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Single amino acid substitutions in either YhjD or MsbA confer viability to 3-deoxy-D-*manno*-oct-2-ulosonic acid-depleted *Escherichia coli*

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Summary

The *Escherichia coli* K-12 strain KPM22, defective in synthesis of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), is viable with an outer membrane (OM) composed predominantly of lipid IV_A, a precursor of lipopolysaccharide (LPS) biosynthesis that lacks any glycosylation. To sustain viability, the presence of a second-site suppressor was proposed for transport of lipid IV_A from the inner membrane (IM), thus relieving toxic side-effects of lipid IV_A accumulation and providing sufficient amounts of LPS precursors to support OM biogenesis. We now report the identification of an arginine to cysteine substitution at position 134 of the conserved IM protein YhjD in KPM22 that acts as a compensatory suppressor mutation of the lethal ΔKdo phenotype. Further, the *yhjD400* suppressor allele renders the LPS transporter MsbA dispensable for lipid IV_A transmembrane trafficking. The independent derivation of a series of non-conditional KPM22-like mutants from the Kdo-dependent parent strain TCM15 revealed a second class of suppressor mutations localized to MsbA. Proline to serine substitutions at either residue 18 or 50 of MsbA relieved the Kdo growth dependence observed in the isogenic wild-type strain. The possible impact of these suppressor mutations on structure and function are discussed by means of a computationally derived threading model of MsbA.

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Introduction

The cell envelope of Gram-negative bacteria contains, in addition to the inner membrane (IM) and the peptidoglycan layer, a bilayered and asymmetrically organized outer membrane (OM). The OM inner leaflet consists of various glycerophospholipids, while the outer leaflet predominantly contains lipopolysaccharide (LPS). LPS is a complex amphiphilic molecule composed of a hydrophilic heteropolysaccharide and the OM-embedded lipid A. The polysaccharide component of many wild-type bacteria can be subdivided into the O-specific polysaccharide chain and an outer and inner core oligosaccharide. Enterobacterial lipid A represents together with the inner core region a rather conserved LPS domain. The structural heterogeneity increases distal to lipid A and the inner core, with the terminal O-specific chain being the most variable part of the LPS molecule (Mamat *et al.*, 1999; Raetz and Whitfield, 2002). The eight-carbon sugar 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) is the only conserved structural element found in all inner core regions investigated so far, linking lipid A to the carbohydrate domain of LPS (Holst, 2007).

Biosynthesis of LPS is a transmembrane process initiated at the cytoplasmic face of the IM. In Wzy-dependent *Escherichia coli* strains, the lipid A-core part and the O-specific chain repeating units are synthesized separately at the cytoplasmic side of the IM, followed by transfer of the units through the IM by specific translocators. At the periplasmic face of the membrane, the O-antigen repeats are ligated to the LPS-core acceptor molecule before the eventual translocation to its final location in the outer leaflet of the OM via ill-defined processes (Raetz and Whitfield, 2002). The transbilayer movement of lipid A-core molecules from the cytoplasmic to the periplasmic face of the IM is dependent on MsbA (Zhou *et al.*, 1998; Doerrler *et al.*, 2004), an essential protein of the ATP-binding cassette (ABC) transporter superfamily (Karow and Georgopoulos, 1993; Polissi and Georgopoulos,

1996), which includes mammalian membrane transporters such as the human multidrug resistance P-glycoprotein MDR1 (Borges-Walmsley and Walmsley, 2001), and the bacterial multidrug transporters LmrA of *Lactococcus lactis* (van Veen *et al*., 1996) and Sav1866 of *Staphylococcus aureus* (Dawson and Locher, 2006). MsbA was originally identified as a multicopy suppressor of the temperature-sensitive phenotype of *htrB* (*lpxL*) mutants (Karow and Georgopoulos, 1993; Polissi and Georgopoulos, 1996) that accumulated tetraacylated lipid A species and phospholipids in the IM at non-permissive temperatures (Zhou *et al*., 1998). Subsequent studies characterized MsbA as highly selective for hexaacylated LPS/lipid A substrates (Doerrler and Raetz, 2002), consistent with earlier observations that *E. coli* and *Salmonella enterica* sv. Typhimurium do not efficiently transport underacylated lipid A species to the OM (Osborn *et al*., 1980; Nishijima and Raetz, 1981).

It has long been recognized that non-conditional Kdo pathway mutants of *E. coli* are not viable and consequently the minimal LPS structure required for growth of *E. coli* cells is two Kdo residues attached to lipid A (Gronow and Brade, 2001; Raetz and Whitfield, 2002). However, we previously reported the isolation of the non-conditional *E. coli* K-12 Kdo pathway null mutant KPM22 from the auxotrophic parent strain TCM15. KPM22, defective in the D-arabinose 5-phosphate isomerases (API) KdsD and GutQ, lacks Kdo and is viable despite predominantly elaborating the lipid A precursor lipid IV_A, thus redefining the minimal LPS structure capable of supporting OM biogenesis in *E. coli* (Meredith *et al*., 2006). Several lines of evidence indicated the development of a compensatory suppressor mutation(s) that enables KPM22 to tolerate null mutations in Kdo pathway genes. It was shown that increased levels of MsbA on a multicopy plasmid can directly rescue the auxotrophic parent strain from the otherwise lethal ΔKdo phenotype without the need to develop the presumed suppressor mutation, thereby indirectly implicating a role for the unmapped suppressor in transport of lipid IV_A to the OM.

In this report, a P1*vir* co-transductional mapping approach has been used to identify the suppressors of Kdo dependence in KPM22 and in a series of independently derived KPM22-like mutants. It is shown that at least two classes of mutations arise to suppress Kdo essentiality. Single amino acid substitutions in either MsbA or the unknown integral IM protein YhjD relegate Kdo pathway genes dispensable in an otherwise isogenic wild-type *E. coli* K-12 background when cultured under standard growth conditions. A putative function for the YhjD suppressor protein in lipid transport is proposed that suggests an MsbA-independent suppressor-facilitated lipid A translocation pathway in KPM22. In addition, a computationally derived three-dimensional model of MsbA is presented to gain insight into the mechanism of LPS/lipid A flip-flop by MsbA.

Results

Mapping of the suppressor mutation of KPM22

Our previous studies on the ΔKdo phenotype indicated that *E. coli* TCM15, the D-arabinose 5-phosphate (A5P) auxotrophic parent strain of KPM22, readily regains its colony-forming ability on solid A5P-free medium in the presence of a presumed suppressor mutation or when multiple copies of MsbA are provided *in trans* (Meredith *et al*., 2006). Accordingly, this selection technique was utilized to map the unknown suppressor mutation in KPM22. A library of kanamycin-marked Tn*903* insertion mutants was generated in KPM22, and a P1*vir* donor lysate was obtained from the pooled transposon library. Selection on kanamycin plates lacking A5P identified co-transductants in which the auxotrophic TCM15 strain had become non-conditional for A5P. Determination of co-transduction frequencies by two-marker P1*vir* transductional mapping from a total of 25 co-transductants revealed a co-transducible linkage of 80% between the Tn*903* insertion and the suppressor mutation in isolate KPM96. The Tn*903* insertion site in KPM96 was mapped to the genomic *gadA-yhjJ* region, which was obtained as two overlapping inserts of 7.1 kb and 15.3 kb in pMMW84 and pMMW86 by subcloning of genomic DNA fragments (Fig. 1). Analysis of the DNA sequence of the 15.8 kb region identified Tn*903* inserted in the cytoplasmic trehalase gene *treF*. More importantly, a C:G to T:A transition at base number 400 of the *yhjD* gene was found in close proximity to the insertion site of the kanamycin resistance cassette. YhjD is a conserved inner membrane protein of unknown function. The point mutation of the *yhjD400* allele causes a substitution of an Arg to a Cys residue at position 134. KPM22 was sequenced directly and confirmed the presence of the mutant *yhjD400* allele, while the mutation was not present in TCM15, which had the wild-type *yhjD* sequence (Fig. 1). Taken together, this suggests that the *yhjD400* allele can function as a compensatory suppressor of Kdo depletion.

Deletion of waaA *in BW30270 carrying the* yhjD400 *allele*

Attachment of two Kdo residues to lipid IV_A by the Kdo transferase WaaA is thought to be essential for viability of *E. coli*, and attempts to isolate non-conditional mutants of Kdo transferase have not been successful (Belunis *et al*., 1995). We therefore examined the capability of the *yhjD400* allele to suppress a *waaA* null

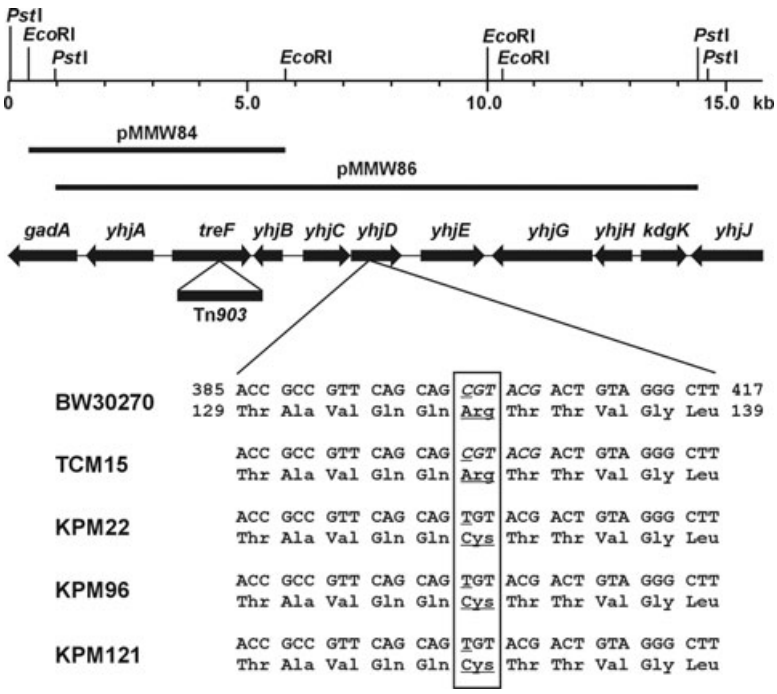

Fig. 1. Genetic organization of the *gadA-yhjJ* region of *E. coli* K-12. The KPM96-derived EcoRI and PstI inserts of pMMW84 and pMMW96, respectively, carry the Tn*903* transposon inserted in *treF*. The C:G to T:A transition at base number 400 of the *yhjD* gene (underlined) causes an Arg to Cys substitution at position 134 (underlined). The BsiWI recognition sequence in the wild-type *yhjD* gene of BW30270 and TCM15, used to screen KPM22-like mutants for the presence of the *yhjD400* allele, is shown in italics.

mutation in the *E. coli* K-12 wild-type strain BW30270. KPM96 was used as the donor for co-transfer of *yhjD400* and *treF*::Tn*903* to BW30270 via P1*vir* transduction to yield strain MAW01, which subsequently served as the host for deletion of the *waaA* gene. The resulting strain KPM121, carrying the *yhjD400* allele (Fig. 1), was indeed viable and capable of maintaining the normally lethal Δ*waaA* mutation. KPM121 showed a growth rate strikingly similar to that of KPM22 (data not shown). Investigation of the LPS precursor of KPM121 by mass spectrometry revealed two prominent LPS related peaks with molecular masses of 1404.85 u and 1527.87 u, consistent with the structures of the tetraacyl-1,4′-bisphosphate LPS precursor lipid IV_A (calculated mass 1404.854 u) and lipid IV_A modified with a phosphoethanolamine (P-EtN) group (calculated mass 1527.863 u), respectively (Fig. 2A). The late acyltransferases LpxL and LpxM exhibit an extremely high specificity for Kdo_2-lipid IV_A substrate both *in vitro* (Brozek and Raetz, 1990) and *in vivo* (Meredith *et al.*, 2006), explaining the lack of the lauroyl and myristoyl chains. Thus, these results strongly support the conclusion that the *yhjD400* allele is a suppressor of the lethal ΔKdo phenotype in *E. coli*. The survival of the BW30270-derived strain KPM121, which elaborates non-glycosylated lipid A intermediates as a result of a Δ*waaA* knockout in the presence of the *yhjD400* allele, suggests that the suppressor is not specifically associated with the ΔAPI background of KPM22 and that no other additional mutations are co-required for suppression.

The yhjD400 *allele is essential to rescue* yhjD*-deficient TCM15 and KPM22 mutants under conditions of A5P depletion*

In order to obtain additional evidence for the decisive role of the *yhjD400* allele in sustaining viability of *E. coli* strains defective in the Kdo pathway, we proceeded to investigate the growth rates of TCM15 and KPM22 derivatives carrying chromosomal *yhjD* deletions that have been complemented with both the wild-type and the mutated allele of *yhjD* on a plasmid (Table 1). Disruption of the *yhjD* and *yhjD400* genes in TCM15 and KPM22 yielded the strains MAW03 and MAW06, respectively, which were viable provided that A5P was included in the medium. Under these conditions, MAW03 and MAW06 displayed almost identical growth rates as their parental strains (TCM15 and KPM22), indicating that *yhjD* in fact is dispensable. Thus, the recent classification of *yhjD* as a non-essential gene in wild-type *E. coli* is confirmed (Baba *et al.*, 2006). However, MAW06, like MAW03, ceased to grow exponentially after two to three generations in LB medium without A5P, suggesting a direct correlation between the recovery of the A5P auxotrophic phenotype and the loss of the *yhjD400* allele in MAW06. To assess the ability of *yhjD400* to subvert the A5P auxotrophic phenotype of MAW03 and MAW06, the strains MAW05 and MAW08 were constructed, each containing the plasmid pT7LOH*yhjD400*. The strains MAW03 and MAW06 were additionally transformed with pT7LOH*yhjD* to generate the strains MAW04 and

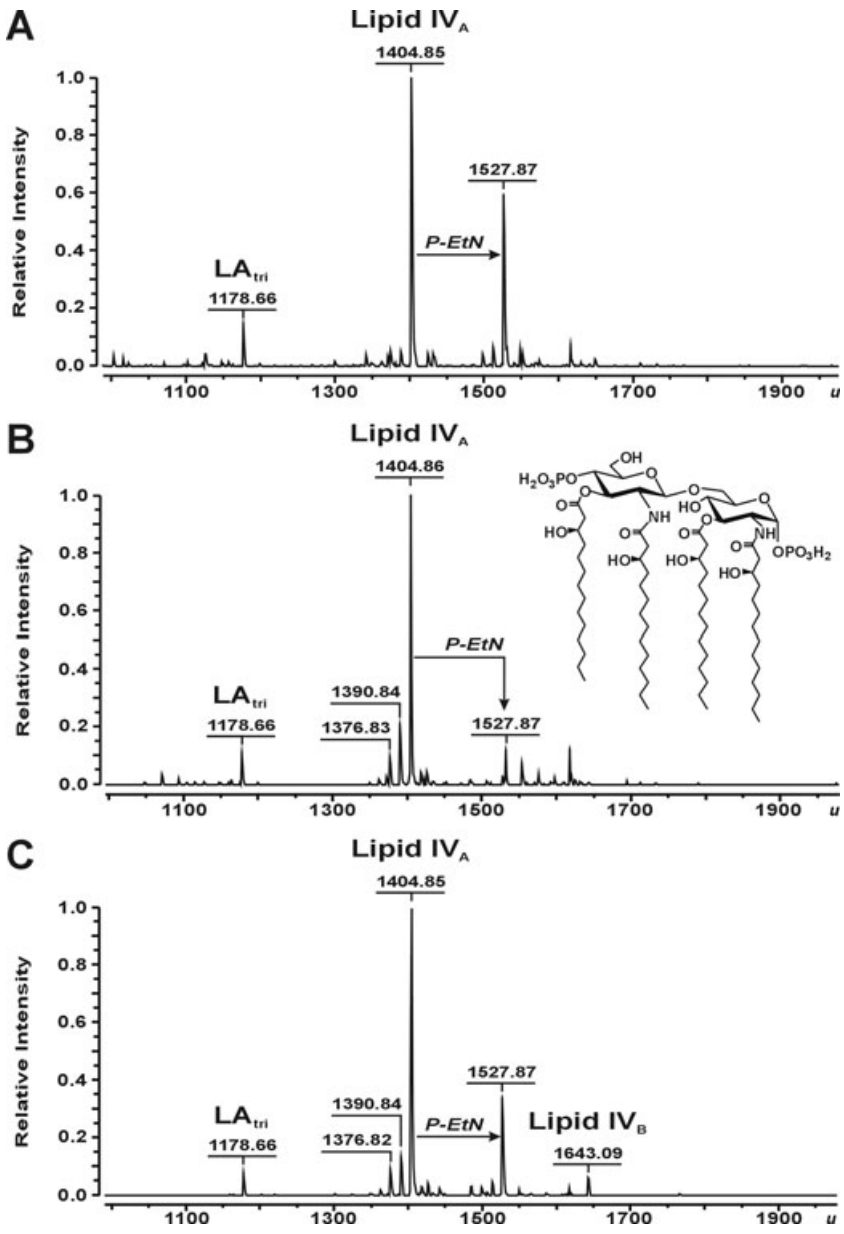

Fig. 2. Charge deconvoluted ESI FT-ICR mass spectra in negative ion mode of LPS molecules isolated from KPM121 (A), KPM22 L1 (inset structure of lipid IV_A) (B) and KPM231 (C). Mass numbers given refer to the monoisotopic masses of neutral lipid A precursor molecules. Lipid IV_A (1404.86 u) modified with a phosphoethanolamine (P-EtN) group (1527.87 u) and with a palmitate chain (lipid IV_B) (1643.09 u), as well as peaks presumably representing lipid IV_A molecules with variations in acyl chain length (1390.84 u and 1376.82 u), are labelled. The peak corresponding to LA_{tri} (1178.66 u) is likely an artefact produced during lipid IV_A isolation and/or ionization as it is not consistent with a known pathway intermediate.

MAW07, respectively, and used as controls in subsequent complementation experiments. As expected, the control strains MAW04 and MAW07 grew only in the presence of exogenous A5P, whereas the strains rapidly ceased to grow when A5P was omitted. In contrast, the *yhjD400* allele was capable of converting MAW05 and MAW08 into non-conditional ΔAPI mutants. Although the strains had a doubling time in LB medium approximately twice as long as in medium supplemented with A5P, the generation time of both the MAW05 and the MAW08 strain was similar to that of KPM22 in LB medium alone. The data provides further evidence that the *yhjD400* allele is essential to sustain viability of A5P auxotrophic strains under non-permissive conditions, whereas multiple copies of the wild-type *yhjD* allele does not exhibit any suppressor activity. Collectively, we conclude that the *yhjD400* mutation imparts suppression not through a loss of protein function, but rather by modifying its activity.

Identification of msbA52 *and* msbA148 *as suppressors of Kdo depletion in KPM22-like mutants*

To address the possibility that mutations other than the *yhjD400* allele may suppress the lethal phenotype associated with Kdo depletion, a series of independent, non-clonal ΔKdo suppressor derivatives of TCM15 that we call KPM22-like mutants were isolated. The *yhjD* loci were

Table 1. Suppression of ΔA5P auxotrophy by the *yhjD400* allele in Δ*yhjD* derivatives of TCM15 and KPM22.

Strain	Generation time (min)	
	Growth in LB + A5P/G6P[a]	Growth in LB
TCM15	23	N/A[b]
MAW03	23	N/A
MAW04	25	N/A
MAW05	23	43
KPM22	22	37
MAW06	23	N/A
MAW07	21	N/A
MAW08	22	40

a. LB medium containing 15 μM A5P and 10 μM G6P.
b. Not applicable. Strains ceased to grow after two to three generations.

amplified from the KPM22-like mutants and then digested with BsiWI to screen for the *yhjD400* allele. The single BsiWI recognition sequence within *yhjD* is destroyed upon conversion to *yhjD400* (see Fig. 1). Besides re-identifying the *yhjD400* mutation in a number of new KPM22-like mutants, we found several strains containing the wild-type allele of *yhjD*. This suggested a second *yhjD*-independent suppression mechanism was operative, and so the representative strain KPM22 L1 was chosen for further analysis. Electrospray ionization Fourier transform ion cyclotron (ESI FT-ICR) mass spectrometry of LPS from KPM22 L1 once again revealed the nearly exclusive peak of lipid IV_A [1404.86 u (Fig. 2B)], supporting the conclusion for the existence of another suppressor that enables KPM22 L1 to survive despite lacking Kdo. To map the suppressor mutation in KPM22 L1, we employed the same P1*vir* co-transductional mapping approach essentially as described above for the identification of the *yhjD400* mutation. Sequence analysis was performed on two overlapping PCR products, covering the 9.6 kb genomic *aroA-msbA* region of the co-transductant KPM129 downstream of the subclone pMMW91 insert (Fig. 3). The Tn*903* integration site was not determined. A C:G to T:A transition at base number 148 was found within the coding region of the ABC transporter gene *msbA*, resulting in a Pro to Ser substitution at position 50. The sequence of the *msbA148* allele in KPM129 perfectly matched that of KPM22 L1, whereas TCM15 was shown to contain the wild-type allele of BW30270 (Fig. 3). Fortuitously, this mutant allele could also be rapidly screened for by DNA restriction analysis in an analogous fashion using the MwoI site at position 145. Amplification of the *msbA* gene from KPM22-like mutants, screening for restriction modification, and DNA sequence analysis identified yet another mutation residing within *msbA* in the non-conditional ΔAPI mutant KPM22 L11 (Fig. 3). The *msbA52* suppressor allele is also a C:G to T:A transition that results in another Pro to Ser substitution but closer to the N-terminus at position 18. To verify that *msbA148* acts as a suppressor of the ΔKdo phenotype, we generated the Δ*waaA* knockout strain KPM231 on the basis of MAW02, which is a derivative of BW30270 obtained by transfer of the *msbA148* allele from KPM129 via P1*vir* transduction. The non-conditional strain KPM231, defective in attachment of Kdo to the lipid A backbone, was viable in the presence of *msbA148*. As a result, only tetraacylated lipid A precursors were detected. In addition to lipid IV_A, lipid

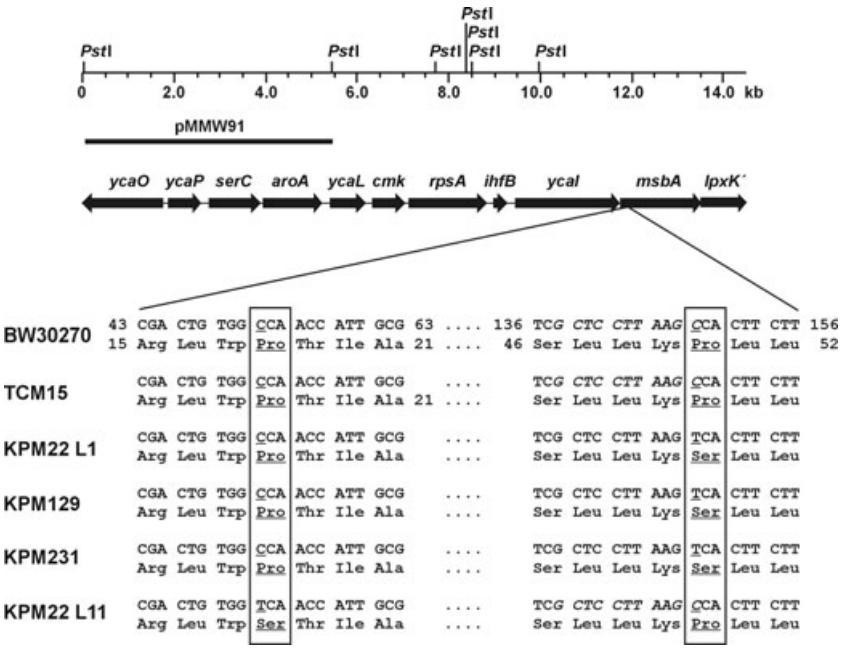

Fig. 3. Genetic organization of the *ycaO-lpxK'* locus of *E. coli* K-12. The PstI insert on pMMW91 and the DNA sequence spanning the region between *aroA* and *msbA* were obtained from KPM129. The C:G to T:A transitions at base numbers 148 and 52, leading in both cases to a Pro to Ser substitution at positions 50 and 18, respectively, are underlined. The MwoI recognition sequence at position 145 of the *msbA* wild-type gene in BW30270 and TCM15 used to screen for the *msbA148* allele in KPM22-like mutants is shown in italics.

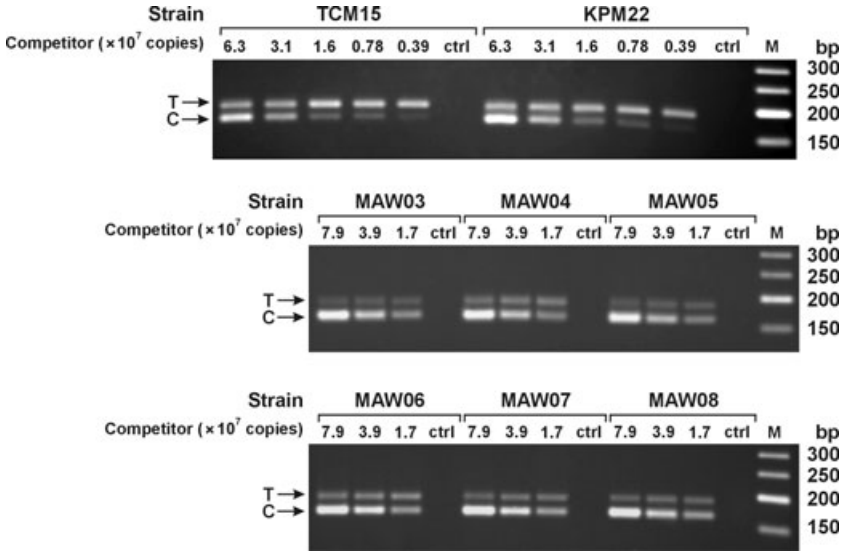

Fig. 4. Quantification of *msbA* gene expression by competitive RT-PCR. Equal amounts of total RNA (1 μg) from each strain were spiked with the indicated copies of *msbA* competitor RNA. For the control experiments, 1 μg of total RNA of the strains was mixed each with 7.9×10^7 copies of *msbA* competitor RNA and subjected to RT-PCR reactions lacking reverse transcriptase. T, PCR product of target cDNA (188 bp); C, PCR product of competitor cDNA (165 bp); M, 50 bp DNA ladder marker.

IV_A modified with either P-EtN or substituted with a palmitate chain [lipid IV_B, calculated mass 1643.084 u (Bishop *et al*., 2000)] could be assigned (Fig. 2C). Taken together, the results allow us to draw the conclusion that both the P18S and the P50S substitution in MsbA are suppressors of the lethal ΔKdo phenotype and hence enable *E. coli* strains to survive without the Kdo pathway.

Quantification of msbA *gene expression*

The capability of multiple MsbA copies to directly rescue the auxotrophic parent strain TCM15 from the lethal ΔKdo phenotype (Meredith *et al*., 2006) raised the question of whether the *yhjD400* suppressor allele may lead to elevated expression levels of the *msbA* mRNA in KPM22 and related strains. Therefore, competitive reverse transcription polymerase chain reaction (RT-PCR) experiments were performed to determine the abundance of the *msbA* transcript relative to the abundance of an *in vitro* synthesized competing *msbA* RNA standard (Fig. 4). The standard RNA, modified by introduction of a deletion of 23 nucleotides into the target region of the *msbA* message, was specifically designed to apply identical experimental conditions for both the target and the competitor RNA in RT-PCRs with identical primers. This establishes nearly indistinguishable amplification kinetics for both RNAs in the same reaction. The initial amount of the *msbA* target in TCM15 and KPM22 was deduced from the ratios of the amounts of competitive amplification products, using 6.3×10^7, 3.1×10^7, 1.6×10^7, 7.8×10^6 and 3.9×10^6 copies of *msbA* competitor RNA and 1 μg of total RNA per cDNA reaction mixture, respectively. Constant amounts of the target cDNA together with decreasing amounts of the competitor cDNA resulted in an increase of the intensity of the upper target-derived bands of 188 bp and a decrease of the intensity of the signals obtained from the competitor cDNA (lower bands of 165 bp). Determination of the competition equivalence points revealed nearly identical initial amounts of the *msbA* target of approximately 4×10^7 copies in both TCM15 and KPM22. The levels of *msbA* in the auxotrophic parent strain and in the suppressor strain are not significantly different. Furthermore, we did not detect any meaningful variations in the amount of the *msbA* target among the TCM15 derivatives MAW03, MAW04 and MAW05, as well as in the KPM22-derived strains MAW06, MAW07 and MAW08. Thus, regardless of whether the *yhjD* gene is deleted or overexpressed on a multicopy plasmid as either the wild-type *yhjD* or the *yhjD400* allele, the mRNA level of *msbA* remains constant.

Deletion of msbA *in KPM22*

In view of underacylated LPS precursors presumably being very poorly translocated across the IM by MsbA (Osborn *et al*., 1980; Nishijima and Raetz, 1981; Zhou *et al*., 1998; Doerrler and Raetz, 2002), it was not unreasonable to assume that contribution of MsbA to lipid IV_A transport is negligible and therefore dispensable in *E. coli* ΔKdo strains containing the *yhjD400* mutation. To test for a non-essential role of MsbA in KPM22, we deleted the entire *msbA* coding sequence of 1749 nucleotides in MAW09, leaving the lipid A 4′-kinase-encoding *lpxK* gene of the *msbA-lpxK* transcriptional unit unaffected. The subsequent transfer of the *msbA* knockout from MAW09 to KPM22 yielded the strain KPM272, which was viable despite lacking the *msbA* gene. Several attempts to additionally delete the *lpxK* gene in KPM22 were unsuccessful (data not shown). This suggested that phosphorylation of the 4′-position of the tetraacyldisaccharide 1-phosphate

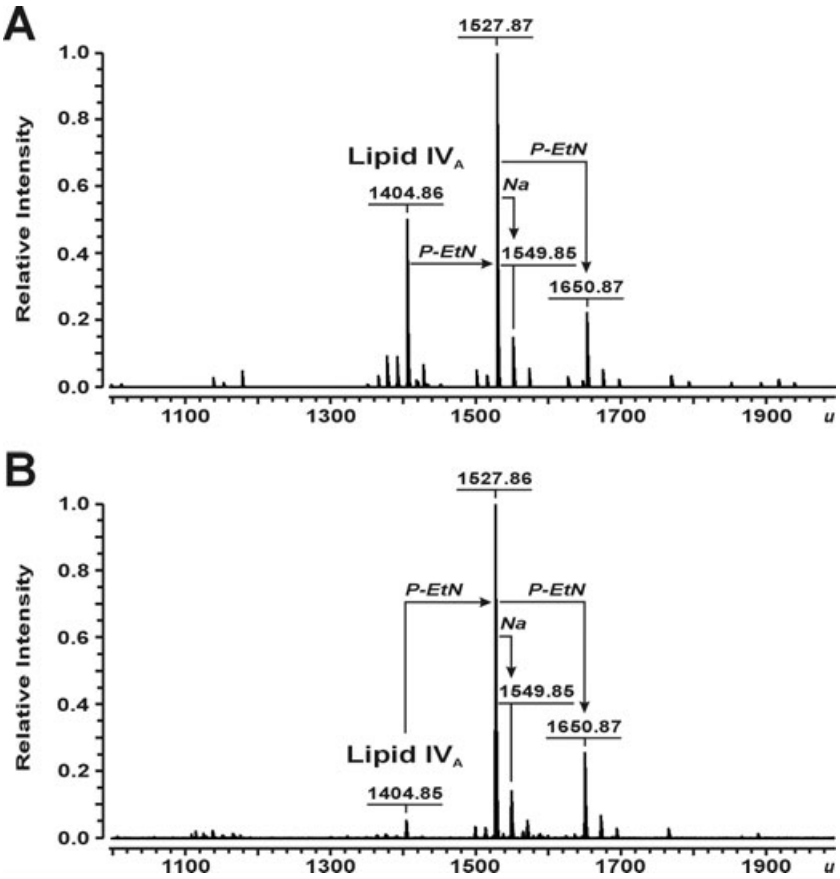

Fig. 5. Charge deconvoluted ESI FT-ICR mass spectra in negative ion mode of LPS molecules isolated from the IM (A) and OM (B) of KPM272. Mass numbers given refer to the monoisotopic masses of neutral lipid A precursor molecules. Lipid IV$_A$ (1404.86 u) substituted with one phosphoethanolamine (P-EtN) group (1527.87 u) and with two P-EtN moieties (1650.87 u) are labelled.

precursor of lipid A is required for viability of KPM22 as in wild-type *E. coli* cells (Garrett *et al*., 1998). To address the subcellular location of lipid IV$_A$ molecules and determine whether they are transported to the OM of KPM272, the LPS precursors were isolated from its IM and OM. ESI FT-ICR analysis of the samples identified in decreasing abundance lipid IV$_A$ substituted with one P-EtN group (1527.87 u), lipid IV$_A$ (1404.86 u), and lipid IV$_A$ modified with two P-EtN moieties (calculated mass 1650.871 u) as constituents of both the IM and OM of KPM272 (Fig. 5). The data collectively indicate MsbA is indeed not responsible for trafficking LPS-related precursors to the OM in strains that harbour the *yhjD400* allele.

Discussion

Original studies on the Kdo8P synthase KdsA of *S. enterica* sv. Typhimurium (Rick and Osborn, 1977; Rick and Young, 1982; Raetz *et al*., 1985) and application of agents specifically targeting the CMP-Kdo synthetase KdsB of *S. enterica* sv. Typhimurium and *E. coli* (Goldman *et al*., 1987; 1988) demonstrated the accumulation of large amounts of underacylated lipid A precursors, the arrest of cell growth and ultimately cell death as a result of the interruption in Kdo biosynthesis. Substantial quantities of LPS precursors accumulated in the IM, although traces of stably integrated precursors could be detected in the OM. It was suggested that the conditionally lethal effects stem from a markedly diminished lipid translocation rate to the OM, rather than an incapability of the temperature-sensitive KdsA mutant of *S. enterica* sv. Typhimurium *per se* to integrate lipid A precursors into the OM (Osborn *et al*., 1980). However, in consideration of the fact that the minimal LPS structure required to sustain viability of *E. coli* cells had been recognized as two Kdo residues attached to lipid A, it remained uncertain whether at least a Kdo$_2$-lipid A LPS substructure is actually required for growth/OM maintenance, or whether the accumulation of LPS precursors causes toxicity. We previously have shown that the Kdo molecule itself is a dispensable structural component of the OM LPS layer of *E. coli* (Meredith *et al*., 2006). The non-conditional ΔAPI strain KPM22 is defective in Kdo biosynthesis and yet remains viable despite lacking the entire LPS-core structure. It was therefore suggested that the suppressor mutation enables the strain to tolerate Kdo depletion by increasing the rate of lipid IV$_A$ transport, resulting in an adequate supply for OM biogenesis and/or removal of lipid IV$_A$ from the IM to attenuate toxic side-effects caused by lipid IV$_A$ accumulation.

Here we report that an Arg to Cys substitution at position 134 of YhjD suppresses the lethal ΔKdo phenotype of KPM22. Four lines of evidence support the conclusion that $YhjD_{R134C}$ is required for viability of KPM22. First, the presence of the *yhjD* wild-type allele in the auxotrophic parent strain TCM15 argues strongly for the development of the suppressor mutation in KPM22 during the extended lag of more than 24 h between the shift to non-permissive conditions and subversion of the A5P auxotrophic phenotype. Second, the *yhjD400* allele was clearly essential as well as sufficient to transform Δ*yhjD* derivatives of both KPM22 and TCM15 without any lag into non-conditional ΔAPI mutants. Third, the independent rederivation of non-conditional TCM15 derivatives yielded phenotypically equivalent KPM22-like mutants that carried the same mutation as in KPM22. Finally, integration of the *yhjD400* allele into the genome of the *E. coli* K-12 wild-type strain BW30270 directly converted the normally essential *waaA* Kdo transferase gene into a non-essential gene in the mutant KPM121.

Although our results implicate $YhjD_{R134C}$ in participation of lipid IV_A removal from the IM of Kdo-depleted *E. coli* K-12 strains, elucidation of the mode of suppression by $YhjD_{R134C}$ must await further investigations. An attractive hypothesis was that the expression of $YhjD_{R134C}$ increases the *msbA* mRNA copy number to compensate for the low affinity of MsbA for lipid IV_A and restores transport to viable rates by simple mass action. However, we could not find any indication of an impact of $YhjD_{R134C}$ on the abundance of the *msbA* transcript, either in KPM22 or in Δ*yhjD* derivatives of KPM22 and TCM15. At present, we cannot rule out the possibility that the expression of MsbA is regulated post-transcriptionally, for example, via the amount of $tRNA_2^{Thr}$ and/or the usage of the rare codon ACG that is frequently found in the *msbA* gene (Mohri *et al*., 2003), and that this is actually the underlying mechanism of suppression. Based on sequence similarities between *yhjD* and *yihY* (b3886), thought to code for the RNase BN of *E. coli* (Callahan and Deutscher, 1996), YhjD has been assigned to a subgroup of the RNase BN-like family of rather unusual hydrophobic RNases with five to six predicted transmembrane spans (Zuo and Deutscher, 2001). However, the apparent incorrect assignment of *yihY* as the gene encoding RNase BN (Ezraty *et al*., 2005) challenges the classification of YhjD as an RNase BN-like protein. Taking into account that YhjD is a putative conserved IM protein (Daley *et al*., 2005) and appears to be orthologously related to members of the major facilitator superfamily of proteins for transport of small molecules [Kyoto Encyclopedia of Genes and Genomes (http://www.genome.ad.jp/kegg/kegg2.html)] (Pao *et al*., 1998), we currently favour the hypothesis of a direct participation of $YhjD_{R134C}$ in transmembrane movement of lipid IV_A. The hypothesis is based on the assumption that the Arg to Cys substitution results in a modified YhjD protein, which can now transit lipid IV_A molecules across the IM. This in turn relieves a lethal bottleneck in lipid IV_A transport, supplementing the insufficient amounts of lipid IV_A being translocated by MsbA alone and restoring viability.

The hypothesis of a direct participation of $YhjD_{R134C}$ in lipid IV_A transmembrane transport in KPM22 is strongly supported by the *msbA* knockout experiments that showed MsbA is non-essential in the presence of the *yhjD400* suppressor mutation. The strain KPM272 (KPM22 Δ*msbA*) is shown to possess an OM composed predominantly of tetraacylated lipid A species (Fig. 5B), which suggests an alternative translocation pathway for lipid IV_A molecules in KPM22 and related strains. We propose $YhjD_{R134C}$ plays a key role in supplying the amounts of underacylated lipid A precursors required for OM biogenesis. Like lipid IV_A previously identified in the IM of KPM22 (Meredith *et al*., 2006), the lipid A precursors remaining localized in the IM of KPM272 (Fig. 5A) appear to have no detrimental effects on the bacterial cells. Recently, a direct role of MsbA in phospholipid transport across the IM of *E. coli* has been suggested (Zhou *et al*., 1998; Doerrler *et al*., 2001; 2004), whereas MsbA apparently did not play any role in translocation of phospholipids in *Neisseria meningitidis* (Tefsen *et al*., 2005). Other studies have reported the ATP-independent transbilayer movement of lipids by hydrophobic membrane-spanning α-helical proteins as an alternative to the concept of lipid flip-flop by the activity of dedicated proteins such as ABC transporters (Kol *et al*., 2003; 2004). The synthesis of an OM by the *msbA* null mutant KPM272 argues against a direct role of MsbA in phospholipid transport also in *E. coli*, although we cannot entirely exclude the possibility that the MsbA-independent supply of phospholipids for OM biogenesis is specifically associated with the genetic background of KPM272, i.e. a compensatory function of $YhjD_{R134C}$ in transmembrane trafficking of phospholipids as well.

We consistently observed varying but elevated levels of P-EtN-modified lipid IV_A in suppressor strains defective in Kdo transfer like KPM121 and KPM231 (Fig. 2A and C). In particular, the Δ*msbA* knockout strain KPM272 expresses lipid IV_A molecules substituted with two P-EtN groups (Fig. 5). Lipid A covalently modified with P-EtN is normally not a constituent of the LPS of *E. coli* K-12 when grown in LB medium, and only polymyxin-resistant mutants of *E. coli* K-12 (Nummila *et al*., 1995) and wild-type cells exposed either to mild acidic conditions (Gibbons *et al*., 2005) or ammonium metavanadate (Zhou *et al*., 1999) have so far been shown to modify lipid A with P-EtN. We did not address the question of which phosphate position of the lipid IV_A in KPM121, KPM231 or KPM272 is P-EtN-substituted. The transfer of P-EtN occurs predominantly to the 1-phosphate group of lipid A at the periplasmic face of the IM (Doerrler *et al*., 2004), whereas temperature-

sensitive KdsA mutants of *S. enterica* sv. Typhimurium were shown to accumulate lipid A precursors upon shift to non-permissive temperatures that are substituted independently of Kdo with a P-EtN group solely at the 4′-phosphate position (Strain *et al.*, 1985; Zhou *et al.*, 2000; 2001). In KPM121, KPM231 and KPM272, however, lipid IV_A presumably is transported with increased rates to the OM. Therefore, we must also consider the possibility that lipid IV_A, accessible at the periplasmic side of the IM while translocated to the OM, is modified with P-EtN at the 1-phosphate group in *E. coli* K-12 suppressor strains lacking Kdo.

The capability to suppress the lethal ΔKdo phenotype is not restricted to $YhjD_{R134C}$. Here we also show that Pro to Ser substitutions either at position 18 or at residue number 50 of MsbA enable *E. coli* K-12 strains to survive non-conditionally with null mutations in Kdo pathway genes, including *gutQ*/*kdsD* and *waaA*. Although the YhjD and MsbA suppressor proteins confer viability, there are likely subtle differences in the OM composition due to varying rates of lipid IV_A transport in the different suppressor strains. Strains defective in LPS biosynthesis translocate phospholipids to the outer leaflet of the OM. As demonstrated with EDTA-treated *E. coli* cells (Jia *et al.*, 2004) and *E. coli* mutants under conditions of Imp/RlpB depletion (Wu *et al.*, 2006), the palmitate transferase PagP on the outer surface of the OM is capable of using these outer leaflet phospholipids as palmitoyl donors to modify LPS molecules (Bishop *et al.*, 2000). We did not detect lipid IV_B in KPM22 (Meredith *et al.*, 2006), KPM121, KPM22 L1 and KPM272 but have identified a palmitoylated fraction of lipid IV_A molecules in KPM231, suggesting the presence of phospholipids patches in the outer leaflet OM that are being used by PagP to modify lipid IV_A.

Our data give reason to suggest that the *msbA52* and *msbA148* suppressor mutations in KPM22-like mutants relax the high substrate specificity of MsbA for mature LPS. The *msbA52* mutation in KPM22 L11 was not further characterized, but $MsbA_{P18S}$ basically confers the same phenotype as found in all investigated $YhjD_{R134C}$ and $MsbA_{P50S}$ suppressor strains, i.e. the cells are viable despite perhaps predominantly synthesizing lipid IV_A and no Kdo. The current data do not allow us to determine if the *msbA* mutations increase substrate promiscuity during active, ATP-dependent transport or facilitate passive transport of LPS precursors. In either case, the substitution of proline residues as a prerequisite for increasing the substrate promiscuity of MsbA supports the key role of proline residues in determining the structure and function of α-helical membrane proteins (Cordes *et al.*, 2002). To gain insight to possible structural and functional consequences of the *msbA52* and *msbA148* suppressor mutations, we generated a threading model of the primary amino acid sequence of wild-type MsbA from

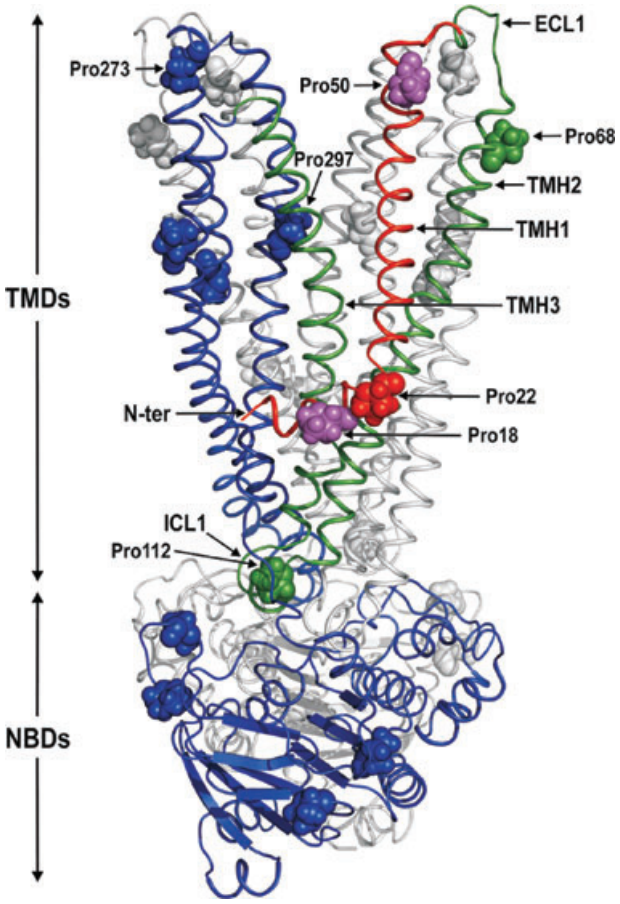

Fig. 6. Threading model of the homodimeric MsbA from *E. coli* in coil representation, with the monomers coloured blue and grey. The primary amino acid sequence of MsbA was threaded onto the crystal structure for the ABC transporter Sav1866 of *S. aureus* in the ADP-bound outward-facing conformation (Dawson and Locher, 2006) as described in *Experimental procedures*. The N-terminal helix and transmembrane helix (TMH) 1 are highlighted in red, TMH2 and TMH3 in green. The Pro residues of both monomers are shown as spheres. Pro18 and Pro50, substituted to Ser in the suppressor proteins $MsbA_{P18S}$ and $MsbA_{P50S}$, respectively, are coloured magenta. All other labelled Pro residues depict the sites suggested for substitution (see *Discussion*). The PyMOL software [DeLano, 2002; The PyMOL Molecular Graphics System (http://www.pymol.org)] was used to present the threading model. TMDs, transmembrane domains; NBDs, nucleotide binding domains; N-ter, N-terminus; ECL, extracellular loop; ICL, intracellular loop.

E. coli onto the crystal structure for the homodimeric *S. aureus* ABC transporter Sav1866 in the ADP-bound outward-facing conformation (Dawson and Locher, 2006) (Fig. 6). A similarly generated model for LmrA was recently published (Federici *et al.*, 2007). As MsbA of *E. coli* and Sav1866 share significant overall sequence similarity of 63% (Fig. S1), the fundamental assumption in the creation of our model is that the structure of MsbA is nearly identical to that of Sav1866. The threading model is not intended as a starting point for quantitative computational studies, but rather to provide a qualitative tool for

generation of hypotheses, illustration and discussion. A more rigorous foundation for future studies must await the release of the corrected MsbA crystal structures (Chang *et al*., 2006).

The homodimeric Sav1866 has been recently suggested as a reliable structural model for the core architecture of all ABC exporters (Dawson *et al*., 2007; Hollenstein *et al*., 2007). Sav1866 comprises two 'half-transporters' with two identical subunits, each containing a hydrophilic cytoplasmic nucleotide-binding domain (NBD) for ATP binding and hydrolysis, and a hydrophobic transmembrane domain (TMD), determining the specificity for the substrate and forming the pathway through which the substrate is transported. Furthermore, the structure of Sav1866 in complex with ADP (Dawson and Locher, 2006) and AMP-PNP (Dawson and Locher, 2007) is thought to be in good agreement with the previously proposed 'ATP-switch' model for the transport cycle of ABC transporters (Higgins and Linton, 2004), which describes the binding of ATP to the NBD and 'closed dimer' formation as the driving force for the conformational changes involved in substrate transport, by conversion of a high-affinity binding site of the substrate on the TMDs at the cytoplasmic side of the membrane into a low-affinity site exposed at the extracellular face of the membrane. Conversely, ATP hydrolysis and ADP-Pi release are expected to reset the transporter to its basal inward-facing 'open dimer' configuration.

The predicted intercalation of Sav1866 in the IM (Dawson and Locher, 2006) suggests the N-terminal helix near Pro18 of the MsbA model on the interface between the cytoplasm and the IM, and Pro50 at the junction between transmembrane helix (TMH) 1 and extracellular loop (ECL) 1 on the periplasmic side of the IM. In an ATP-independent model for passage of lipid IV_A, the Pro to Ser substitutions may simply confer enough flexibility to MsbA to facilitate passive translocation of lipid IV_A across the IM. The Pro50 residue is located at a position in the model structure where a substitution to Ser could decrease rigidity and relax substrate discrimination. A decrease in the rigidity of TMH1 or perhaps a change in the bend of ECL1 may contribute to substrate promiscuity. As there could be an electrostatic contribution by the hydroxyl group of Ser, substitutions of Pro50 to Gly, Ala or Val should provide additional understanding as to whether hydrogen bonding plays a role. We suppose that mutations in any of the TMD prolines could provide new insights into MsbA function and selection of lipid A species for trafficking. Substitution of Pro68, which is not located in the extrusion cavity, could decrease the rigidity of ECL1 in the same area as Pro50, whereas Pro297 and Pro273 would be interesting to substitute because of their analogous arrangement relative to Pro50 and Pro68, inside the cavity but lower down and outside the chamber but higher up respectively. The intracellular loops (ICLs) represent the shared interface between the NBDs and the TMDs (Dawson and Locher, 2006). Pro112 at the junction of TMH2 and ICL1, and basically located at the base of the extrusion cavity between TMH2 and TMH3 would be another interesting candidate for substitution.

The location of the Pro to Ser substitution at residue number 18 favours currently two, though not altogether unrelated possibilities of structural perturbation. First, the perpendicular orientation of the N-terminal α-helical segment at the interface between the cell interior and the IM seems to hold the bundles of transmembrane helices in a fixed position. The P18S mutation could 'loosen' the bundle to allow the entry of lipid IV_A into the translocation pathway. In addition, provided that the N-terminal helix is positioned at the entry site into the channel between the two MsbA monomers, lipid IV_A could enter the channel via a less rigid 'gate' in $MsbA_{P18S}$. Mutating Pro22 could shed further light on this.

As mentioned, it must be considered that lipid IV_A remains subject to an ATP-dependent transport cycle. This requires the specific binding of the substrates to one or more substrate binding sites located on the TMDs (Higgins and Linton, 2004). While it is not surprising that point mutations may alter the substrate specificity of ABC transporters (Armandola *et al*., 1996; Ozvegy *et al*., 2002), it is currently difficult to explain why the Pro to Ser substitutions at different sites in MsbA confer one and the same phenotypes on the KPM22-like suppressor strains. As substrate binding and conformational changes are assumed to be required for ATP binding (Higgins and Linton, 2004), the most straightforward explanation for our data is that the initiation of the ATP-dependent transport cycle is actually accomplished by binding of lipid IV_A to two different sites in $MsbA_{P18S}$ and $MsbA_{P50S}$, either to modulated high-affinity binding site(s) for hexaacylated lipid A and LPS molecules, or to cryptic binding site(s) for the tetraacylated lipid A precursor. On the other hand, it is attractive to speculate that the proposed structural changes at the sites of Pro to Ser substitutions, discussed in the context of passive lipid IV_A translocation, are sufficient to act as continuous inducing signals for ATP binding. This poses the question of whether (i) an initial high-affinity binding of lipid IV_A to $MsbA_{P18S}$ and $MsbA_{P50S}$ is required at all to trigger its ATP-dependent transport, and (ii) the suggested modes for lipid IV_A translocation may overlap or even complement one another.

The previous construction of the Kdo-deficient strain KPM22 (Meredith *et al*., 2006) has not only challenged the Kdo_2-lipid A LPS dogma of *E. coli*, but also affords the unique opportunity to obtain new insights into lipid A trafficking and OM biogenesis by identification and analysis of mutations that suppress Kdo dependence (Meredith *et al*., 2006; Raetz *et al*., 2007). In addition to the strains

Table 2. Bacterial strains and plasmids used in this study.

Strains or plasmids	Description	Source or reference
Strains		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^R)]	Stratagene
DY378	*E. coli* K-12 W3110 λ*cI857* Δ(*cro-bioA*)	Yu *et al.* (2000)
BW30270	*E. coli* K-12 MG1655 *rph*$^+$*fnr*$^+$	CGSC#7925
TCM15	BW30270 Δ*gutQ* Δ*kdsD*; A5P auxotroph	Meredith and Woodard (2005)
KPM22	Non-conditional TCM15 derivative; *yhjD400*	Meredith *et al.* (2006)
KPM22 L1	Non-conditional TCM15 derivative; *msbA148*	This study
KPM22 L11	Non-conditional TCM15 derivative; *msbA52*	This study
KPM96	TCM15 *yhjD400 treF*::Tn*903*	This study
KPM121	MAW01 Δ*waaA*	This study
KPM129	TCM15 *msbA148* Tn*903*$^+$	This study
KPM231	MAW02 Δ*waaA*	This study
KPM272	KPM22 Δ*msbA*	This study
MAW01	BW30270 *yhjD400 treF*::Tn*903*	This study
MAW02	BW30270 *msbA*148 Tn*903*$^+$	This study
MAW03	TCM15 Δ*yhjD*	This study
MAW04	MAW03 with pT7LOH*yhjD*	This study
MAW05	MAW03 with pT7LOH*yhjD400*	This study
MAW06	KPM22 Δ*yhjD*	This study
MAW07	MAW06 with pT7LOH*yhjD*	This study
MAW08	MAW06 with pT7LOH*yhjD400*	This study
MAW09	DY378 Δ*msbA*::*kan* with pK-Cla	This study
Plasmids		
pNK2859	Amp^R, Km^R; transposon delivery plasmid; carries miniTn*10* derivative Tn*903*	Kleckner *et al.* (1991)
pKD46	Amp^R; λ Red recombinase expression plasmid	Datsenko and Wanner (2000)
pKD3	Amp^R, Cm^R; template plasmid for chloramphenicol resistance cassette	Datsenko and Wanner (2000)
pKD4	Amp^R, Km^R; template plasmid for kanamycin resistance cassette	Datsenko and Wanner (2000)
pCP20	Amp^R, Cm^R; FLP recombinase expression plasmid	Datsenko and Wanner (2000)
pUC18	Amp^R; high-copy-number general cloning vector	Invitrogen
pT7LOH	Amp^R; T7 expression vector for N-terminal HisTag fusions	Muda *et al.* (2002)
pK-Cla	Amp^R; pREG153 carrying a 3.8 kb KpnI–ClaI insert with *msbA* and *lpxK* of *E. coli*	Karow and Georgopoulos (1993)
pT7LOH*yhjD*	pT7LOH carrying the *yhjD* gene of BW30270	This study
pT7LOH*yhjD400*	pT7LOH carrying the *yhjD400* gene of KPM22	This study
pMMW84	pUC18 carrying a 7.1 kb EcoRI insert of KPM96 with *gadA′*, *yhjA*, *treF*::Tn*903* and *yhjB*	This study
pMMW86	pUC18 carrying a 15.3 kb PstI insert of KPM96 with *gadA′*, *yhjA*, *treF*::Tn*903*, *yhjB*, *yhjC*, *yhjD400*, *yhjE*, *yhjG*, *yhjH*, *kdgK* and *yhjJ′*	This study
pMMW91	pUC18 carrying a 7.2 kb PstI insert of KPM129 with *ycaO′*, *ycaP*, *serC*, *aroA*, *ycaL′* and Tn*903*	This study

that carry the herein reported suppressor mutations in the *yhjD* and *msbA* genes, we have also isolated a third class of KPM22-like mutants that retain wild-type alleles for both *yhjD* and *msbA* genes (U. Mamat and R.W. Woodard, unpublished). This suggests the existence of a number of suppressors capable of compensating for the lethal ΔKdo phenotype, and should further illuminate the complex process of LPS trafficking in *E. coli*.

Experimental procedures

Bacterial strains, plasmids and growth conditions

All strains and plasmids used in the present study are described in Table 2. Bacteria were routinely grown aerobically with shaking (250 r.p.m.) at 37°C in standard Luria–Bertani (LB) medium containing 10 g l^{-1} of NaCl to maintain a non-mucoid phenotype in ΔKdo strains (Meredith *et al.*, 2007). For growth of TCM15, containing deletions in the API genes *gutQ* and *kdsD*, 15 μM A5P was added along with 10 μM D-glucose 6-phosphate (G6P) to induce the Uhp sugar phosphate transport system (Eidels *et al.*, 1974; Meredith and Woodard, 2005). Non-conditional derivatives of TCM15 were derived in MOPS-minimal medium with 0.2% glycerol as has been described (Meredith *et al.*, 2006). Growth rates were determined using cultures in early exponential growth phase by monitoring the OD_{600} of the cell suspensions. In order to induce LPS biosynthesis (Meredith *et al.*, 2006) and therefore restore the receptor for phage P1 adsorption, KPM strains used as donors in P1*vir* transductions were grown in the presence of 15 μM A5P and 10 μM G6P prior to phage infection.

Mapping of suppressor mutations

Genetic mapping of suppressor mutations was performed by P1*vir* transduction according to standard protocols (Miller,

Table 3. Primers.

Primer	Sequence	Source
ECOyhjDH1	ATGACGCAGGAAAACGAGATCAAACGTCCCATCCAGGATCTGGAGCACGAGTGTA GGCTGGAGCTGCTTC[a]	Invitrogen
ECOyhjDH2	TTAAGGCTGCGTTTTCCCCGGCATTCGCGGGTCGTCTTTATATTCGGCGGCATATG AATATCCTCCTTAG[a]	Invitrogen
ECO5NdeIyhjD	GATTCTAGAATT*CATATG*ACGCAGGAAAACGAGATCAAACG[b]	Invitrogen
ECO3BamHIyhjD	GAATTCAAGCTT*GGATCC*TTAAGGCTGCGTTTTCCCCGGC[c]	Invitrogen
ECOycaOP1	TTATTTTGCCCAGAATGCTGCTT	Invitrogen
ECOycaLP1	TTACTTACCAGAGGCGATACGATC	Invitrogen
ECOycaLP2	GCGCTATCAATGCTAAATACTCC	Invitrogen
ECOlpxKP1	GGGATTCACCAGACCAGATTTT	Invitrogen
ECOyhjDP1	GCTGGTTTATCCGCAGCGTCG	Invitrogen
ECOyhjDP2	CGAGTTTCAAGAGGTTATGTGC	Invitrogen
ECOmsbAP1	ATGCATAACGACAAAGATCTCTCTA	MWG
ECOmsbAP2	TCATTGGCCAAACTGCATT	MWG
ECOmsbAH1	TGGATAACGGGTAGAATATGCGGCTATTTCAACAAATGCTGGTTTTTTGAGTGTA GGCTGGAGCTGCTTC[a]	MWG
ECOmsbAH2	AGCAATAGCCGCCACAAAGGGGATTCACCAGACCAGATTTTTTCGATCATCATATG AATATCCTCCTTAG[a]	MWG
ECOwaaAH1	ACAGCTAAATACATAGAATCCCCAGCACATCCATAAGTCAGCTATTTACTGTGTAG GCTGGAGCTGCTTC[a]	MWG
ECOwaaAH2	TAATGGGATCGAAAGTACCCGGATAAATCGCCCGTTTTTGCATAACAACCCATATG AATATCCTCCTTAG[a]	MWG
5T7msbAcRTPCR	gcgtaatacgactcactatagggagaggagATGCTGAAGGGCCACAAAGGAAGTGGAAACGAAACGCTT[d]	MWG
3msbAcRTPCR	GACGCATCAGTGCAATCATTGA	MWG
5msbALC1	ATGCTGAAGGGCCACAAA	MWG
3msbALC1	AAGCTCGCCGCATACAGA	MWG

a. Homology regions are underlined.
b. NdeI site is shown in italics.
c. BamHI site is shown in italics.
d. T7 promoter sequence is indicated in lower case letters.

1992). Tn*903* transposon libraries of strains KPM22 and KPM22 L1 harbouring the transposon delivery plasmid pNK2859 were constructed as described previously (Kleckner *et al.*, 1991), followed by generation of transducing P1*vir* lysates from pools of the donors carrying randomly inserted chromosomal Tn*903* insertions. Transduction of TCM15 recipient cells with P1*vir* lysates, followed by dual marker selection for kanamycin resistance (30 μg ml^{-1} kanamycin) and loss of A5P auxotrophy on LB agar, yielded the co-transductants KPM96 and KPM129, respectively. The kanamycin resistance marker along with the suppressor mutations were then moved from KPM96 and KPM129 into the parent *E. coli* K-12 wild-type strain BW30270 genetic background by P1*vir* transduction to obtain strains MAW01 and MAW02, respectively. Co-transduction frequencies were estimated in all experiments by transduction of TCM15 using P1*vir* lysates from potential co-transductants and selected for resistance to kanamycin on LB agar containing 30 μg ml^{-1} kanamycin, 15 μM A5P and 10 μM G6P, followed by scoring of the kanamycin-resistant transductants for loss of A5P auxotrophy on LB agar only.

DNA manipulations

Standard recombinant DNA methods were used for nucleic acid preparation and analysis (Sambrook and Russell, 2001). Primer sequences are listed in Table 3. To localize the integration sites of the Tn*903*-derived kanamycin resistance cassette, EcoRI- or PstI-digested genomic DNA fragments from KPM96 and KPM129 were ligated into the EcoRI or PstI site of pUC18. XL1-Blue transformants were selected on LB agar containing 30 μg ml^{-1} kanamycin and 100 μg ml^{-1} ampicillin. The entire DNA insert contained in two KPM-96 derived clones [pMMW84 (7.1 kb) and pMMW86 (15.3 kb)] was directly sequenced, whereas the sequence of the chromosomal region of KPM129 downstream of the pMMW91 insert was determined on overlapping PCR products obtained with the primer pairs ECOycaOP1/ECOycaLP1 and ECOycaLP2/ECOlpxKP1. In mapping subsequent suppressor mutants, clones were initially screened for known mutant alleles by BsiWI and MwoI restriction analysis, respectively, prior to sequencing. Primer pairs ECOyhjDP1/ECOyhjDP2 and ECOmsbAP1/ECOmsbAP2 were used to amplify the *yhjD* and *msbA* genes for sequencing.

Chromosomal *yhjD* and *waaA* deletions were constructed using the phage λ Red recombinase procedure as described (Datsenko and Wanner, 2000), except chloramphenicol was used at 10 μg ml^{-1} for selection. Primer pairs ECOyhjDH1/ECOyhjDH2 with pKD4 (kanamycin) or ECOwaaAH1/ECOwaaAH2 with pKD3 (chloramphenicol) as templates were used to construct the insert cassettes targeting *yhjD* of TCM15 and KPM22, and *waaA* of MAW01 and MAW02, respectively. Antibiotic resistance markers were excised by the FLP recombinase system essentially as described (Datsenko and Wanner, 2000), except plasmids pKD46 and pCP20 were cured at 37°C to accommodate the temperature-sensitive phenotype of KPM22-related strains (Meredith

et al., 2006). The resulting strains were designated MAW03 (TCM15 Δ*yhjD*), MAW06 (KPM22 Δ*yhjD*), KPM121 (MAW01 Δ*waaA*) and KPM231 (MAW02 Δ*waaA*). Both the *yhjD* gene and the *yhjD400* allele were amplified using the primers ECO5NdeIyhjD and ECO3BamHIyhjD, followed by cloning of the NdeI- and BamHI-treated PCR products into the expression vector pT7LOH to construct the complementation vectors pT7LOH*yhjD* and pT7LOH*yhjD400*, respectively.

To delete the *msbA* gene in KPM22, the kanamycin resistance cassette targeting *msbA* was amplified from pKD4 (Datsenko and Wanner, 2000) with primers ECOmsbAH1 and ECOmsbAH2. The resulting PCR product was inserted into the chromosome of λ Red strain DY378 (Yu *et al*., 2000) containing a wild-type *msbA* allele on plasmid pK-Cla to sustain viability (Karow and Georgopoulos, 1993). The Δ*msbA*::*kan* insert was transferred subsequently from MAW09 to KPM22 by P1*vir* transduction, followed by excision of the kanamycin resistance marker in the presence of pCP20 (Datsenko and Wanner, 2000) and removal of the helper plasmid to yield strain KPM272. Strain genotypes are listed in Table 2.

Competitive RT-PCR

The *msbA* competitor RNA was synthesized *in vitro* by transcription with T7 RNA polymerase in accordance with the instructions of the supplier (Fermentas). The template for *in vitro* transcription was generated by PCR using genomic DNA of BW30270 as a template and the primer pair 5T7msbAcRTPCR/3msbAcRTPCR to fuse a T7 promoter to the PCR product and introduce a 23 bp deletion into the *msbA* target sequence spanning the region between nucleotides 646 and 668. Total RNA stabilized with RNAprotect reagent (Qiagen) was isolated from late-exponential-phase cultures using the RNeasy mini kit system as recommended by the manufacturer (Qiagen). RNA samples were further purified by treatment with 4 U RNase-free DNase (Amplification Grade, Invitrogen), phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The integrity of the RNA samples was inspected by agarose gel electrophoresis under denaturing conditions in glyoxal as described (Burnett, 1997). For quantification of the *msbA* target mRNA, serial dilutions of *msbA* competitor RNA were spiked with equal amounts of total RNA (1 μg) and subjected to cDNA synthesis in reaction mixtures containing primer 3msbAcRTPCR, 40 U RiboLock RNase inhibitor and 200 U RevertAid H Minus M-MuLV reverse transcriptase according to the manufacturer's protocol (Fermentas). Subsequent DNA amplifications of the target and competitor sequences were performed using the primers 5msbALC1 and 3msbALC1. Aliquots of the PCR products were separated on 2% agarose gels and visualized by staining with ethidium bromide. Quantitative densitometry of the digitized agarose gels was performed with the Quantity One software (Bio-Rad). The amount of the target was calculated by determining the competition equivalence point of target and competitor amplification products as described (Zimmermann and Mannhalter, 1996).

Generation of an MsbA threading model

The primary amino acid sequence of wild-type MsbA from *E. coli* was threaded onto the crystal structure for the *S. aureus* ABC transporter Sav1866 (Dawson and Locher, 2006), using the homology modelling tools of the MOE software package [The Molecular Operating Environment (MOE), version 2007.05, Chemical Computing Group, Montreal, Quebec, Canada]. The amino acid sequences of MsbA and Sav1866 were aligned using default settings in MOE with the only constraint that the Arg4 of Sav1866 aligned with the Arg15 of MsbA to generate the alignment shown in Fig. S1. The 3.0 Å resolution crystal structure for Sav1866 was obtained from the Protein Data Bank (PDB ID: 2HYD). Models were generated for each monomer separately while maintaining the relative co-ordinate frame of the 2HYD structure. The default settings of MOE were used with the exceptions that the number of intermediate models was increased from 10 to 50 and the best intermediate was minimized to 'fine', a more stringent criterion than the default setting. On completion, the two monomers were joined together to form the complete MsbA dimer. The AMBER99 force field, as implemented in MOE (Ponder and Case, 2003), was used in a series of energy minimizations to relax the threaded structure. First hydrogen atoms, then hydrogen and side-chain atoms were allowed to relax while keeping all other atoms fixed. Backbone atoms were then also allowed to relax using a series of minimizations in which they were tethered with decreasing force to their starting positions. Finally, all atoms were allowed to relax with no constraints to a final gradient of 0.0001 kcal mol^{-1} $Å^{-1}$ producing the model displayed in Fig. 6.

Lipid IV_A isolation

The biomass of stationary-phase cultures grown in 2 l of LB medium (KPM22 L1) or LB medium containing 30 μg ml^{-1} kanamycin (KPM121 and KPM231) at 37°C with vigorous shaking (250 r.p.m.) was extracted using a modified phenol-chloroform-light petroleum (PCP) protocol (Galanos *et al*., 1969) in which lipid IV_A is recovered from the organic phase by extensive dialysis against distilled water before lyophilization (Meredith *et al*., 2006).

Separation of the IM and OM

Isolation of the membrane fraction from a late-exponential-phase culture of KPM272 grown in 2 l of LB medium at 37°C with vigorous shaking (250 r.p.m.), as well as separation of the IM and OM by discontinuous sucrose gradient centrifugation, was performed as described recently (Meredith *et al*., 2006). Sucrose within the pooled IM and OM fractions was removed by extensive dialysis against distilled water before lyophilization and lipid IV_A isolation by the modified PCP procedure (Meredith *et al*., 2006).

ESI FT-ICR mass spectrometry

ESI FT-ICR mass spectrometry was performed in the negative ion mode using an APEX II and a hybrid Apex Qe Instrument (Bruker Daltonics) equipped with a 7 Tesla actively shielded magnet. Details on sample preparation and mass spectrometry characteristics of LPS have been published (Kondakova and Lindner, 2005).

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Note added in proof

After the acceptance of our manuscript, corrected MsbA structures were published [Ward, A. *et al.* (2007) *Proc Natl Acad Sci USA* **104:** 19005–19010]. Our predicted MsbA model is in excellent agreement with the crystal structure for MsbA of *S. enterica* sv. Typhimurium; RMSD of 2.7 Å, well under the structure resolution of 3.7 Å. We therefore believe that the crystal structure in no way alters the interpretation of our experimental data using the computationally derived MsbA model.

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Supplementary material

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