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Proton motive force drives the interaction of the inner membrane TolA and outer membrane Pal proteins in *Escherichia coli*

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Summary

The Tol–Pal system of the *Escherichia coli* envelope is formed from the inner membrane TolQ, TolR and TolA proteins, the periplasmic TolB protein and the outer membrane Pal lipoprotein. Any defect in the Tol–Pal proteins or in the major lipoprotein (Lpp) results in the loss of outer membrane integrity giving hypersensitivity to drugs and detergents, periplasmic leakage and outer membrane vesicle formation. We found that multicopy plasmid overproduction of TolA was able to complement the membrane defects of an *lpp* strain but not those of a *pal* strain. This result indicated that overproduced TolA has an envelope-stabilizing effect when Pal is present. We demonstrate that Pal and TolA formed a complex using *in vivo* cross-linking and immunoprecipitation experiments. These results, together with *in vitro* experiments with purified Pal and TolA derivatives, allowed us to show that Pal interacts with the TolA C-terminal domain. We also demonstrate using protonophore, K^+ carrier valinomycin, nigericin, arsenate and fermentative conditions that the proton motive force was coupled to this interaction.

Introduction

The cell envelope of Gram-negative bacteria acts as a barrier against many noxious compounds. These compounds can overcome cell envelope integrity by three main methods: diffusion through the outer membrane porins; disruption of the lipopolysaccharide (LPS) leaflet; or when the active efflux systems are defective (Nikaido, 1996). The alteration of the outer membrane's effectiveness as a permeability barrier and its integrity have been compared previously in *tol–pal*, *tolC*, *rfaD* and *lpp* *Escherichia coli* strains. These strains were found to be hypersensitive to drugs and detergents, but only strains having a defect in one of the TolA–B–Q–R proteins or in the outer membrane lipoproteins Pal or Lpp were found to release outer membrane vesicles and periplasmic proteins (Suzuki *et al*., 1978; Yem and Wu, 1978; Webster, 1991; Bernadac *et al*., 1998). Lpp is covalently attached to the peptidoglycan and thus fixes it to the outer membrane (Braun, 1975; Yakushi *et al*., 1997). Similarly, although in the absence of a covalent link, the peptidoglycan-associated lipoprotein Pal mediates interaction between the murein layer and the outer membrane (Leduc *et al*., 1992). The Tol–Pal proteins have been shown to form an inner membrane TolQ–R–A complex through transmembrane segment interactions (Derouiche *et al*., 1995; Lazzaroni *et al*., 1995; Germon *et al*., 1998; Journet *et al*., 1999) and an outer membrane TolB–Pal complex (Bouveret *et al*., 1995). The co-fractionation of the TolQ–R–A–B proteins in sucrose density gradient analyses of cell membranes suggested the existence of a transenvelope Tol complex (Guihard *et al*., 1994). Furthermore, Lpp and OmpA, two major structural envelope proteins, were found to bind TolB in a Pal-dependent manner (Clavel *et al*., 1998). Besides these interactions, there is no direct evidence for an interaction between the inner and outer membrane Tol–Pal complexes. Although nothing is known about the precise function of the Tol–Pal system, these proteins are believed to play an important role because *tol–pal* gene clusters have been found in many other Gram-negative bacteria (Sturgis, 2001). A possible function was hypothesized from *in vitro* experiments showing that the central domain of TolA and the TolB proteins interact with trimeric outer membrane porins (Derouiche *et al*., 1996; Rigal *et al*., 1997). These results, taken together with the outer membrane defects of *tol–pal* mutations, suggested their potential role in the transport of outer membrane components through the periplasm. It is noteworthy that the Tol proteins have been found to be implicated in the virulence of *Salmonella typhimurium* (Bowe *et al*., 1998) and in the import of the cholera toxin phage into *Vibrio cholerae* (Heilpern and Waldor, 2000).

The Tol–Pal system shows homologies with the TonB system, in which ExbB and ExbD proteins are homologous in their amino acid sequences and topologies with TolQ and TolR proteins respectively (Eick-Helmerich and

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Braun, 1989; Kampfenkel and Braun, 1992; 1993; Müller *et al*., 1993; Vianney *et al*., 1994). Moreover, the interactions of the transmembrane domains of ExbB–D and TonB (Larsen *et al*., 1994; Braun *et al*., 1996; Higgs *et al*., 1998) share similarity with those of the TolQ–R–A proteins. Cross-talk of TonB with TolQ–R was demonstrated (Braun and Hermann, 1993; Larsen *et al*., 1999), and TonB could be cross-linked *in vivo* to outer membrane siderophore or cobalamin receptors (Skare *et al*., 1993; Moeck *et al*., 1997; Cadieux and Kadner, 1999). High-affinity receptors for the energy-dependent transport of cobalamin and iron-siderophore require the proton motive force (Bradbeer, 1993) and the presence of TonB protein (Reynolds *et al*., 1980). TonB functions in transducing energy from the cytoplasmic membrane to specific receptors of the outer membrane for the active transport of nutrients (Braun, 1995). Although TolA and TonB sequences are dissimilar, the exchange of the N-terminal anchor of TonB for that of TolA suggested that the four conserved residues found in their inner membrane anchors were functionally significant (Karlsson *et al*., 1993). Another striking similarity between these systems is the fact that they have both been parasitized and are required for the import of macromolecules such as colicins and phage DNA through the cell envelope (Webster, 1991; Lazdunski *et al*., 1998).

Until now, no link between TolA and outer membrane proteins has been demonstrated *in vivo*. In this study, we found that overproducing TolA protein stabilized the cell envelope defects of an *lpp* mutant but had no effect in a *pal* mutant. Cross-linking and co-immunoprecipitation experiments demonstrated the *in vivo* interaction of TolA with the outer membrane Pal lipoprotein. Cell energization was found to be required to detect this interaction, indicating a new analogy between the TolA and TonB proteins.

Results

Outer membrane defects of lpp, pal *and* tolA *cells*

To compare the outer membrane alterations of *lpp*, *pal* and *tolA* cells, we have investigated three distinct criteria: first, hypersensitivity to antibiotics and detergents; secondly, the membrane defect reflected by periplasmic leakage; and thirdly, the formation of outer membrane microvesicles. The presence of microvesicles was observed by electron microscopy; cells were analysed for the release of periplasmic RNase I and their susceptibility to SDS and vancomycin. SDS, in common with lipophilic antibiotics, is pumped out of cells by active efflux systems. Vancomycin, a hydrophilic glycopeptide that inhibits the transpeptidation step of cell wall synthesis, is too large to diffuse through the major porins and is presumed to be imported through the TolC pore (Schlör *et al*., 1997). All three parental strains used in this study, designated as wild type (wt), presented identical membrane integrity according to these different criteria (Table 1). By way of comparison, the vancomycin-resistant *tolC* cells (growth not affected at 200 μg ml^{-1}), which did not grow at 0.1% SDS, never showed vesicle formation or periplasmic leakage (Bernadac *et al*., 1998). The *lpp*, *pal* and *tolA* mutants showed similar cell envelope defects as far as the release of RNase I and SDS sensitivity are concerned. As observed previously (Bernadac *et al*., 1998), the *pal* mutant showed fewer vesicles than the *lpp* mutant. This might be related to the abundance of the structural lipoproteins in the cell envelope. However, although *tolA–B* cells have been found previously to be hypersensitive to vancomycin (Bernstein *et al*., 1972), the higher sensitivity to vancomycin found in *tolA* and *pal* cells indicates that they have different alterations from *lpp* cells.

Overexpressed TolA protein suppresses effects caused by an lpp *mutation*

Two plasmids overproducing TolA derivatives were transformed into *tolA*, *lpp* and *pal* cells, and the cell envelope integrity was analysed (Table 1). TolA is a three-domain protein with an N-terminal inner membrane anchor and a long periplasmic region formed of two domains (TolAII and TolAIII) (Levengood *et al*., 1991). pTolA and pTolAI–IIr plasmids express N-terminal histidine$_6$-tagged TolA (H_6TolA) and TolA devoid of its C-terminal domain (H_6TolAI–IIr) respectively. H_6TolAI–IIr corresponds to a construct in which the TolA domain III

Table 1. Outer membrane defects of *lpp*, *pal* and *tolA* cells and plasmid complementations.

Strains	OMV[a]	RNase[b]	SDS[c]	Van[c]
Wild type	0	0	> 2	180
tolA	+ +	+	0.1	20
tolA pTolA	0	0	2	190
tolA pTolAI–IIr	+ +	+	0.1	30
lpp	+ +	+	0.1	100
lpp pTolA	+	0	1.1	150
lpp pTolAI–IIr	+ +	+	0.1	120
pal	+	+	0.1	20
pal pTolA	+	+	0.1	30

a. Amounts of outer membrane vesicles observed by electron microscopy after negative staining of cells grown on agar plates (classified according to Bernadac *et al*., 1998). + +, many vesicles on all the cells; +, some vesicles on most cells; 0, no vesicles on most cells.
b. RNase I leakage. + indicates a clear zone of RNA hydrolysis around the cell colony (0, no leakage).
c. SDS (% w/v) and vancomycin (μg ml^{-1}) concentrations required for a 50% decrease in cell turbidity measured after 3 h of culture (average values from duplicate experiments ± 10%).

was replaced by the periplasmic domain of RseA protein (a regulator of σ^E activity). We observed that pTolA restored the outer membrane integrity of the *tolA* strain, as was expected from previous results with this plasmid (Bernadac *et al.*, 1998). Moreover, pTolA was also able to suppress the SDS and vancomycin susceptibility and, to a lesser extent, the periplasmic release and microvesicle formation of the *lpp* mutant but not of the *pal* mutant (Table 1). pTolAI–IIr was unable to suppress any of the three mutants. Upon arabinose induction of the two TolA derivatives, similar amounts of outer membrane vesicles to those observed in uninduced conditions in *lpp* pTolA and *lpp* pTolAI–IIr cells and an absence of RNase I leakage was observed in *lpp* pTolA (not shown). The levels of TolA and TolA derivatives immunodetected in *tolA*, *pal* and *lpp* cells transformed or not with pTolA and pTolAI–IIr were analysed in the absence of arabinose induction. Western blot analyses indicated that, in the membrane fractions, the TolA derivatives were at least three times more abundant in the transformed strains than TolA in *pal*, *lpp* or parental strains. Upon 0.05% arabinose induction, H_6TolA was found to be increased in the membrane fraction of the *lpp* pTolA cells compared with uninduced conditions (not shown). Thus, we conclude that overproduction of H_6TolA suppresses the membrane-perturbing effects of the *lpp* deletion but not that of the *pal* deletion and, furthermore, that there was a limiting level of H_6TolA sufficient to produce this effect. Therefore, these results suggested that Pal, together with overproduced TolA, has an outer membrane-stabilizing effect and that this can compensate for the absence of Lpp.

TolA C-terminal domain interacts with Pal

In vitro analyses. Co-purification experiments using purified Pal and the purified C-terminal domain of TolA, either TolAIII1 (N-terminal His_6-tagged TolAIII) or TolAIII3 (C-terminal His_6-tagged TolAIII) indicated that the amount of Pal that could be collected by cobalt affinity chromatography was greater than that non-specifically bound in the absence of TolAIII derivatives (not shown). In order to stabilize the TolAIII–Pal interaction, *in vitro* cross-linking with formaldehyde (FA) followed by SDS–PAGE and Coomassie blue staining analyses were performed (Fig. 1A). In the absence of FA, the monomeric forms of the TolAIII3 and Pal purified proteins were detected. In the presence of FA, Pal monomer was still detected together with bands having electrophoretic mobilities of Pal multimers (P2 and P3). Moreover, upon cross-linking of the TolAIII3 and Pal mixture, two protein complexes, C1 (the major complex) and C2, were specifically detected. Western blot immunodetections were performed to identify the C1 and C2 complexes (Fig. 1B). Purified Pal immunodetections with the anti-Pal antibody detected a

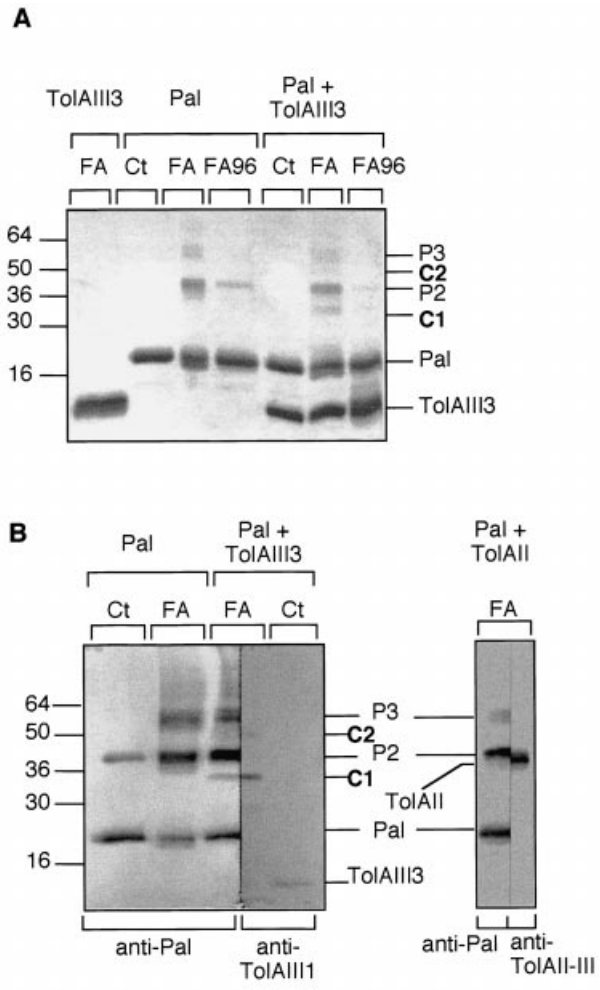

Fig. 1. Pal interacts *in vitro* with TolAIII.
A. Coomassie blue-stained gel of the purified proteins, Pal, Pal multimers (P2 and P3), TolAIII3, and protein complexes (C1 and C2) cross-linked for 20 min with 1% formaldehyde (FA) or without FA treatment (Ct).
B. Western blot immunodetections of purified proteins and their complexes obtained after FA cross-linking. The antibodies used for the immunodetections are indicated. Proteins were used at a concentration of 10 μM and corresponded to about 2, 3 and 4 μg of TolAIII3, Pal and TolAII respectively [(B), although samples were twofold overloaded in (A)]. All samples were incubated at room temperature, except when indicated at 96°C (FA96). Molecular mass standards are reported in kDa.

monomer of about 20 kDa, together with another band having an electrophoretic mobility close to that of a Pal dimer (P2). Cross-linking of Pal increased the amount of P2 and revealed a possible Pal trimer (P3), as found before from the analysis of Coomassie blue-stained gels. The two protein complexes C1 and C2 were simultaneously immunodetected by anti-Pal and anti-TolAIII1 antibodies (Fig. 1B). Based on their electrophoretic mobilities, the major complex C1 could correspond to a Pal-TolAIII3 heterodimer, whereas C2 could correspond to a Pal-TolAIII3 multimer. As a negative control for FA

cross-linking, TolA central domain (TolAII) did not give any complex with Pal (Fig. 1B).

In vivo analyses. We could not directly detect any TolA–Pal or TolAIII3–Pal complex upon *in vivo* cross-linking with FA and Western blot immunodetections of *tolA* cells transformed with pQRA (encoding TolQ–R–A proteins) or pTolAIII3 (encoding periplasmic TolAIII3 protein) (not shown). We also checked the possible co-immunoprecipitation of Pal using anti-TolAII–III antibodies. However, in the absence of a cross-linking agent, we could not clearly evaluate the immunodetection of Pal. This result could be caused by a possible dissociation of the complex during immunoprecipitation washing steps. However, *in vivo* cross-linking and immunoprecipitation experiments performed with FA cross-linked cells showed that Pal specifically co-immunoprecipitates with TolA in *tolA* pQRA cells (Fig. 2A). *tolA* cells analysed by the same procedure gave, at best, only a very faint signal of Pal, indicating that TolAII–III antibody did not cross-react with Pal. As can be observed, the anti-rabbit IgG immunodetections did not interfere with the detection of Pal. Different cells producing TolA derivatives were analysed by FA cross-linking followed by TolA immunoprecipitations (Fig. 2B). Although Pal can be detected under chromosomal production of TolA in parental cells, Pal was faintly detected when TolAI–III (from pQRAI–III encoding TolA devoid of its long central domain) was produced. This faint signal was reproducibly detected and was significant when compared with that of *tolA* cells. Using *tolA* pTolA and *tolA* pTolAI–IIr cells, Pal was found to co-immunoprecipitate with H_6TolA, but not with H_6TolAI–IIr. In order to confirm that the absence of Pal co-immunoprecipitation was not caused by a problem of TolA stability or reactivity, all the experiments were analysed for the presence of the TolA protein or TolA derivatives (not shown). These results indicated that Pal and TolA interact *in vivo* and that this interaction requires the TolAIII domain. This conclusion was confirmed using *tolA* pTolAIII3 cells, producing periplasmic TolAIII3, and observing the co-immunoprecipitation of Pal (Fig. 3C).

TolA–Pal interaction is energy dependent

In vivo cross-linking and co-immunoprecipitations of Pal were analysed with respect to cell energization. Using *tolA* pQRA cells grown in rich medium under aerobic conditions to exponential growth phase and treated further for 25 min with 0.1 mM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), the detection of Pal was abolished or identical to that of a *tolA* strain (Fig. 3A). This result indicates that CCCP inhibited the TolA–Pal interaction. As cell treatments with low CCCP concentrations have been shown to deplete the cells' energy reserves (Possot *et al*., 1997), we hypothesized that the energy provided by ATP hydrolysis or the proton motive force (pmf) was required for the interaction of TolA with Pal. Furthermore, the TolA–TolQ and TolA–TolR protein complex immunodetections, previously detected after cross-linking and Western blot (Derouiche *et al*., 1995), were still observed irrespective of the absence or presence of 0.1 mM CCCP (not shown). The TolA–TolR complex was analysed after cell cross-linking and TolA immunoprecipitations using TolR immunodetections. The results confirmed that the addition of CCCP had no secondary effect on the co-immunoprecipitation of TolR and therefore on the TolA–TolR interaction (Fig. 3A). In order to avoid possible secondary effects resulting from the high concentration and long incubation with CCCP, further experiments were carried out using lower concentrations of CCCP and shorter, 5 min incubation (CCCP was removed during the cross-linking reaction; Fig. 3B). The level of ATP in *tolA* pTolA cells treated with 10 and 100 μM CCCP was measured and found to decline to 5% and 0% of the initial level respectively. High CCCP concentrations, previously found to abolish the TolA–Pal interaction, still induce similar effects for the H_6TolA–Pal interaction, whereas 10 μM CCCP almost totally inhibited the interaction

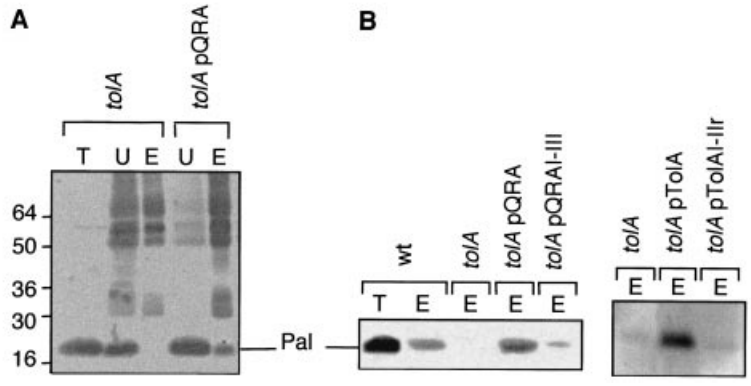

Fig. 2. Pal interacts *in vivo* with TolA. *tolA* cells harbouring or not Tol plasmids and parental cells (wt) treated with FA were immunoprecipitated with anti-TolAII–III antibody. Immunoprecipitated samples were heat denatured and analysed by Western blotting.
A. Anti-Pal immunodetections from *tolA*, *tolA*pQRA cells overproducing TolA.
B. Immunodetections of Pal from parent, *tolA*, *tolA* cells overproducing TolA, TolAI–III, H_6TolA, and H_6TolAI–IIr (from pQRA, pQRAI–III, pTolA and pTolAI–IIr plasmids respectively). T, U and E correspond to total sample (before immunoprecipitation), unbound and eluted material respectively.

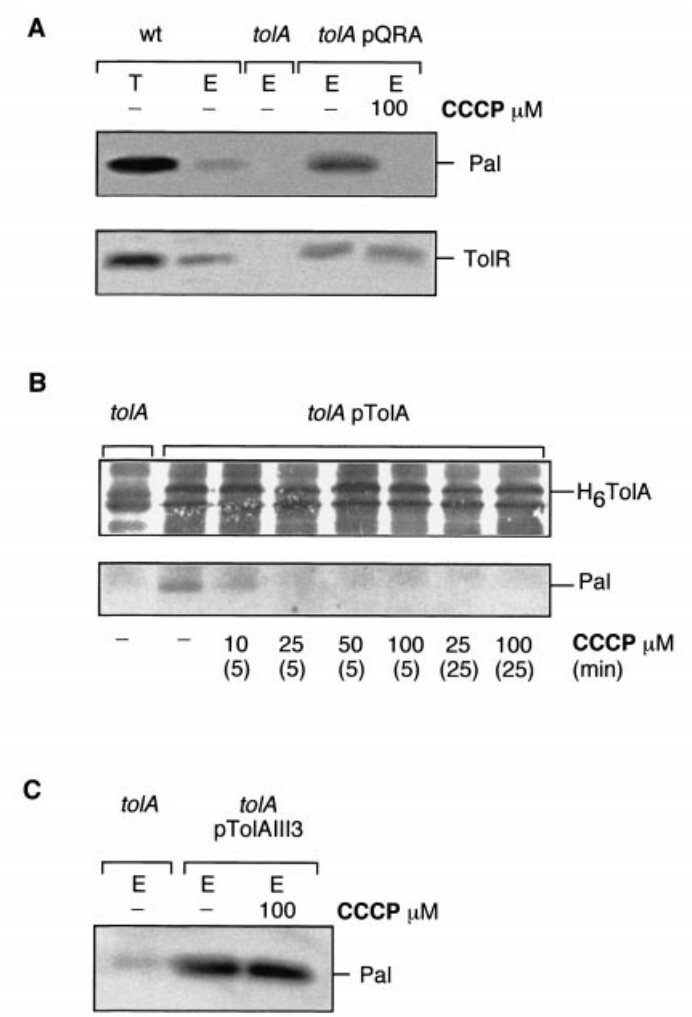

Fig. 3. The TolA–Pal, but not the TolAIII–Pal. interaction is energy dependent.
A. Parental *tolA* and *tolA* cells overexpressing TolA (from pQRA plasmid) were treated or not with CCCP and then cross-linked with FA. Anti-TolAII–III-immunoprecipitated samples were heat denatured and analysed by Western blotting using anti-Pal and anti-TolR antibodies.
B. Similarly, Pal immunodetections were analysed using *tolA*pTolA cells treated with various amounts of CCCP and incubation times. Pal and H_6TolA were immunodetected on the bottom and top parts of the same Western blot with anti-Pal and anti-TolAIII1 antibodies respectively.
C. *tolA*pTolAIII3 cells treated or not with CCCP were analysed by the same approach for the immunodetection of Pal. T and E correspond to total sample and eluted material.

(Fig. 3B). As a further control, all the experiments were concomitantly checked for the levels of H_6TolA immunoprecipitations, H_6TolA being detected with anti-TolAIII1 antibody together with immunodetections of the anti-TolAII–III rabbit IgG. The position of H_6TolA was clearly observed compared with the IgG background immunodetections of *tolA* cells, and similar levels were detected (Fig. 3B).

We were intrigued to know whether periplasmic TolAIII3–Pal interaction was affected by CCCP. Interestingly, periplasmic TolAIII3, devoid of the inner membrane anchor, was found to interact with Pal even in the presence of 0.1 mM CCCP (Fig. 3C). Moreover, this experiment indicates that CCCP does not hinder the interaction with membrane-anchored TolA by any secondary effect.

TolA–Pal interaction is proton motive force dependent

To characterize better the energy source required for the TolA–Pal interaction, FA cross-linking followed by TolA immunoprecipitations were carried out using sodium arsenate and ionophores (Fig. 4A) or anaerobic conditions with glucose as carbon source (Fig. 4B). In addition, *tolA* pTolA cells under these different conditions were analysed to verify similar immunodetections of H_6TolA (not shown) and to measure intracellular ATP levels. These were measured with the luciferase luminescence assay, whereas FA cross-linking followed by TolA immunoprecipitations were performed on separate aliquots of the same samples. Using 1–50 mM sodium arsenate and 10–60 min incubation periods, ATP values of *tolA* pTolA cells were found to be between 45% and 0% of the initial ATP level (not shown). Increasing the amounts of sodium arsenate was found to decrease the ATP levels, as observed previously by Possot *et al.* (1997). Two conditions giving about 30% and 0% of the initial ATP level, corresponding to 15 min incubation with 1 and 50 mM arsenate, were used for further analysis by FA cross-linking followed by TolA immunoprecipitations. In addition, nigericin and valinomycin with different KCl concentrations were added to *tolA* pTolA cells treated or not with EDTA. The results of Pal immunodetections are

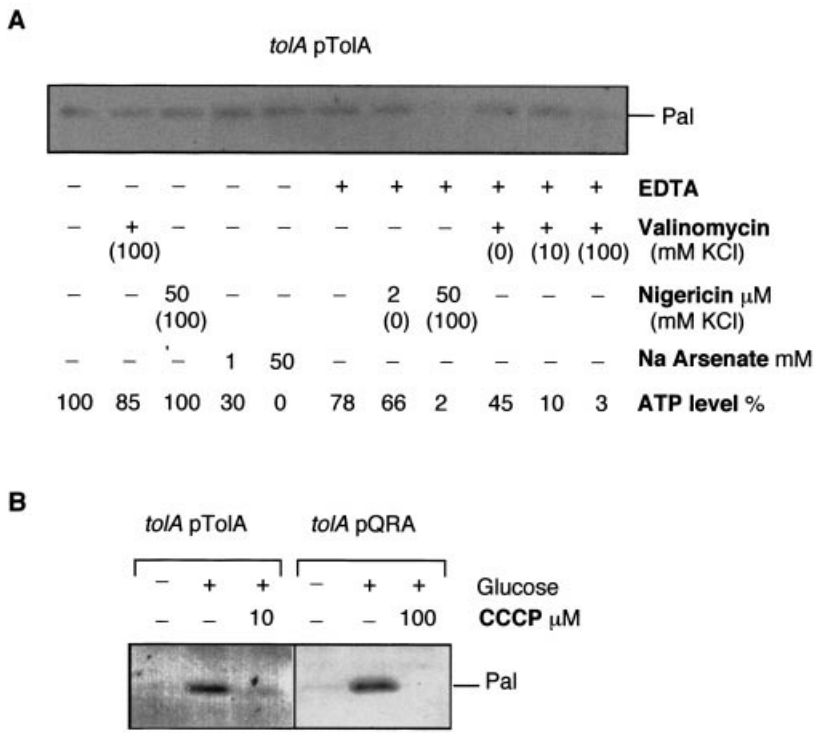

Fig. 4. Pmf is required for TolA–Pal interaction. Western blot immunodetection of Pal from *in vivo* cross-linked cells immunoprecipitated with anti-TolAII–III antibody.
A. *tolA*pTolA cells permeabilized or not with 1 mM EDTA were treated with poisons and ionophores. ATP levels (corresponding to the ratio of the initial level) are indicated, together with the concentrations of nigericin, sodium arsenate and KCl added. Valinomycin was used at a concentration of 70 μg ml^{-1}.
B. *tolA*pQRA and *tolA*pTolA cells incubated in M9 minimal medium under anaerobic conditions were analysed by the same approach for the immunodetection of Pal. CCCP treatment and the presence or absence of glucose as carbon source are indicated.

presented in Fig. 4A. In the absence of EDTA treatment, we observed that nigericin and valinomycin with 100 mM KCl did not perturb the cellular ATP level or the co-immunoprecipitation of Pal. The sodium arsenate treatments that reduced the ATP level to either 30% or 0% also did not change the detection of Pal. In the permeabilized *tolA* pTolA cells, we found that EDTA treatment had a small, but significant effect on the intracellular ATP level. The addition of 2 μM nigericin, which mediates an electroneutral exchange of cations for protons and thus dissipates the ΔpH (Ahmed and Booth, 1983), had no effect on the immunodetection of Pal. Higher concentrations of nigericin (50 μM) plus 100 mM KCl, reported to collapse both $\Delta\psi$ and ΔpH (Ahmed and Booth, 1983), were found to dissipate the intracellular ATP level and also to affect the detection of Pal. Similarly, in EDTA-permeabilized cells treated with valinomycin and a high KCl concentration (100 mM), Pal could only be faintly immunodetected, whereas in the absence of KCl or with low KCl concentrations, Pal was easily detected. However, these results were not quantitative and only indicated that concentrations of CCCP, nigericin and valinomycin/K^+ that are known to reduce the pmf and ATP level also interfere with Pal immunodetections and, consequently, with the TolA–Pal interaction. In order to confirm that ATP was not the energy source required to detect the TolA–Pal interaction, as suggested by the results with arsenate, *tolA* pQRA and *tolA* pTolA cells grown under fermentative anaerobic conditions were analysed by FA cross-linking followed by TolA immunoprecipitations. In these cells, we observed that either 10 or 100 μM CCCP affected the detection of Pal, thus inhibiting the interaction of Pal with TolA or H_6TolA (Fig. 4B). However, under these conditions, we found that the ATP levels of 10 μM CCCP-treated cells was about 85% that of untreated cells. These results indicated that the ATP, obtained by fermentation, was not able to act as the energy source necessary for the interaction to occur. On the other hand, when cells were deprived of glucose, in the absence of CCCP, we did not detect Pal, as the cells were starved and the ATP level was found to be reduced to about 10% in the absence of any energy source. Taken together, these results showed that, in the absence of ATP but in the presence of a pmf, as in sodium arsenate-treated cells, the TolA–Pal interaction is preserved, whereas in the presence of ATP but in the absence of a pmf, as in CCCP-treated fermenting cells, the interaction is inhibited. Thus, it appears that the pmf drives the TolA–Pal interaction.

Discussion

The results presented here demonstrate that TolA interacts with the Pal lipoprotein, thus forming a link between the inner and outer membranes. Pal, with a number of other structural proteins, contains the sequence motif implicated in murein interaction (Lazzaroni and Portalier, 1992; Koebnik, 1995; Bouveret *et al.*, 1999). Numerous lipoproteins can be cross-linked to the peptidoglycan (Leduc *et al.*, 1992). Among these lipoproteins, the absence of

Lpp or Pal was shown to induce outer membrane alterations (Suzuki *et al*., 1978; Bernadac *et al*., 1998). A structural role in maintaining the cell envelope integrity was also proposed for OmlA lipoprotein in *Pseudomonas aeruginosa*, which shares homology with SmpA of unknown function in *E. coli* (Ochsner *et al*., 1999). Other outer membrane lipoproteins were found to possess important functions: transglycosidase activity for MltA and EmtA (Lommatzsch *et al*., 1997; Kraft *et al*., 1998); export of group 1 capsular polysaccharides to the cell surface by Wza (Drummelsmith and Whitfield, 2000); and outer membrane targeting of lipoproteins with LolB (Yokota *et al*., 1999). Besides the structural role of the Lpp covalently attached to the peptidoglycan (Braun, 1975), other lipoproteins found associated with it have no definite function. Pal belongs to the Tol system, in agreement with its interaction with TolB (Bouveret *et al*., 1995) and according to the *tol–pal* operon organization (Vianney *et al*., 1996; Sturgis, 2001). Pal and Lpp belong to the structural network of proteins linking the outer membrane to the murein sacculus. Lpp was cross-linked to OmpA (Choi *et al*., 1986), Pal interacts with OmpA, TolB interacts with Lpp and with OmpA in the presence of Pal (Clavel *et al*., 1998). Defects in the expression of any of the TolQ–R–A proteins result in the production of more outer membrane vesicles than in the absence of Pal or TolB (Bernadac *et al*., 1998). TolA might be considered as an essential stabilizing protein with respect to its low abundance within the cell (400–800 copies; Levengood *et al*., 1991), compared with TolR (2000–3000 copies; Müller *et al*., 1993). The stoichiometry of the TolQ–R–A complex is still unknown, but TolR dimerization has been reported (Journet *et al*., 1999). In this study, we demonstrated the interaction of Pal with the C-terminal domain of TolA both *in vivo and in vitro*, and a TolA–Pal complex was observed in various isogenic backgrounds including the *lpp* strain (not shown). Overproduction of TolA from pQRA and pTolA multicopy plasmids was found to increase the co-immunoprecipitation of Pal. These results indicated that Pal is present in large excess over the TolA protein, as we have observed following the quantification of Pal (E. Cascales, M. Gavioli, A. Bernadac and R. Lloubès, unpublished). The decrease in the amounts of Pal co-precipitating with overproduced TolA deleted of its long periplasmic central domain (TolAI–III) suggested a less efficient interaction of the C-terminal domain of TolA (TolAIII). However, these results were not quantitative, as TolAI–III was immunoprecipitated with the antibodies directed against the whole periplasmic form of TolA. Nevertheless, in both cases, TolA or TolAI–III must cross the periplasm through the murein sacculus to reach outer membrane-anchored Pal. The overexpression of TolA protein found to suppress the effects caused by an *lpp* mutation might indicate that this stabilizing effect occurred through an increase in TolA–Pal complexes. Thus, Pal involved in an outer membrane–murein structural network is also involved in an inner and outer membrane structural network. It remains to be determined whether TolA interacts with the TolB–Pal complex or with Pal alone. The measurement of affinity constants for the different interactions using purified proteins should also provide an alternative analysis. Preliminary experiments indicate that the TolAIII3–Pal interaction can be monitored by following tyrosine fluorescence, whereas no change occurred when TolAII was added to Pal (not shown). This result agrees with the *in vitro* interaction described in this study.

One attractive aspect of the TolA–Pal interaction was its relation to the cellular energy resources. We demonstrated that the pmf induces the TolA–Pal interaction. Which of $\Delta\psi$ or ΔpH is required for the interaction remains to be determined, although the preliminary results with nigericin suggest an important role for $\Delta\psi$. Experiments using *tolA* cells producing periplasmic TolAIII3 indicated that the presence of CCCP has no effect on this interaction. Thus, it appeared that the absence of an inner membrane anchor abolished the CCCP effect and that TolA was energized through its transmembrane domain. Recently, TonB protein was shown to undergo conformational changes dependent on the pmf, ExbB and ligand-bound FepA (Larsen *et al*., 1999). The TolA protein has been reported to undergo conformational modifications in the presence of CCCP (Lazzaroni *et al*., 1999). It might be that the energization of TolA induces a conformation of the C-terminal domain that is accessible to Pal, as in TolAIII3, or that TolA can reach Pal only after conformational changes. Moreover, we observed that CCCP has no effect on the *in vivo* interaction of TolA with TolQ and TolR, as TolA–Q and TolA–R complexes were formed. From our qualitative results, we reproducibly observed an increase in Pal immunodetections when TolQ–R–A or TolAIII3 proteins were overproduced in *tolA* cells compared with H_6TolA. This observation could be explained by the free diffusion of TolAIII3 in the periplasm, whereas with anchored TolA, the simultaneous overproduction of the TolQ–R protein might be required for maximum levels of TolA–Pal interaction. New data using *in vivo* cell radiolabelling analysed by cross-linking followed by TolA immunoprecipitations could assess quantitative values for these interactions, allowing further investigation of the requirement for the TolQ–R proteins in relation to the TolA–Pal interaction. The simultaneous or sequential interactions of Pal and TolA with their numerous partners are currently being analysed using different tagging sequences in the TolQ–R proteins as, until now, attempts to use anti-histidine immunoprecipitations with H_6TolA have been unsuccessful.

The physiological significance of the TolA–Pal interaction

remains to be elucidated and, in common with the interactions of energized TonB, which has been shown to be required for channel opening upon ligand binding by specific receptors (Moeck *et al*., 1997; Larsen *et al*., 1999), the TolA–Pal interaction may well be dependent on dynamic events. In view of the cell energization required to follow the TolA–Pal interaction, we might suspect that only part of the cellular TolA complement is energized and involved in this interaction; the remaining TolA that does not interact with Pal could have another conformation or interacting partner, which prevents it from reaching and interacting with Pal in the absence of energization. The TolA protein might be involved in transducing energy from the cytoplasmic membrane to the Pal lipoprotein or via Pal lipoprotein to some other periplasmic or outer membrane component. Previous data have suggested that the Tol proteins are involved in the surface expression of O antigen (Whitfield and Valvano, 1993) or in the dynamic assembly of outer membrane trimeric porins (Derouiche *et al*., 1996; Rigal *et al*., 1997). TolA could drive newly synthesized outer membrane components across the periplasm (Levengood-Freyermuth *et al*., 1993) through its C-terminal interaction with Pal. As has been suggested, its long amphipathic coiled-coil central domain (Levengood *et al*., 1991; Derouiche *et al*., 1999) might interact with hydrophobic compounds and drive them through the peptidoglycan network. The crystal structure of the TolB protein has been obtained, and its possible biological implications in peptidoglycan processing have been suggested (Abergel *et al*., 1999). The results concerning the increased sensitivity to vancomycin of *tolA* and *pal* cells compared with that of *lpp* cells remains to be elucidated. As vancomycin diffusion might occur through the TolC pore (Schlör *et al*., 1997), immunodetections of TolC were analysed and found to be similar in the three strains (not shown), indicating that this effect is unrelated to the amount of this protein. The effect of each of the *tol–pal* mutations would be a result of an absence of the Tol–Pal complex linking the inner and outer membranes, leading to the desynchronization of outer membrane and peptidoglycan synthesis and to the formation of outer membrane vesicles during cell elongation. This process could be assisted by the cell wall turnover, which has been proposed to cause outer membrane turgor and blebbing of numerous Gram-negative bacteria (Zhou *et al*., 1998).

Experimental procedures

Bacterial strains, plasmids and growth conditions

lpp (KS303) and *pal* (JC8056*pal892*) strains were generous gifts from J. Beckwith and J. C. Lazzaroni respectively. KS303 is a *lpp5508* derivative of KS272 (Strauch and Beckwith, 1988), and JC8056*pal892* is a *pal* (stop after codon 41) mutant of JC8056 (Clavel *et al*., 1998). JC7782 is a *tolA* (stop after codon 40) derivative of 1292 (Derouiche *et al*., 1996). Strains were grown aerobically in Luria–Bertani (LB) medium on LB plates or in minimal M9 medium (Miller, 1992) supplemented or not with 0.4% glucose. For anaerobic conditions, Eppendorf tubes filled with 2.0 A_{600} of cells (recovered at the exponential growth phase) were grown for 2 h in M9 medium. When required, ampicillin (100 $\mu g\ ml^{-1}$), chloramphenicol (30 $\mu g\ ml^{-1}$), IPTG (0.1 mM) and arabinose (0.5 $mg\ ml^{-1}$) were added.

pTolA (*tolA* His_6, Cm^R) (previously called pARTolA; Bernadac *et al*., 1998), pQRA (*orf1*, *tolQ–R–A*, Amp^R) and its derivative pQRAI–III plasmids (encoding TolQ, TolR and TolAI–III, previously called pQRAΔh; Derouiche *et al*., 1995) have been described. pTolAI–IIr plasmid was constructed by exchanging the *Not*I–*Fsp*I DNA fragment of pTolA (encoding the C-terminal domain of TolA) for the polymerase chain reaction (PCR) DNA fragment *Eag*I–*Stu*I encoding the 96 C-terminal periplasmic residues of RseA protein using the following PCR primers: 5′-cgttggcgtccggccgataatggac and ggcaaaccaaaaggccttcattactgcg (restriction sites underlined). pTolAIII1 and pTolAIII3 plasmids encoded the C-terminal 93 and 97 residues of TolA respectively. The proteins were tagged with six histidines located at their N-terminal (TolAIII1) or C-terminal (TolAIII3) extremity. TolA derivative expressions were regulated by AraC (pTolA, pTolAI–IIr and TolAIII1) or LacI (TolAIII3). pTolAIII1 and pTolAIII3 plasmids will be described elsewhere (C. Deprez *et al*., unpublished). Pal lipoprotein was purified from the *lpp* strain using the protocol described for the purification of the peptidoglycan-associated lipoprotein from *Haemophilus influenzae* (Zlotnick *et al*., 1988). Pal was extracted at 55°C for 1 h in 50 mM phosphate buffer, pH 8.0, 1% Na deoxycholate. The detergent was exchanged for 0.08% Triton X-100 using a PD-10 column (Pharmacia). TolAII protein was purified as described previously (Derouiche *et al*., 1999).

Permeability and vesicle assays

Periplasmic RNase I leakage was estimated from LB plates containing 1.5% yeast RNA (purchased from Sigma Chemical) according to the method described previously (Lazzaroni and Portalier, 1981).

Tolerance for SDS and vancomycin was checked in liquid medium assay. Cells grown at A_{600} of about 0.5 were 100-fold diluted in LB supplemented with various concentrations of these substances. The values used for comparing strains correspond to the toxic compound concentration necessary for a twofold decrease in turbidity after 180 min of growth.

Outer membrane vesicles were observed by electron microscopy analyses after negative staining, as described previously (Bernadac *et al*., 1998).

Co-precipitation experiments

About 2 μg of purified TolAIII1 or TolAIII3 was immobilized on cobalt beads (Clontech) and incubated for 60 min with 3 μg of purified Pal in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl buffer (TN) containing either 0.08% Triton X-100 or

0.2 mg ml^{-1} BSA. The same conditions were used, but in the absence of TolAIII3, to detect the non-specific binding of Pal. Beads were washed with the same buffer, and the immobilized material was eluted with 25 μl of 500 mM imidazole in TN buffer. Samples were heat denatured with loading buffer for 10 min at 96°C before SDS–PAGE analyses.

In vitro *and* in vivo *cross-linking*

In vitro cross-linking experiments were performed with formaldehyde (FA; Merck). Proteins were mixed in a 10 mM sodium phosphate buffer (pH 6.3) at a concentration of 10 μM and incubated for 15 min at room temperature. Formaldehyde (1%) was added, and the mixture was incubated further for 20 min at room temperature. The samples, kept at room temperature or heated at 96°C for 10 min (to break the chemical cross-links), were treated in Laemmli loading buffer without reducing agent and analysed by SDS–PAGE and Coomassie blue (R250) staining or Western blotting.

In vivo cross-linking experiments were performed on exponentially growing cells. Cells were washed and resuspended at 0.5 A_{600} in 10 mM sodium phosphate buffer (pH 6.3) and incubated further for 20 min with the addition of 1% FA (Bouveret *et al*., 1995). Cells were solubilized in loading sample buffer for 15 min at 37°C with the addition of benzonase (Merck) and were analysed further by Western blotting. Cells harbouring the pTolAIII3, pTolA and pTolAI–IIr plasmids were induced with IPTG (0.1 mM) or arabinose (0.5 mg ml^{-1}) for 30 min before cross-linking.

Immunoprecipitation experiments

Cells treated (or not) with FA were solubilized under mild conditions by heating at 37°C for 15 min in TES (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% SDS) with the addition of protease inhibitors (Complete; Boehringer Mannheim). Then they were diluted 15-fold in TNE (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl) supplemented with 1% Triton X-100. For some experiments, TES was exchanged for TET (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100). After incubation at room temperature with vigorous shaking, the extract was centrifuged for 15 min at 18 000 *g* in order to remove unsolubilized material. The supernatant was incubated for 60 min at room temperature with antibody coupled to protein A–sepharose CL-4B (Pharmacia). The supernatant was trichloroacetic acid (TCA) precipitated and corresponded to the unbound fraction. Beads were then washed twice with TNE supplemented with 1% Triton X-100 and once with TNE supplemented with 0.1% Triton X-100. Unbound proteins were precipitated by TCA addition on ice (10% final volume), and immunoprecipitates were resuspended in Laemmli loading buffer without reducing agent and heated at 96°C for 20 min in order to remove the antigen and to dissociate the cross-linked proteins. Proteins were separated on 12.5% or 15% SDS–PAGE before Western blot analyses.

Cell treatments

Bacterial cultures were grown to A_{600} of 0.8 in LB medium, and CCCP (10–100 μM) was added for 5 min before cross-linking incubations. In some cases, CCCP was also added during FA cross-linking. Nigericin and valinomycin cell treatments were performed using various amounts of KCl essentially as described previously (Ahmed and Booth, 1983). Briefly, 1 ml of cells grown in LB to A_{600} of 0.7, washed and resuspended in the same volume of 100 mM Tris-HCl pH 7.5 were permeabilized (or not) with 1 mM EDTA for 3 min. Cells were incubated further at 37°C for 10 min with the addition of valinomycin (70 μg ml^{-1}) or nigericin (2 or 50 μM). At this stage, 20 μl of the suspension was analysed for ATP content, while the remaining cell suspension was washed in phosphate buffer for FA cross-linking treatment. Sodium arsenate was added at a concentration of 1–50 mM for 10 to 60 min periods, and the ATP levels were measured according to Possot *et al*. (1997) using the ATP bioluminescence assay kit (CLSII; Boehringer Mannheim). Briefly, 20 μl of cells was mixed with 80 μl of dimethyl sulphoxide (DMSO) and further diluted in 1 ml of ice-cold water. Luminescence was measured with 20 μl of the cell mixture using a Spex Fluorolog III fluorimeter equipped with a cooled detector (Jobin Yvon). The remaining treated cells were cross-linked with FA and analysed further by FA cross-linking followed by TolA immunoprecipitations.

The overproductions of TolA and derivatives were analysed by Western blot using total membrane fractions. These fractions were obtained from cell spheroplasts treated five times by freeze and thaw. Membranes were collected by ultracentrifugation (100 000 *g* for 30 min), further treated in 8 M urea, 10 mM Tris-HCl, pH 8.0, for 10 min (to remove aggregates and peripheral membrane proteins) and washed in the same buffer in the absence of urea.

Antibodies

The anti-TolAII–III (Derouiche *et al*., 1995), anti-Pal (Bouveret *et al*., 1999) and anti-TolR (Journet *et al*., 1999) antibodies have been described previously. Antiserum was raised in rabbits using TolAIII1 purified protein. About 150 μg of purified TolAIII1 protein mixed with Freund's adjuvant was injected three times over a period of 40 days.

Miscellaneous

Standard methods were used for DNA manipulations (Sambrook *et al*., 1982). PCR amplifications were performed as described previously (Ho *et al*., 1989) using Expand PCR polymerases (Boehringer Mannheim). SDS–PAGE and electrotransfer onto nitrocellulose (Towbin *et al*., 1979) were performed as described. Prestained protein markers (See-Blue; Novex) were used. After transfer, the nitrocellulose membrane was treated with antisera. Secondary antibodies coupled to alkaline phosphatase were revealed using bromo-chloro-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). The amounts of cell samples used for Western blot immunodetections correspond to 0.4 A_{600}. All the FA cross-linking followed by TolA immunoprecipitations

were performed using 0.6 or 0.8 A_{600} of cells overproducing TolA (and its derivatives) or parental cells respectively.

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References

Abergel, C., Bouveret, E., Claverie, J., Brown, K., Rigal, A., Lazdunski, C., and Bénédetti, H. (1999) Structure of the *E. coli* TolB protein determined by MAD methods at 1.95 A resolution. *Structure* **7**: 1291–1300.

Ahmed, S., and Booth, I. (1983) The use of valinomycin, nigericin and trichlorocarbaniline in control of the proton-motive force in *E. coli*. *Biochem J* **212**: 105–112.

Bernadac, A., Gavioli, M., Lazzaroni, J.C., Raina, S., and Lloubès, R. (1998) *E. coli tol–pal* mutants form outer membrane vesicles. *J Bacteriol* **180**: 4872–4878.

Bernstein, A., Rolfe, B., and Ononera, K. (1972) Pleiotropic properties and genetic organization of the *tolA, B* locus of *E. coli* K12. *J Bacteriol* **112**: 74–83.

Bouveret, E., Derouiche, R., Rigal, A., Lloubès, R., Lazdunski, C., and Bénédetti, H. (1995) Peptidoglycan-associated lipoprotein–TolB interaction. *J Biol Chem* **270**: 11071–11077.

Bouveret, E., Bénédetti, H., Rigal, A., Loret, E., and Lazdunski, C. (1999) *In vitro* characterization of peptidoglycan-associated lipoprotein (Pal)–peptidoglycan and Pal–TolB interactions. *J Bacteriol* **181**: 6306–6311.

Bowe, F., Lipps, C., Tsolis, R., Groisman, E., Heffron, F., and Kusters, J. (1998) At least four percent of the *Salmonella typhimurium* genome is required for fatal infection of mice. *Infect Immun* **7**: 3372–3377.

Bradbeer, C. (1993) The proton motive force drives the outer membrane transport of cobalamin in *E. coli*. *J Bacteriol* **175**: 3146–3150.

Braun, V. (1975) Covalent lipoprotein from the outer membrane of *E. coli*. *Biochim Biophys Acta* **415**: 335–377.

Braun, V. (1995) Energy-coupled transport and signal transduction through the gram-negative outer membrane via TonB-ExbB-ExbD-dependent receptor proteins. *FEMS Microbiol Rev* **16**: 295–307.

Braun, V., and Hermann, C. (1993) Evolutionary relationship of uptake of biopolymers in *E. coli:* cross-complementation between the TonB-ExbB-ExbD and the TolA-TolQ-TolR proteins. *Mol Microbiol* **8**: 261–268.

Braun, V., Gaisser, S., Herrmann, C., Kampfenkel, K., Killmann, H., and Traub, I. (1996) Energy-coupled transport across the outer membrane of *E. coli:* ExbB binds ExbD and TonB *in vitro*, and leucine 132 in the periplasmic region and aspartate 25 in the transmembrane region are important for ExbD activity. *J Bacteriol* **178**: 2836–2845.

Cadieux, N., and Kadner, R. (1999) Site-directed disulfide bonding reveals an interaction site between energy-coupling protein TonB and BtuB, the outer membrane cobalamin transporter. *Proc Natl Acad Sci USA* **96**: 10673–10678.

Choi, D.S., Yamada, H., Mizuno, T., and Mizushima, S. (1986) Trimeric structure and localization of the major lipoprotein in the cell surface of *E. coli*. *J Biol Chem* **261**: 8953–8957.

Clavel, T., Germon, P., Vianney, A., Portalier, R., and Lazzaroni, J.C. (1998) TolB protein of *E. coli* K-12 interacts with the outer membrane peptidoglycan-associated proteins Pal, Lpp and OmpA. *Mol Microbiol* **29**: 359–367.

Derouiche, R., Bénédetti, H., Lazzaroni, J.C., Lazdunski, C., and Lloubès, R. (1995) Protein complex within *E. coli* inner membrane. *J Biol Chem* **270**: 11078–11084.

Derouiche, R., Gavioli, M., Bénédetti, H., Prilipov, A., Lazdunski, C., and Lloubès, R. (1996) TolA central domain interacts with *E. coli* porins. *EMBO J* **15**: 6408–6415.

Derouiche, R., Lloubès, R., Sasso, S., Bouteille, H., Oughideni, R., Lazdunski, C., and Loret, E. (1999) Circular dichroism and molecular modeling of the *E. coli* TolA periplasmic domains. *Biospectroscopy* **5**: 189–198.

Drummelsmith, J., and Whitfield, C. (2000) Translocation of group 1 capsular polysaccharide to the surface of *E. coli* requires a multimeric complex in the outer membrane. *EMBO J* **19**: 57–66.

Eick-Helmerich, K., and Braun, V. (1989) Import of biopolymers into *E. coli*: nucleotides sequences of the *exbB* and *exbD* genes are homologous to those of *tolQ* and *tolR* genes, respectively. *J Bacteriol* **171**: 5117–5126.

Germon, P., Clavel, T., Vianney, A., Portalier, R., and Lazzaroni, J.-C. (1998) Mutational analyses of the *E. coli* K-12 TolA N-terminal region and characterization of its TolQ-interacting domain by genetic suppression. *J Bacteriol* **180**: 6433–6439.

Guihard, G., Boulanger, P., Bénédetti, H., Lloubès, R., Besnard, M., and Letellier, L. (1994) Colicin A and the Tol proteins involved in its translocation are preferentially located in the contact sites between the inner and outer membrane of *E. coli* cells. *J Biol Chem* **269**: 5874–5880.

Heilpern, A., and Waldor, M. (2000) CTXΦ infection of *Vibrio cholerae* requires the tolQRA gene products. *J Bacteriol* **182**: 1739–1747.

Higgs, P., Myers, P., and Postle, K. (1998) Interactions in the TonB-dependent energy transduction complex: ExbB and ExbD form homomultimers. *J Bacteriol* **180**: 6031–6038.

Ho, S., Hunt, H., Horton, R., Pullen, J., and Pease, L. (1989) Site-directed mutagenesis by overlap extinction using the polymerase chain reaction. *Gene* **77**: 51–59.

Journet, L., Rigal, A., Lazdunski, C., and Bénédetti, H. (1999) Role of TolR N-terminal, central and C-terminal domains in its dimerization and interaction with TolA and TolQ. *J Bacteriol* **181**: 4476–4484.

Karlsson, M., Hannavy, K., and Higgins, C. (1993) A sequence-specific function for the N-terminal signal-like sequence of the TonB protein. *Mol Microbiol* **8**: 379–388.

Kampfenkel, K., and Braun, V. (1992) Membrane topology of the *E. coli* ExbD protein. *J Bacteriol* **174**: 5485–5487.

Kampfenkel, K., and Braun, V. (1993) Topology of the ExbB protein in the cytoplasmic membrane of *E. coli*. *J Biol Chem* **268**: 6050–6057.

Koebnik, R. (1995) Proposal for a peptidoglycan-associating alpha-helical motif in the C-terminal regions of some

bacterial cell-surface proteins. *Mol Microbiol* **16**: 1269–1270.

Kraft, A., Templin, M., and Höltje, J.-V. (1998) Membrane-bound lytic endotransglycosylase in *E. coli*. *J Bacteriol* **180**: 3441–3447.

Larsen, R., Thomas, M., Wood, G., and Postle, K. (1994) Partial suppression of an *E. coli* TonB transmembrane domain mutation (D17V) by a missense mutation in ExbB. *Mol Microbiol* **13**: 627–640.

Larsen, R., Thomas, M., and Postle, K. (1999) Proton motive force, ExbB and ligand-bound FepA drive conformational changes in TonB. *Mol Microbiol* **31**: 1809–1824.

Lazdunski, C., Bouveret, E., Rigal, A., Journet, L., Lloubès, R., and Bénédetti, H. (1998) Colicin import in *E. coli* cells. *J Bacteriol* **180**: 4993–5002.

Lazzaroni, J.-C., and Portalier, R. (1981) Genetic and biochemical characterization of periplasmic-leaky mutants of *E. coli* K-12. *J Bacteriol* **145**: 1351–1358.

Lazzaroni, J.-C., and Portalier, R. (1992) The *excC* gene of *E. coli* K-12 required for cell envelope integrity encodes the peptidoglycan-associated lipoprotein (Pal). *Mol Microbiol* **6**: 735–742.

Lazzaroni, J.-C., Vianney, A., Popot, J.-L., Bénédetti, H., Samatey, F., Lazdunski, C., *et al.* (1995) Transmembrane α-helix interactions are required for the functional assembly of the *E. coli* Tol complex. *J Mol Biol* **246**: 1–7.

Lazzaroni, J.-C., Germon, P., Ray, M.-C., and Vianney, A. (1999) The Tol proteins of *E. coli* and their involvement in the uptake of biomolecules and outer membrane stability. *FEMS Microbiol Lett* **177**: 191–197.

Leduc, M., Ishidate, K., Shakibai, N., and Rothfield, L. (1992) Interactions of *E. coli* membrane lipoproteins with the murein sacculus. *J Bacteriol* **174**: 7982–7988.

Levengood, S., Beyer, W., and Webster, R. (1991) TolA, a membrane protein involved in colicin uptake contains an extended helical region. *Proc Natl Acad Sci USA* **88**: 5939–5943.

Levengood-Freyermuth, S., Click, E.-M., and Webster, R. (1993) Role of the carboxy-terminal domain of TolA in protein import and integrity of the outer membrane. *J Bacteriol* **175**: 222–228.

Lommatzsch, J., Templin, M., Kraft, A., Vollmer, W., and Höltje, J.-V. (1997) Outer membrane localization of murein hydrolases: MltA, a third lipoprotein lytic transglycosylase in *E. coli*. *J Bacteriol* **179**: 5465–5470.

Miller, J. (1992) *A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for E. coli and Related Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Moeck, G., Coulton, J., and Postle, K. (1997) Cell envelope signaling in *E. coli*. Ligand binding to the ferrichrome-iron receptor FhuA promotes interaction with the energy-transducing protein TonB. *J Biol Chem* **272**: 28391–28397.

Müller, M., Vianney, A., Lazzaroni, J.-C., Webster, R., and Portalier, R. (1993) Membrane topology of the *E. coli* TolR protein required for cell envelope integrity. *J Bacteriol* **175**: 6059–6061.

Nikaido, H. (1996) Outer membrane. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*. Neidhardt, F.C. (ed.). Washington, DC: American Society for Microbiology Press.

Ochsner, U., Vasil, A., Johnson, Z., and Vasil, M. (1999) *P. aeruginosa fur* overlaps with a gene encoding a novel outer membrane lipoprotein, OmlA. *J Bacteriol* **181**: 1099–1109.

Possot, O., Letellier, L., and Pugsley, A. (1997) Energy requirement for pullulanase secretion by the main branch of the general secretory pathway. *Mol Microbiol* **24**: 457–464.

Reynolds, P., Mottur, G., and Bradbeer, C. (1980) Transport of vitamin B12 in *E. coli*. Some observations on the roles of the gene products of *btuC* and *tonB*. *J Biol Chem* **255**: 4313–4319.

Rigal, A., Bouveret, E., Lloubès, R., Lazdunski, C., and Bénédetti, H. (1997) The TolB protein interacts with the porins of *E. coli*. *J Bacteriol* **179**: 7274–7279.

Sambrook, J., Fritsch, E., and Maniatis, T. (1982) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schlör, S., Schmidt, A., Maier, E., Benz, R., Goebel, W., and Gentschev, I. (1997) *In vivo* and in *vitro* studies on interactions between the components of the hemolysin (HlyA) secretion machinery of *E. coli*. *Mol Gen Genet* **256**: 306–319.

Skare, J., Ahmer, B., Seachord, C., Darveau, R., and Postle, K. (1993) Energy transduction between membranes: TonB, a cytoplasmic membrane protein, can be chemically cross-linked *in vitro* to the outer membrane receptor FepA. *J Biol Chem* **268**: 16302–16308.

Strauch, K., and Beckwith, J. (1988) An *E. coli* mutation preventing degradation of abnormal periplasmic proteins. *Proc Natl Acad Sci USA* **85**: 1576–1580.

Sturgis, J. (2001) Organization and evolution of the *tol-pal* gene cluster. *J Mol Microb Biotech (*in press).

Suzuki, H., Nishimura, Y., Yasuda, S., Nishimura, A., Yamada, M., and Hirota, Y. (1978) Murein-lipoprotein of *E. coli:* a protein involved in the stabilization of bacterial cell envelope. *Mol Gen Genet* **167**: 1–9.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354.

Vianney, A., Lewin, T., Beyer, W., Lazzaroni, J.-C., Portalier, R., and Webster, R. (1994) Membrane topology and mutational analysis of the TolQ protein of *E. coli* required for the uptake of macromolecules and cell envelope integrity. *J Bacteriol* **176**: 822–829.

Vianney, A., Müller, M., Clavel, T., Lazzaroni, J.-C., Portalier, R., and Webster, R. (1996) Characterization of the *tol-pal* region of *E. coli* K-12: translational control of *tolR* expression by TolQ and identification of a new open reading frame downstream of *pal* encoding a periplasmic protein. *J Bacteriol* **178**: 4031–4038.

Webster, R. (1991) The *tol* gene products and the import of macromolecules into *E. coli*. *Mol Microbiol* **5**: 1005–1011.

Whitfield, C., and Valvano, M. (1993) Biosynthesis and expression of cell-surface polysaccharides in gram-negative bacteria. *Adv Microb Physiol* **35**: 135–246.

Yakushi, T., Tajima, T., Matsuyama, S.I., and Tokuda, H. (1997) Lethality of the covalent linkage between mislocalized major outer membrane lipoprotein and the peptidoglycan of *E. coli*. *J Bacteriol* **179**: 2857–2862.

Yem, D., and Wu, H. (1978) Physiological characterization of an *E. coli* mutant altered in the structure of murein-lipoprotein. *J Bacteriol* **133**: 1418–1426.

Yokota, N., Koruda, T., Matsuyama, S., and Tokuda, H. (1999) Characterization of the LolA–LolB system as the general lipoprotein localization mechanism of *E. coli*. *J Biol Chem* **274**: 30995–30999.

Zhou, L., Srisatjaluk, R., Justus, D., and Doyle, R. (1998) On the origin of membrane vesicles in gram-negative bacteria. *FEMS Microbiol Lett* **163**: 223–228.

Zlotnick, G., Sanfilippo, V., Mattler, J., Kirkley, D., Boykins, R., and Seid, J. (1988) Purification and characterization of a peptidoglycan-associated lipoprotein from *H. influenzae*. *J Biol Chem* **263**: 9790–9794.