Fig. 1B). Substitution of the 1- and 4'-phosphate groups with one or two 4-amino-4-deoxy-L-arabinose (L-Ara4N) moieties in *S. Typhimurium* was found to be dependent on the enzyme ArnT (Trent et al., 2001b). Recently, the PmrC protein was identified to mediate the addition of phosphoethanolamine to lipid A in *S. enterica* (Lee et al., 2004). Another enzyme, designated LpxO, catalyses the O$_2$-dependent hydroxylation of lipid A (Gibbons et al., 2000), and a lipid A 1-phosphatase was identified in *Rhizobium leguminosarum*.
Fig. 1 Lipid A architecture. (A) E. coli lipid A consists of a bisphosphorylated glucosamine disaccharide substituted with four R-3-hydroxymyristoyl moieties, of which the 2'- and 3'-fatty-acyl chains are esterified with laurate and myristate, respectively. (B) regulated modifications of Salmonella lipid A. Substitution of the phosphate moieties with L-Ara4N or phosphoethanolamine is mediated by ArnT and PmrC, respectively, the formation of a 2-hydroxymyristate-modified lipid A by LpxO, the addition of a secondary palmitoyl chain at the 2 position by PagP, and the removal of the 3-hydroxymyristoyl moiety at the 3 position by PagL are shown.
(Karbarz et al., 2003). All these enzymes are thought to reside within the inner membrane or periplasmic space (Trent et al., 2001b; Lee et al., 2004; Gibbons et al., 2000; Karbarz et al., 2003). Recently, a new class of outer membrane-localised lipid A-modifying enzymes was discovered. One of them is the palmitoyl transferase PagP (Bishop et al., 2000). Palmitoylation of lipid A leads to an increased resistance to cationic antimicrobial peptides (Guo et al., 1998). Furthermore, palmitoylated lipid A antagonises LPS-induced activation of human cells (Tanamoto and Azumi, 2000). Homologs of PagP are found, among others, in S. Typhimurium, Bordetella pertussis, Bordetella bronchiseptica, Bordetella parapertussis, Legionella pneumophila, E. coli, and Y. pestis (Bishop et al., 2000; Robey et al., 2001). Another outer membrane-localised lipid A-modifying enzyme is the 3-O-deacylase PagL (Trent et al., 2001a). This enzyme was discovered in S. Typhimurium and shown to hydrolyse the ester bond at the 3 position of lipid A, thereby releasing the primary 3-hydroxymyristoyl moiety (Trent et al., 2001a). At that time, no obvious homologs of this protein could be found in the nonredundant or unfinished microbial databases, except in the closely related strains S. enterica serovars Typhi and Paratyphi (Trent et al., 2001a). Nevertheless, some other Gram-negative bacteria, including P. aeruginosa (Ernst et al., 1999b), R. leguminosarum (Bhat et al., 1994), Helicobacter pylori (Moran et al., 1997), and Porphyromonas gingivalis (Kumada et al., 1995) contain 3-O-deacylated lipid A species, suggesting that these organisms contain enzymes with an activity similar to that of PagL. We report now the identification of PagL homologs in a variety of Gram-negative bacteria. The limited sequence similarity among the various proteins was used to identify active-site residues.

Materials and Methods

Bacterial strains and growth conditions

All bacterial strains used in this study are described in Table 1. Unless otherwise notified, the E. coli and P. aeruginosa strains were grown at 37°C and 30°C, respectively, in a modified Luria-Bertani broth, designated LB (Tommassen et al., 1983), supplemented with 0.2% glucose, or in minimal medium (SV) (Winkler and de Haan, 1949) supplemented with 0.5% glucose, while shaking at 200 rpm. To induce expression of the pagL genes cloned behind the T7 promoter, the bacteria were grown in LB supplemented with glucose until an absorbance at 600 nm ($A_{600}$) of 0.4–0.6 was reached. Expression of the pagL genes was then induced by adding 1 mM isopropyl-β-D-galactopyranoside (IPTG), and incubation at 37°C was continued. When appropriate, bacteria were grown in the presence of 100 μg/ml ampicillin, 50 μg/ml kanamycin, 10 μg/ml tetracycline, or
100 μg/ml streptomycin, for plasmid maintenance. *S. Typhimurium* SR11 was grown on LB agar plates at 37°C. *B. bronchiseptica* and *B. pertussis* strains were grown at 35°C on Borduet-Gengou agar (Difco) supplemented with 15% defibrinated sheep blood (Biotrading). Stain JG101, carrying a tetracycline-resistance transposon insertion in *pagP*, was obtained by P1 transduction by using *E. coli* BL21 Star™ (DE3) and *E. coli* SK2257 (Table 1) as the acceptor and donor, respectively.

### TABLE 1

| Bacterial strains and plasmids used in this study |
|--------------------------------------------------|
| **Strain or plasmid** | **Genotype or description** | **Source or reference** |
| **Strains** | | |
| *B. bronchiseptica* | | |
| B505 | Wild-type strain | N.V.I. a |
| BP509 | Dutch vaccine strain | N.V.I. a |
| BP134 | Dutch vaccine strain | N.V.I. a |
| *B. pertussis* | | |
| BP509 | Dutch vaccine strain | N.V.I. a |
| BP134 | Dutch vaccine strain | N.V.I. a |
| *P. aeruginosa* | | |
| PAO1 | Wild-type strain | Jacobs et al., 2003 |
| PAO25 | PAO1 leu arg | Haas and Holloway, 1976 |
| #32751 | PA4661 (*pagL*) mutant-derivative of PAO1 | Jacobs et al., 2003 |
| *S. Typhimurium* | | |
| SR11 | Wild-type strain | Pace et al., 1993 |
| **E. coli** | | |
| TOP10F* | F’ (lacIq Tn10 (TetR)) mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 (ara-leu)7697 galU galK rpsL endA1 nupG | Invitrogen |
| DH5α | Δ(lacZA-algF)U169 thi-1 hsdR17 gyrA96 recA1 endA1 supE44 relA1 phoA Φ80 ΔlacZΔM15 | Hanahan, 1983 |
| BL21 Star™ (DE3) | F’ ompT hsdS B (rB mB) gal dcm me131 | Invitrogen |
| SK2257 | F’ crcA280::Tn10 thyA6 rpsL120(StrR) deoC1 | CGSC b |
| JG101 | BL21 Star™ (DE3) crcA280::Tn10 | This study |
| **Plasmids** | | |
| pCRII-TOPO | *E. coli* cloning vector AmpR KanR | Invitrogen |
| pET-11a | *E. coli* high-copy expression vector, AmpR, T7 promoter | Novagen |
| pPagL(Pa) | pET-11a derivative harboring *P. aeruginosa* *pagL* | This study |
| pPagL(Bb) | pET-11a derivative harboring *B. bronchiseptica* *pagL* | This study |
| pPagL(St) | pET-11a derivative harboring *S. Typhimurium* *pagL* | This study |
| pPagL(Pa)(−) | pET-11a derivative encoding *P. aeruginosa* *PagL* without signal sequence | This study |
| pPagL(Pa)(H81A) | pPagL(Pa) encoding PagL(Pa) with H81A substitution | This study |
| pPagL(Pa)(H81N) | pPagL(Pa) encoding PagL(Pa) with H81N substitution | This study |
| pPagL(Pa)(S84A) | pPagL(Pa) encoding PagL(Pa) with S84A substitution | This study |
| pPagL(Pa)(S84C) | pPagL(Pa) encoding PagL(Pa) with S84C substitution | This study |
| pPagL(Pa)(H149A) | pPagL(Pa) encoding PagL(Pa) with H149A substitution | This study |
| pPagL(Pa)(H149N) | pPagL(Pa) encoding PagL(Pa) with H149N substitution | This study |
| pPagL(Pa)(S151A) | pPagL(Pa) encoding PagL(Pa) with S151A substitution | This study |
| pPagL(Pa)(S151C) | pPagL(Pa) encoding PagL(Pa) with S151C substitution | This study |

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*a* Netherlands Vaccine Institute, Bilthoven, The Netherlands  
*b* *E. coli* genetic stock center, Yale university, New Haven (CT)  
*c* *pagP* is also known as *crcA*
Recombinant DNA techniques

The plasmids used are described in Table 1. Plasmid DNA was isolated using the Promega Wizard® Plus SV Minipreps system. Calf intestine alkaline phosphatase and restriction endonucleases were used according to the instructions of the manufacturer (Fermentas). DNA fragments were isolated from agarose gels using the Qiagen quick gel extraction kit. Ligations were performed by using the rapid DNA ligation kit (Roche Applied Science).

The pagL genes from S. Typhimurium SR11 (pagL\textsubscript{(St)}), B. bronchiseptica B505 (pagL\textsubscript{(Bb)}), and P. aeruginosa PAO25 (pagL\textsubscript{(Pa)}) were cloned into pET-11a (Novagen) behind the T7 promoter. The genes were amplified by PCR using chromosomal DNA as template. Template DNA was prepared by resuspending ~10^9 bacteria in 50 μl of distilled water, after which the suspension was heated for 15 min at 95°C. The suspension was then centrifuged for 1 min at 16,100 x g, after which the supernatant was used as template DNA. The sequences of the forward primers, which contained an NdeI site (underlined), including an ATG start codon, were 5'-AACATATGAAGAGAATATTTATATC-3' (pagL\textsubscript{(St)}), 5'-AACATATGAAGAAACTACTTCCGCTGG-3' (pagL\textsubscript{(Pa)}), and 5'-AACATATGCAATTTCTCAAGAAAAACA-3' (pagL\textsubscript{(Bb)}). The sequences of the reverse primers, which contained a BamHI site (underlined) and included a stop codon, were 5'-AAGGATCCTCAGAAAT TATAACTAATT-3' (pagL\textsubscript{(St)}), 5'-AAGGATCCCTAGATCGGGATCTTGTAG-3' (pagL\textsubscript{(Pa)}), and 5'-AAGGATCCTCAGAACTGGTACGTATA-G-3' (pagL\textsubscript{(Bb)}). The PCRs were done under the following conditions: 50-μl total reaction volume, 25 pmol of each primer, 0.2 mM dNTPs, 3 μl of template DNA solution, 1.5% dimethyl sulfoxide, 1.75 units of Expand High Fidelity enzyme mix with buffer supplied by the manufacturer (Roche Applied Science). The temperature program was as follows: 95°C for 3 min, a cycle of 1 min at 95°C, 1 min at 60°C, and 1 min 30 s at 72°C repeated 30 times, followed by 10 min at 72°C and subsequent cooling to 4°C. The PCR products were purified from agarose gel and subsequently cloned into pCRII-TOPO. Plasmid DNA from correct clones was digested with NdeI and BamHI, and the PagL-encoding fragments were ligated into NdeI/BamHI-digested pET-11a. The ligation mixture was used to transform E. coli DH5α using the CaCl\textsubscript{2} method (Sambrook et al., 1989). Plasmid DNA from transformants was checked for presence of the correct PagL-encoding insert by digestion with NdeI and BamHI. Plasmids that gave a correct digestion profile were designated pPagL\textsubscript{(Pa)}, pPagL\textsubscript{(Bb)} and pPagL\textsubscript{(St)} (Table 1). The correct coding sequences of the cloned pagL genes were confirmed by nucleotide sequencing in both directions. Mutations were introduced in pagL by using the QuikChange site-directed mutagenesis kit (Stratagene).
and the primers listed in Table 2. Plasmid pPagL(Pa) was used as the template in which the mutations were created. The presence of the correct mutations was confirmed by nucleotide sequencing in both directions.

### Table 2

| Name   | Sequence (5'-3') |
|--------|-----------------|
| H81A_FW | GAAGGCGCGCGCAAGGCGTCTCGTTCGCT |
| H81A_REV | AGCGAAGCAGACGCAGCCCTTGCCTC |
| H81N_FW | GAAGGCGCGCAGCAGAAGACTCGTCTCGTTCGCT |
| H81N_REV | AGCGAAGCAGACGCAGCTTGCCTCCTT |
| S84A_FW | GGCAGGCTCCGTCGGTTCGCT |
| S84A_REV | AGCGAACGAGCGCCAGCAGCTGC |
| S84C_FW | GGCAAGCGTGCTGTTCGCT |
| S84C_REV | AGCGAACGAGCGCCAGCAGCTGC |
| H149A_FW | GCAGGCTCGCTGGTTCGCT |
| H149A_REV | AGCGAACGAGCGCCAGCAGCTGC |
| H149N_FW | GCAGGCTCGCTGGTTCGCT |
| H149N_REV | AGCGAACGAGCGCCAGCAGCTGC |
| S151A_FW | CGGCCGATCCACTACGACGCGCGTCTGAA |
| S151A_REV | GCCGGGCTGCCGATCCACTACGACGCGCGTCTGAA |
| S151C_FW | CGGCCGATCCACTACGACGCGCGTCTGAA |
| S151C_REV | GCCGGGCTGCCGATCCACTACGACGCGCGTCTGAA |

*a The primer name gives the amino acid substitution, e.g. H81A_FW indicates that the oligonucleotide shown was used as the forward primer in a site-directed mutagenesis procedure to substitute the histidine at position 81 of the precursor PagL(Pa) by an alanine.

*b Introduced mutations are underlined.

### Isolation of cell envelopes

Cells were harvested by centrifugation for 10 min at 1,500 x g and washed once in 50 ml of cold 0.9% sodium chloride solution. The cell pellets were frozen for at least 15 min at -80°C and then suspended in 20 ml of 3 mM EDTA, 10 mM Tris-HCl (pH 8.0) containing Complete protease inhibitor mixture (Roche Applied Science). The cells were disrupted by sonication, after which unbroken cells were removed by centrifugation for 10 min at 1,500 x g. The cell envelopes were pelleted from the supernatant by centrifugation for 1.5 h at 150,000 x g and resuspended in 2 mM Tris-HCl (pH 7.4). The cell envelopes were stored at -80°C in aliquots.

### SDS-PAGE and immunoblotting

Proteins were analysed by SDS-PAGE (Laemmli, 1970) with 0.2% SDS in the running gel using the Bio-Rad Mini-PROTEAN®3 apparatus. Samples were applied to a 13% polyacrylamide gel with a 4% stacking gel and subjected to electrophoresis at 150 V. Proteins were stained with Coomassie Brilliant Blue. Prestained or unstained Precision
Plus Protein™ Standard from Bio-Rad was used to determine the relative molecular mass. For Western blotting, proteins were transferred from SDS-polyacrylamide gels onto nitrocellulose membranes. The membranes were blocked overnight in phosphate-buffered saline (pH 7.6), 0.5% nonfat dried milk, 0.1% Tween 20 and incubated with guinea pig antibodies directed against PagL\textsubscript{(Pa)} in blocking buffer followed by an incubation with horseradish peroxidase-conjugated rabbit anti-guinea pig IgG antibodies (Sigma) in blocking buffer. Blots were developed using SuperSignal® WestPico Chemiluminescent Substrate (Pierce).

**Polyclonal antibodies**

For antibody production, the \textit{pagL} gene from \textit{P. aeruginosa} PAO25 without the signal sequence-encoding part was PCR amplified by using the forward primer (5’-AAC\underline{AT}ATGGCGGACGTCTCGGCCGCG-3’), which contained an Ndel site (underlined), including an ATG start codon, and the reverse primer (5’-\underline{AAG}GATCGTCTAGATCGGGATCTTGTAG-3’), which contained an BamHI site (underlined) and included a stop codon. The PCR product was cloned into pET-11a, and the resulting plasmid, pPagL\textsubscript{(Pa)}(-), was used to transform \textit{E. coli} BL21 Star\textsuperscript{TM} (DE3) to allow for expression of the truncated \textit{pagL} gene. The PagL\textsubscript{(Pa)} protein, accumulating in inclusion bodies, was isolated (Dekker \textit{et al.}, 1995), purified from a preparative SDS-polyacrylamide gel, and used for immunisation of guinea pigs at Eurogentec.

**Microsequencing**

Proteins were transferred from SDS-polyacrylamide gels to an Immobilon-P polyvinylidene difluoride membrane (Millipore Corp.) in 192 mM glycine, 25 mM Tris (pH 8.3), 10% methanol (v/v) at 100 V for 1 h using the Bio-Rad Mini-PROTEAN\textsuperscript{®}2 blotting apparatus. After transfer, the membrane was washed three times for 15 min with distilled water. Transferred proteins were stained with Coomassie Brilliant Blue. The membrane was dried in the air, and the putative PagL bands were excised and subjected to microsequencing at the Sequencing Center Facility, Utrecht University, The Netherlands.

**LPS analysis by Tricine-SDS-PAGE**

Approximately \(10^9\) bacteria were suspended in 50 \(\mu\text{l}\) of sample buffer (Laemmli, 1970), and 0.5 mg/ml proteinase K (end concentration) was added. The samples were incubated for 60 min at 55°C followed by 10 min at 95°C to inactivate proteinase K. The samples were then diluted 10-fold by adding sample buffer, after which 2 \(\mu\text{l}\) of
each sample was applied to a Tricine-SDS-polyacrylamide gel (Lesse et al., 1990). The bromphenol blue was allowed to run into the separating gel at 35 V, after which the voltage was increased to 105 V. After the front reached the bottom of the gel, the samples were left running for another 45 min. The gels were fixed overnight in water/ethanol/acetic acid 11:8:1 (v/v/v) and subsequently stained with silver as described previously (Tsai and Frasch, 1982).

Gas chromatography-mass spectrometry (GC/MS) and electrospray ionisation-mass spectrometry (ESI/MS)

LPS was isolated using the hot phenol/water extraction method (Westphal and Jann, 1965). For fatty acid analysis by GC/MS, a 5-fold volume excess of acetone was added to an aliquot of the isolated LPS (0.2 mg/ml), after which the solution was dried at 60°C under a nitrogen flow. Subsequently, 10 μg of 2OH C12 (1 mg/ml in ethanol) was added as an internal standard, as well as 100 μl of acetylchloride/ethanol 1:9 (v/v), after which the samples were derivatised for 1 h at 90°C. After cooling, the reaction was stopped by adding 200 μl of 1 M K2HPO4 (pH 8.0), followed by extraction of the acylethyl esters with 200 μl of ethyl acetate. A 1-μl volume of the upper phase was used for analysis by GC/MS on a Finnigan MAT SSQ in the electron-impact mode. For ESI/MS, an aliquot of isolated LPS was freeze-dried and taken up in 1.8 ml of 12.5 mM sodium acetate (pH 4.5) containing 1% SDS. The mixture was boiled for 30 min to hydrolyse the LPS and release the lipid A moiety, after which the mixture was cooled to room temperature and converted into a two-phase Bligh and Dyer mixture by adding 2 ml of methanol and 2 ml of chloroform. Phases were separated by centrifugation, after which the lower phase was collected and washed twice with the upper phase of a fresh two-phase Bligh and Dyer mixture, consisting of chloroform/methanol/water (2:2:1.8, v/v). Structural analysis of purified lipid A was performed by nanoelectrospray tandem MS on a Finnigan LCQ in the negative ion mode (Wilm and Mann, 1996).

Results

Identification of PagL homologs in various Gram-negative bacteria

The 187-amino acid sequence of the S. Typhimurium PagL precursor protein (GenBank accession no. AAL21147) was used as a lead to identify putative PagL homologs in other Gram-negative bacteria, by searching all completed and unfinished genomes of Gram-negative bacteria present in the NCBI data base (www.ncbi.nlm.nih.gov/sutils/ genom_table.cgi). BLAST search (Altschul et al., 1990) revealed the
presence of putative homologs in the *Bordetella* spp. *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis* (Fig. 2). The PagL homologs of *B. bronchiseptica* and *B. parapertussis* are two mutually identical 178-amino acid polypeptides (Fig. 2) with, as predicted by the SignalP server (Nielsen et al., 1999), a 25-amino acid N-terminal signal peptide. A gene for a PagL homolog was also found in the genome of the *B. pertussis* Tohama I strain (Parkhill et al., 1999), but this open reading frame (ORF) was disrupted by a frame shift. Nucleotide sequencing of the PagL ORFs from *B. pertussis* strains BP509 and BP134 also showed the presence of the same frameshift,¹ which indicates that disruption of the PagL ORF might be a common feature in *B. pertussis* strains. By using the newly identified *B. bronchiseptica* pagL homolog as a probe for further BLAST analysis, additional putative pagL homologs could be identified in the genomes of *P. aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas syringae*, *Pseudomonas putida*, *Ralstonia metallidurans*, *Ralstonia solanacearum*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Burkholderia fungorum*, and *Azotobacter vinelandii* (Fig. 2). Together, all PagL homologs exhibited a low overall mutual sequence identity but contained a clear homologous domain near the C terminus.

**Cloning of *pagL* and heterologous expression in *E. coli***

To verify their putative lipid A deacylase activity, we cloned the *pagL* homologs of *P. aeruginosa* (*pagL*<sub>(Pa)</sub>) and *B. bronchiseptica* (*pagL*<sub>(Bb)</sub>). We included in these studies *pagL* from *S. Typhimurium* (*pagL*<sub>(St)</sub>) as a reference. These *pagL* genes were amplified from the chromosomes by PCR and eventually cloned into pET-11a under the control of the T7 promoter, resulting in plasmids, pPagL<sub>(Pa)</sub>, pPagL<sub>(Bb)</sub>, and pPagL<sub>(St)</sub>. To investigate expression and membrane localisation of PagL in *E. coli*, *E. coli* BL21 Star<sup>TM</sup> (DE3) containing the empty vector pET-11a or the pPagL plasmids was grown overnight in LB, after which cell envelopes were isolated. Analysis by SDS-PAGE revealed the presence of prominent additional bands with molecular masses of 15,000–18,000 in the cell envelopes of the cells expressing PagL (Fig. 3). This was consistent with the expected molecular masses of the mature PagL proteins, i.e., PagL<sub>(Pa)</sub> 16.1 kDa, PagL<sub>(Bb)</sub> 17.2 kDa, and PagL<sub>(St)</sub> 18.2 kDa. To identify the additional protein bands, they were subjected to microsequencing. The sequences of the first 5 amino acid residues of PagL<sub>(Pa)</sub>, PagL<sub>(Bb)</sub>, and PagL<sub>(St)</sub> were ADVSA, QPTQG, and NDNVF, respectively, indicating that cleavage of the signal peptide by leader peptidase occurs between amino acid residues 23 and 24 (AQA and ADV), 25 and 26 (AQA and QPT), and between 20 and 21 (CSA and NDN), respectively. Particularly in the case of expression of PagL<sub>(Bb)</sub>, an additional band with a higher molecular mass was visible on the gel (Fig. 3). The N-terminal sequence of this band, MQFLK, corresponded with that of the precursor of PagL<sub>(Bb)</sub>.

¹ H. J. Hamstra and P. van der Ley, unpublished observation.
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Fig. 2  Multiple sequence alignment of the PagL proteins. Sequences were aligned using ClustalW (www.ch.embnet.org/software/ClustalW.html). Hyphens indicate gaps introduced for optimal alignment. Absolutely conserved residues are marked with asterisks. Colons and dots indicate strongly and weakly conserved residues, respectively. The pagL ORF in B. pertussis is disrupted by a frameshift, which was restored for this alignment by adding two nucleotides in codon 33. The GenBank™ protein accession nos. for the PagL homologs are: S. Typhimurium, AAL21147; B. bronchiseptica, NP_890306; B. pertussis, NP_885487; P. aeruginosa, NP_253350; P. fluorescens, NZ_AAAT0300006; P. putida, NC_002947; P. syringae, ZP_00125465; R. metallidurans, ZP_00274744; R. solanacearum, ZP_00274744; A. vinelandii, ZP_00089534.

The symbol § indicates GenBank™ accession numbers of whole (unfinished) genomes, in which the PagL homologs were identified manually.
In vivo modification of *E. coli* LPS by PagL

To study whether the cloned PagL homologs were active on *E. coli* LPS, IPTG was added to exponentially growing *E. coli* BL21 Star™ (DE3) cells containing the empty vector pET-11a or the pPagL plasmids, and after various incubation periods, samples equivalent to one $A_{600}$ unit were collected, and their LPS content was analysed by Tricine-SDS-PAGE. In accordance with the expected hydrolysis of the R-3-hydroxy-myristate at the 3 position of lipid A, expression of any of the three *pagL* homologs converted the LPS into a form with a higher electrophoretic mobility (Fig. 4). The conversion was almost complete within 75 min after PagL$_{(Pa)}$ or PagL$_{(Bb)}$ was induced but took somewhat longer in the case of PagL$_{(St)}$.

To test whether the efficiency of *in vivo* modification in *E. coli* was dependent on growth temperature (30, 37, or 42°C), the presence of magnesium chloride (10 mM), time of PagL induction during the growth phase (early log phase, mid log phase, or stationary phase), or nutrient availability (rich medium (LB), or minimal medium (SV)), PagL$_{(Pa)}$ was expressed in *E. coli* under various conditions, after which the LPS profile was analysed by Tricine-SDS-PAGE. Under all conditions tested, no obvious changes in deacylation efficiency could be observed (data not shown).
Structural analysis of PagL-modified LPS

To determine its fatty acid content, LPS was isolated from bacteria that were grown in the presence of 10 mM MgCl₂ to suppress PhoP/PhoQ-regulated modifications of lipid A and analysed by GC/MS. The C14/3OH C14 ratio in the PagL-modified LPS samples was increased compared with that in the wild-type LPS (Fig. 5), consistent with the expected removal of a 3OH C14 from lipid A. To confirm these data, the lipid A moieties were isolated and analysed by ESI/MS in the positive ion mode, which revealed the presence of four major lipid A species in wild-type LPS (Fig. 6A). The peak at m/z 1797 represents the characteristic hexaacylated bis-phosphate species that is typically found in E. coli, whereas the peak at m/z 1928 corresponds to a hexaacylated bis-phosphate species replaced with an L-Ara4N moiety. The two remaining peaks at m/z 1716 and m/z 1847 most likely represent fragment ions of the two former species missing a phosphate group. Upon expression of PagL_(St) (Fig. 6B), PagL_(Pa) (Fig. 6C), or PagL_(Bb) (Fig. 6D), the major lipid A species were present at m/z 1622 and m/z 1490, which correspond to the loss of one β-hydroxymyristate residue and one phosphate group from the major species at m/z 1928 and m/z 1797 present in the empty vector control, respectively. Also here, the loss of the phosphate group is probably an artifact of the ionisation procedure. Based upon the GC/MS and ESI/MS data, it can be concluded that the identified PagL homologs of P. aeruginosa and B. bronchiseptica, like that of S. Typhimurium, are active lipid A deacylases. Furthermore, the data suggest that the deacylation is not dependent upon the absence or presence of an L-Ara4N moiety because both species were deacylated efficiently.

Subsequent in vivo modification of PagL-deacylated LPS

In the course of these experiments, it was observed that after prolonged PagL expression, PagL-modified LPS was no longer detectable on Tricine-SDS-PAGE and that the LPS migrated again at the position of wild-type LPS, as illustrated for the strain.
Fig. 5 GC/MS analysis of wild-type and PagL-modified E. coli BL21 Star™ (DE3) LPS. GC/MS analysis of purified E. coli BL21 Star™ (DE3) wild-type LPS (WT), PagL_{St}-modified LPS (L(St)), PagL_{Bb}-modified LPS (L(Bb)), and PagL_{Pa}-modified LPS (L(Pa)) (t = time after induction). Indicated are the normalised C14/30A C14 ratios with wild-type LPS set at 100 (values shown above bars).

Fig. 6 Structural analysis by ESI/MS of wild-type and PagL-modified E. coli BL21 Star™ (DE3) LPS. Lipid A species from wild-type E. coli BL21 Star™ (DE3) containing empty vector pET-11a (A) and lipid A species modified by PagL_{St} (B), PagL_{Pa} (C), and PagL_{Bb} (D) were analysed by ESI/MS. Major peaks at m/z 1797, 1928, 1622, and 1490 were interpreted as the characteristic hexaacylated bis-phosphate species that is typically found in E. coli, a hexaacylated bis-phosphate species substituted with an L-Ara4N moiety, a 3-O-deacylated mono-phosphate species substituted with an L-Ara4N moiety, and a 3-O-deacylated mono-phosphate species, respectively. The major peaks at m/z 1716 and 1847 probably represent fragment ions of the species at m/z 1797 and 1928.
Lipid A deacylases in Gram-negative bacteria expressing PagL(Bb) (Fig. 7A). The PagL protein was still abundantly present at this time point, as revealed on SDS-PAGE (data not shown). Furthermore, analysis by GC/MS revealed that the C14/3OH C14 ratio was not decreased again for the LPS isolated after a 5-h induction of PagL(Bb) (Fig. 7B). Thus, the secondary modification observed on the Tricine-SDS-polyacrylamide gel (Fig. 7A) was not the consequence of restoration of the PagL modification, but the result of (an) additional modification(s) that restored the electrophoretic mobility to that of wild-type LPS. Therefore, other fatty acid ratios were compared. A striking increase in the C16/C14 ratio was found in the LPS of cells induced 5 h for PagL production (Fig. 7C), suggesting that the PagL-deacylated LPS was subsequently palmitoylated.

A protein that adds palmitate to lipid A is the outer membrane protein PagP (Bishop et al., 2000) (Fig. 1). Therefore, we hypothesised that the secondary modification of PagL-modified LPS might have been the result of endogenous PagP activity. To investigate this possibility, we transformed wild-type E. coli BL21 Star™ (DE3) and its pagP mutant derivative JG101 with the pPagL(Pa) plasmid. The secondary modification of PagL-modified LPS was again observed in the case of the wild-type strain, but not in that of the mutant strain (Fig. 7D). This result strongly suggests that the secondary modification of PagL-modified LPS (Fig. 7A) was indeed the consequence of endogenous PagP activity.

**Identification of PagL active-site residues**

The mutual sequence identity between the identified PagL homologs is very low (Fig. 2). Among the few totally conserved residues are a histidine and a serine, which, we hypothesise, might be part of a “classical” Asp/Glu-His-Ser catalytic triad of serine hydrolases. These putative active-site residues are located at the lipid-exposed side near the top of a β-strand in a topology model we propose (Fig. 8). Interestingly, in the outer membrane phospholipase A (OMPLA), the active-site His and Ser are located in a similar position (Snijder et al., 1999). To test whether these residues, located at positions 149 and 151 of the PagL(Pa) precursor protein, respectively, are indeed important for catalytic activity, they were replaced by alanine or asparagine, and by alanine or cysteine, respectively. As a control, the same substitutions were made for a non-conserved histidine and serine residue, located at positions 81 and 84 of the PagL(Pa) precursor, respectively. The protein and LPS profiles of E. coli BL21 Star™ (DE3) cells carrying the relevant plasmids and induced for 75 min with IPTG were analysed by immunoblotting (Fig. 9A) and Tricine-SDS-PAGE (Fig. 9B), respectively. Whereas substitution of the non-conserved His-81 and Ser-84 did not affect LPS deacylation, deacylation of LPS
Fig. 7 In vivo remodification of deacylated LPS and the role of endogenous PagP. (A), exponentially growing *E. coli* BL21 Star\textsuperscript{TM} (DE3) cells containing the empty pET-11a vector or the pPagL\textsubscript{(Bb)} plasmid were induced with IPTG for the indicated time period. Samples corresponding to 1 \(A_{600}\) unit were collected and analysed by Tricine-SDS-PAGE. (B) and (C), the fatty acid content of purified *E. coli* BL21 Star\textsuperscript{TM} (DE3) wild-type LPS (WT) and PagL\textsubscript{(Bb)} modified LPS (L(Bb)), isolated at the indicated time after induction of pagL expression, was analysed by GC/MS. Indicated are the normalised C14 30H C14 (B) and C16/C14 (C) ratios with wild-type LPS set at 100 (values shown above bars). (D), exponentially growing wild-type *E. coli* BL21 Star\textsuperscript{TM} (DE3) or *E. coli* BL21 Star\textsuperscript{TM} (DE3) and its pagP mutant derivative JG101, containing pPagL\textsubscript{(Pa)}, were induced with IPTG for the indicated time period, after which 1 \(A_{600}\) unit culture samples were collected and analysed on Tricine-SDS-PAGE gel.
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was no longer observed when the conserved His-149 and Ser-151 were replaced (Fig. 9B), even though the expression of these mutant proteins was not affected (Fig. 9A). These results strongly support the hypothesis that the conserved histidine at position 149 and serine at position 151 of the precursor PagL(Pa) protein are active-site residues and that PagL mechanistically functions as a serine hydrolase.

PagL expression in *P. aeruginosa* and phenotypic characterisation of a *pagL* mutant

Because *pagL* homologs were identified in many Gram-negative bacteria, including non-pathogenic soil bacteria (Fig. 2), a primary role for the enzyme in pathogenesis appears unlikely. To gain insight in the possible function of PagL in *P.
aeruginosa, we tested whether endogenous pagL expression levels in P. aeruginosa can be influenced by the growth conditions. Therefore, wild-type P. aeruginosa PAO1 was grown in LB supplemented or not with 0–5% ethanol, 10 mM magnesium chloride, or 5 μg/ml EDDHA (to create iron-limiting conditions), or in minimal medium, or on solidified media (LB agar, and SV agar). Endogenous PagL levels were analysed by immunoblotting. The result showed that under all conditions tested, PagL was expressed, and the expression levels were similar (data not shown). Furthermore, we compared the growth characteristics of a P. aeruginosa pagL transposon-insertion mutant (32751) and its parental strain (PAO1). Both strains were tested for their ability to grow in LB medium at different temperatures (25, 30, or 37°C) or in LB medium supplemented with 0–4 M...
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sodium chloride, 0–1,500 μg/ml chloramphenicol, 0–5% ethanol, or 0–1% chloroform. The results showed that both strains had similar growth characteristics under most circumstances. Only when chloramphenicol was present in the medium, a difference in the ability to grow was observed. This difference was most pronounced at a concentration of 650 μg/ml chloramphenicol, where the absorbance of the wild-type culture after overnight growth was 1.7-fold higher than that of the PagL mutant (data not shown). This suggests that PagL probably does not function in the adaptation to different growth temperatures or in the resistance against osmotic stress or organic solvents but that its activity affects the permeability of the outer membrane for hydrophobic compounds, such as chloramphenicol.

Discussion

A lipid A 3-O-deacylase, PagL, was originally identified in S. Typhimurium (Trent et al., 2001a). Although similar activity was detected in some other bacteria, no homologs of pagL were identified (Trent et al., 2001a). Now, we identified pagL homologs in a range of Gram-negative bacteria, and we showed by cloning and expression of the corresponding genes in E. coli that at least two of them, those of P. aeruginosa and B. bronchiseptica, are functional lipid A deacylases. The newly identified PagL homologs are from organisms with genomes with a high GC content, and no other homologs were identified in Enterobacteriaceae, which suggested that Salmonellae might have acquired the pagL gene by horizontal gene transfer from an organism with a high GC content. However, although all newly identified pagL genes have a relatively high GC content (55–66%), pagL of S. Typhimurium has a GC content of only 39.5%, which is considerably lower than the S. Typhimurium average chromosomal GC content of 53% (McClelland et al., 2001). This observation suggests that S. Typhimurium has indeed acquired the pagL gene by horizontal gene transfer but not from an organism with a high GC content.

In previous work, it was demonstrated that outer membranes prepared from P. aeruginosa PAO1 harboured lipid A 3-O-deacylase activity (Basu et al., 1999). Furthermore, it is known that P. aeruginosa contains partially 3-O-deacylated lipid A species (Kulshin et al., 1991). The P. aeruginosa PagL homolog identified here is most likely responsible for the 3-O-deacylase activity found previously in P. aeruginosa membranes. However, we did not find an intact PagL homolog in the R. leguminosarum genome sequence, although 3-O-deacylase activity in its membranes has been described (Basu et al., 1999). BLAST searches with the unfinished R. leguminosarum
genome sequence did show the presence of two pagL homologs; however, the ORFs encoding the homologs were found to be disturbed by premature stop codons. Possibly, the available sequence still contains some errors. Also in other Gram-negative bacteria that are known to contain partially 3-O-deacylated lipid A, such as H. pylori (Bhat et al., 1994), no pagL homologs could be found. It is possible that the PagL homologs in these bacteria show only very low sequence similarity to the PagL family described in this paper, or 3-O-deacylation in these bacteria is mediated by entirely different proteins. On the other hand, we have also identified PagL homologs in bacteria that have not previously been described to contain 3-O-deacylated lipid A, for example, in the genomes of Bordetella spp. Strikingly, the pagL ORF in the B. pertussis strains appears to be disrupted by a frame-shift mutation, whereas the closely related species B. parapertussis and B. bronchiseptica have an intact pagL gene. Perhaps inactivation of pagL in B. pertussis is an example of host-specific adaptation. Large scale analysis of the capacity of Bordetella strains of expressing PagL will probably give insight in the relevance of the absence of intact pagL in B. pertussis.

We observed that E. coli lipid A upon deacylation by PagL was subsequently modified by the addition of palmitate, which was the consequence of endogenous PagP activity. An interesting question is whether the additional modification by PagP is actively triggered by lipid A deacylation or whether it is the consequence of a changed physiological state of the outer membrane, e.g., by the accumulation of fatty acids resulting from PagL activity. In the recently described NMR structure of E. coli PagP, the active-site was found to be located at the outer surface of the outer membrane, where phospholipids are normally not present (Hwang et al., 2002). The authors suggested that PagP activity might depend on the aberrant migration of phospholipids into the outer leaflet of the outer membrane. Thus, the observed secondary modification might be a consequence of the PagL activity, resulting in the migration of phospholipids from the inner leaflet to the outer leaflet of the outer membrane, where they can become a substrate for PagP. Interestingly, it was reported recently that some membrane-spanning proteins passively induce the flip-flop of phospholipids across lipid bilayers (Kol et al., 2003). Thus, the mere overproduction of PagL in our studies may already be sufficient to induce phospholipids transport. This notion is consistent with the appearance of a slower migrating LPS form, even when inactive PagL proteins were expressed (Fig. 9B).

The high expression of the PagL homologs in E. coli allowed the determination of the processing sites by N-terminal sequence analysis. The identified leader peptidase I cleavage sites for PagL_{Pa} and PagL_{Bb} correspond to the location predicted by the SignalP server (Nielsen et al., 1999). However, for PagL_{St}, the cleavage site did
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corresponded neither to the SignalP predicted site (between amino acids 17 and 18 (AFA and CSA)) (Nielsen et al., 1999) nor to the position that was identified in earlier work, in which the cleavage position was determined to be between residues 22 and 23 (AND and NVF) (Trent et al., 2001a). Because the -1 residue of the latter cleavage site does not conform to the consensus sequence $S_nXA$ ($S_n$ stands for an amino acid with a small neutral side chain), which is necessary for recognition by leader peptidase (von Heijne, 1983), this cleavage site is unlikely to be correct. The SignalP predicted site does match the consensus sequence for the -3 and -1 position, but the C domain, comprising the last six residues of the signal sequence does not include polar residues as one would expect it to do (von Heijne, 1983). The cleavage position identified here between residues 20 and 21 (CSA and NDN) does match both criteria, with a cysteine at the -3 ($S_n$) position and a serine at -2, increasing the polarity of the C domain.

The poor conservation of the PagL sequence allowed us to speculate about the catalytic mechanism. Among the few totally conserved residues were a histidine and a serine, which are located in our topology model in a position similar to that of the active-site residues in OMPLA (Snijder et al., 1999), i.e., at the lipid-exposed side of a $\beta$-strand, close to the cell surface. By means of amino acid substitutions, we demonstrated that these conserved residues are indeed essential for catalytic activity. The position of the active-site residues at the lipid-exposed side of the $\beta$-barrel suggests that PagL, like OMPLA, may be active as a dimer to be able to form a substrate binding pocket. Good candidates for the acidic component of the catalytic triad are the highly conserved aspartate and glutamate at positions 129 and 163 of the PagL(Pa) precursor sequence, respectively, which, in our model, are located in the $\beta$-strands flanking the one containing the active-site His and Ser residues (Fig. 8). The Asp-129 is conserved in all homologs, except in S. Typhimurium, where it is replaced by glutamate. The Glu-163 varies more, with possible substitutions by an aspartate, or an asparagine. Although rare, asparagine can substitute for the acidic residue in serine hydrolases, as it does, for instance, in E. coli OMPLA (Snijder et al., 1999).

The physiological role of PagL-mediated deacylation of lipid A remains to be elucidated. The identification of PagL homologs in a variety of Gram-negative bacteria, including non-pathogenic ones, such as P. putida and R. metallidurans, suggests that lipid A deacylation does not have a dedicated function in pathogenicity. To gain insight in its function, we tested whether pagL expression in P. aeruginosa can be influenced by the growth conditions. Supplementation of the growth medium with Mg$^{2+}$ ions did not affect pagL expression, consistent with the observation that 3-O-deacylase activity in P. aeruginosa membranes is not affected by the Mg$^{2+}$ concentration in the medium nor
by a phoP mutation (Trent et al., 2001a). Also, other growth conditions tested did not appear to affect pagL expression. Furthermore, we compared the growth of wild-type P. aeruginosa and a pagL mutant derivative under different conditions. The results showed a decreased resistance of the pagL mutant to chloramphenicol, suggesting that PagL activity, at least in P. aeruginosa, affects the permeability barrier of the outer membrane to hydrophobic compounds. The PagL family described here probably includes many more proteins because new BLAST searches just before submission of this paper (December 2004) using the newly identified homologs as leads revealed several additional PagL homologs in the genomes of, for example, Agrobacterium tumefaciens (GenBank™ accession no. AAK87616), Methylobacillus flagellatus (GenBank™ accession no. ZP_00173991), and Geobacter sulfurreducens (GenBank™ accession no. AAR36806). Again, these proteins have low overall sequence similarity but a clearly conserved C-terminal region, including the histidine-serine couple that was identified here as part of the active-site.
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