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Dual orientation of the outer membrane lipoprotein Pal in *Escherichia coli*

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Peptidoglycan associated lipoprotein (Pal) of *Escherichia coli* (*E. coli*) is a characteristic bacterial lipoprotein, with an N-terminal lipid moiety anchoring it to the outer membrane. Since its discovery over three decades ago, Pal has been well studied for its participation in the Tol–Pal complex which spans the periplasm and has been proposed to play important roles in bacterial survival, pathogenesis and virulence. Previous studies of Pal place the lipoprotein in the periplasm of *E. coli*, allowing it to interact with Tol proteins and the peptidoglycan layer. Here, we describe for the first time, a subpopulation of Pal which is present on the cell surface of *E. coli*. Flow cytometry and confocal microscopy detect anti-Pal antibodies on the surface of intact *E. coli* cells. Interestingly, Pal is surface exposed in an ‘all or nothing’ manner, such that most of the cells contain only internal Pal, with fewer cells (<20 %) exhibiting surface Pal.

INTRODUCTION

Like all Gram-negative bacteria, *Escherichia coli* (*E. coli*) contains two distinct membranes, an outer membrane and an inner membrane, separated by an aqueous layer commonly referred to as the periplasm. The periplasm contains peptidoglycan (also known as murein), a mesh-like layer of amino acids and sugars which provide the cell with structural integrity (Vollmer & Bertsche, 2008) and a unique opportunity to ‘connect’ the two membranes. For example, in the Tol–Pal complex, a combination of lipoproteins, integral membrane proteins and periplasmic proteins interact with each other and the peptidoglycan, forming a web of covalent and noncovalent contacts between the outer and inner membranes (Yeh *et al.*, 2010). The outer membrane peptidoglycan associated lipoprotein (Pal; tethered to the outer membrane via its N-terminal lipid moiety) is one of the key components in the Tol–Pal complex (Godlewska *et al.*, 2009; Gerding *et al.*, 2007) interacting noncovalently with the peptidoglycan layer (Leduc *et al.*, 1992; Bouveret *et al.*, 1999), outer membrane proteins OmpA (Clavel *et al.*, 1998) and Lpp

(Cascales *et al.*, 2002), periplasmic protein TolB (Bouveret *et al.*, 1995, 1999) and the inner membrane protein TolA (Cascales *et al.*, 2000; Deprez *et al.*, 2005). Although the exact function of Pal is unknown, Pal deletion mutants in *E. coli* exhibit cell envelope defects and greater susceptibility to the antibiotic vancomycin (Bernadac *et al.*, 1998; Cascales & Lloubès, 2004). Pal has also been shown to dimerize in the presence of Lpp and OmpA (Cascales *et al.*, 2002), although the physiological significance of dimerization is unknown.

While its periplasmic interactions have been well established, we considered the possibility that Pal may have a second orientation allowing surface exposure. We have recently shown that a homologue protein of Pal called P6 in nontypable *Haemophilus influenzae* (NTHi) has a dual orientation, existing as a surface-exposed protein and a periplasmic protein (Michel *et al.*, 2013). Cowles *et al.* (2011) were the first to describe a bacterial lipoprotein existing in two distinct orientations. They studied Lpp in *E. coli*. It had been known for some time that Lpp existed in two distinct forms – one covalently bound to the peptidoglycan, and a ‘free’ population that did not interact with peptidoglycan (Braun & Rehn, 1969; Braun & Bosch, 1972). The novel observation made by Cowles *et al.* (2011) was the discovery that the ‘free’ population of Lpp spans the outer

Abbreviations: Pal, peptidoglycan associated lipoprotein.

Four supplementary figures are available with the online Supplementary Material.

membrane, with one end of its trimeric structure exposed to the extracellular space.

In light of these recent Lpp and P6 dual orientation studies, we sought to determine if Pal might be another *E. coli* protein with a dual orientation. Here we describe experiments that show Pal is a third example of a dual oriented lipoprotein in Gram-negative bacteria, with a smaller population of Pal being surface exposed compared to its periplasmic population. We also show that the transmembrane protein OmpA does not play an important role in surface exposure of Pal. Pal has been shown to be released from *E. coli* in experimental models of sepsis (Hellman *et al.*, 2000, 2002). We discuss the possibility that the surface form of Pal may allow for its release from the outer membrane during sepsis, thus potentially implicating dual orientation in an important pathological mechanism.

METHODS

Bacterial strains and cell culture conditions. All *E. coli* cultures were grown on Luria Broth (LB) media. The following *E. coli* strains were utilized for experiments: XL1-Blue Supercompetent cells (Agilent); K1 RS218 cells and OmpA deletion cells (an OmpA-deficient derivative of K1 RS218), which were gifts from Dr Kwang Sik Kim, Johns Hopkins Children's Center (Wang & Kim, 2002); K12 JC1292 and JC7752 (a Pal-deficient derivative of JC1292) cells, which were a gift from Dr Jean-Claude Lazzaroni, University of Lyon (Hellman *et al.*, 2000); Top10 competent cells (Life Technologies); and JM109 competent cells (Agilent or Sigma Aldrich). Cells were cultured on LB under aerobic conditions, shaking (200 r.p.m.) at 37 °C for 2–3 h until the OD₆₀₀ reached 0.8 (exponential phase). Cells were pelleted gently (5000 g) and washed before further sample preparation.

SDS-PAGE and immunoblot assay. Samples for the 10% SDS-PAGE experiments were prepared in non-reducing sample buffer (2 × recipe: 0.12 M Tris/HCl pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue) and boiled for 10 min. Proteins were transferred to a nitrocellulose membrane (Pierce) and blocked with 5% milk in Tris buffered saline (TBS). The membrane was incubated with anti-Pal sera (Valentine *et al.*, 2006) at a 1:4000 dilution, anti-alkaline phosphatase (Millipore) at a 1:1000 dilution or anti-RNA polymerase B (Santa Cruz Biotechnology) at a 1:200 dilution in 1% milk and TBS. The membrane was then incubated with HRP-conjugated goat anti-mouse IgG (Bethyl Laboratories) at a 1:12 000 dilution in 1% milk and TBST (TBS with 0.05% Tween-20). The membrane was washed with TBS or TBST between antibody incubations. The blot was visualized using the Lumiglo Reserve HRP chemiluminescent substrate kit (KPL) according to the manufacturer's instructions.

Flow cytometry. Bacterial samples were cultured as described above and used for surface expression analysis by flow cytometry as described in the literature (Michel *et al.*, 2013). Briefly, approximately 250 µl of cells (OD₆₀₀ of approximately 0.8) were washed twice with PBS/1% BSA pH 7.2, and then incubated with 250 µl of primary antibody (anti-Pal) in serum at a 1:200 dilution for 1 h at room temperature. The cells were washed twice and then incubated with either 250 µl of either Alexa Fluor 488-labelled (Invitrogen) or Alexa Fluor 647-labelled (Life Technologies) goat anti-mouse IgG at 1:167 or 1:350 dilutions, respectively. After two additional washes, the cells were fixed using 250 µl of 4% paraformaldehyde fixative reagent (BioLegend) for 30 min at room temperature. For the vancomycin experiments, cells were incubated with 17 µg ml⁻¹ of BODIPY FL vancomycin (Life Technologies) for 30 min prior to fixation. Per-

meabilized cells were treated with 0.1% Triton X-100 (US Biologicals) in PBS (final concentration of approximately 1.7 mM Triton X-100) for 30 min at room temperature prior to fixation. All samples were resuspended in 200–250 µl of PBS and read on a LSR II flow cytometer (BD Biosciences).

Confocal microscopy. Bacterial samples were cultured and prepared as described above for flow cytometry. The confocal images were collected on a Leica TCS SP5 II AOBs Filter-free Tunable Spectral Confocal Research Microscope with Resonant Scanner and Hybrid Detectors (Leica Microsystems) attached to a Leica DMI6000 Fully Automated Microscope using Leica LAS system software and a 60 × oil immersion objective.

Biotinylation experiment: Sulfo-NHS-SS-biotin. A Cell Surface Protein Isolation kit (Pierce) was used according to the manufacturer's instructions, with some modifications. *E. coli* bacteria were cultured as described above. Ten millilitres of cells was pelleted (5000 g), washed in PBS and incubated with 10 ml of freshly prepared Sulfo-NHS-SS-biotin (0.6 mg ml⁻¹) for 30 min. Quenching solution (1.5 ml) was added to the cells which were then washed with TBS. Cells were resuspended in 10 ml TBS and lysed via sonication (15 s on, 45 s off, for 20 cycles) in the presence of 30 µl of Triton X-100. The cells were pelleted (15 000 g), and the supernatant was further concentrated to 500 µl using an Amicon Ultra-15 Centrifugal Filter unit (10 kDa MWCO). The supernatant was then added to a NeutrAvidin Agarose column (500 µl of beads, prepared as described in the manufacturer's instructions). After a 1 h incubation, the nonbiotinylated proteins were eluted off the column, added to 500 µl of 2 × SDS-PAGE sample buffer and stored at 4 °C. The biotinylated proteins were eluted off the column after a 1 h incubation with 500 µl of 1 M DTT and 2 × SDS-PAGE sample buffer. Before running the nonbiotinylated and biotinylated samples on a 10% SDS-PAGE gel, the biotinylated sample was brought to a final volume of 1 ml with water. Both 1 ml (nonbiotinylated and biotinylated) samples were boiled for 10 min and stored in the -20 °C freezer. Samples were run on an SDS-PAGE gel and detected using anti-Pal sera in an immunoblot, as described above. To serve as a control, the same samples were also detected using anti-RNA polymerase B or anti-alkaline phosphatase. To quantify the nonbiotinylated and biotinylated proteins, the chemiluminescence of the immunoblot was detected using a ChemiDoc XRS system (Bio-Rad) and band volumes were calculated using Quantity One 1-D Analysis software (Bio-Rad).

Biotinylation experiment: NHS-LC-LC-biotin. Biotinylation of surface proteins using NHS-LC-LC-biotin was performed as described in the literature (Cowles *et al.*, 2011), with some modifications. Briefly, *E. coli* bacteria were cultured as described above until the OD₆₀₀ reached approximately 0.8. Cell culture (10 ml) was gently pelleted, washed with PBS twice, and resuspended in 1 ml of PBS. A 25 mg ml⁻¹ stock of NHS-LC-LC-biotin was prepared in DMSO (Pierce) and added to a final concentration of 2% v/v (20 µl per 1 ml of cell suspension). After a 20 min incubation at room temperature (rocking), the NHS-LC-LC-biotin was capped using 500 µl of the quenching solution from a Cell Surface Protein Isolation kit (Pierce). The cells were washed several times with TBS, and resuspended to a final volume of 10 ml of TBS. Cells were lysed via sonication (15 s on, 45 s off, for 20 cycles) in the presence of 30 µl of Triton X-100. After the cells were pelleted (15 000 g), the supernatant was concentrated in an Amicon Ultra-15 Centrifugal Filter unit (10 kDa MWCO) to a final volume of 500 µl. The supernatant was added to a NeutrAvidin Agarose column and allowed to incubate with 500 µl of NeutrAvidin Agarose beads (washed and spun dry prior to addition of the supernatant) for 60 min (as described above). The nonbiotinylated proteins were eluted off the column, added to 500 µl of 2 × SDS sample buffer, and boiled for 10 min. The NeutrAvidin beads and bound biotinylated proteins were washed four times with Wash buffer

(provided in the Pierce Cell Surface Protein Isolation kit). Finally, 500 ml of $2 \times$ SDS sample buffer was added to the beads and the mixture was boiled for 10 min to release the biotinylated proteins. The samples were brought to their final volume using TBS. Samples were separated and quantified as described in the other biotinylation experiment.

RESULTS

Pal is expressed on the surface of *Escherichia coli*

The periplasmic population of Pal has been well documented (Godlewska *et al.*, 2009). Therefore, our focus was to determine if Pal was surface exposed. To indirectly visualize surface Pal, we used confocal microscopy to detect Pal after monoclonal antibody (Valentine *et al.*, 2006) and AlexaFluor 488-conjugated secondary antibody labelling. Surface Pal was observed and its expression was 'all or nothing'; that is, *E. coli* (XL1-Blue) cells displayed either no visible surface Pal or were highly illuminated, as seen in Fig. 1(a). Confocal images demonstrated that only a few *E. coli* cells exhibited fluorescent staining (we estimate between 2.5–7% of the total cells), suggesting that the cells with exposed Pal were scarce in a given population. The fluorescently labelled cells appeared to be of normal morphology and size compared to non-fluorescent *E. coli* cells.

To ensure that the cells that exhibited surface Pal (as seen by confocal microscopy) were not the result of fragile or perforated bacterial membranes, similar experiments were performed with *E. coli* (XL1-Blue) cells using green fluorescently labelled vancomycin (BODIPY FL vancomycin)

which targets the peptidoglycan of Gram-positive bacteria. Since vancomycin is too large to diffuse through the major porins of the outer membrane in Gram-negative cells (Cascales *et al.*, 2000), sensitivity to vancomycin could only occur if the outer membranes of the *E. coli* cells were fragile or easily penetrable. We did visualize Pal-labelled cells that did not exhibit vancomycin labelling (Fig. 1b). As a control, we observed that the same concentration of vancomycin was able to readily label *E. coli* cells that were pretreated with 0.1% Triton X-100 detergent (Fig. 1c). We analysed the same Pal-labelled cell samples with vancomycin using flow cytometry, and observed that 1.82% of the cells were labelled with anti-Pal only, 1.25% of cells were labelled with vancomycin only, and 0.44% of cells were labelled with both anti-Pal and vancomycin (Fig. 2; controls in Fig. S1 and S2, available in the online Supplementary Material).

As an additional control, we performed labelling experiments using the Pal deletion strain JC7752 and compared them with experiments using the parent *E. coli* strain JC1292 (Hellman *et al.*, 2000). The parent strain exhibited labelling with Pal and secondary antibody conjugated to Alexa Fluor 488 (green) or Alexa Fluor 647 (red), while the Pal deletion strain showed low Pal labelling above background (Fig. 3). Fig. 3 contains only single snapshots of the cells, but these snapshots are representative of what we saw throughout the entire sample. We performed flow cytometry on the same samples (parent strain: with anti-Pal and 488 or 647; Pal deletion strain: with anti-Pal and 488 or 647) (Fig. 4; controls in Fig. S3). The Pal deletion strain consistently showed some Pal labelling, but labelling was only approximately three times the background level.

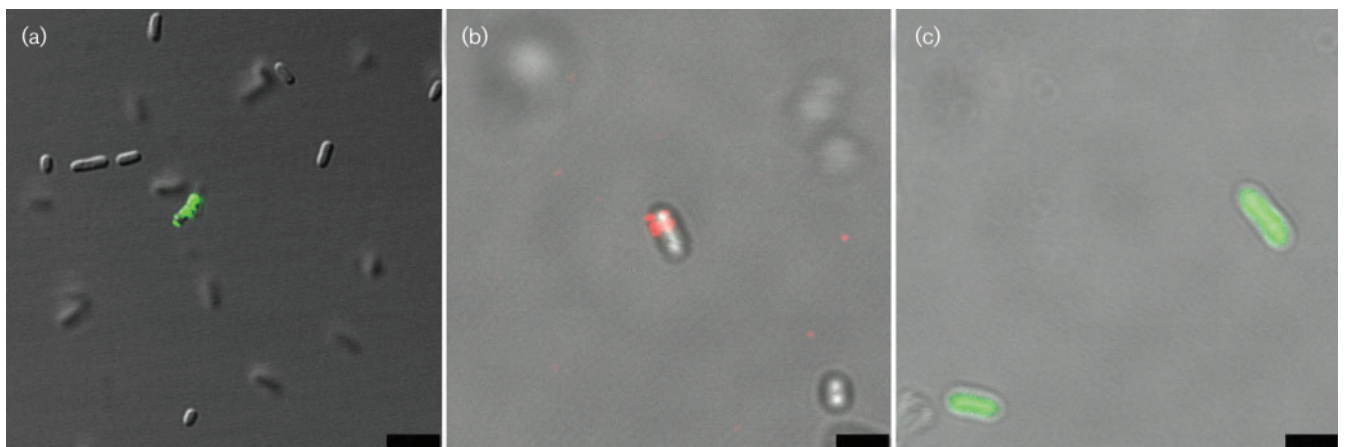


Fig. 1. Confocal images of *E. coli* stained with anti-Pal and vancomycin. (a) This pseudo-colour confocal image shows that only a small percentage of whole *E. coli* cells are stained with anti-Pal and Alexa Fluor 488 (green). Scale bar, 5 μ m. (b) This merged pseudo-colour confocal image shows an *E. coli* cell stained with Pal and Alexa Fluor 647 (red), but not BODIPY FL vancomycin (green). Scale bar, 2.5 μ m. (c) This pseudo-colour confocal image shows that BODIPY FL vancomycin (green) readily stains *E. coli* cells that were permeabilized by pretreatment with 0.1% Triton X-100. Scale bar, 2.5 μ m.

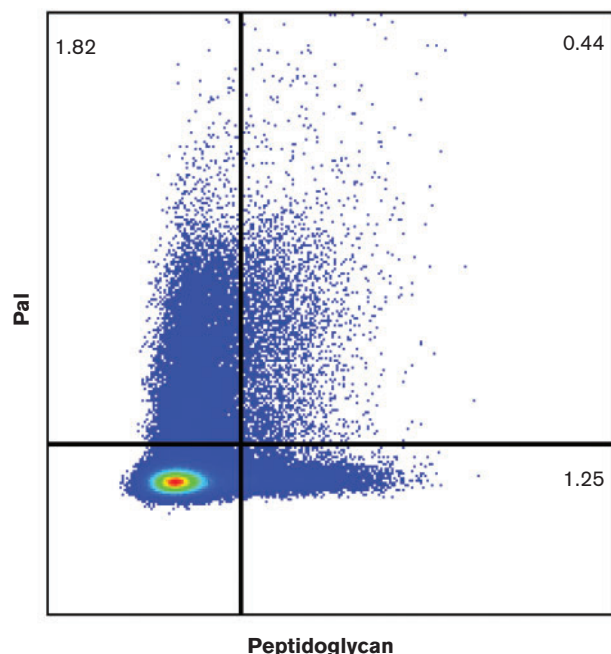


Fig. 2. Flow cytometric detection of *E. coli* cells stained with anti-Pal and vancomycin. Flow cytometry data demonstrate that a small percentage of *E. coli* cells are stained with BODIPY FL vancomycin (peptidoglycan) only (1.25%), a small percentage with Alexa Fluor 647 (Pal) only (1.82%), and an even smaller percentage of cells with both stains (0.44%). The percentages of labeled bacteria (from total bacteria) are noted in the quadrants.

In contrast, the parent strain showed labelling approximately 23 times the background level.

The majority of Pal is periplasmic

As determined with confocal microscopy and flow cytometry, Pal appeared to be on the surface of only a limited number of *E. coli* cells, in an ‘all or nothing’ manner. We estimated using confocal microscopy that between 2.5% and 7% (mean average of 5%) of the total cells had surface Pal; not surprisingly, our flow cytometry experiments yielded similar values (approximately 3% of total cells were labelled with anti-Pal). To quantify the average surface versus periplasmic Pal populations using an alternate method, we employed a biotinylation technique previously described in the literature (Cowles *et al.*, 2011). The hydrophobic, primary amine-reactive biotinylation reagent, NHS-LC-LC-biotin, was shown to be impermeable to the *E. coli* outer membrane due to its hydrophobicity and relatively high molecular mass (Cowles *et al.*, 2011). We employed the same reagent to label surface Pal in *E. coli* with biotin, thus allowing us to quantify internal and surface Pal populations. Monoclonal anti-Pal Western blots of the two populations showed that the majority of Pal was internally localized, while a much smaller population was surface

exposed [*E. coli* K-12 RS218 cells: Fig. 5a (A and B) and Table 1]. Several other *E. coli* strains were also tested using the same methodology (Fig. S4 and Table 1), all yielding similar results with internal Pal population around 72% and surface Pal around 28%.

To confirm the NHS-LC-LC-biotin results, we used a second biotinylation reagent, Sulfo-NHS-SS-biotin (Lei *et al.*, 2011) to quantify internal and surface Pal. The Sulfo-NHS-SS-biotin labelling results were similar to those with NHS-LC-LC-biotin. Internal Pal was present as 74% of the total population, and surface Pal was present as 26% of the population [Fig. 5a (C and D) and Table 1].

To ascertain that the biotin experiments were not a result of a compromised membrane, we used antibodies to two non-exposed proteins in *E. coli* to test the leakiness of the biotin reagents (Fig. 5b, c). We could not detect any labelling using our periplasmic control antibody (anti-alkaline phosphatase) (Fig. 5b), and the cytoplasmic control antibody (anti-RNA polymerase β) was only minimally leaky (NHS-LC-LC: 14%, Sulfo: 5%) (Fig. 5c), indicating that the quantification of the surface Pal populations using both Sulfo-NHS-SS-biotin and NHS-LC-LC-biotin were, at most, overestimates by 14% and 5%, respectively.

OmpA does not play a role in Pal surface exposure

OmpA is a large transmembrane protein with a periplasmic domain that has been shown to undergo a temperature-sensitive transition (Zakharian & Reusch, 2005) where it combines with the transmembrane region to form a larger β -barrel pore at elevated temperatures. The structure of the OmpA periplasmic domain was recently solved (Park *et al.*, 2012) and, as suggested by its sequence homology to Pal (29% sequence identity), its 3D structure is similar to the structure of Pal. We, therefore, wanted to assess whether or not OmpA played an integral important role in ‘flipping’ Pal to the cell surface. We biotinylated surface Pal using the NHS-LC-LC-biotin protocol on an *E. coli* *ompA* knockout mutant. The results of this experiment showed that Pal is surface exposed in the OmpA knockout mutant cells at the same level as in the WT *E. coli* parent strain (K1 RS218): 71% internal Pal and 29% surface Pal (Fig. 6). Neither biotinylated RNA polymerase β , nor biotinylated alkaline phosphatase, were detected in the same samples.

DISCUSSION

Since its discovery in 1981 (Mizuno, 1981), Pal has been well studied (Godlewska *et al.*, 2009); mostly notably for its interactions with other bacterial proteins (Bouveret *et al.*, 1995; Cascales *et al.*, 2000, 2002; Clavel *et al.*, 1998), its proposed role in maintaining the integrity of the outer membrane of *E. coli* (Cascales *et al.*, 2002) and its other physiological interaction with the peptidoglycan layer (Lazzaroni & Portalier,

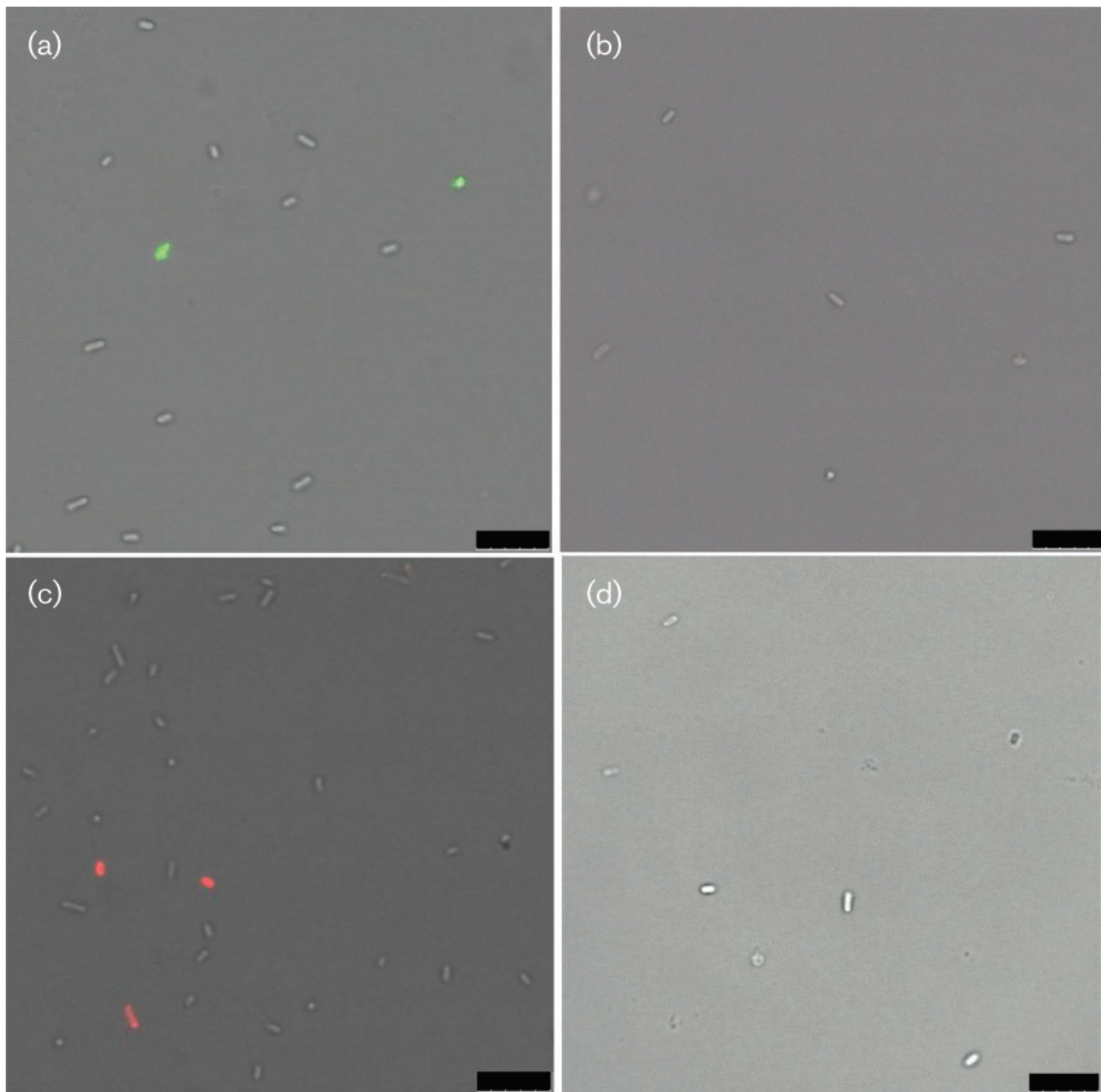


Fig. 3. Confocal images comparing the Pal deletion strain with WT *E. coli*. Pseudo-colour confocal images show that the *E. coli* Pal deletion strain does not fluoresce when incubated with anti-Pal and Alexa Fluor 488 (b) or Alexa Fluor 647 (d). (a) and (c) show the *E. coli* parent strain fluorescing under the same incubation conditions, respectively. Bars, 10 μm .

1992). TolB and peptidoglycan compete for the same binding site on Pal, which happens to be on the opposite end (structurally) of the N-terminal lipid anchor (Abergel *et al.*, 1999; Bouveret *et al.*, 1995; Cascales & Lloubès, 2004; Parsons *et al.*, 2006), thus making the interactions of Pal with TolB and peptidoglycan mutually exclusive. These interactions all point to the necessity for Pal to be localized in the periplasm of the cell. This study describes, for the first time, a subpopulation of Pal that is localized to the cell surface of *E. coli*.

The discovery that the homologue of Pal in NTHi, P6, is expressed both on the cell surface and in the periplasm (Michel *et al.*, 2013) led us to the hypothesis that Pal might also be dual oriented. As corroborative evidence, it

was also suggested that both Lpp and Pal epitopes were exposed on the surface of *E. coli* that had been incubated in human serum (Hellman *et al.*, 1997). Here we have shown with flow cytometry and confocal microscopy that Pal is exposed on the surface of uncompromised *E. coli* cells. We also demonstrate that Pal is surface exposed in an ‘all or nothing’ manner, with a few cells seemingly covered with surface Pal and most other cells with undetectable levels of surface Pal. Biotinylation of surface Pal allowed us to separate the ‘inside’ and ‘outside’ Pal populations to estimate that less than 25% of the total Pal population is surface exposed (taking into account leakiness, we estimate that between 10–20% of Pal may be surface exposed).

Table 1. Biotinylation of Pal in several *E. coli* strains

Average biotinylated (B) and nonbiotinylated (NB) Pal populations, using Sulfo-NHS-SS-biotin and NHS-LC-LC-biotin with different *E. coli* strains, suggest that most of the Pal populations are nonbiotinylated.

<i>E. coli</i> strain	Biotin	NB (% population)	B (% population)
RS218	Sulfo-NHS-SS-biotin	74	26
RS218	NHS-LC-LC-biotin	74	26
XL1-Blue	NHS-LC-LC-biotin	72	28
Top10	NHS-LC-LC-biotin	69	31
JM109	NHS-LC-LC-biotin	72	28
JC1292	NHS-LC-LC-biotin	73	27

The biotinylation experiment yields slightly higher percentages of surface Pal compared to estimates using confocal microscopy and flow cytometry. This discrepancy is likely to be the result of our different labelling techniques. For the biotinylation experiment, our biotin reagents label any/all exposed lysine residues on Pal (six lysine residues available on the Pal structure). In contrast, the confocal and flow experiments rely on monoclonal antibody binding to a specific epitope on Pal. The monoclonal antibody interaction can be hindered by crowding on the cell surface, thus yielding lower estimates for surface Pal.

OmpA is a large transmembrane protein that exhibits a unique temperature-sensitive folding transition where, at elevated temperatures, its periplasmic C-terminal tail is incorporated into the membrane-spanning domain thus

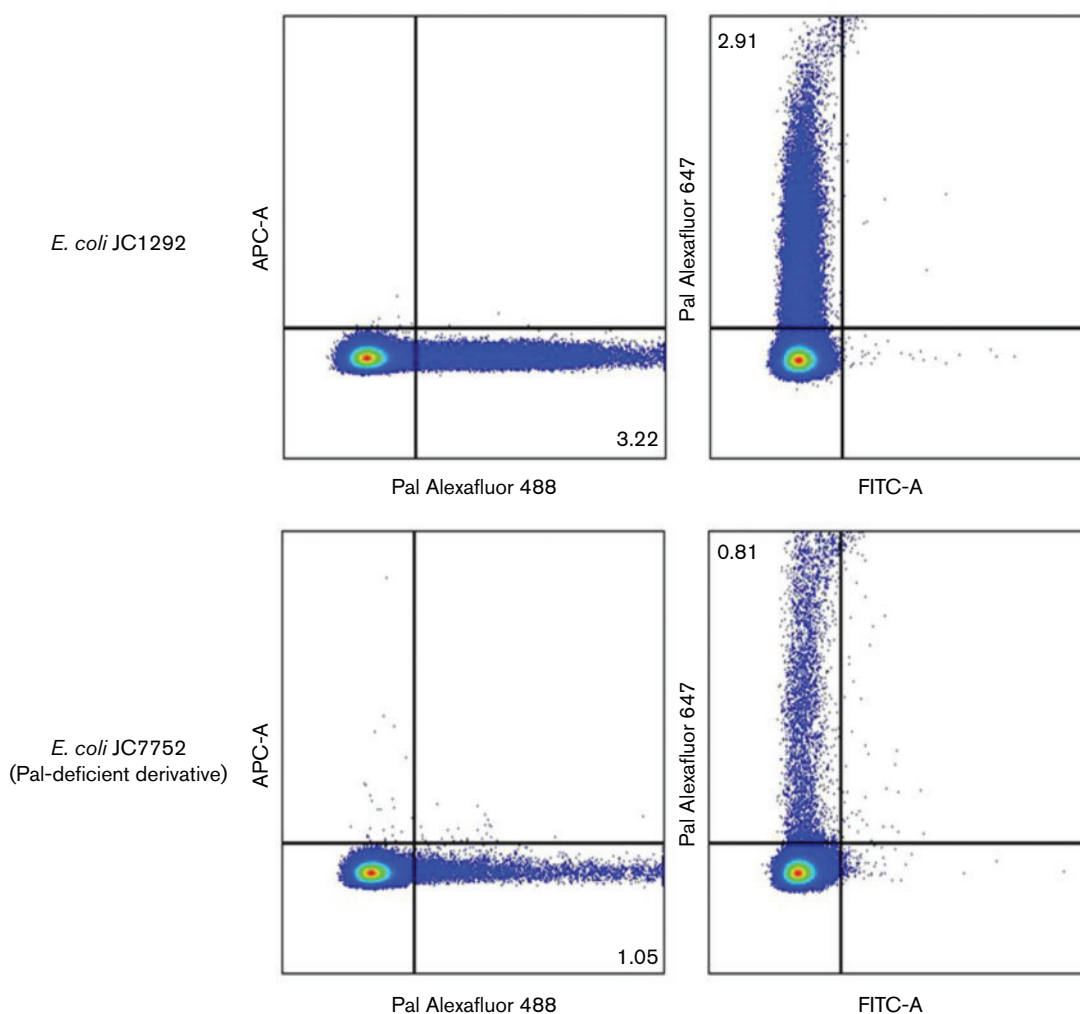


Fig. 4. Flow cytometric detection of Pal in a Pal deletion strain and WT *E. coli*. *E. coli* JC1292 cells (top) and JC7752 (Pal-deficient derivative) cells (bottom). The left panels are cells stained with Alexa Fluor 488 (Pal) and the right panels are stained with Alexa Fluor 647 (Pal). These data were representative of the three replicates that were collected for the experiment. The percentages of labeled bacteria (from total bacteria) are noted in the quadrants.

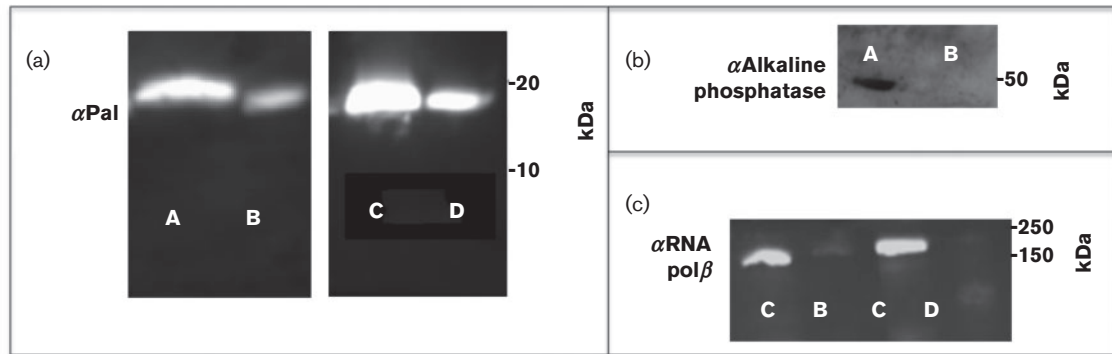


Fig. 5. Biotinylation of surface proteins in *E. coli*. (a) Representative immunoblots of Pal from *E. coli* K1 RS218: nonbiotinylated Pal (A) and Sulfo-NHS-SS-biotinylated Pal (B); nonbiotinylated Pal (C) and NHS-LC-LC-biotinylated Pal (D). (b) The immunoblot for alkaline phosphatase from *E. coli* K1 RS218 shows that all of the protein is nonbiotinylated (A) and no biotinylated protein (by NHS-LC-LC-biotin) was detected (B). (c) The immunoblot for RNA polymerase β from *E. coli* K1 RS218 shows that only a very small percentage of RNA polymerase β is biotinylated with NHS-LC-LC-biotin (14%) (B) or Sulfo-NHS-SS-biotin (5%) (D), while most of RNA polymerase β is nonbiotinylated (A, C).

forming a large ‘megapore’ (Zakharian & Reusch, 2005). Since the periplasmic tail of OmpA is similar in both sequence and structure to Pal (Park *et al.*, 2012), we considered the possibility that Pal might interact with the periplasmic domain of OmpA such that it could be ‘flipped’ to the surface during the OmpA temperature transition. Our experimental data, however, showed that surface Pal was biotinylated in the OmpA knockout (in *E. coli*), suggesting that OmpA is not required for surface exposure of Pal.

We know that lipoproteins are transported (as dictated by their signal sequences) to the outer membrane via the Lol transport system (Narita *et al.*, 2004; Narita & Tokuda, 2010). Therefore, we consider only two ways for Pal to be inserted into the outer membrane. Either the Lol system inserts Pal into both the inner and outer leaflets of the outer membrane, or Lol inserts Pal into the inner leaflet of the outer membrane and then Pal ‘flips’ to the cell surface sporadically or in response to a signal/event. Since it would be energetically unfavourable for Pal to flip to the surface on its own, the latter hypothesis would require a lipoprotein ‘flippase’ to accomplish the task.

In summary, we determined that less than 25% of Pal is surface exposed, and that Pal surface exposure occurs in an ‘all or nothing’ manner. Although currently we have no hypotheses as to how or why Pal is dual oriented, with this report we add Pal to Lpp and NTHi P6 as dual oriented lipoproteins in Gram-negative bacteria. It has also been shown that Pal is released from bacteria in experimental models of sepsis, and that a proportion of released Pal was tightly associated with LPS, OmpA and Lpp (Hellman *et al.*, 2000, 2002). A second population of released Pal, however, was not associated with LPS, OmpA or Lpp (Hellman *et al.*, 2000). Furthermore, serum-released Pal was preferentially bound by IgG in J5 antiserum, which has been shown to protect in sepsis (Liang *et al.*, 2005). Pal has also been reported to cause inflammation and death in LPS-hypo-responsive mice, and Pal-deficient *E. coli* mutants are less virulent than their WT progenitors. Taken together, these reports suggest that Pal may be a bacterial mediator of Gram-negative sepsis (Hellman *et al.*, 2002). We speculate that serum-released Pal (perhaps more specifically the unassociated population of released Pal) may be derived from bacteria with surface expressed Pal.

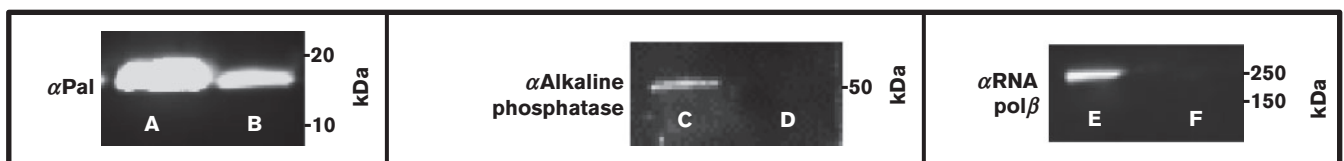


Fig. 6. Biotinylation of surface proteins in an OmpA deletion strain of *E. coli*. Immunoblots from the same biotinylation experiment with *E. coli* OmpA deletion cells: nonbiotinylated (A) and biotinylated Pal (B); nonbiotinylated (C) and biotinylated alkaline phosphatase (D); nonbiotinylated (E) and biotinylated RNA polymerase β (F).

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