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Defective O-Antigen Polymerization in *tolA* and *pal* Mutants of *Escherichia coli* in Response to Extracytoplasmic Stress

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We have previously shown that the TolA protein is required for the correct surface expression of the *Escherichia coli* O7 antigen lipopolysaccharide (LPS). In this work, Δ*tolA* and Δ*pal* mutants of *E. coli* K-12 W3110 were transformed with pMF19 (encoding a rhamnosyltransferase that reconstitutes the expression of O16-specific LPS), pWQ5 (encoding the *Klebsiella pneumoniae* O1 LPS gene cluster), or pWQ802 (encoding the genes necessary for the synthesis of *Salmonella enterica* O:54). Both Δ*tolA* and Δ*pal* mutants exhibited reduced surface expression of O16 LPS as compared to parental W3110, but no significant differences were observed in the expression of *K. pneumoniae* O1 LPS and *S. enterica* O:54 LPS. Therefore, TolA and Pal are required for the correct surface expression of O antigens that are assembled in a *wzy* (polymerase)-dependent manner (like those of *E. coli* O7 and O16) but not for O antigens assembled by *wzy*-independent pathways (like *K. pneumoniae* O1 and *S. enterica* O:54). Furthermore, we show that the reduced surface expression of O16 LPS in Δ*tolA* and Δ*pal* mutants was associated with a partial defect in O-antigen polymerization and it was corrected by complementation with intact *tolA* and *pal* genes, respectively. Using derivatives of W3110Δ*tolA* and W3110Δ*pal* containing *lacZ* reporter fusions to *fkpA* and *degP*, we also demonstrate that the RpoE-mediated extracytoplasmic stress response is upregulated in these mutants. Moreover, an altered O16 polymerization was also detected under conditions that stimulate RpoE-mediated extracytoplasmic stress responses in *tol*$^+$ and *pal*$^+$ genetic backgrounds. A Wzy derivative with an epitope tag at the C-terminal end of the protein was stable in all the mutants, ruling out stress-mediated proteolysis of Wzy. We conclude that the absence of TolA and Pal elicits a sustained extracytoplasmic stress response that in turn reduces O-antigen polymerization but does not affect the stability of the Wzy O-antigen polymerase.

Gram-negative bacteria have a unique envelope, consisting of a plasma membrane surrounded by the cell wall peptidoglycan and an outer membrane. One major component of the outer membrane is the lipopolysaccharide (LPS), which consists of lipid A, core oligosaccharide, and in some bacteria an O-specific polysaccharide that extends from the cell surface. The biogenesis of LPS is a complex, multistep process occurring at both sides of the plasma membrane, which is followed by the translocation of LPS molecules to the outer membrane cell surface (for recent reviews see references 44 and 54). LPS biosynthesis requires the participation of many enzymes and assembly proteins, encoded by more than forty genes (20, 21, 23, 44). The core oligosaccharide is assembled by the sequential transfer of monosaccharides onto preformed lipid A, while the O antigen is assembled onto undecaprenol-phosphate (Und-P), a polyisoprenoid lipid, to which is linked via a phosphodiester bond (44). These two pathways eventually converge by the ligation of the O antigen onto the outer core domain of the lipid A-core OS acceptor, with the concomitant release of Und-PP (54).

Three different pathways for the synthesis of the O polysaccharide have been described (44, 54). One of them involves the synthesis of O subunits by addition of monosaccharides at the nonreducing end of the molecule; this process takes place in the inner side of the plasma membrane. The subunits are then translocated across the membrane and polymerized by a mechanism involving the addition of the reducing end of the growing polysaccharide to the nonreducing end of Und-PP-linked subunits. The Und-PP-linked polymer is then ligated as a whole to preformed lipid A-core on the periplasmic face of the cytoplasmic membrane. This pathway, referred to as the *wzy* (polymerase)-dependent pathway, is found in the synthesis of the majority of O antigens, especially in those with repeating units made of different sugars (heteropolymeric O antigens), such as *Escherichia coli* O7 and O16 among others. The pathway also requires the putative flippase Wzx (17, 30) that is always encoded by O-antigen biosynthesis clusters containing the *wzy* gene. A second O-antigen assembly pathway involves the formation and elongation of the O repeating units in the cytoplasmic face of the inner membrane, followed by the transport of the O polysaccharide across the inner membrane by an ABC transporter and the subsequent ligation to the lipid A-core. This pathway is predominantly found in homopolymeric O antigens such as *E. coli* O8 and O9 (whose O repeats are composed of mannose residues) and *Klebsiella pneumoniae* O1 (whose O repeat is composed of galactose residues) (7, 25, 51). The third pathway is known as the synthase-dependent pathway, and the only known example is the plasmid-encoded O:54 antigen of *Salmonella enterica* serovar Borreze (44). It is believed that in this pathway the product of a single gene catalyzes a vectorial polymerization reaction, simultaneously ex-

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tending the polysaccharide chain and extruding the nascent polymer across the plasma membrane (44).

The outer membrane protects the bacterial cell against rapid entry of lipophilic compounds such as bile salts, detergents, fatty acids, and antibiotics and larger molecules such as bacteriophage DNA and bacteriocins (40). Also, certain beneficial compounds, such as vitamins and iron chelators, which cannot readily cross the outer membrane, bind to specific receptors and are internalized by two distinct import systems. One of these systems involves the TonB, ExbB, and ExbD proteins, which interact with outer membrane receptors for a certain bacteriocins and bacteriophages, iron chelators, vitamins, and antibiotics, promoting their energy-dependent passage across the outer membrane (42). The other system is the Tol import system, a multiprotein complex that allows the translocation of a different class of bacteriocins and bacteriophages from outer membrane surface receptors to the periplasmic space and intracellular targets (27). The Tol proteins are encoded by a cluster of seven genes, *orf1-tolQRAB-pal-orf2*, organized into two transcriptional units (56). The Tol proteins have been extensively studied (4–6, 8, 9, 15, 16, 22, 29, 55). TolA, -Q, and -R are integral membrane proteins. However, TolA also extends into the periplasmic space, and recently it has been shown to physically interact with the peptidoglycan-associated lipoprotein Pal, which is located in the outer membrane (8). TolB and Orf2 (of unknown function) are periplasmic proteins. Orf1, also known as YbgC, is a cytosolic protein that displays a thioesterase activity (58), but its involvement with the function of the other Tol proteins is not clear.

Mutations in some of the *tol* genes are associated with tolerance to certain bacteriophages and bacteriocins, and they also cause profound changes in the permeability of the outer membrane (57). The precise physiological role of the Tol system has not been established, but the accumulated evidence suggests that it plays a general role in maintaining the organization and normal function of the outer membrane (4, 18, 34). In a previous study, we showed that a *tolQ* mutation with strong polar effects on TolA protein expression compromises the surface expression of polymeric O7 antigen (18). We proposed that TolA, and possibly Pal, could have a role in modulating the surface expression of O antigen by an involvement in the processing of the O-antigen subunits, during either the process of membrane translocation of O antigen or the subsequent stages of LPS assembly at the periplasm. In the present study, we investigated whether a similar phenomenon occurs in *E. coli* K-12, using a genetic system that allows for the reconstitution of *E. coli* K-12's own O antigen (17, 31). The biosynthesis of O16-specific LPS in the *E. coli* K-12 strain W3110 is prevented by an IS*5* insertion mutation in *wbbL*, the most distal gene of the O-antigen synthesis cluster. *wbbL* encodes a rhamnosyltransferase that adds the second sugar to the nascent O subunit (17, 31), thus permitting the completion of the O16 subunit synthesis. We show in this study that TolA and Pal are required for the normal expression of O antigens that are only assembled by the *wzy*-dependent pathway. We also demonstrate that mutations in *tolA* and *pal* are associated with a sustained extracytoplasmic stress response, which in turn impairs O-antigen polymerization, a process that involves the Wzy protein.

MATERIALS AND METHODS

Strains and plasmids. The properties of strains and plasmids used in this study are described in Table 1. Bacteria were cultured in LB, supplemented (final concentrations) with ampicillin (100 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}), kanamycin (50 μg ml^{-1}), spectinomycin (80 μg ml^{-1}), and 0.5% (wt/vol) arabinose as appropriate, at 37°C. For some experiments, bacteria were cultured in LB at 30°C.

Construction of mutant strains and plasmids. Mutagenesis was carried out according to the method described by Datsenko and Wanner (13) to disrupt specific chromosomal genes using PCR products. Accordingly, *E. coli* K-12 W3110 was transformed with pKD46, a temperature-sensitive plasmid carrying the Red recombinase system from the λ bacteriophage under the control of the arabinose-inducible P_{BAD} promoter. The Red recombinase system mediates the replacement of the target chromosomal sequence with an antibiotic resistance cassette obtained by PCR amplification using primers carrying homologies to the vicinity on the gene targeted for disruption. *E. coli* W3110 carrying pKD46 was transformed by electroporation with the PCR product generated using either plasmid pKD3 or pKD4 as the template (13). Transformants were plated in LB agar, containing the appropriate antibiotic, and mutants were confirmed by PCR. Primer pairs 483 (5′-GCGAACAGTTTTTGGAAACCGAGAGTGTCAAAGGCAACCGTGTAGGCTGGAGCTGCTTCG-3′) and 484 (5′-TGCCTGATGTTGACCGTCCGAACAGTCAACATCGCGATTACATATGAATATCCTCCTTAG-3′), 487 (5′-GAATAGTAAAGGAATCATTGAAATGCAACTGAACAAAGTGTAGGCTGGAGCTGCTTCG-3′) and 488 (5′-ACGACAGACTCAATAGTTGATGTCTGAAGTTACTGCTCATATGAATATCCTCCTTAG-3′), 719 (5′-TAGCATTCACGAGGATTATCGCTAAACTATGCGGACTTGGGTGTAGGCTGGAGCTGCTTCG-3) and 720 (5′-CCTTTTTCTTTAAAACCGAAAAGATTACTTCGCGTTGTAATTCATATGAATATCCTCCTTAG-3′), 1171 (5′-TCGAGACTGAAATACATGAAAAAAACCACATTAGCACTGAGTGTGTAGGCTGGAGCTGCTTCG-3′) and 1172 (5′-GTTGAGGGAGATTACTGCATTAACAGGTAGATGGTGCTGTCGCATATGAATATCCTCCTTAG-3′), and 1221 (5′-CGTTGACGATAGCGGGATACTGGATAAGGGTATTAGGCATGGTGTAGGCTGGAGCTGCTTCG-3′) and 1222 (5′-CTACCTGTCACTAATGACATGGCAAACCAAAGTTGCTTCATATGAATATCCTCCTTAG-3′) were used to construct *tolA*, *pal*, *wzz*, *degP*, and *rseA* deletion mutants, respectively (the common region from the pKD3 and pKD4 templates is underlined).

The cloning of the *tolA* gene was carried out by amplifying its coding region using the direct primer 481 (5′-GTAGAATTCCCGAGAGTGTCAAAGGCAA-3′) carrying an EcoRI site (underlined) located 6 bases upstream of the *tolA* start codon (double underlining) and the reverse primer 482 (5′-AAGGTACCGCGATTACGGTTTGAAGTCCA-3′), which included the stop codon. The PCR product was digested with EcoRI and cloned into pBAD24 digested with EcoRI and SmaI. The cloning of *pal*, *wzz*, and *wzy* was done in a similar manner as above using primers 485 (5′-CCCGAATTCCATTGAAATGCAACTGAAC-3′) and 486 (5′-TAGTTGATGTCTGAAGTTACTGCTCAT-3′), 717 (5′-ATCGGAATTCTCGCTAAACTATGCGGACT-3′) and 718 (5′-AAACCGAAAAGATTACTTCGCGTTG-3′), and 721 (5′-ATCGGAATTCGTACGGATTAATGATCTA-3′) and 708 (5′-AATCCAGCATCGCGTCTAGAGAAATT-3′), respectively.

The cloning of the *rseA* gene was carried out by amplifying its coding region using the direct primer 1219 (5′-AGGCATGCAGAAAGAACAACTTTC-3′) that includes the *rseA* gene start codon (double underlining), and the reverse primer 1220 (5′-CGGGGTACCTTACTGCGAATTGCGTTCCTAA-3′), which included the stop codon and a KpnI site (underlined). The PCR product was digested with KpnI, and cloned into pBAD24 which had been digested with EcoRI, blunted with T4 DNA polymerase, and digested with KpnI.

LPS analysis. LPS was extracted as described previously (39). Briefly, cells from overnight plate cultures were suspended in a lysis buffer containing proteinase K, followed by hot phenol extraction and a subsequent extraction of the aqueous phase with ether. LPS was resolved by electrophoresis in 14% polyacrylamide gels using a Tricine-sodium dodecyl sulfate (SDS) system (28, 48) and visualized by silver staining. Densitometry analysis of the gels was performed using Odyssey software (Li-Cor Biosciences). The concentration of LPS was measured by the keto-deoxy-octulosonic assay (41). LPS from strains expressing *S. enterica* O:54 was resolved in 14% polyacrylamide gels and transferred to a nitrocellulose membrane using standard procedures. The membrane was incubated with a polyclonal rabbit antiserum against O:54 as a primary antibody. An IRDye 800 goat anti-rabbit immunoglobulin G (IgG) (Rockland Immunochemicals) was used as a secondary antibody. Detection was performed by infrared imaging, using the Odyssey Infrared Imager (Li-Cor Biosciences). Detection of

TABLE 1. Relevant characteristics of the strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
W3110	*rph-1* 1N(*rrnD-rrnE*)	Laboratory stock
EVV8	W3110 Δ*tolA*	This study
EVV9	W3110 Δ*pal*	This study
EVV16	W3110 Δ*wzzB*::Kan	This study
EVV17	EVV8 Δ*wzzB*::Kan	This study
EVV18	EVV9 Δ*wzzB*::Cam	This study
EVV19	W3110 Δ*degP*::Kan	This study
EVV20	EVV8 Δ*degP*::Kan	This study
EVV21	EVV9 Δ*degP*::Kan	This study
EVV22	W3110 *wzy*-FLAG3x	This study
EVV23	EVV8 *wzy*-FLAG3x	This study
EVV24	EVV9 *wzy*-FLAG3x	This study
EVV30	W3110 Δ*rseA*::Kan	This study
EVV31	GL113 Δ*rseA*::Kan	This study
EVV50	GL111 Δ*tolA*::Kan	This study
EVV51	GL111 Δ*pal*::Kan	This study
EVV52	GL112 Δ*tolA*::Kan	This study
EVV53	GL112 Δ*pal*::Kan	This study
EVV54	GL113 Δ*tolA*::Kan	This study
EVV55	GL113 Δ*pal*::Kan	This study
GL111	W3110 Δ(*argF-lac*)U169 λRS88(*porfA-dsbA-lacZ*)	26
GL112	W3110 Δ(*argF-lac*)U169 λRS88(*fkpA-lacZ*)	26
GL113	W3110 Δ(*argF-lac*)U169 λRS88(*degP-lacZ*)	26
GL123	W3110 *pldA*1 Δ(*argF-lac*)U169 λRS88(*degP-lacZ*)	26
Plasmids		
pBAD24	Cloning vector inducible with arabinose; Ap^r	19
pCM237	wzx_{EcO16} cloned into pBADNTF; Ap^r	38
pEV2	1.3-kb PCR amplicon containing the *tolA* gene from *E. coli* W3110 cloned into pBAD24; Ap^r	This study
pEV3	0.5-kb PCR amplicon containing the *pal* gene from *E. coli* W3110 cloned into pBAD24; Ap^r	This study
pEV6	1.0-kb PCR amplicon containing the *wzz* gene from *E. coli* W3110 cloned into pBAD24; Ap^r	This study
pEV7	1.2-kb PCR amplicon containing the *wzy* gene from *E. coli* W3110 cloned into pBAD24; Ap^r	This study
pEV30	0.65-kb PCR amplicon containing the *rseA* gene from *E. coli* W3110 cloned into pBAD24; Ap^r	This study
pKV1	*wecA* gene expressing WecA with a C-terminal FLAG-His_6 fusion cloned in pBAD24	K. Vigeant
pMF19	0.9-kb PCR amplicon containing the *wbbL* gene (rhamnosyltransferase) cloned into pEXT21; Sp^r	17
pWQ5	7.2-kb insert including the rfb_{KpO1} gene cluster cloned from *K. pneumoniae* O1:K20 into pBluescript KS(+); Ap^r	10
pWQ802	Naturally occurring plasmid containing $rfb_{O:54}$ with the Km^r cassette from pUC4K inserted at EcoRI site	24

[a] Ap, ampicillin; Km, kanamycin; Sp, spectinomycin; Cam, chloramphenicol.

O16 antigen was also carried out in a similar manner using a polyclonal rabbit antiserum against O16 as the primary antibody.

β-Galactosidase assay. Strains carrying *lacZ* fusions were grown overnight at 30°C, subcultured to an optical density at 600 nm (OD_{600}) of 0.01, and grown to an OD_{600} of 0.2 to 0.3. Enzymatic activity was determined as described elsewhere (43).

Construction of chromosomal Wzy_{FLAG3X}. Mutagenesis was carried out according to the method described by Uzzau et al. (52) to tag specific chromosomal genes using PCR products. The epitope tag and downstream kanamycin resistance cassette were obtained by PCR amplification using primers 1264 (5′-ATCATAGTATTCTCTCAATTTCTTAAGGCCCAGAAAATAAAGGACTACAAAGACCATGACGGT-3′) and 710 (5′-CAGCATCGCGTCTAGAGAAATTTAAATCATTCAAAAAATACATATGAATATCCTCCTTAG-3′), which carry homologies to the 3′ end of the gene targeted (underlined) and to the 5′ end of the downstream gene (double underlined). *E. coli* W3110 and Δ*tolA* and Δ*pal* derivatives, all carrying pKD46, were transformed by electroporation with the PCR product generated using plasmid pSUB11 as a template (52). Transformants were plated in LB agar containing kanamycin, and the insertion was confirmed by PCR.

Detection of Wzy_{FLAG3X}, $WecA_{FLAGHis}$, and Wzx_{FLAG} proteins. Strains expressing WzyFLAG3X, $WecA_{FLAGHis}$, or Wzx_{FLAG} were grown overnight at 37°C and subcultured in 500 ml of LB at an OD_{600} of 0.2. Growth was continued with vigorous aeration for 5 to 6 h. The cells were collected by centrifugation at 5,900 × *g* for 10 min, resuspended in 15 ml of 25% sucrose in 25 mM HEPES, pH 7.4, containing Complete broad-spectrum protease inhibitors (Roche), and then lysed by two passages through a French press cell at 10,000 lb/in^2. The cell lysates were centrifuged at 27,200 × *g* for 15 min to separate debris and unbroken cells, the clear supernatants were layered on a 60% (wt/wt) sucrose cushion (25 mM HEPES, pH 7.4) followed by a 2-h centrifugation at 270,000 × *g*. Cell membranes were collected from the interface of the sucrose cushion. This suspension was brought to 60% sucrose by adding granulated sucrose and placed on the bottom of a tube. A 32 to 56% (wt/wt) sucrose step gradient was layered on top of the sample. The gradient was centrifuged at 288,000 × *g* for 48 h, and fractions were collected from the top of the tube. Samples from the inner membrane fractions were mixed with 3× protein tracking dye and loaded in SDS-14% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane according to standard procedures, and the membrane was incubated with the FLAG M2 monoclonal antibody (Sigma). An Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes) was used as a secondary antibody. Detection was performed by infrared imaging, using the Odyssey Infrared Imager (Li-Cor Biosciences).

RESULTS AND DISCUSSION

***tolA and pal* genes play a role in the expression of O16 LPS.** Transformation of W3110 with pMF19, which carries the complete *wbbL* gene, restores expression of O16 LPS (17), providing us with a robust system to investigate the role of *tolA* and *pal* in this process. We constructed the strains EVV8

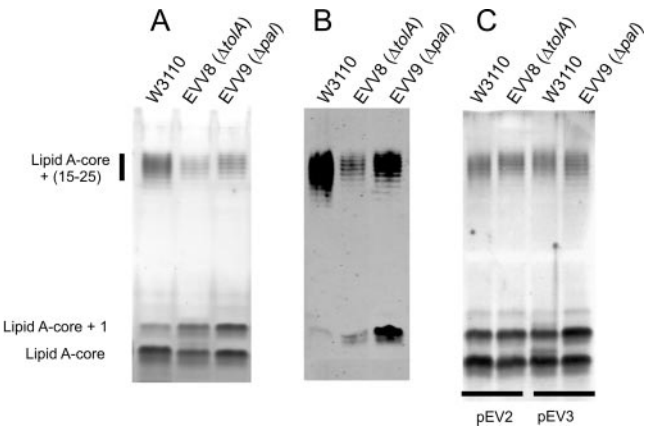

FIG. 1. Analysis of O16 LPS expression in *E. coli* K-12 strain W3110 and Δ*tolA* and Δ*pal* isogenic mutants. All strains carry the plasmid pMF19 for O16 antigen expression. LPS preparations in panels A and C were analyzed by tricine-SDS-PAGE followed by silver staining as described in Materials and Methods. (A) Effect of Δ*tolA* and Δ*pal* on O16 LPS surface expression. (B) Western blot of a similar gel as in panel A using O16-specific antiserum. (C) Complementation analysis of *E. coli* W3110 and the Δ*tolA* and Δ*pal* mutants with pEV2 and pEV3 carrying functional *tolA* and *pal* genes, respectively.

(W3110Δ*tolA*) and EVV9 (W3110Δ*pal*), which carry nonpolar deletions of these genes. In the presence of pMF19, EVV8 and EVV9 displayed a reduced amount of O16 LPS when compared to the parental W3110 strain (Fig. 1A and B). The LPS defect was corrected by transforming each mutant with pEV2 or pEV3, which carry wild-type *tolA* and *pal* genes, respectively, under the control of the P_{BAD} promoter (Fig. 1C). The differences in O-antigen LPS expression were carefully investigated by normalizing the gel loading according to various parameters. These parameters were (i) the bacterial density at the starting point of the LPS preparations, (ii) the concentration of 3-deoxy-D-*manno*-octulosonic acid, a conserved sugar component in the lipid A-core, and (iii) the amount of protein present in the whole-cell lysates before treatment with proteinase K. Similar differences in the O16 LPS expression of Δ*tolA* and Δ*pal* mutants, relative to parental *E. coli* W3110, were observed in all cases (data not shown). We concluded from these data that TolA and Pal independently contribute to modulate the surface expression of O16 LPS. The observed phenotypes were similar to previous findings in the *E. coli* O7 strain VW187, containing a polar *tolQ* mutation that also affected *tolA* gene expression (18). Since both O16 and O7 polysaccharides are structurally different (35, 49), we also concluded that TolA and Pal must play a role in the biogenesis of O antigens that is independent of the chemical nature of the O-antigen subunits.

Δ*tolA* and Δ*pal* mutants have a partial defect in O-antigen polymerization. The banding pattern of O16 LPS in Δ*tolA* and Δ*pal* mutants (Fig. 1A) exhibited a smaller amount of highly polymerized O antigen and a larger amount of the band consistent with the core plus one O-antigen subunit. The identity of this band was confirmed by a Western blot with O16-specific antiserum (Fig. 1B), which does not react with lipid A-core oligosaccharide (17). Also, the immunoblot results indicate that the O antigens produced in the *tolA* and *pal* mutants

TABLE 2. Densitometry analysis of LPS banding profiles in W3110, and Δ*tolA* and Δ*pal* mutants

	% Pixels in lane containing lipid A-core[a]:		
	+ 15–25 O units	+ 1 O unit	Alone
W3110(pMF19)	56	10	34
W3110Δ*tolA*(pMF19)	25	32	43
W3110Δ*pal*(pMF19)	31	31	38

[a] Densitometry was performed on each of the lanes show in Fig. 1A, counting the pixels from the bands corresponding to lipid A-core, lipid A-core plus 1 O unit, and lipid A-core plus 15 to 25 O units. The relative amount of pixels in each region of the gel is expressed as percent values of the total pixels in each lane. The results were linear within the loading range used in these experiments (data not shown).

contain a larger proportion of the band consistent with lipid A-core plus one O-antigen subunit than the O antigen produced in the parental W3110 strain. The altered banding distribution in Δ*tolA* and Δ*pal* mutants suggested a possible defect in O-antigen polymerization. The relative intensities of the bands corresponding to lipid A-core, lipid A-core plus one O-antigen subunit, and lipid A-core plus 15 to 25 O-antigen subunits (Fig. 1A) in each lane were quantitatively analyzed by densitometry. Data in Table 2 show that *tolA* and *pal* mutants exhibited a significant reduction in the relative amounts of polymerized O-antigen subunits and a corresponding increment in the amount of the band representing lipid A-core plus one O-antigen subunit. Standardization of the relative intensities of the LPS bands as a function of the total amount of LPS in each lane revealed a linear relationship over the concentrations ranges used for gel loading in these experiments (data not shown). We concluded from these data that the polymerization of O antigen is altered in the Δ*tolA* and Δ*pal* mutants. These results cannot be explained by differences in the expression levels of the *wzy* gene, since in a previous study with the O7 LPS we demonstrated that the LPS phenotype in the absence of TolA function was not due to reduced gene expression of the O-antigen biosynthesis cluster (18), and both O7 and O16 LPS biosynthesis gene clusters have a very similar gene organization and regulation (36, 37, 50).

Wzz and Wzy proteins expressed from high-copy-number plasmids rescue the defective O-antigen polymerization phenotype of Δ*tolA* and Δ*pal* mutants. Polymerization of *wzy*-dependent O antigens requires the activities of two proteins, the Wzy polymerase and the Wzz regulator of O chain length (44). We examined the effect of a mutation in the *wzz* gene in the O16 antigen biosynthesis in W3110Δ*tolA* by constructing a double Δ*tolA* Δ*wzz* mutant. The resulting strain, EVV17 (W3110Δ*tolA* Δ*wzz*), as well as the control strain, EVV16 (W3110Δ*wzz*), were transformed with pMF19, and the O16 LPS production was examined by silver staining. Figure 2A shows that deletion of *wzz* caused a typical loss in the modality of the polymerization of O antigen, resulting in a monomodal ladder-like banding profile of the O-antigen chains. However, the Δ*tolA* Δ*wzz* double mutant displayed a lower level of polymerization (Fig. 2A) than that observed in its Δ*tolA* mutant counterpart (Fig. 1A). A similar result was obtained with a double mutant W3110Δ*pal* Δ*wzz* containing pMF19 (Fig. 2B). These data demonstrated that in the absence of the Wzz protein, the partial defect in O-antigen polymerization shown by

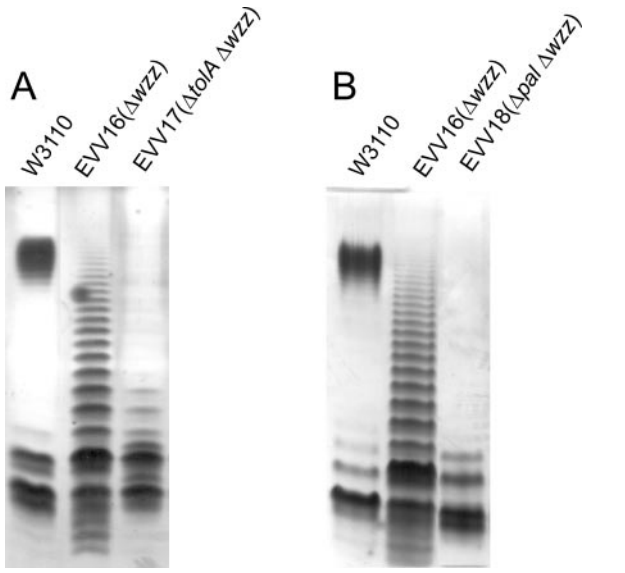

FIG. 2. (A) Analysis of O16 LPS expression in *E. coli* K-12 strain W3110 and the isogenic Δ*wzz* and Δ*tolA* Δ*wzz* mutants. LPS preparations were analyzed by tricine-SDS-PAGE followed by silver staining. (B) Analysis of O16 LPS expression in *E. coli* K-12 strain W3110 and the isogenic Δ*wzz* and Δ*pal*Δ*wzz* mutants. All strains in panels A and B carry the plasmid pMF19 for the expression of the O16 polysaccharide.

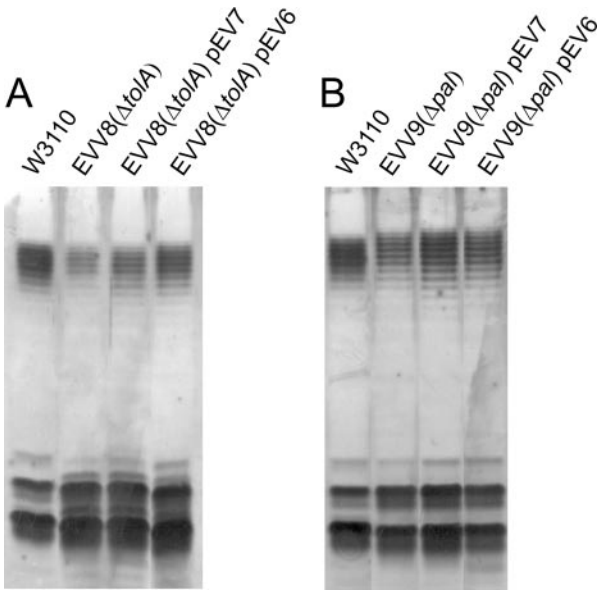

FIG. 3. Complementation of the Δ*tolA* (A) and Δ*pal* (B) phenotypes with plasmids carrying functional *wzz* or *wzy* genes under the control of the P_{BAD} promoter. All strains carry the plasmid pMF19 for the expression of the O16 polysaccharide and were grown in the presence of 0.1% arabinose.

both Δ*tolA* and Δ*pal* mutants is more accentuated, suggesting the possibility of an increased instability of the Wzy protein in these mutants.

We also conducted the converse experiment by investigating the effect of Wzz overexpression in Δ*tolA* and Δ*pal* mutants. Strains EVV8 and EEV9, both containing pMF19, were transformed with pEV6. This plasmid carries a functional *wzz* gene that was placed under the control of the arabinose-inducible P_{BAD} promoter. The transformants showed increased O-antigen polymerization when incubated in the presence of 0.1% arabinose (Fig. 3A and B). Similar results were obtained in a parallel experiment conducted with pEV7, which carries a functional *wzy* gene also under the control of the control of P_{BAD} (Fig. 3A and B). Therefore, increased dosage of Wzz or Wzy can correct the altered O-antigen polymerization in the *tolA* and *pal* mutants, suggesting that this alteration could be attributed to a reduced function or stability of the Wzy protein or a putative Wzy-Wzz complex.

TolA and Pal proteins are not required for the correct polymerization of O antigens synthesized by the *wzy*-independent pathway. The biogenesis of LPS O antigens can be distinguished into *wzy*-dependent and *wzy*-independent pathways (44, 54). We examined whether TolA and Pal are involved in determining the surface expression of a *wzy*-independent O antigen, by transforming *E. coli* W3110, EVV8 (W3110Δ*tolA*), and EVV9 (W3110Δ*pal*) with pWQ5. This plasmid encodes the proteins required for the biosynthesis and ABC transporter-mediated translocation of the *Klebsiella pneumoniae* O1 polysaccharide (10). The experiment showed that the polymerization of *K. pneumoniae* O1 LPS seems to be independent of TolA or Pal (Fig. 4A). In the case of the Δ*pal* mutant EVV9(pWQ5), a novel band reproducibly appeared in the area corresponding to lipid A-core plus one or two O-antigen subunits. However, the banding pattern of higher-molecular-weight O-antigen polymers was comparable with that seen in parental and Δ*tolA* strains. In addition, *E. coli* W3110, EVV8, and EVV9 were transformed with pWQ802. This plasmid car-

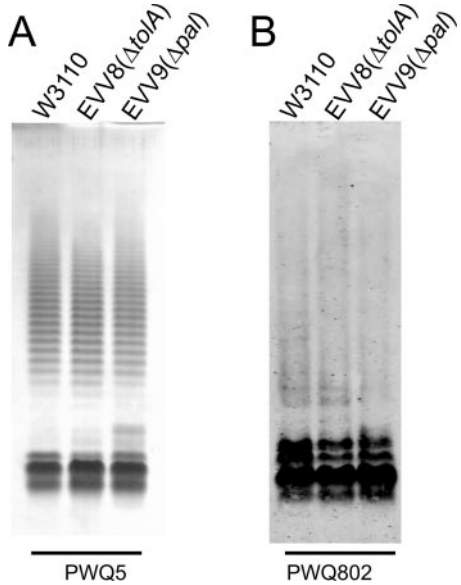

FIG. 4. (A) Analysis of LPS in *E. coli* W3110 and the Δ*tolA* and Δ*pal* isogenic mutants, each containing pWQ5, which encodes the synthesis of *K. pneumoniae* O1 antigen. LPS preparations were analyzed by tricine-SDS-PAGE followed by silver staining. (B) Analysis of LPS in *E. coli* W3110 and the Δ*tolA* and Δ*pal* isogenic mutants, each containing pWQ802, which encodes the synthesis of *S. enterica* serovar Borreze O:54 antigen. LPS preparations were separated by tricine-SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting with O:54-specific antiserum.

ries the genes necessary for the synthesis and surface expression of *S. enterica* serovar Borreze O:54 antigen (24), which is synthesized by the synthase-dependent pathway. The polymerization degree of *S. enterica* O:54 polysaccharide was also unaffected by the absence of TolA or Pal when compared to the parental strain (Fig. 4B), although there was a small decrease in the amount of O antigen detected in the Δ*tolA* and Δ*pal* mutants. These results suggest that the absence of TolA and Pal proteins does not significantly alter the polymerization process in *wzy*-independent systems. Also, these results suggest that the defect observed in the absence of TolA and Pal proteins cannot be due to an impaired WecA function, since the biosynthesis of *E. coli* O16, *K. pneumoniae* O1, and *S. enterica* serovar Borreze O:54 antigens is initiated in all cases by the same biochemical reaction catalyzed by WecA, which involves the formation of a GlcNAc-PP-Und intermediate (2, 54).

***tolA* and *pal* mutations elicit an extracytoplasmic stress response.** Mutations in *tolA* or *pal* are associated with pronounced alterations in the cell envelope of gram-negative bacteria (4, 32, 34, 57). Defects in the bacterial cell envelope cause the activation of regulatory cascades known as extracytoplasmic stress response pathways. There are two well-described systems regulating extracytoplasmic stress responses (46). One of them involves the activation of the RpoE alternative sigma factor (11), while the other requires the CpxAR two-component system (47). Also, a third envelope stress response in *E. coli* that is controlled by the sensor kinase BaeS and the response regulator BaeR has been recently reported (45). Therefore, we investigated whether mutations in *tolA* and *pal* genes are associated with extracytoplasmic stress responses. We generated Δ*tolA* and Δ*pal* derivatives of the *E. coli* K-12 W3110 strains GL111, GL112, and GL113, carrying *lacZ* fusions to the genes *porfA-dsbA*, *fkpA*, and *degP*, respectively (Table 1). The former two genes are regulated by CpxAR and RpoE, respectively, while *degP*, which encodes a periplasmic chaperone/protease, is upregulated by overlapping signals that activate RpoE (11), CpxAR (47), and BaeSR (45) signal transduction pathways. No significant differences in β-galactosidase production were observed in the strains containing *porfA-dsbA-lacZ*. On the other hand, the Δ*tolA* and Δ*pal* mutants carrying *fkpA-lacZ* fusions showed 9% and 18% increases, respectively, in β-galactosidase production ($P < 0.005$) as compared to the parental isolates (Fig. 5). The transcription of *degP-lacZ* was sevenfold higher in the Δ*tolA* mutant and eightfold higher in the Δ*pal* mutant than in the parental strain. The isogenic strain GL123 that carries a mutation in the outer membrane phospholipase A gene *pldA* was used as a positive control for these experiments (Fig. 5), since Cpx- and RpoE-mediated extracytoplasmic stress responses are induced in this strain (26). Altogether these experiments demonstrated that the transcription of genes controlled by the RpoE extracytoplasmic stress response is upregulated in the Δ*tolA* and Δ*pal* mutants.

Extracytoplasmic stress responses affect O-antigen polymerization independent from mutations in *tolA* or *pal*. To further study the effect of extracytoplasmic stress on the polymerization of O16 antigen in *E. coli*, we exposed bacterial cells to indole and diphenylamine. Indole has been reported to elicit extracytoplasmic stress responses (45), while diphenylamine has been shown to reduce the polymerization of O antigen in *E. coli* strains (53). Figure 6A shows that increasing concentrations of indole or diphenylamine were associated with decreased polymerization of O antigen in *E. coli* W3110 containing pMF19. Figure 6B shows that treatment with indole or diphenylamine induced an extracytoplasmic stress response in *E. coli* GL113 (*degP-lacZ*), as evidenced by the increase in β-galactosidase activity. These results demonstrate that extracytoplasmic stress responses alone can cause defects in O-antigen polymerization in bacterial cells that do not have mutations in *tolA* or *pal*.

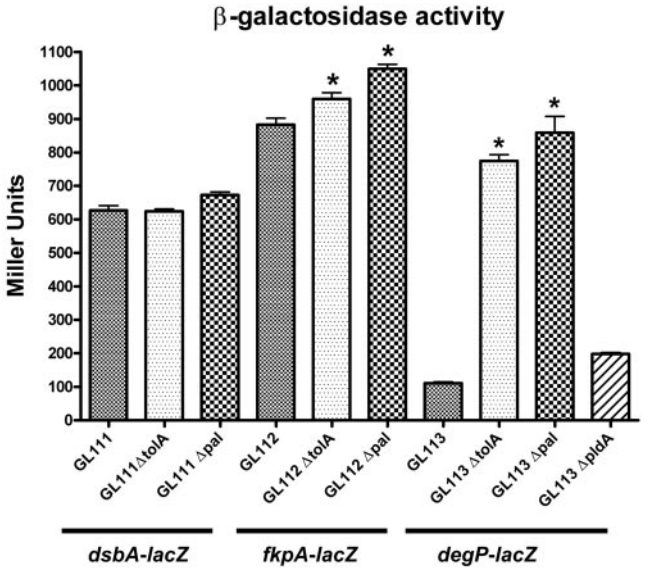

FIG. 5. β-Galactosidase activities of W3110 and Δ*tolA* and Δ*pal* derivatives containing *dsbA-lacZ*, *fkpA-lacZ*, or *degP-lacZ* fusions. Bars depict the mean units of activity calculated from five repeated experiments. Asterisks indicate statistically significant induction ($P < 0.005$).

RpoE-mediated extracytoplasmic stress response impairs O-antigen polymerization. To determine whether the RpoE-

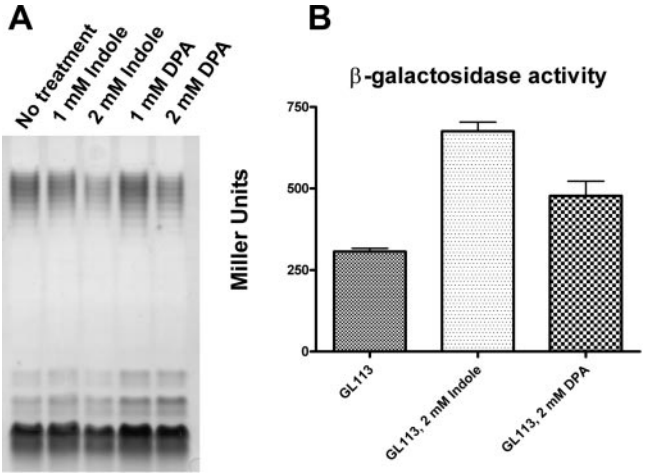

FIG. 6. (A) Effect of indole and diphenylamine (DPA) on LPS expression in *E. coli* K-12 W3110(pMF19). LPS preparations were analyzed by tricine-SDS-PAGE followed by silver staining. (B) Effect of indole and diphenylamine on the β-galactosidase activity of the W3110 derivative GL113 containing a *degP-lacZ* fusion. Bars depict the mean units of activity calculated from three repeated experiments.

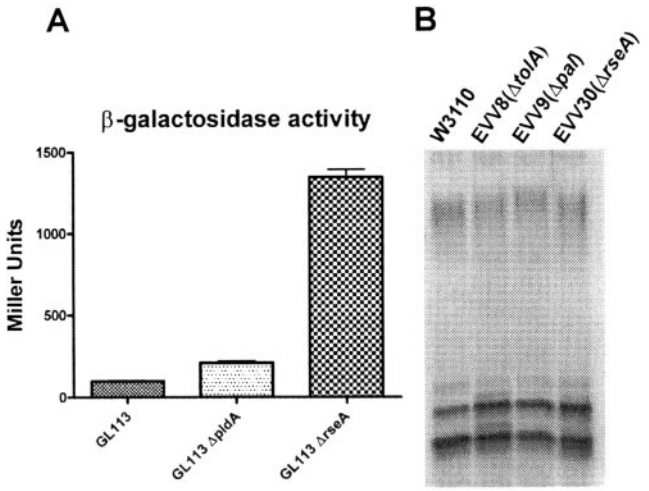

FIG. 7. (A) Effect of an *rseA* deletion on the β-galactosidase activity of the W3110 derivative GL113 containing a *degP-lacZ* fusion. Bars depict the mean units of activity calculated from three repeated experiments. (B) Effect of the *rseA* deletion on the LPS expression in *E. coli* W3110, as compared with the LPS expression in the parental W3110, EVV8 (Δ*tolA*), and EVV9 (Δ*pal*) strains. All strains carry the plasmid pMF19 for the expression of the O16 polysaccharide. LPS preparations were analyzed by tricine-SDS-PAGE followed by silver staining.

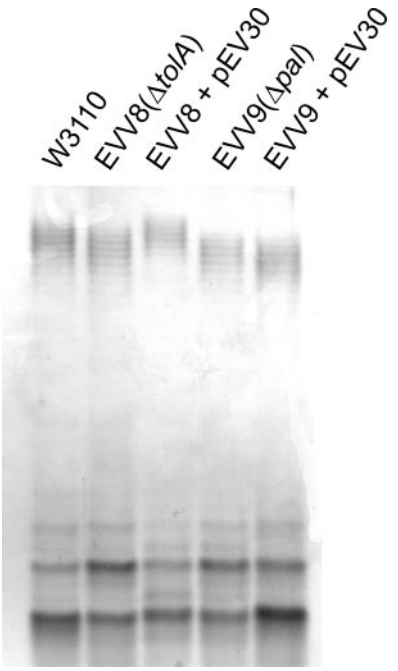

FIG. 8. Effect of overexpression of *rseA* encoded by pEV30 on LPS expression of Δ*tolA* and Δ*pal* mutants in *E. coli* K-12. All strains carry the plasmid pMF19 for O-antigen expression and were also grown in the presence of 0.3% arabinose.

mediated extracytoplasmic stress response is associated with the *tolA* or *pal* LPS phenotype, we constructed strains EVV30 (W3110) and EVV31 (W3110; *degP-lacZ*), which carried a nonpolar deletion of the *rseA* gene, which encodes the anti-sigma factor RseA (1). Lack of RseA should cause a constitutive RpoE-dependent extracytoplasmic stress response (14). We confirmed the activation of the RpoE extracytoplasmic stress response by measuring β-galactosidase activity of strain EVV31 and comparing it with the activity from strains GL113 (*degP-lacZ*) and GL123 (Δ*pldA degP-lacZ*). Figure 7A shows that upon deletion of *rseA*, the activity of the *degP-lacZ* fusion in strain EVV31 was induced approximately 12-fold when compared to that in the parental strain GL113. To assess the effect of the *rseA* deletion on the LPS expression in *E. coli*, we transformed strain EVV30 with pMF19 to reconstitute O-antigen expression and analyzed the LPS profile by Tricine-SDS-polyacrylamide gel electrophoresis (PAGE), followed by silver staining. Figure 7B shows that the deletion of *rseA* was associated with a defect in O-antigen polymerization that is very similar to that found in the *tolA* deletion mutant.

To further study the participation of the RpoE-mediated extracytoplasmic stress response pathway in the *tolA* or *pal* LPS phenotype, we transformed strains EVV8 (Δ*tolA*) and EVV9 (Δ*pal*) with plasmid pEV30, which carries the *rseA* gene under the control of the P_{BAD} inducible promoter. LPS was prepared from the different strains under inducing conditions and was analyzed by Tricine-SDS-PAGE followed by silver staining. The overexpression of RseA was associated with increasing amounts of the highly polymerized O antigen in strains EVV8 and EVV9 (Fig. 8).

The Δ*tolA* or Δ*pal* defect in O-antigen polymerization is not associated with overexpression of *degP* or instability of the Wzy polymerase. The considerable induction of the *degP* gene upon deletion of either *tolA* or *pal* genes prompted us to further study the relationship between the extracytoplasmic stress response and the defect in the polymerization of O antigen. We thus constructed strains EVV19, EVV20, and EVV21, which carry a nonpolar deletion of the *degP* gene in the wild-type, Δ*tolA*, and Δ*pal* backgrounds. These strains were transformed with pMF19, and the LPS profiles for each mutant were investigated. No significant differences were observed when LPS profiles from strains EVV20 (Δ*tolA* Δ*degP*) and EVV21(Δ*pal* Δ*degP*) (Fig. 9) were compared to those from strains EVV8 and EVV9. We concluded from these results that the effect of the *tolA* and *pal* mutations on the expression of the O16 polysaccharide is independent of DegP.

Extracytoplasmic stress responses involve increased proteolysis of periplasmic and membrane proteins (46), and although the experiments described in the previous section demonstrated that DegP was not involved, it could still be possible that other proteases could compromise the stability of the Wzy polymerase in Δ*tolA* and Δ*pal* mutants. Therefore, we constructed strains EVV22 (W3110), EVV23 (W3110Δ*tolA*), and EVV24 (W3110Δ*pal*), all carrying a chromosomal copy of a modified *wzy* gene encoding a Wzy protein fused to a FLAG3x epitope at its C-terminal end. Inner membrane fractions from these strains were isolated, and Wzy_{FLAG3x} was visualized by immunoblotting as described in Materials and Methods. Initial experiments in which samples were boiled prior to electrophoresis did not yield any detectable protein band in the immunoblots (data not shown). Similar difficulties with boiling have been previously encountered with other integral membrane proteins, including WecA and Wzx (3, 12, 38). In contrast, a polypeptide with an apparent molecular mass of 40

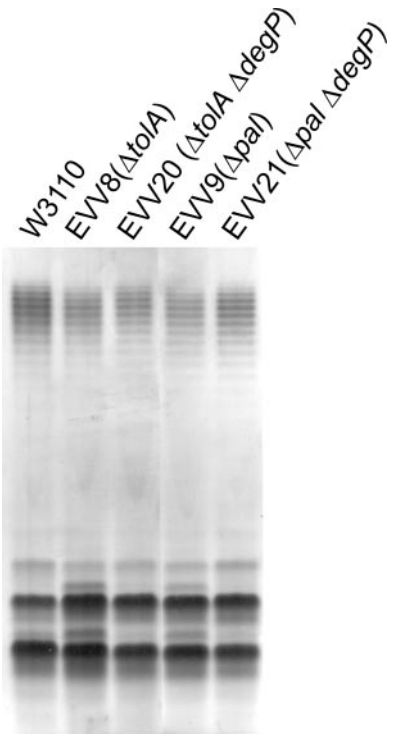

FIG. 9. Analysis of O16 LPS expression in *E. coli* K-12 strain W3110 and the Δ*tolA*, Δ*pal*, Δ*degP*, Δ*tolA* Δ*degP*, and Δ*pal* Δ*degP* isogenic mutants. LPS preparations were analyzed by tricine-SDS-PAGE followed by silver staining. All strains carry the plasmid pMF19 for the expression of the O16 polysaccharide.

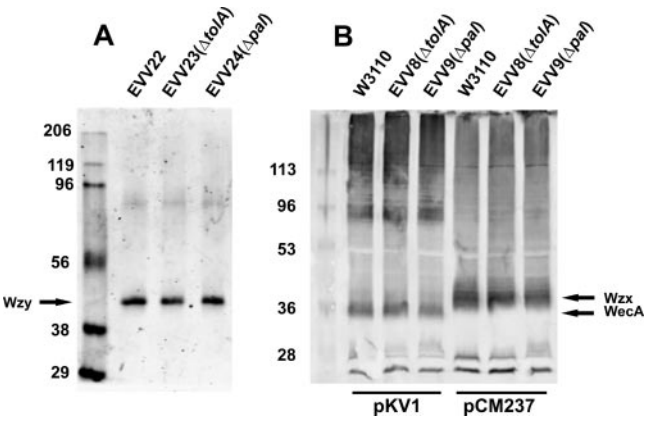

FIG. 10. Western blot analysis of proteins involved in O-antigen assembly. Inner membrane fractions were obtained as described in Materials and Methods. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were reacted with the anti-FLAG M2 monoclonal antibody. (A) Detection of Wzy_{FLAG3X} protein expression. Prestained molecular mass standards in kilodaltons are indicated: myosin (206 kDa), β-galactosidase (119 kDa), bovine serum albumin (96 kDa), ovalbumin (56 kDa), carbonic anhydrase (38 kDa), and soybean trypsin inhibitor (29 kDa). The arrow indicates the position of Wzy_{FLAG3X}. (B) Detection of $WecA_{FLAG6xHis}$ and Wzx_{FLAG}. Prestained molecular mass standards in kilodaltons are indicated: β-galactosidase (113 kDa), bovine serum albumin (96 kDa), ovalbumin (53 kDa), carbonic anhydrase (36 kDa), and soybean trypsin inhibitor (28 kDa). The differences with respect to the markers in panel A are due to different lots of markers used for the two gels. The arrows indicate the positions of Wzx_{FLAG} and $WecA_{FLAG6xHis}$.

kDa, consistent with the expected mass of Wzy_{FLAG3X}, was clearly visualized by incubating samples at 45°C for 30 min (Fig. 10). These conditions were previously used in our laboratory to successfully visualize WecA and Wzx, other integral membrane proteins involved in O-antigen synthesis (3, 38). No significant differences were found in the amounts of Wzy_{FLAG3x} protein produced by these strains (Fig. 10A). Since all the lanes were loaded with an equal amount of protein, we conclude that the stability of Wzy is not compromised in Δ*tolA* and Δ*pal* mutants. Similar experiments were conducted using W3110, EVV8, and EVV9 strains transformed with pKV1 and pCM237 (Table 1). These plasmids encode FLAG epitope fusions of WecA and Wzx proteins (Table 1), which are involved in the initiation of O-antigen subunit synthesis and the translocation of O subunits across the plasma membrane, respectively. No differences among these strains were observed in the amounts of $WecA_{FLAG}$ and Wzx_{FLAG} detected, suggesting that the stability of these proteins is also not affected in Δ*tolA* and Δ*pal* mutants (Fig. 10B).

Concluding remarks. This study shows that the absence of TolA or Pal proteins in *E. coli* K-12 causes a detectable alteration of the polymerization of O16-specific LPS but does not significantly compromise the polymerization of *K. pneumoniae* O1 LPS and *S. enterica* serovar Borreze O:54 LPS. The biogenesis of *K. pneumoniae* O1 LPS and *S. enterica* O:54 requires *wzy*-independent pathways, while the synthesis of O16 LPS requires the *wzy*-dependent pathway (44, 54). The reduced O16 polysaccharide expression in *tolA* or *pal* deletion mutants could not be explained by defects in the biosynthesis of O-antigen subunits, as also shown previously with the O7 antigen system (18).

The abnormal distribution of O16 polysaccharide bands in the *tolA* and *pal* deletion mutants pointed towards a defect in O-antigen polymerization. Overexpression of functional *wzy* and *wzz* genes corrected the O16 LPS phenotype in these mutants, suggesting that O-antigen polymerization, a process that involves Wzy, was compromised in the absence of TolA or Pal. However, we demonstrated that the amount of Wzy protein produced by Δ*tolA* and Δ*pal* mutants is comparable to that observed in the wild-type strain, suggesting that the stability of the Wzy polymerase is not affected.

The Tol import system was first described as involved in tolerance to colicins and filamentous phages. Mutations in its components are associated with a variety of pleiotropic effects, such as increased sensitivity to detergents, outer membrane blebbing, and leakage of periplasmic components (27). Although its physiological role still remains to be elucidated, it is clear that the Tol-Pal system plays a key role in maintenance of the integrity of the gram-negative envelope (27, 32). In this work, we demonstrate that *tolA* and *pal* deletion mutants exhibit an extracytoplasmic stress response that is characterized by an dramatic increase in the transcription of *degP*, a gene encoding a chaperone/protease which is activated by overlapping stress signals (1). This observation prompted us to investigate whether the *tolA* or *pal* O-antigen defect could be found under conditions of extracytoplasmic stress not involving TolA or Pal proteins. We demonstrate a similar phenotype in the presence of indole and diphenylamine, compounds that acti-

vate extracytoplasmic stress responses, and in a strain with a deletion of the *rseA* gene, which exhibits a constitutive extracytoplasmic stress response. Therefore, several conditions leading to RpoE-mediated extracytoplasmic stress, in addition to the absence of TolA and Pal proteins, can compromise the polymerization of O antigen. Recently, an intriguing new phenotype has been described in *tol* mutants of *Pseudomonas putida* and in *E. coli* suggesting that Tol-Pal(OprL) proteins are also necessary for the appropriate function of several uptake systems at the level of the cytoplasmic membrane (33). These observations are consistent with our finding that mutations in the Tol-Pal system cause cell envelope stress responses. These responses may affect the function of membrane proteins, like Wzy, whose active sites are located at the periplasmic face of the plasma membrane. This model also explains why the expression of O antigens assembled by *wzy*-independent pathways, where the polymerization occurs in the bacterial cytosol, is not affected in *tolA* or *pal* mutants. To our knowledge, a connection between extracytoplasmic stress and O-antigen polymerization has not been reported before. Further studies are under way in our laboratory to elucidate the molecular mechanism by which extracytoplasmic stress alters the polymerization of O antigen.

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