O-antigens are cell surface polysaccharides of many Gram-negative pathogens that aid in escaping innate immune responses. A widespread O-antigen biosynthesis mechanism involves the synthesis of the lipid-anchored polymer on the cytosolic face of the inner membrane, followed by transport to the periplasmic side where it is ligated to the lipid A core to complete a lipopolysaccharide molecule. In this pathway, transport to the periplasm is mediated by an ATP-binding cassette (ABC) transporter, called Wzm–Wzt. Here we present the crystal structure of the Wzm–Wzt homologue from *Aquifex aeolicus* in an open conformation. The transporter forms a transmembrane channel that is sufficiently wide to accommodate a linear polysaccharide. Its nucleotide-binding domain and a periplasmic extension form 'gate helices' at the cytosolic and periplasmic membrane interfaces that probably serve as substrate entry and exit points. Site-directed mutagenesis of the gates impairs in vivo O-antigen secretion in the *Escherichia coli* prototype. Combined with a closed structure of the isolated nucleotide-binding domains, our structural and functional analyses suggest a processive O-antigen translocation mechanism, which stands in contrast to the classical alternating access mechanism of ABC transporters.

Microorganisms commonly use cell surface polysaccharides to establish extended barriers that protect against the defence machineries of their hosts. O-antigens help bacteria to evade innate immune responses including phagocytosis and complement-mediated lysis. Polymers are hypervariable polysaccharides up to approximately 100 sugar units long and most reach the periplasm by one of two convergent pathways. In the widespread ABC transporter-dependent pathway, the O-antigen is fully synthesized as an undecaprenyl diphosphate (UND-PP)-linked intermediate, before being transported to the periplasmic leaflet of the inner membrane by Wzm–Wzt and ligated to the lipid A core (Extended Data Fig. 1). PglK, an oligosaccharide ABC transporter from a bacterial protein N-glycosylation system, provided the first example of an exporter translocating UND-PP-linked substrates.

Some systems signal completion of O-antigen biosynthesis by modifying the growing (non-reducing) end of the polysaccharide chain with, for example, phosphate, methyl, or sugar moieties. The corresponding ABC transporter recognizes the modified terminus via a carbohydrate-binding domain (CBD) fused to the C terminus of its nucleotide-binding domain (NBD) to accomplish transport (Extended Data Fig. 1). In other systems, export of uncapped glycans, such as O-antigens and teichoic acids, occurs without the involvement of CBDs.

ABC transporters usually cycle between inward- and outward-facing conformations to facilitate substrate transport. However, this 'alternating access' model may not apply to transporters translocating high molecular mass polymers, such as polypeptides, O-antigens, and capsular polysaccharides. To elucidate the O-antigen translocation mechanism, we determined the crystal structure of *A. aeolicus* (Aa)Wzm–Wzt, which is homologous to the prototypical *E. coli* Wzm–Wzt and *Staphylococcus aureus* wall teichoic acid transporters (Extended Data Fig. 2). In a nucleotide-free state, Wzm–Wzt forms a continuous channel across the membrane. The similarity of the transporter to the *E. coli* O9a transporter allowed testing of functional predictions in vivo with an established prototype. Combined with structures of the transporter's isolated NBDs in a closed conformation, our structural and functional analyses suggest substrate entry and exit pathways and a model for O-antigen membrane translocation.

We initially expressed and purified the full-length AaWzm–Wzt transporter, with Wzm and Wzt forming the transmembrane domain and NBD, respectively. For crystallization, the C-terminal CBD of Wzt was removed, generating a construct including residues 1–235 (WztN). Similar constructs of *E. coli* O9a and *Klebsiella pneumoniae* O12 Wzt...
proteins are fully functional in vivo if the CBD is expressed in trans\(^{8,10}\). The 3.85 Å-resolution Wzm–WztN structure includes residues 2–255 of Wzm and 2–235 of WztN (Extended Data Table 1 and Extended Data Fig. 3).

In a nucleotide-free state, Wzm–WztN adopts a compact structure containing a Wzm dimer interacting with a WztN dimer in an open conformation (Fig. 1a). The transmembrane domains closely interact over the entire length of their transmembrane regions and surround a central transmembrane channel, formed by transmembrane helices 1, 2, and 5, which is open to the intra- and extracellular milieu (see below).

Wzm does not contain any cysso helices that would interact with WztN of the neighbouring half-transporter, unlike previously described bacterial exporters\(^{12}\). At its N terminus, Wzm forms an amphipathic interface helix that runs parallel to the Wzm–WztN interface, followed by six transmembrane helices. The loop connecting transmembrane helices 2 and 3 (TM2 and TM3) couples Wzm with WztN (coupling helix) and the periplasmic connection between TM5 and TM6 forms two re-entrant helices, PG1 and PG2. Overall, the Wzm architecture resembles the type-II ABC exporter topology, which has so far been observed only in human lipid exporters\(^{13,14}\) (Fig. 1b and Extended Data Fig. 4a). Notably, the Wzm–WztN architecture, and thus probably its translocation mechanism, differs markedly from PglK, which translocates UND-PP-linked oligosaccharides\(^{9}\) (Extended Data Fig. 4b).

The NBDs of the transporter are separated by about 8 Å between the Walker A and signature motifs, sufficient for nucleotide diffusion\(^{12}\) (Figs 1a and 2a). A defining feature of WztN is an extension of the β-strand 1/2 loop (residues 13–32), which forms a short ‘gate helix’ (residues 18–26) that rests near the Wzm–WztN protomer interface at the putative water–lipid boundary (Fig. 2a). The gate helix packs against a loop connecting TM4 and TM5 of Wzm of the same half-transporter. This loop contains a conserved F-X-R/K-D motif, of which Phe164 interacts with Arg20 of the gate helix and Asp167 sits directly at the Wzm–WztN interface (Fig. 2a). Additional interactions occur with backbone residues of the loop connecting the N-terminal interface helix and TM1 in the opposing Wzm subunit.

Because the C terminus of the gate helix is rotated away from the transmembrane region, the helix creates a wedge-shaped path towards the Wzm dimer interface, probably forming a substrate-binding pocket (Fig. 2a). At its centre, the gate helix contains a conserved positively charged residue (Arg20), which could be implicated in binding the UND-PP (Fig. 2a and Extended Data Fig. 2a). Strikingly, a preceding Tyr residue (Tyr14) packs against and stabilizes the gate helix on its membrane-distal side (Fig. 2a). Primary sequence alignments of homologous transporters reveal that the gate helix and the Tyr residue are characteristic features of all known O-antigen and wall teichoic acid ABC transporters (Extended Data Fig. 2a), which accept substrates synthesized as UND-PP-linked intermediates\(^{11,13}\).

We also determined the structure of the isolated NBD of WztN in two different crystal forms at 2.05 and 3.5 Å resolution (Extended Data Table 1). Despite the absence of a stabilizing nucleotide, both structures represent a WztN dimer in a closed conformation, with only about 4.0 Å between the hydroxyl group of Ser161 (Walker A) and the backbone amide nitrogen of Ser143 (signature) (Fig. 2c and Extended Data Fig. 5). This closed conformation is in agreement with the adenosine 5′-((3′,5′-imido)triphosphate-stabilized closed state of the NBDs of the maltose transporter\(^{16}\), and probably reflects the nucleotide-bound conformation of Wzm–Wzt (Fig. 2b, c and Extended Data Fig. 5).

The transporter’s transmembrane domain is formed by two closely interacting Wzm protomers that contact each other through TM1 and TM5 (Fig. 3a). TM5 is capped at the C terminus by a cluster of conserved aromatic residues that pack against the C-terminal end of TM1 of the opposing Wzm protomer. Strikingly, the Wzm protomers enclose a large channel spanning the entire membrane (Fig. 3b). The channel is constricted near the periplasmic exit as well as the Wzm–WztN interface, yet continuously accessible to a 3.5 Å radius probe, thus capable of accommodating a polysaccharide. The structure of the native substrate of AaWzm–Wzt is currently unknown but a model of the E. coli O9a polymannose antigen can be accommodated, with eight to ten sugar units spanning the channel (Fig. 3c).

The channel is lined with aromatic residues that are organized in three layers. First, Tyr18, Trp27 and Trp31 reside at the cytosolic Wzm–WztN interface where the channel is widest. Second, Tyr39, Phe69, Trp71, Phe72, Phe180 and Trp181 form a central layer halfway across the membrane, and Phe43, Tyr60 and Phe195 surround the periplasmic channel exit (Fig. 3b, d). Protein–carbohydrate interactions are frequently mediated by CH–π stacking interactions between aromatic residues and the sugar rings\(^{17}\). Clustering of these residues within the channel strongly suggests a role in O-antigen coordination during transport. Indeed, a continuous and mostly conserved ‘aromatic path’ runs from the putative cytosolic substrate entrance to the periplasmic channel exit (Fig. 3d and Extended Data Fig. 2). Similar paths have been described in cellulose synthase, cellobiohydrolase I and maltoporin\(^{18–20}\).

As discussed for the maltoporin channel, hydrophobic interactions with aromatics are often combined with a continuous pattern of hydrogen-bond donors and acceptors that contact the hydroxyl groups of the polymers and probably minimize translocation energy barriers\(^{20}\). Tyr39, Ser75, Asn76, Ser79, Arg80, Glu110 and Gln177 may serve this purpose in Wzm–WztN (Fig. 3d).

At the periplasmic channel exit, the PG1 helix is also preceded by a conserved aromatic residue, usually a tyrosine (Tyr187), similar to the gate helix on the cytosolic side (Fig. 3c). It is thus likely that PG1 forms the gate towards the periplasmic membrane leaflet. The functional importance of the gate helices was addressed by introducing...
point mutations into the *E. coli* Wzm–Wzt O9a transporter and monitoring O-antigen export in vivo. Tyr14 and 187 of *Aa* Wzm–Wzt correspond to Tyr15 and 192 in *E. coli* Wzm–Wzt, respectively (Extended Data Fig. 2). As shown in Fig. 3e and Extended Data Fig. 6, replacing Tyr15 at the cytosolic gate of Wzt with Trp or Phe supports O-antigen export similar to wild-type levels, whereas replacing it with the hydrophobic β-branched residues Val and Ile abolishes export. Among the charged residues, Lys and Arg support some export, requiring longer incubation periods (post-induction) before reaching detectable levels, whereas the Y15E mutant is inactive. Replacing Tyr15 with Ala or Leu only shows a kinetic effect, the exported O-antigen levels reach wild-type levels about 30 min after initiating transport. This is possibly due to a precise orientation of the CBD relative to the NBD remains unknown. Possibly occurs on the surface of the jelly-roll fold9,10 (Fig. 4a), but the precise orientation of the CBD relative to the NBD remains unknown.

In the absence of the CBD, Wzm–WztN hydrolyses ATP in a detergent-solubilized state in a tested temperature range from 27 to 65 °C, with an apparent Michaelis constant (*Kₘ*) for ATP of about 350 μM at 27 °C (Fig. 4b and Extended Data Fig. 8). Strikingly, the full-length transporter hydrolyses ATP about seven times faster than the truncated

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**Figure 3** | The polysaccharide translocation channel. **a.** The Wzm interface. One Wzm protomer is shown as a surface and the opposing subunit is shown as a cartoon. Both subunits are shown as cartoons in the close-up view. TM1 and TM5 are coloured red and green, respectively, and the interface helix is coloured beige. Conserved residues are shown as sticks. **b.** Surface representation of the Wzm–WztN channel. The channel volume accessible to a 3.5 Å-radius probe is shown as a green surface and aromatic residues lining the channel are shown as brown spheres. Selected residues are labelled. **c.** Cytosolic and periplasmic gate helices at the Wzm protomer interface. A model of the *E. coli* O9a antigen containing ten mannose units was manually placed in the channel and is shown as a red surface. **d.** Putative translocation path (red dotted line). Channel-exposed aromatic and polar residues are shown as brown sticks. **e.** In vivo O-antigen translocation. The indicated point mutations were introduced into *E. coli* O9a Wzm–Wzt. O-antigen export was detected after inducing transporter expression by silver staining (Ag) of whole-cell lysates, detecting exported and lipopolysaccharide-linked O-antigens only. Western blots detecting Wzt and maltose-binding protein (MBP) were performed to monitor transporter expression and as a loading control, respectively. All results have been confirmed at least three times as technical replicates. Time, period after inducing Wzt–Wzm expression.
version, but a similar apparent $K_m$ for ATP suggests that the CBD accelerates the rate-limiting step of ATP hydrolysis (Extended Data Fig. 8c).

To investigate a direct interaction of Wzm–WztN with the CBD, we measured its hydrolytic activity in the presence of increasing CBD concentrations in detergent-solubilized and liposome-reconstituted states. The ATPase activity of Wzm–WztN increases with increasing CBD concentrations and reaches maximum rates at an approximately threefold molar excess of CBD over Wzm–WztN, consistent with a direct CBD–NBD interaction (Fig. 4b). Control experiments in which purified CBD was added to the full-length transporter did not increase its hydrolytic activity. Instead, we observed a slight reduction in ATPase activity, perhaps because of non-specific interactions of the isolated and NBD-attached CBDs (Extended Data Fig. 8b).

Compared with detergent-solubilized states, the hydrolytic activities of the transporters increase significantly upon reconstitution into liposomes (Extended Data Fig. 8c). Assuming similar concentrations of catalytically active transporters, the apparent catalytic rates in liposomes increase about 3- and 20-fold for full-length and truncated Wzm–Wzt, respectively, relative to the detergent-solubilized states. These data suggest that the transporter adopts a different, perhaps closed, conformation in a lipid bilayer environment or the presence of the CBD, thereby affecting its hydrolytic activity. These properties could be modulated by the O-antigen to facilitate translocation.

In the absence of a translocating substrate in vivo, the transporter’s transmembrane channel must be closed to prevent leakage of small solutes across the membrane. Channel closure probably correlates with closing of the transporter’s NBDs, perhaps through rigid body movements of the Wzm–Wzt half transporters relative to one another. This state can be modelled by superimposing the NBDs of the Wzm–WztN transporter halves with the closed structure of the isolated WztN dimer (Fig. 2c). In this model, the transmembrane channel is closed because the Wzm subunits pack tightly against each other without any significant backbone clashes (Extended Data Fig. 9). However, significant overlaps occur at the putative cytosolic substrate-binding site, where the gate helix of WztN contacts the interface helix–TM1 loop of the opposing Wzm subunit (Fig. 2). This region probably undergoes additional conformational changes during channel closure to facilitate substrate translocation (discussed below). The predicted rigid body movement of the transporter halves is supported by disulfide cross-linking of the Wzm subunits. Cys residues introduced into periplasmic loops predicted to be in close proximity in the closed conformation indeed form disulfide bridges under oxidizing conditions (Extended Data Fig. 9c).

The channel-forming conformation of the ABC transporter is consistent with its biological function. However, in the absence of a polysaccharide, mechanisms must exist that prevent spontaneous transporter opening. It is possible that channel formation is tightly coupled to substrate recognition and insertion, such that the translocating polymer seals the channel (Fig. 5).

It is unknown which end of the O-antigen enters the transporter first. Our structural and functional data argue that the gate helix of Wzt functions in substrate binding, most probably by recognizing the pyrophosphate group of UND-PP, together with the first sugar unit. Accordingly, some ABC transporters for O-antigens and teichoic acids that operate without the fine specificity imposed by CBDs can export glycans with different repeat-unit structures. Yet, all substrates contain an acetylated amino sugar as the connector between UND-PP and the repeat-unit glycan. Our data suggest that Wzm–Wzt specifically recognizes this motif, in contrast to PglK, which has been proposed to recognize the undecaprenyl moiety (Extended Data Fig. 4b).

Substrate binding to the cytosolic entrance probably leads to opening of the transporter and insertion of the lipid head group into the channel through a gate between the Wzm subunits (Fig. 5). Following insertion, the lipid anchor may spontaneously re-orient to the periplasmic side, possibly facilitated by the proton-motive force across the inner membrane. During this transition, the hydrophobic cap.
part of the lipid anchor probably remains in the membrane, similar to the model proposed for PglK⁶. After this passive flipping, the transporter contains the polysaccharide in the channel proper. Export could be achieved in a single cycle or require several steps of ATP hydrolysis, however, these alternatives are currently impossible to distinguish.

We speculate that the loop connecting the interface helix of Wzm with TM1 near the cytosolic face (Figs 1 and 2) contacts the polysaccharide during NBD closure. The gate helix, upon ATP binding, probably pushes against this loop, such that it moves horizontally towards the channel (Fig. 2). The loop contains several polar residues, including Thr21, which could interact with and move the polysaccharide during this transition, similar to the translocation mechanism proposed for cellulose synthase²³. Conformational changes at the gate could mediate the translocation of about one or two sugar units at a time. As such, Wzm–Wzt would combine the functions of a lipid flippase and polysaccharide translocase.

ABC transporters exporting uncapped O-antigens (for example, K. pneumoniae O2a) do not contain C-terminal CBDs²². In these systems, polymer export is dependent on simultaneous synthesis, whereas in E. coli O9a, O-antigen synthesis and export can be temporally uncoupled²². Both types of transporter share structural features key to our model (Extended Data Fig. 2). Whereas uncapped O-antigens may be synthesized and exported by multi-subunit complexes including the transporter, CBD-containing ABC transporters probably function independently during or after O-antigen biosynthesis. In this scenario, the CBD may ensure a sufficient local substrate concentration.

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METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Cloning and protein expression. The wzm and wzt genes were PCR amplified from genomic A. aeolicus VF5 DNA and sequentially cloned into an engineered pETDuet expression vector (Novagen) with a C-terminal histidine tag on Wzt. A second construct containing only residues 1–235 of Wzt (WztN) was cloned in PhimiphiBlue. The transporters were expressed in E. coli C43 cells in Luria broth (LB) medium upon induction with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) at an absorbance of 600 nm of 0.6. Cells were harvested by centrifugation after incubation at 37 °C for 4 h. The cells were resuspended in RB-1 buffer containing 20 mM Tris HCl pH 7.5, 0.1 M NaCl, and 5 mM β-mercaptoethanol (β-ME) and then lysed in a microfluidizer. The crude membranes were collected by centrifugation for 60 min at 200,000 g in a Beckman Ti45 rotor and solubilized for 60 min at 4 °C in RB-2 buffer containing 50 mM sodium phosphate pH 7.2, 0.1 M NaCl, 20 mM imidazole, 5 mM β-ME, and 2% polyoxyethylene(8)-dodecyl ether (C12E8). The insoluble material was cleared by centrifugation for 30 min at 200,000 g in a Beckman Ti45 rotor and the membrane extract was batch incubated with Ni-NTA agarose (Qiagen) for 60 min at 4 °C. The resin was packed in a gravity flow chromatography column, washed with 50 ml WB1 buffer (RB-1 buffer containing 20 mM imidazole and 5 mM dodecyl-N,N-dimethylamino-N-oxide (LDAO)), 50 ml WB2 buffer (RB-1 buffer containing 40 mM imidazole and 5 mM LDAO), and 50 ml WB3 buffer (RB-1 buffer containing a total of 1.5 M NaCl, 20 mM imidazole and 5 mM LDAO), and the transporter was eluted in 50 ml EB buffer containing 20 mM Tris HCl pH 7.5, 0.1 M NaCl, 300 mM imidazole, 5 mM β-ME, and 5 mM LDAO. The eluted protein was purified by an analytical Superdex 200 gel filtration column (GE Healthcare) equilibrated in buffer containing 20 mM Tris HCl pH 7.5, 0.15 M NaCl, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP), and 5 mM LDAO. The peak fraction was concentrated to 15 mg ml−1 final concentration in a 50-kDa cut-off centrifugal filter (Amicon) before crystallization in the presence of 5 mM MgCl2. To guide model building, Thr128 in TM3 of Wzm was replaced with a Cys residue for derivatization with ethylmercurithiosalicylic acid. This mutant was generated by QuikChange mutagenesis and purified as described for the wild-type transporter. Selenomethionine-derivated Wzm–WztN was prepared as described above with the exception that the cells were grown in the M9 minimal medium supplemented with 60 μg ml−1 l-selenomethionine (Se-Met). The CBD of Wzt (residues 235–394) was expressed from a PET30α vector (Novagen) in E. coli BL21 (DE3) cells (Invitrogen). The E. coli cells were cultured in LB medium at 37 °C and protein expression was induced at an optical density at 600 nm of 0.6 with 0.5 mM IPTG. The cells were harvested after centrifugation after 4 h of incubation at 37 °C. Subsequently, the cells were resuspended in RB-1 buffer containing 20 mM sodium phosphate pH 7.5, 0.05 M NaCl and 5 mM β-ME and then lysed in a microfluidizer. The insoluble material was cleared by centrifugation for 30 min at 200,000 g in a Beckman Ti45 rotor. The supernatant was batch incubated with Ni-NTA agarose (Qiagen) for 60 min at 4 °C. The protein was then purified by Ni-NTA affinity chromatography at 4 °C, washed with 50 ml WB1 buffer containing 25 mM Tris HCl pH 8.5, 0.5 M NaCl, 30 mM imidazole, and 5 mM β-ME, 50 ml WB2 buffer (WB1 buffer containing a total of 50 mM imidazole and 50 mM NaCl) and eluted with EB buffer, consisting of 25 mM Tris HCl pH 8.5, 50 mM NaCl, 300 mM imidazole, and 5 mM β-ME. The protein was further purified by gel filtration chromatography (Superdex-200) in 25 mM Tris, pH 8.5, 50 mM NaCl, 5 mM β-ME.

Crystallization. The truncated Wzm–WztN transporter was crystallized by combining 1 μl of well solution (32% polyethylene glycol (PEG) 400, 0.05 M sodium acetate pH 4.5, and 1 M magnesium acetate) with 1 μl of protein solution and sitting-drop vapour diffusion at 22 °C. Addition of 3.6 mM decyl-β-maltoside to the crystallization mixture significantly improved diffraction. Crystallization trials with full-length Wzm–WztN were unsuccessful.

WztN crystallized from a Wzm–WztN sample set up under different conditions, each producing a P3_2_1 crystal form but with different unit cell dimensions. Crystals with a smaller unit cell contained a WztN monomer in the crystallographic asymmetric unit and were obtained by sitting-drop vapour diffusion in the presence of 41% PEG 400, 0.05 M sodium acetate pH 5.4, and 0.15 M magnesium acetate, and 47 mM octyl-glucoside at 17 °C. Crystals with a larger unit cell contained a WztN dimer per asymmetric unit and grew in the presence of 0.4 M magnesium nitrate, 17.5% PEG 8000, and 0.1 M Tris pH 8.5 by sitting-drop vapour diffusion at 17 °C. Crystallization of Wzm–WztN–WztN does not exhibit any detectable ATPase activity. ADP formation was quantified by following the decrease in NADH fluorescence at 450 nm (excitation at 340 nm) using a Fluoromax 3 (Horiba) fluorimeter at 22 °C. For ATPase assays in proteoliposomes, purified Wzm–Wzt was reconstituted into 3 mg ml−1 E. coli total lipid extract at a protein concentration...
of 0.3 mg ml\(^{-1}\). Vesicles were formed upon detergent removal using SM-2 Bio-beads (Bio-rad). For CBD titration experiments, the CBD of Wzt was pre-mixed with transporter for 3 h at 4 °C before adding ATP and MgCl\(_2\). Decrease in NADH fluorescence was converted to molar concentrations on the basis of measurements of known standards. All experiments were repeated at least three times and data were fitted to Michaelis–Menten kinetics to calculate \(K_m\) and \(V_{max}\) values using GraphPad Prism 6. Error bars are deviations from the means.

**Disulfide cross-linking of Wzm–WztN.** For disulfide cross-linking experiments, Wzm and WztN were co-expressed from pETDuet and pACYC vectors, respectively, with an N-terminal Flag-tag on Wzm and C-terminal His-tag on WztN. Protein purification was as described above with the exception that the gel filtration buffer contained 1 mM DTT instead of TCEP. Disulfide cross-linking experiments confirming the modelled closed conformation of Wzm–WztN were performed with purified detergent-solubilized transporter. Oxidation was induced with copper-phenanthroline or sodium tetraethionate (STT). A copper-phenanthroline stock solution was prepared by combining 0.36 M 1,10-phenanthroline monohydrate (VWR) (in 50% ethanol) with 0.24 M copper sulfate (Sigma) at a 2:1 volume ratio. STT was dissolved in double-distilled H\(_2\)O at 80 mM concentration, and N-ethylmaleimide to block free cysteines was prepared at 1 M concentration in dimethylsulfoxide. Wild-type or Cys-introduced Wzm–WztN at 0.09 mM concentration was incubated with 4 mM copper-phenanthroline or STT and incubated for 40 min at room temperature followed by addition of 25 mM N-ethylmaleimide and incubation at 4 °C for 30 min. Samples oxidized in the presence of ADP/Mg\(^{2+}\) were pre-incubated with 2 mM ADP and 2 mM MgCl\(_2\) for 20 min at room temperature. The oxidized protein was resolved by non-reducing SDS–PAGE and protein bands were visualized by western blotting against an N-terminal Flag-tag on Wzm.

**Size-exclusion multi-angle light scattering.** Mass measurements of the CBD of Wzt were performed on a Dionex UltiMate3000 HPLC system with a UV detection module (ThermoFisher), connected to a miniDAWN TRESOS static light-scattering detector (Wyatt Technology) and Optilab T-rEX differential refractometer (Wyatt Technology). A 100-μl sample at 0.1 mM concentration was loaded onto a Superdex 200 HR 10/300 GL column (GE Healthcare) equilibrated in 25 mM Tris, pH 8.5, 50 mM NaCl, 5 mM β-ME at a flow rate of 0.4 μl min\(^{-1}\). Data were recorded and processed using ASTRA software (Wyatt Technology).

**E. coli O9 antigen modelling.** An E. coli O9a antigen containing ten mannose units was modelled using the GLYCAM carbohydrate builder and manually placed into the AaWzm–WztN transmembrane channel (http://glycam.org/tools/molecular-dynamics/oligosaccharide-builder/build-glycan?id=1).

**In vivo O-antigen export assays.** Growth conditions. Bacterial cultures (Supplementary Information) were grown with aeration in LB base (Invitrogen) at 37 °C. Broth was supplemented with 100 mg ml\(^{-1}\) ampicillin, 0.4% d-mannose, and/or 0.1% L-arabinose where appropriate. Unless otherwise stated, cells were grown in the presence of 0.4% d-mannose to repress mannose uptake.

**DNA methods.** Oligonucleotide primers were custom designed and obtained from Sigma Aldrich (Supplementary Information). PfuUltra DNA polymerase (Agilent) was used for PCR amplification, according to the manufacturer’s instructions, and PCR product was treated with DpnI (New England Biolabs). DNA sequencing was performed by the Genomics Facility at the University of Guelph Advanced Analysis Center.

**Complementation.** E. coli CWG638 transformants containing plasmids with wild-type or variant wzt, along with wzm in the native chromosomal organization, were used to ensure equal protein expression levels. Cultures were grown overnight in the presence of 0.4% d-glucose. E. coli CWG638 cannot produce its own GDP-mannose, the substrate of glycosyltransferases responsible for O9a O-antigen assembly; owing to a deletion of mar\(_A\); therefore, O-antigen production relies upon mannose uptake. Accumulation of UND-PP-O9a intermediates in the absence of export results in growth defects that are alleviated by second-site mutations that repress O-antigen synthesis. Growth in glucose represses uptake of any trace amounts of mannose in the medium and prevents harmful O9a synthesis. Overnight cultures were diluted 1/10 in fresh LB supplemented with 0.4% glucose and grown for 4 h to an absorbance at 600 nm of approximately 1.0. Cells were subjected to centrifugation at 5,000g for 10 min, resuspended in fresh LB containing 0.4% d-mannose to induce O-antigen biosynthesis, and grown for 15 min at 37 °C with aeration. After 15 min, 0.1% L-arabinose was added to induce protein expression. Aliquots of culture were taken immediately and after 5, 10, 20, 30, and 60 min and put immediately on ice to suppress further cell growth and O-antigen export. An equivalent of 1 optical density units of cultures was harvested by centrifugation at 13,000g and resuspended in 100 μl of SDS–PAGE loading buffer. Cells were lysed by boiling for 10 min. For western immunoblots, samples were subjected to SDS–PAGE using 12% acrylamide resolving gels in Tris-glycine buffer. For lipopolysaccharide analysis, samples were first treated with 500 μg ml\(^{-1}\) proteinase K for 1 h at 55 °C before SDS–PAGE. Lipopolysaccharide was visualized by silver staining.

For immunoblot analyses, material resolved by SDS–PAGE was transferred to nitrocellulose membranes (Protran; PerkinElmer Life Sciences). Wzt was detected using anti-Wzt primary antisera, generated in rabbits, and cross-reactive material adsorbed against E. coli CWG708 whole-cell lysate. Goat-anti-rabbit secondary antibody conjugated to alkaline phosphatase (Cedarlane Laboratories) was used to facilitate detection with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science). MBP was detected using monoclonal anti-MBP mouse primary antibody (New England Biolabs) with secondary alkaline phosphatase-conjugated goat-anti-mouse antibody (Jackson Immunoresearch Laboratories) for detection with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

**Data availability.** Atomic coordinates for the atom models have been deposited in the Protein Data Bank under accession numbers 6AN7 for Wzm–WztN, and 6AMX and 6AN5 for WztN.

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Extended Data Figure 1 | ABC transporter-dependent O-antigen biosynthesis. In this pathway, O-antigens are completely synthesized on the cytosolic leaflet of the plasma membrane. Undecaprenyl-phosphate (black line and yellow circle) serves as the lipid acceptor and is modified by the addition of an acetylated amino sugar phosphate (frequently N-acetylglucosamine-1-P, white hexagon) as well as two or more additional sugar residues (grey hexagons) to generate a biosynthesis primer. The polymerizing enzyme(s) extend the primer with tens to hundreds of O-antigen repeat units (light blue hexagons). In some species, termination of O-antigen biosynthesis is achieved by modifying the polymer’s non-reducing end (black star). An ABC transporter translocates the UND-PP-linked O-antigen intermediate to the membrane’s periplasmic side, where it forms a substrate for glycosylation of the lipopolysaccharide (LPS) core. Only transporters translocating terminally modified O-antigens contain CBDs that bind the polysaccharide’s modified terminus.
Extended Data Figure 2 | Sequence alignment of O-antigen and wall teichoic acid transporters. a, b, Alignments of the nucleotide-binding (a) and transmembrane domains (b). The conserved tyrosines preceding the cytosolic gate helix of the NBD and the periplasmic gate are highlighted with a red arrow and red box in a and b, respectively. Transmembrane helices and cytosolic and periplasmic gate helices are shown as green and beige cylinders, respectively. Blue sequence labels indicate predicted teichoic acid transporters. All O-antigen transporter NBDS except for K. pneumoniae O2a contain predicted CBDs at their C termini, which are not shown. c, Alignment of the C-terminal region of AaWzt with the corresponding domains from the E. coli O9a (Protein Data Bank accession number 2R5O) and R. terrigena (Protein Data Bank accession number 5HNO) transporters. Sequences were aligned in CLUSTAL Omega and displayed in Jalview coloured by sequence identity.
Extended Data Figure 3 | Anomalous difference and experimental electron density maps. 

**a.** Heavy atom positions used for experimental phasing and model building. Five native cysteines in the NBDs as well as an engineered Cys at the C terminus of TM3 (T128C) were modified with ethylmercurithiosalicylic acid, shown as green and red meshes and contoured at 4.5\(\sigma\) and 3\(\sigma\), respectively. Only the mercury sites shown in green were used for mercury-single anomalous dispersion phasing.

**b.** Unbiased experimental sigma-A-weighted electron density after NCS and cross-crystal averaging and phase extension to 3.85 Å, contoured at 1\(\sigma\).

The transmembrane domain contains three native Met residues, which were identified upon substitution with seleno-methionine (cyan mesh, contoured at 3\(\sigma\)). Shown are sigma-A-weighted anomalous difference electron densities; AaWzm–WztN is shown as a grey ribbon.
Extended Data Figure 4 | Overview of type-II ABC exporters and PglK.

a. The structures of the transmembrane domains of *A. aeolicus* Wzm, *Homo sapiens* ABCG5 and *H. sapiens* ABCA1 are shown as cylindrical cartoons. One subunit of the dimers is coloured in rainbow colours from blue to red, N terminus to C terminus. b. Structure of PglK, an ABC transporter translocating UND-PP-linked oligosaccharides across the plasma membrane. PglK probably recognizes the polyprenyl moiety of the substrate via a conserved periplasmic helix (shown in magenta), which is missing in Wzm.
Extended Data Figure 5 | Closed conformation of the isolated WztN-NBD.

a, The isolated WztN dimer structure was aligned by secondary matching in Coot with the NBDs of the adenosine 5′-(3,γ-imido)triphosphate-stabilized maltose transporter (Protein Data Bank accession number 3RLF). The WztN dimer is shown in cyan and light blue, and the NBDs of the maltose transporter are shown in light and dark grey. Right: the Walker A (S61) and signature (S143) motifs in the closed WztN dimer structure are separated by approximately 4 Å. b, Comparison of WztN dimer structures. The structure shown in dark blue was obtained from a crystal form containing a WztN dimer in the crystallographic asymmetric unit. The structure shown in grey was obtained from a crystal form with a monomeric WztN per crystallographic asymmetric unit related to the other protomer by two-fold crystallographic symmetry. The signature motifs are coloured cyan and yellow, and the Walker A motifs are coloured magenta and red for the crystallographic monomeric and dimeric WztN structures, respectively.
Extended Data Figure 6 | Impact of conserved tyrosine residues of the cytosolic and periplasmic gates on O-antigen translocation.
The indicated point mutations were introduced into the *E. coli* O9a Wzt–Wzm transporter and O-antigen transport was assayed by silver staining of the whole-cell lysate. Ag, silver-stained SDS–PAGE. Wzt and MBP were detected immunologically to monitor transporter expression and as a loading control, respectively. All results showing a phenotype have been confirmed at least three times as technical replicates. Time, period after inducing Wzt–Wzm expression in minutes.
Extended Data Figure 7 | Dimerization of the isolated CBD of Wzt.
Multi-angle static light scattering coupled to size-exclusion chromatography was used to determine the molecular mass of the purified CBD of Wzt (one representative experiment is shown). The molecular mass of a monomeric Wzt-CBD is 20 kDa, including a C-terminal 6 × His-tag and linker region. Inset, Coomassie-stained SDS–PAGE of the purified CBD of Wzt.
Extended Data Figure 8 | Hydrolytic activity of the Wzm–Wzt ABC transporter. ATP hydrolytic activity was measured by following the decrease of NADH fluorescence in an enzyme-coupled assay upon excitation at 340 nm and emission at 450 nm in a temperature range from 4 to 65 °C. a, Temperature dependence of the ATPase activity of Wzm–WztN. Shown is the difference in NADH fluorescence between control reactions in the absence of Wzm–WztN and reactions in its presence. b, Hydrolytic activity of full-length Wzm–Wzt in the presence of isolated Wzt-CBD measured at 27 °C. Shown are fluorescence intensity differences (calculated as for Fig. 4b) but not converted to apparent catalytic rates. Dashed line, ATP titration in the presence of only the CBD of Wzt. Hydrolytic activity of Wzm–WztN in the absence of the CBD of Wzt is shown for comparison. c, Comparison of ATPase activities of full-length (green) and truncated (black) Wzm–Wzt. Shown are apparent catalytic rates in detergent-solubilized and liposome-reconstituted states. Data points represent the mean of three independent repeats with s.d. CPS, counts per second.
Extended Data Figure 9 | Model of the Wzm–WztN closed conformation. **a**, Rigid body alignment of the Wzm–WztN transporter halves with the corresponding NBDs of the closed WztN dimer structure. The closed WztN dimer is shown in grey, and Wzm–WztN is coloured in red and green for Wzm and cyan and blue for WztN. Residues replaced with Cys are shown with spheres at their Cα carbons. Observed disulfide cross-links are indicated with a dashed line. **b**, Cartoon illustration of the open to closed transition of the transporter. **c**, Disulfide cross-linking of Wzm protomers. Purified Wzm–WztN transporters harbouring the indicated Cys mutations were oxidized with either copper phenanthroline (Co-Phen) or sodium tetrathionate (STT), blocked with N-ethylmaleimide (NEM), and analysed by western blotting against the N-terminal Wzm Flag-tag. Experiments were repeated three times with similar results. M and D, Wzm monomer and dimer.
# Extended Data Table 1 | Crystallographic data collection and refinement statistics

|                        | WzmWztN (Hg) | WzmWztN-T128C (Hg) | WzmWztN (Se-Met) | WztNBD (monomer) | WztNBD (Hg) | WztNBD (dimer) |
|------------------------|--------------|---------------------|------------------|-----------------|------------|----------------|
| **Data collection**     |              |                     |                  |                 |            |                |
| Space group            | P432         | P432                | P432             | P432            | P321       | P321           |
| Wavelength (Å)         | 0.9895       | 1.0052              | 1.0052           | 0.9895          | 0.9895     | 1.0052         |
| **Cell dimensions**    |              |                     |                  |                 |            |                |
| a, b, c (Å)            | 228.1, 228.1 | 233.5, 233.5        | 232.0, 232.0     | 230.8, 230.8    | 96.2, 96.2 | 97.0, 97.0     |
|                       | 228.1        | 233.5               | 232.0            | 230.8           | 60.9       | 60.9           |
|                       | 90, 90, 90   | 90, 90, 90          | 90, 90, 90       | 90, 90, 120     | 90, 90, 120| 90, 90, 120    |
| Resolution (Å)         | 24.9–3.85    | 30.5–8.50           | 29.46–7.09       | 24.89–5.21      | 31.5–2.65  | 42.0–3.69      |
|                        | (4.22–3.85)† | (9.51–8.5)          | (7.93–7.09)      | (5.82–5.21)     | (2.11–2.05)| (4.04–3.69)    |
| R<sub>merge</sub>      | 0.23 (2.19)  | 0.12 (0.18)         | 0.12 (1.16)      | 0.19 (1.38)     | 0.08 (1.22)| 0.09 (0.15)    |
| R<sub>free</sub>       | 0.08 (0.73)  | 0.01 (0.02)         | 0.03 (0.26)      | 0.04 (0.31)     | 0.04 (0.61)| 0.02 (0.03)    |
| CC<sub>1/2</sub>       | 0.991 (0.43) | 0.999 (0.99)        | 0.997 (0.81)     | 0.997 (0.78)    | 0.997 (0.82)| 0.996 (0.99)   |
| R<sub>i</sub> of I     | 7.6 (1.3)    | 45.0 (35.5)         | 20.4 (3.0)       | 12.8 (3.0)      | 9.5 (1.5)  | 30.3 (20.8)    |
| Completeness (%)       | 99.6 (100.0) | 97.8 (100.0)        | 97.5 (96.1)      | 99.1 (100.0)    | 98.3 (99.3)| 99.9 (100.0)   |
| Redundancy             | 9.6 (9.9)    | 76.7 (81.6)         | 20.3 (19.5)      | 19.0 (20.1)     | 4.8 (4.7)  | 20.9 (20.9)    |
| **Refinement**         |              |                     |                  |                 |            |                |
| Resolution (Å)         | 24.9–3.85    | 27.8–2.05           |                  |                 | 27.1–3.5   |               |
| No. reflections        |              |                     |                  |                 |            |                |
| Total                  | 36222        | 37942               |                  |                 | 13640      |               |
| R<sub>free</sub>       | 1824         | 1766                |                  |                 | 722        |               |
| R<sub>free</sub> / R<sub>free</sub> (%) | 25.8/32.1 | 20.2/23.4           |                  |                 | 24.5/30.5  |               |
| No. atoms              | 7962         | 1919                |                  |                 | 3579       |               |
| Protein                | 7962         | 1919                |                  |                 | 3579       |               |
| PEG-400                |              |                     |                  |                 |            |                |
| B-factors (Å<sup>2</sup>) | 204        |                     |                  |                 |            |                |
| Chain A                | 172.9        | 67.3                |                  | 107.8           |            |                |
| Chain B                | 184.5        |                     |                  | 128.2           |            |                |
| Chain C                | 130.3        |                     |                  |                 |            |                |
| Chain D                | 145.8        |                     |                  |                 |            |                |
| PEG-400                |              |                     |                  | 68.4            |            |                |
| R.m.s deviations      | 0.005        | 0.008               |                  | 0.005           |            |                |
| Bond lengths (Å)       | 0.856        | 0.859               |                  | 0.813           |            |                |

*Values in parentheses refer to the highest-resolution shell.
†Correlation between intensities from random half-data sets.{}

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Experimental design

1. Sample size
   Describe how sample size was determined.
   Sample sizes were triplicates to ascertain correct values and statistical methods were only used to calculate standard deviations.

2. Data exclusions
   Describe any data exclusions.
   No experimental data was excluded.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All replicas of the data shown produced similar results.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   Samples were not randomized as this is not applicable to the kind of biochemical and structural data generated.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   No blinding was applied to the data used in this study as this is not applicable to the kind of biochemical and structural data generated.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a Confirmed

   □ □ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   □ □ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   □ □ A statement indicating how many times each experiment was replicated
   □ □ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   □ □ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   □ □ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
   □ □ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   □ □ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

| Software Package | Version | Notes |
|------------------|---------|-------|
| CCP4 v7          |         | Aimless/Refmac5 |
| Phenix v1.11.1-2575 | Autosol/Refine |
| XDS              |         | Diffraction data processing package |
| CLUSTAL Omega    |         | Multiple sequence alignment tool |
| Jalview          |         | Version 14.0, multiple sequence alignment tool |
| CLUSTAL Omega    |         | Multiple sequence alignment tool |
| Astra            |         | Multi-angle light scattering data processing software, Wyatt Technology |
| Coot             |         | Version 0.8.2, Crystallographic model building software |

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No unique materials were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Wzt was detected using previously described anti-Wzt primary antisera.

MBP was detected using a commercial anti-MBP antibody (New England Biolabs)

FLAG-tagged AaWzm was detected with a commercially available anti-FLAG antibody (Sigma).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

No eukaryotic cells were used for this study.

b. Describe the method of cell line authentication used.

NA

c. Report whether the cell lines were tested for mycoplasma contamination.

NA

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

NA

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

No research animals were used for this study.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

No human participants were used for this study.