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Identification of the lipopolysaccharide O-antigen biosynthesis priming enzyme and the O-antigen ligase in Myxococcus xanthus: critical role of LPS O-antigen in motility and development

María Pérez-Burgos,1 Inmaculada García-Romero,2 Jana Jung,1 Miguel A. Valvano2 and Lotte Søgaard-Andersen1*

1 Department of Ecophysiology, Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch Str. 10, 35043, Marburg, Germany.
2 Wellcome-Wolfson Institute for Experimental Medicine, Queen’s University Belfast, Belfast, BT9 7BL, UK.

Summary

Myxococcus xanthus is a model bacterium to study social behavior. At the cellular level, the different social behaviors of M. xanthus involve extensive cell–cell contacts. Here, we used bioinformatics, genetics, heterologous expression and biochemical experiments to identify and characterize the key enzymes in M. xanthus implicated in O-antigen and lipopolysaccharide (LPS) biosynthesis and examined the role of LPS O-antigen in M. xanthus social behaviors. We identified WbaP Mx (MXAN_2922) as the polyisoprenyl-phosphate hexose-1-phosphate transferase responsible for priming O-antigen synthesis. In heterologous expression experiments, WbaPMx complemented a Salmonella enterica mutant lacking the endogenous WbaP that primes O-antigen synthesis, indicating that WbaPMx transfers galactose-1-P to undecaprenyl-phosphate. We also identified WaaLMx (MXAN_2919), as the O-antigen ligase that joins O-antigen to lipid A-core. Our data also support the previous suggestion that Wzm Mx (MXAN_4622) and Wzt Mx (MXAN_4623) form the Wzm/Wzt ABC transporter. We show that mutations that block different steps in LPS O-antigen synthesis can cause pleiotropic phenotypes. Also, using a wbaPMx deletion mutant, we revisited the role of LPS O-antigen and demonstrate that it is important for gliding motility, conditionally important for type IV pilus-dependent motility and required to complete the developmental program leading to the formation of spore-filled fruiting bodies.

Introduction

The Gram-negative deltaproteobacterium Myxococcus xanthus is a model organism to study social behavior in bacteria. Social behaviors of M. xanthus include the formation of saprophytically feeding colonies in which cells spread outward in a highly coordinated fashion, predation and starvation-induced development with the formation of multicellular spore-filled fruiting bodies (Berleman and Kirby, 2009; Konovalova et al., 2010; Cao et al., 2015). At the cellular level, these social behaviors of M. xanthus require extensive and diverse cell–cell contact-dependent interactions.

The rod-shaped M. xanthus cells harbor two systems for motility: one for type IV pilus (T4P)-dependent motility and one for gliding motility (Zhang et al., 2012; Schumacher and Søgaard-Andersen, 2017). T4P supports the movement of groups of cells in a cell–cell contact-dependent manner and not only depends on T4P (Kaiser, 1979), but also on exopolysaccharide (EPS) (Shimkets, 1986; Arnold and Shimkets, 1988). In addition, T4P-dependent motility may also depend on the O-antigen moiety of the lipopolysaccharide (LPS), although this is still debated. Using different mutants and motility assays, several groups reported that O-antigen is important for T4P-dependent motility (Bowden and Kaplan, 1998; Yang et al., 2000; Youderian and Hartzell, 2006; Vassallo et al., 2015) while Fink and Zissler (1989b) reported that the O-antigen is not required. In the current model, contact by a T4P on one cell to EPS on a neighboring cell triggers pilus retraction, enhancing the movement of cells within groups (Li et al., 2003). By contrast, gliding motility promotes the movement of single cells and depends on the Agl/Git machinery that assembles at the leading cell pole, adheres to the substratum, moves rearwards as cells move and finally disassembles at the lagging cell pole.
Lipopolysaccharide biosynthesis in *Myxococcus xanthus* (Zhang et al., 2012; Faure et al., 2016; Schumacher and Søgaard-Andersen, 2017). LPS O-antigen has also been implicated in gliding motility (Fink and Zissler, 1989b; Yang et al., 2000; Yu and Kaiser, 2007), while Bowden and Kaplan (1998) reported that O-antigen is not important for gliding. The process referred to as ‘outer membrane exchange’ also involves cell–cell contact. Here, the outer membrane (OM) of neighboring cells are thought to fuse to allow the bidirectional exchange of OM lipids, lipoproteins and LPS (Nudleman et al., 2005; Wei et al., 2011; Pathak et al., 2012; Vassallo et al., 2015; 2017). This exchange can have positive effects on recipient cells, i.e., LPS transfer can help recipient cells with damaged OMs to regain fitness (Vassallo et al., 2015) and transferred lipoproteins can stimulate assembly of the T4P and Agl/Glt machineries for motility (Nudleman et al., 2005; 2006; Jakobczak et al., 2015). Conversely, transfer of lipoproteins may also have negative effects on recipients, as is the case for the transfer of toxins that kill non-immune recipient cells (Vassallo et al., 2017). Other toxins that kill non-immune cells are transferred in a contact-dependent manner by the type VI secretion system (Gong et al., 2018; Troselj et al., 2018). Finally, during the starvation-induced formation of spore-filled fruiting bodies, transmission of the cell–cell contact-dependent C-signal is essential for the completion of this developmental program (Kim and Kaiser, 1990a; 1990b).

Here, we focused on the elucidation of the pathway for O-antigen and LPS biosynthesis to better understand the role of LPS in contact-dependent social behaviors in *M. xanthus*. LPS is the main component of the outer leaflet of the OM of most Gram-negative bacteria and is also found in a few diderm phyla that belong to the Firmicutes while the inner leaflet of the OM is composed of phospholipids (Raetz and Whitfield, 2002; Valvano, 2011; Antunes et al., 2016). Generally, LPS has a protective function and helps maintain OM stability, relative impermeability and also plays an important role in virulence (Raetz and Whitfield, 2002; Valvano, 2011; Okuda et al., 2016). LPS molecules encompass three regions: the hydrophobic lipid A, a core oligosaccharide that is attached to lipid A, and the highly variable O-antigen polysaccharide that is attached to the core. The O-antigen is composed of repeating oligosaccharide units. While the lipid A-core is structurally conserved, the composition of the repeat units and the length of the O-antigen chain vary within and between species (Raetz and Whitfield, 2002; Whitfield and Trent, 2014). Unlike lipid A-core, the O-antigen is typically not essential for viability (Raetz and Whitfield, 2002; Whitfield and Trent, 2014). Biosynthesis and membrane translocation of lipid A-core and O-antigen occur in separate pathways, and the two moieties are joined at the periplasmic side of the inner membrane (IM) followed by the transport of the complete LPS molecules to the OM via the Lpt system (Raetz and Whitfield, 2002; Ruiz et al., 2009; Valvano, 2011; Whitfield and Trent, 2014; Okuda et al., 2016).

Lipid A-core biosynthesis begins with the synthesis of the lipid A-Kdo\(_2\) intermediate at the cytoplasmic side of the IM via the conserved Raetz pathway (Whitfield and Trent, 2014). Subsequently, heptosyl- and glycosyltransferases add sugars onto lipid A-Kdo\(_2\) followed by translocation of lipid A-core to the periplasmic side of the IM by the MsbA flippase (Whitfield and Trent, 2014). O-antigen synthesis and assembly follows one of two pathways (Raetz and Whitfield, 2002; Valvano, 2011; Kalynych et al., 2014; Whitfield and Trent, 2014). These pathways share the same mechanism for initiation of synthesis of the repeating units, but differ in how these units are extended, joined and transported to the periplasm. The initiation of O-antigen synthesis involves a reaction in which a sugar-1-phosphate (sugar-1-P) from an activated sugar-nucleotide donor is transferred to the lipid carrier undecaprenyl-phosphate (Und-P) giving rise to an Und-PP sugar intermediate. Und-P is also used as a sugar lipid carrier for the synthesis of EPS, capsular polysaccharides and peptidoglycan. The priming enzymes that initiate O-antigen biosynthesis, also referred to as polyisoprenyl phosphate C-1-phosphoglycosyltransferases (Lukose et al., 2017), can be broadly placed into two protein families, the polyisoprenyl-phosphate hexasose-1-phosphate transferases (PHPTs) or the polyisoprenyl-phosphate N-acetylleucosamine-1-phosphate transferases (PNPTs) (Valvano, 2011). The synthesis of the rest of the O-antigen requires specific glycosyltransferases and also depends on the specific membrane translocation pathway. In the Wzx/Wzy pathway, specific glycosyltransferases act sequentially to transfer the relevant sugar building blocks from nucleotide-sugar donors to the Und-PP-sugar primer molecule to generate the Und-PP-O-repeat unit. The Wzx flippase translocates individual Und-PP-O-repeat units to the periplasmic side of the IM. There, the O-antigen polymerase Wzy joins and polymerizes the repeat units. O-antigen chain length is regulated by the protein Wzz resulting in the formation of O-antigen molecules with a range of lengths. Finally, the O-antigen chain is ligated to the lipid A-core by the WaaL O-antigen ligase in a reaction in which the O-antigen chain is transferred from Und-PP and the proximal sugar joined to a sugar molecule in the lipid A-core acceptor. The Und-PP molecules arising after ligation are dephosphorylated into Und-P in the periplasm, translocated to the cytoplasmic leaflet of the IM by an unknown mechanism, and then reused (Tatar et al., 2007; Valvano, 2008; Manat et al., 2015). In the ABC transporter-dependent pathway, the full-length O-antigen is synthesized on the cytoplasmic side of the IM by various glycosyltransferases. Termination of extension may involve addition of a methyl or methyl-phosphate residue to the non-reducing terminus of the O-antigen by...
homologs of the methyltransferase/kinase-methyltransferase WbdD (Clarke et al., 2004; Greenfield and Whitfield, 2012). By terminating extension, WbdD is also involved in controlling O-antigen chain length (Clarke et al., 2004; Greenfield and Whitfield, 2012). Translocation of the O-antigen across the IM depends on an ABC transporter composed of the membrane-spanning permease Wzm and the Wzt ATPase; subsequently O-antigen is ligated to the lipid A-core by the WaaL ligase following the same scheme as in the Wzx/Wzy pathway. In systems with a modification at the non-reducing end of the O-antigen, transport to the periplasm depends on recognition of this modification by a C-terminal carbohydrate binding domain in Wzt (Cuthbertson et al., 2005).

M. xanthus synthesizes an LPS molecule composed of lipid A, core and O-antigen (Fink and Zissler, 1989a). Four proteins implicated in O-antigen synthesis have been identified in M. xanthus (Fig. 1A). WzmMx (MXAN_4622) and WztMx (MXAN_4623) were suggested to form the Wzm/Wzt ABC transporter while the glycosyltransferases WbgA (MXAN_4621) and WbgB (MXAN_4619) were suggested to be involved in the formation of the O-antigen unit (Guo et al., 1996; Bowden and Kaplan, 1998; Yang et al., 2000).

Here, we identify two additional key proteins for M. xanthus LPS synthesis, MXAN_2922 and MXAN_2919, as the PHPT homolog responsible for priming O-antigen synthesis and the O-antigen ligase respectively. Heterologous expression experiments in Salmonella enterica support that MXAN_2922 transfers galactose-1-P (Gal-1-P) to Und-P. We renamed MXAN_2922 and MXAN_2919 as WbaPMx and WaaLMx respectively (Reeves et al., 1996). Finally, using a mutant that lacks WbaPMx, we demonstrate that LPS O-antigen is important for gliding, conditionally important for T4P-dependent motility and required for development.

Results

Identification of PHPT and ligase candidates for LPS O-antigen synthesis

Prior studies by transposon mutagenesis identified genes potentially required for T4P-dependent and/or gliding motility that by sequence homology could be involved in LPS synthesis (Youderian and Hartzell, 2006; Yu and Kaiser, 2007). These genes map to two regions on the M. xanthus genome, one of which encodes the proposed Wzm/Wzt ABC transporter (Fig. 1A).

In LPS gene cluster I (Fig. 1A; Table S1), the genes MXAN_4623-MXAN_4619 form a putative operon encoding the proposed Wzm/Wzt ABC transporter, the WbgA (MXAN_4621) and WbgB (MXAN_4619) glycosyltransferases, and a putative sugar methyltransferase SgmR (MXAN_4620). Interestingly, genes encoding glycosyltransferases and sugar methyltransferases involved in O-antigen biosynthesis are often found in close association with Wzm/Wzt ABC transporter encoding genes (Greenfield and Whitfield, 2012). According to the CAZy database, WbgA contains two glycosyltransferase domains of the GT2 and one of the GT4 family, while WbgB contains a single GT2 glycosyltransferase domain. SgmR has an N-terminal methyltransferase domain (Pfam domain PF13489), similar to WbdD homologs such as Escherichia coli WbdD08 involved in the terminal methylation of O8-antigen synthesis (Clarke et al., 2004) (Fig. S1), suggesting this protein could be involved in sugar methylation.

LPS gene cluster II (Fig. 1A; Table S1) revealed that MXAN_2920 and MXAN_2921 encode putative glycosyltransferases and MXAN_2922 encodes a PHPT homolog. In agreement with (Vassallo et al., 2015), MXAN_2919 encodes a protein with a Wzy_C domain. This domain is present in O-antigen ligases, Wzy O-antigen polymerases and O-linked oligosaccharyltransferases (Schild et al., 2005). MXAN_2917 and MXAN_2918 encode homologs of LptF and LptG, respectively that form part of the ABC transporter involved in translocating LPS to the OM (Okuda et al., 2016). The remaining homologs of Lpt proteins were identified using combined orthology searches in the KEGG database and BlastP searches (Experimental procedures). We identified three additional genomic regions containing lpt genes (Fig. S2A), which together with the lptFG genes in LPS gene cluster II encode a complete Lpt pathway (Fig. S2B).

To better understand LPS O-antigen biosynthesis in M. xanthus, we focused on the Wzm/Wzt ABC transporter encoded by cluster I, and the MXAN_2922 and MXAN_2919 proteins encoded by cluster II. We carried out a detailed domain analysis of MXAN_4623 and MXAN_4622, renamed WzmMx and WztMx respectively. WzmMx has six predicted trans-membrane helices (TMH) and the ABC2_membrane domain (Pfam domain PF01061) characteristic of Wzm proteins (Fig. 1B). WztMx contains the ABC transporter domain (Pfam domain PF00005) and the Wzt_C domain involved in recognition of the terminal modification of the O-antigen chain (Pfam domain PF14524) (Fig. 1C). Similar domain architectures are found in the E. coli Wzm08 and Wzt08 proteins involved in O8-antigen transport (Cuthbertson et al., 2005; 2007) (Fig. 1C).

MXAN_2922 is a PHPT homolog with five TMH, a CoA binding domain (Pfam domain PF02629) and a Bacterial Sugar Transferase domain (Pfam domain PF02397) similar to the two best-studied PHPTs WbaP of Salmonella enterica (WbaP96) that synthesizes Und-PP-Gal and WcaJ of E. coli (WcaJ_ec) that synthesizes Und-PP-glucose (Und-PP-Glc) (Fig. 1D) (Saldías et al., 2008; Furlong et al., 2015).

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Fig. 1. Bioinformatic analysis of gene clusters and proteins involved in LPS synthesis. (A) LPS gene cluster I and II. Genes are drawn to scale and MXAN number or gene name indicated (Table S1). Coordinates are relative to the first nucleotide of each corresponding gene except for MXAN_4622, _4621, _4620 and _4619 for which coordinates are relative to the first nucleotide of MXAN_4623. DNA fragments comprising promoter and structural gene used in complementation experiments are indicated by a blue, green or red line above the corresponding region. Note that MXAN_2919 is likely in an operon with the upstream genes and, therefore, the promoter used for ectopic expression of MXAN_2919 is the region upstream of MXAN_2914. (B-E) Domain and TMH prediction of Wzm Mx and WzmO8 from E.coli (B), WztMx and WztO8 from E. coli (C), WbaPMx (MXAN_2922) and WcaJ Ec (D) and WaaLMx (MXAN_2919) and WaaL Ec (E). Grey rectangles indicate TMH, red and black lines indicate periplasmic and cytoplasmic domains respectively. In D and E, the lower schematics indicate topology predictions for MXAN_2922 and MXAN_2919. Domains are indicated in blue and green. Conserved amino acids important for structure or activity of the protein are marked with orange and red respectively. Sequence alignment of the C-terminal region of MXAN_2922 with WcaJ Ec and WbaPSe, or MXAN_2919 with WaaL Ec (sequences in boxes) are shown in Fig. S3.
The fifth TMH of WcaJEc does not fully span the IM but has a helix-break-helix structure resulting in the cytoplasmic localization of the C-terminal domain (Furlong et al., 2015). In WbaPSe, the C-terminal domain also localizes to the cytoplasm and is sufficient for catalytic activity (Wang et al., 1996; Saldías et al., 2008; Patel et al., 2010). The residue P291 in WcaJEc has been implicated in the helix-break-helix structure and together with D278, forms part of a DX₁₂P motif that is conserved among PHPTs (Furlong et al., 2015). Both residues are conserved in MXAN_2922 (P295 and D282) suggesting that the C-terminal domain of this protein is also cytoplasmic (Figs 1D and S3A). In the C-terminal, catalytic domain of WbaPSe, several amino acids have been identified that are essential for activity and conserved among PHPTs (Patel et al., 2010). All these residues are conserved in MXAN_2922 (Figs 1D and S3B). Based on these comparisons, we suggest that MXAN_2922 has a membrane topology similar to WcaJEc and WbaPSe (Fig. 1D) and a C-terminal domain with PHPT activity. We hypothesized that MXAN_2922 is the PHPT that primes the first step in O-antigen synthesis in M. xanthus.

MXAN_2919 is a membrane protein with eight putative TMHs and a C-terminal Wzy_C domain (Pfam domain PF04932) containing a relatively large predicted periplasmic loop (Fig. 1E). O-antigen synthesis in M. xanthus depends on the proposed Wzm/Wzt ABC transporter, and, therefore, is predicted to not involve a Wzy O-antigen polymerase. Therefore, we speculated that MXAN_2919 is an O-antigen ligase. The amino acid sequences of WaaL ligases are not highly conserved, but they are all predicted integral IM proteins with eight or more TMH (Raetz and Whitfield, 2002). The WaaL ligases of Pseudomonas aeruginosa and E. coli (WaaLPa and WaaLEc respectively) contain a partially periplasmic Wzy_C domain, responsible for catalytic activity and 12 TMHs (Islam et al., 2010; Pan et al., 2012; Ruan et al., 2012; Ruan et al., 2018) (Fig. 1E). Three amino acid residues in the Wzy_C domain of WaaLPa, Arg215, Arg288 and His338, are important for activity and His338 is also part of the conserved H³⁹[^NSQ]X₀GXX[GTY] motif in the last TMH of the Wzy_C domain; moreover, Asp389 in the TMH following the Wzy_C domain is important for activity (Perez et al., 2008; Ruan et al., 2012). MXAN_2919 contains all these residues with the exception of that corresponding to Asp389 (Figs 1E and S3C). Similarly, WaaLPa also contains all these residues except for the residue corresponding to Asp389 (Ruan et al., 2012). Based on these comparisons, we hypothesized that MXAN_2919 is the M. xanthus O-antigen ligase.

Lack of MXAN_2919 (WaaLMx), MXAN_2922 (WbaP_Mx), and Wzm_Mx affects LPS synthesis

To evaluate the role of MXAN_2919, MXAN_2922 and the proposed Wzm/Wzt ABC transporter in LPS synthesis, we generated in-frame deletions in the MXAN_2919, MXAN_2922 and wzm_Mx genes in the wild-type strain DK1622. If MXAN_2922 is responsible for initiating O-antigen synthesis, the ΔMXAN_2922 mutant should lack LPS O-antigen, but still synthetize lipid A-core. If MXAN_2919 is the O-antigen ligase, a ΔMXAN_2919 mutant should accumulate Und-PP-linked O-antigen in the periplasm and produce LPS devoid of O-antigen polysaccharide. LPS extracted from the WT strain DK1622 and the various mutants, separated by SDS-PAGE, was visualized using Emerald staining (Marolda et al., 2006; Davis and Goldberg, 2012). In contrast to WT, none of the three mutants made LPS O-antigen while they all made lipid A-core (Fig. 2A). We investigated these phenotypes in more detail by immunoblot analysis with two monoclonal antibodies (MAbs), MAb783 and MAb2254 that specifically recognize O-antigen and lipid A-core oligosaccharide respectively (Gill and Dworkin, 1986; Fink and Zissler, 1989a).

In cell extracts from the WT strain separated by SDS-PAGE, MAb783 detected polymeric O-antigen forming a characteristic ladder, as previously reported (Fink and Zissler, 1989a), while no O-antigen was detected in the extract of the Δwzm_Mx mutant, also in agreement with previous results (Guo et al., 1996) (Fig. 2B). O-antigen was absent in the extract of the ΔMXAN_2922 mutant but detected in extract of the ΔMXAN_2919 mutant (Fig. 2B).

In the extract from WT, MAb2254 recognized the fast migrating lipid A-core band devoid of O-antigen, as well as the polymeric LPS O-antigen (Fig. 2C). As expected based on previous research (Guo et al., 1996), only the lipid A-core band was detected in the Δwzm_Mx mutant (Fig. 2C). Lack of polymeric LPS O-antigen was also observed in the ΔMXAN_2922 and ΔMXAN_2919 mutants.

From the combined results of LPS detection by Emerald staining and immunoblotting, we concluded that the ΔMXAN_2922 and Δwzm_Mx mutants do not synthesise O-antigen. These results support the hypothesis that MXAN_2922 is the PHPT enzyme for the initiation of O-antigen synthesis. The absence of O-antigen in Δwzm_Mx confirms previous findings indicating that loss of the Wzm_Mx/Wzt_Mx ABC transporter affects O-antigen synthesis (Guo et al., 1996). By contrast, the ΔMXAN_2919 mutant synthesizes O-antigen that is not linked to lipid A-core, explaining its lack of detection with MAb2254 and Emerald staining and the detection of O-antigen bands when LPS was examined with MAb783. This phenotype is consistent with the absence of O-antigen ligase function resulting in Und-PP-linked O-antigen polysaccharide accumulation and demonstrating that MXAN_2919 is the O-antigen ligase.

The loss of O-antigen synthesis in the ΔMXAN_2922 and Δwzm_Mx mutants was generally corrected by the ectopic expression of the full-length proteins from their native promoters on plasmids integrated in single copy at...
the Mx8 attB site (Figs 1A and 2). The total amount of LPS in the ΔMXAN_2922/MXAN_2919 strain was lower than in WT (Fig. 2A and C), which could be due to reduced expression of the ectopic MXAN_2922 gene. Also, the differences in the migration of LPS O-antigen polysaccharides in the Δwzm_Mx/wzm_Mx strain, that appeared of higher molecular mass than in WT (Fig. 2), could be due to an altered accumulation level of Wzm_Mx in the complementation strain compared to WT (see discussion).

Ectopic expression of MXAN_2919 complemented the defect in LPS O-antigen synthesis in the ΔMXAN_2919 mutant (Fig. 2A and C). The differences in the O-antigen bands between the ΔMXAN_2919 mutant and the complemented strain (Fig. 2B) could be explained by the different nature of the O-antigen link, Und-PP in the former and lipid A-core in the latter. Together, the results of the experiments described above support the notion that MXAN_2922 and MXAN_2919 are the PHPT O-antigen initiating enzyme and O-antigen ligase respectively. These proteins were herein renamed WbaP_Mx (see details below) and WaaL_Mx respectively.

**WbaP_Mx transfers galactose-1-P to Und-P**

The majority of PHPTs utilize either UDP-galactose or UDP-glucose (Valvano, 2011; Lukose et al., 2017). To functionally determine the specificity of WbaP_Mx, we performed complementation experiments using *S. enterica* serovar Typhimurium ΔwbaP_Se and *E. coli* ΔwcaM_Ec mutants. WbaP_Se initiates O-antigen synthesis in *S. enterica* by catalysing the transfer of Gal-1-P onto Und-P, while WcaM_Ec initiates synthesis of the colanic acid exopolysaccharide by transferring glucose-1-phosphate (Glc-1-P) onto Und-P generating Und-PP-Glc.

Disruption of *wbaP_Se* in *S. enterica* (Δ*wbaP_Se*) results in the loss of LPS O-antigen (Fig. 3A). As described previously (Saldías et al., 2008), this defect is partially corrected by complementation with plasmid pJD132, which encodes the *E. coli* O9:K30 WbaP homolog (WbaP_Ec O9:K30), and with the plasmid pSM13, which encodes WbaP_Mx (Fig. 3A). The *wbaP_Mx* gene was cloned into pBADNTF resulting in plasmid pMP139, which encodes WbaP_Mx with an N-terminal FLAG tag (FLAG_WbaP_Mx) to facilitate detection by immunoblot and under the control of an arabinose inducible promoter. pMP139 was introduced into the *S. enterica* Δ*wbaP_Se* mutant. In the presence of 0.2% arabinose, FLAG_WbaP_Mx expressed from pMP139 resulted in an LPS banding profile similar to that obtained with pSM13 (Fig. 3A, left panel), while the pBADNTF vector control did not affect the LPS profile. These results were further validated by immunoblotting with *Salmonella* O-antigen rabbit antibodies. The specificity of the antibodies was verified by the lack of reactivity with O-antigen and lipid A-core in the Δ*wbaP_Se* mutant with no plasmid and in the presence of the vector control with no insert (Fig. 3A, right panel). Importantly, WbaP_Se FLAG_WbaP_Mx and WbaP_Ec O9:K30 restored O-antigen synthesis in the Δ*wbaP_Se* mutant (Fig. 3A, right panel). The difference in the O-antigen profile between the strains complemented with WbaP_Se and FLAG_WbaP_Mx compared to the strain
complemented with WbaP\textsubscript{Ec}\textsubscript{O9-K30} are likely due to different processing of the O-antigen, as previously reported (Saldías \textit{et al.}, 2008), and not to a defect in the initiation of its synthesis. We conclude from these experiments that WbaP\textsubscript{Mx} transfers Gal-1-P onto Und-P.

An \textit{E. coli} \Delta wcaJ\textsubscript{Ec} mutant was used to test whether FLAG-WbaP\textsubscript{Mx} can transfer Glc-1-P to Und-P. Colanic acid formation is readily apparent by a strong mucoid phenotype in \textit{wcaJ}\textsuperscript{+} cells containing the plasmid pWQ499, which encodes the positive regulator RcsA of the colanic acid biosynthesis gene cluster (Furlong \textit{et al.}, 2015). In the presence of arabinose, a mucoid phenotype was detected in the \Delta wcaJ\textsubscript{Ec} (pWQ499) mutant complemented with the plasmid pLA3, which encodes FLAG-WcaJ\textsubscript{Ec} under the
control of the arabinose inducible promoter (Fig. 3B). By contrast, no complementation was observed in the presence of arabinose with pMP139, and this strain had the same phenotype as ΔwcaJEc (pWQ499) containing the vector control pBADNTF (Fig. 3B). This result suggests that WbaPmx does not have UDP-Glc transferase activity.

When grown in the presence of arabinose, FLAG-WbaPmx was detected in the S. enterica ΔwbaPSe mutant with α-FLAG antibodies at similar expression levels as in ΔwcaJEc (pWQ499) E. coli strain (Fig. 3C). However, compared to FLAG-WcaJEc, FLAG-WbaPmx was less abundant, which could be due to differences in codon usage, as previously shown for other PHPT proteins heterologously expressed in E. coli or in S. enterica (Steiner et al., 2007; Patel et al., 2012). FLAG-WcaJEc as well as FLAG-WbaPmx showed the characteristic oligomeric and monomeric bands of similar apparent mass, as previously reported for PHPTs (Saldías et al., 2008). The combined results presented above support the notion that WbaPmx transfers Gal-1-P in contrast to the WcaJmx mutant, which does not accumulate Und-PP1 (Yang et al., 2014), as a negative control for spore coat accumulation. Compared to WT, cells lacking WbaPmx or WaaLmx were not affected in the formation of resistant, phase bright spherical spores while cells lacking WzmMx formed spores at a reduced level and most of which were less spherical than those formed by WT (Fig. 4B). This defect in spore morphology and formation was not complemented by ectopic expression of wzmMx (Fig. 4B).

We used cell morphology and length as a readout to determine whether the ΔwbaPmx, ΔwaaLmx and ΔwzmMx mutants had altered peptidoglycan synthesis. Cells of all three mutants had a rod-shaped morphology (Fig. 4B). In the ΔwbaPmx mutant, the cell length distribution was shifted marginally but significantly toward shorter cells and this effect was slightly exacerbated in the complemented strain (Fig. 4C). In the ΔwaaLmx mutant, the cell length distribution was shifted marginally but significantly toward longer cells and this defect was partially corrected in the complemented strain (Fig 4C). Cells of the ΔwzmMx mutant were significantly longer than WT cells and this defect was not corrected in the complemented strain (Fig. 4C).

Together, these observations suggest that lack of WbaPmx and WaaLmx neither causes defects in EPS synthesis nor in spore coat synthesis, while the ΔwbaPmx and ΔwaaLmx mutants have slightly abnormal cell length. By contrast, lack of WzmMx causes pleiotropic effects and reduced EPS synthesis and also had an effect on spore formation and cell length.

Loss of WaaLmx and WbaPmx do not affect EPS and spore coat formation

In addition to LPS, M. xanthus produces two other surface polysaccharides, EPS and spore coat (Dworkin and Gibson, 1964; Fink and Zissler, 1989a; Lu et al., 2005; Holkenbrink et al., 2014). We investigated whether lack of WaaLmx, WzmMx or WbaPmx affect EPS synthesis, spore coat formation and cell morphology.

We determined EPS production using a plate-based colorimetric assay with Congo red that binds EPS. The ΩdiffE mutant, which does not accumulate EPS (Yang et al., 1998), was used as a negative control. Cells lacking WbaPmx or WaaLmx produced EPS as the WT, while cells lacking WzmMx showed reduced EPS (Fig. 4A). The defect in EPS production in the ΔwzmMx mutant was complemented by ectopic expression of wzmMx (Fig. 4A).

The spore coat is essential for spore formation (Licking et al., 2000; Müller et al., 2012). To follow spore coat synthesis, we used an assay in which spore formation is induced in response to a high concentration of glycerol (Dworkin and Gibson, 1964). Although these chemically-induced spores are not identical to the spores formed in response to starvation, their morphogenesis results from the same cellular remodeling process in which rod-shaped cells are remodeled into spherical cells and the composition of the spore coat is similar in both types of spores (Kottel et al., 1975; Inouye et al., 1979a; 1979b; McCleary et al., 1991; Otani et al., 1998; Müller et al., 2012). We generated an in-frame deletion mutation in exoE, which encodes the PHPT suggested to be responsible for initiation of spore coat polysaccharide synthesis (Holkenbrink et al., 2014), as a negative control for spore coat accumulation. Compared to WT, cells lacking WbaPmx or WaaLmx were not affected in the formation of resistant, phase bright spherical spores while cells lacking WzmMx formed spores at a reduced level and most of which were less spherical than those formed by WT (Fig. 4B). This defect in spore morphology and formation was not complemented by ectopic expression of wzmMx (Fig. 4B).

Loss of WzmMx displays small groups of cells at the colony edge, while the ΔwzmMx mutant displayed a more spherical phenotype with single cells and colony expansion was strongly reduced compared to WT (Fig 5A). On 0.5% of agar, WT displayed the long flares at the edge.
of colonies characteristic of T4P-dependent motility while the ΔpilA mutant, which lacks the major pilin of T4P (Wu and Kaiser, 1996) and served as a negative control, generated a colony edge without flares; by contrast, the ΔwbaP<sub>Mx</sub> mutant only formed short flares (Fig. 5A).

Because motility defects observed in the two previous assays can be caused by either <i>bona fide</i> motility defects or improper regulation of the reversal frequency, we analyzed the motility characteristics of the ΔwbaP<sub>Mx</sub> mutant at the single cell level. On 1.5% of agar, the ΔaglQ mutant was strongly reduced in single cell gliding motility (Fig. 5B). Less than 50% of cells of the ΔwbaP<sub>Mx</sub> mutant displayed active movement and cells moved a significantly shorter cumulative distance than WT cells but reversed like WT cells (Fig. 5B).

Because <i>M. xanthus</i> does not move as single cells by means of T4P on agar surfaces, cells were placed on a polystyrene surface and covered with 1% of methylcellulose (Sun <i>et al.</i>, 2000) to analyze motility of single cells moving by T4P. ΔpilA cells showed very little movement on this surface (Fig. 5C). Surprisingly, the ΔwbaP<sub>Mx</sub> mutant moved similarly to WT under this condition. Moreover, both strains had the same reversal frequency as WT cells. In an assay in which T4P were sheared off the cell surface, the ΔwbaP<sub>Mx</sub> mutant contained slightly more PilA protein in the sheared T4P fraction and in total cell extracts compared to WT (Fig. 5D). Thus, the ΔwbaP<sub>Mx</sub> mutant accumulates PilA and assemble T4P.

We conclude that WbaP<sub>Mx</sub> and, therefore, LPS O-antigen, is important for gliding motility, while WbaP<sub>Mx</sub>.
and therefore, LPS O-antigen is conditionally important for T4P-dependent motility.

Different O-antigen deficient mutants have been described to be deficient in fruiting body formation and sporulation (Fink and Zissler, 1989b; Bowden and Kaplan, 1998). While WT cells had aggregated to form darkened mounds after 24 h of starvation, the \( \Delta \text{wbaP}_{\text{Mx}} \) mutant had only aggregated to form abnormally shaped translucent mounds after 24 h and even after 120 h, the \( \Delta \text{wbaP}_{\text{Mx}} \) mutant had not formed regular and darkened...
fructing bodies (Fig. 5E). In addition, the ΔwbaPMx mutant was strongly reduced in sporulation compared to WT.

Discussion

This study focused on elucidating key steps of LPS O-antigen biosynthesis in M. xanthus and determining the functional consequences of LPS O-antigen loss. We demonstrated that MXAN_2919 encodes the WaaLMx O-antigen ligase, which contains all the critical residues for O-antigen ligase activity found in other members of the family. Further, the ΔwaaLMx mutant produced Und-PP-linked O-polysaccharide that was not transferred to lipid A-core, and LPS O-antigen synthesis was restored by ectopic expression of waaLMx. Therefore, we also suggest that WaaLMx is the sole O-antigen ligase in M. xanthus. MXAN_2922 encodes the WbaP homolog for the initiation of O-antigen synthesis in M. xanthus. This is based on three lines of evidence. First, the predicted protein contains all the residues known to be important for enzymatic activity in the PHPT family. Second, a mutant lacking WbaPMx synthesized lipid A-core but lacked polymeric O-antigen. LPS O-antigen synthesis was restored by ectopic expression of wbaPMx except that the complemented strain displayed a lower level of LPS O-antigen than WT, which could be due to differences in expression associated with the ectopic expression of wbaPMx. The absence of LPS O-antigen in the ΔwbaPMx mutant supports the idea that WbaPMx is the only PHPT involved in O-antigen synthesis. Moreover, neither WaaLMx nor WbaPMx are required for EPS and spore coat synthesis, suggesting they exclusively function in LPS O-antigen synthesis.

MXAN_4623 and _4622 have been suggested to form the Wzrn/Wzt ABC transporter for translocation of O-antigen polysaccharide across the IM (Guo et al., 1996). A priori, a mutant lacking the Wzrn/Wzt ABC transporter would have been expected to accumulate O-antigen in the cytoplasm, as has been reported for E. coli lacking the ABC transporters for O8- and O9a-antigen translocation (Cuthbertson et al., 2005) and for Klebsiella pneumoniae lacking the ABC transporter for O2a-antigen translocation (Kos et al., 2009). However, whole cell extracts from mutants lacking WzrnMx (here), containing a loss-of-function point mutation in wzrnMx (Guo et al., 1996) or lacking WzrnMx/WztMx and the glycosyltransferase WbgA (Bowden and Kaplan, 1998), do not detectably accumulate O-antigen polymers by immunoblot analysis. Upon complementation with wzrnMx+, O-antigen chains longer than in the WT were observed (Fig. 2A–C). We speculate that this may be due to an altered accumulation level of WzrnMx in the complementation strain which could result in an unbalance between O-antigen translocation and chain length control (see below).

Heterologous expression in E. coli and S. enterica serovar Typhimurium indicated that WbaPMx can functionally replace WbaPSer, which transfers Gal-1-P to Und-P in S. enterica serovar Typhimurium, but not WcaJEc, which transfers Glc-1-P to Und-P in E. coli. The structure of the M. xanthus LPS core oligosaccharide has been successfully determined and the O-antigen repeat unit has been determined as the 1→4 linked disaccharide α-D-Glcp-(1→4)-α-GalpNAc, in which a fraction of the GalNac residues are methylated (Maclean et al., 2007). However, the linkage between the core and O-antigen was not established. Since the M. xanthus O-antigen is assem-
could be differently regulated in *M. xanthus*. Furthermore, the Δ*wzm*<sub>Mx</sub> mutant does not have the same dramatic growth defects and abnormal cell morphologies as those found in *E. coli* O8- and O9a-antigen ABC transporter mutants (Cuthbertson *et al.*, 2005). Although it is possible that second site suppressors preventing O-antigen synthesis in the Δ*wzm*<sub>Mx</sub> mutant could arise, this interpretation does not agree with the complementation experiment in which O-antigen synthesis is restored. An alternative explanation could be that accumulation of unprocessed (untransported) O-antigen may allosterically inhibit WbaPM<sub>Mx</sub> activity, shutting down O-antigen synthesis. It is possible that this inhibition involves the WbaP<sub>Mx</sub> large cytoplasmic loop next to the C-terminal catalytic site. This region in Cps2E, the PHPT homolog of *Streptococcus pneumoniae*, has been implicated in regulation of polysaccharide synthesis and modulation of polysaccharide length by suppressing transferase activity in *vivo* and in *vitro* (Xayarath and Yother, 2007; James *et al.*, 2013).

Loss of WbaP<sub>Mx</sub> or WaaL<sub>Mx</sub> function had marginal effects on the cell length that were not fully restored in the complementation strains. Several non-mutually exclusive scenarios may explain these effects. In one scenario, the altered OM in the mutants together with accumulation of O-antigen in the periplasm in the case of the Δ*waaL*<sub>Mx</sub> mutant could give rise to these defects. Alternatively, mutants with a defect in O-antigen and LPS synthesis can cause sequestration of Und-P resulting in altered peptidoglycan synthesis and, consequently, cell length defects (Burrows and Lam, 1999; Valvano, 2008; Jorgenson and Young, 2016). In cells lacking WbaP<sub>Mx</sub>, Und-P is not expected to be titrated because the step that consumes Und-P is blocked. Therefore, we consider this scenario unlikely in this mutant. By contrast, the level of accumulation of unligated O-antigen in cells lacking WaaL<sub>Mx</sub> is similar to that of LPS O-antigen in WT (Fig. 2B). Therefore, Und-P might become limiting in Δ*waaL*<sub>Mx</sub> cells. Accordingly, an *E. coli* mutant that lacks the O-antigen ligase also has morphological defects including an increased cell length and this effect is counteracted by deletion of *wecA*, which encodes the PNPT that initiates biosynthesis of O-antigen (Jorgenson and Young, 2016). The partial complementation of the cell length defect in the Δ*waaL*<sub>Mx</sub> and Δ*wbaP*<sub>Mx</sub> mutants may be caused by altered accumulation levels of WbaP<sub>Mx</sub> and WaaL<sub>Mx</sub> in the complementation strains compared to WT.

In contrast to the Δ*waaL*<sub>Mx</sub> and Δ*wbaP*<sub>Mx</sub> mutants, lack of Wzm<sub>Mx</sub> caused significant pleiotropic effects such as reduced EPS accumulation, formation of significantly elongated cells, and elongated spores upon chemical induction. Three lines of evidence support that Wzm<sub>Mx</sub> is not directly involved in translocation of EPS, spore coat or peptidoglycan precursors to the periplasm. First, the eps locus, which encodes the genes for EPS synthesis and export (Lu *et al.*, 2005), encodes homologs of the Wzx/ Wzy pathway. Second, the exo locus involved in spore

![Fig. 6. Model of LPS biosynthesis in *M. xanthus*.](image-url)
coat formation encodes a Wzz homolog that is required for spore coat formation and, therefore, spore coat synthesis has been suggested to depend on a Wzx/Wzy pathway (Holkenbrink et al., 2014). Third, MXAN_3558 encodes a homolog of the lipid II MurJ flippase, also a member of the Wzx flippase family. Therefore, we speculate that the sporulation defect upon chemical induction is a consequence of the increased cell length of growing cells. How then may lack of WzmMx cause defects in EPS and peptidoglycan synthesis? One possibility is that the \( \Delta wzm_{Mx} \) mutant synthesizes short O-antigens that are not detected by the MAb against O-antigen and which are not transported across the IM (due to lack of WzmMx) and, therefore, not ligated to lipid A-core. In this case, synthesis of the short O-antigen chains could cause sequestration of Und-P as reported for other systems with a defect in O-antigen transport and synthesis and, therefore, reduced EPS and peptidoglycan synthesis (Burrows and Lam, 1999; Valvano, 2008; Jorgenson and Young, 2016). To test the idea that Und-P is sequestered in the \( \Delta wzm_{Mx} \) mutant, we tried to generate a \( \Delta wbaP_{Mx} \Delta wzm_{Mx} \) double mutant; however, for reasons that we do not understand, we were unable to obtain this mutant. Another possibility is that the \( \Delta wzm_{Mx} \) mutant has accumulated mutations causing an effect on peptidoglycan synthesis. Supporting this scenario, ectopic expression of \( wzm_{Mx} \) in the \( \Delta wzm_{Mx} \) mutant largely complemented the defect in O-antigen synthesis and fully complemented the EPS synthesis defect. However, the cell morphology and sporulation defects were not complemented. Undoubtedly, more research is needed to clarify the mechanisms causing lack of O-antigen synthesis as well as reduced EPS and peptidoglycan synthesis and morphology defects in the \( \Delta wzm_{Mx} \) mutant.

Because previous experiments to determine the importance of LPS O-antigen for \( M. \) xanthus motility provided different results, we readdressed this question. We specifically used the mutant lacking WbaPMx because the \( \Delta wbaP_{Mx} \) mutation only has a minor pleiotropic effect on cell length and does not accumulate intermediates in O-antigen synthesis. We observed that LPS is conditionally required for T4P-dependent motility: \( \Delta wbaP_{Mx} \) cells that moved by T4P-dependent motility: \( \Delta wbaP_{Mx} \) cells that moved by means of T4P had a motility defect on 0.5% of agar, but not on a polystyrene surface covered with methacrylate. In agreement with the observation that the triple \( wzm_{Mx} \) \( wzb_{Mx} \) \( wbqA \) mutant accumulated PilA and T4P (Bowden and Kaplan, 1998), the \( \Delta wbaP_{Mx} \) mutant also accumulated PilA and T4P, albeit at slightly higher levels than WT. On polystyrene surfaces with methacrylate, mutants lacking EPS show restored T4P-dependent motility, and polystyrene was suggested to serve as an anchor for T4P adhesion (Hu et al., 2011). How polystyrene/methacrylate restores the motility defect of the \( \Delta wbaP_{Mx} \) mutant remains to be clarified because this mutant accumulates WT levels of EPS. We also observed that LPS is important for gliding motility on 1.5% of agar. Gliding motility depends on the Agl/Glt machinery. Moreover, gliding cells deposit a slime trail. The function and composition of slime are unknown; however, slime may promote the adhesion of cells to the substratum and may contain polysaccharides and OM vesicles (Ducret et al., 2012; 2013; Gloag et al., 2016). We conclude that LPS is (conditionally) important for both motility systems. We speculate that the different results previously reported regarding the involvement of LPS O-antigen in motility can be explained by its conditional importance for T4P-dependent motility and the pleiotropic effects of certain mutations that affect LPS O-antigen synthesis.

Mutants with a defect in LPS synthesis have been suggested to have defects in development (Fink and Zissler, 1989b; Bowden and Kaplan, 1998; Yang et al., 2000). However, in those experiments, neither the accumulation of EPS nor cell length was reported. We re-evaluated the connection between LPS O-antigen and development using the \( \Delta wbaP_{Mx} \) mutant and observed that lack of WbaPMx caused a defect in aggregation and sporulation, in agreement with previous reports. Because \( \Delta wbaP_{Mx} \) cells sporulate in response to chemical induction with glycerol, we suggest that the defect in sporulation during starvation-induced development is a consequence of the aggregation defect. Mutants affected in motility have developmental defects (Hodgkin and Kaiser, 1979). Therefore, it remains possible that the developmental defects caused by lack of O-antigen are indirect effects of the motility defects caused by lack of O-antigen.

In summary, we report that mutants blocked in different steps in LPS synthesis have very different phenotypes. With the detailed characterization of these mutants, the tools are now available to analyze in detail the function of LPS O-antigen in motility and development.

**Experimental procedures**

**Strains and cell growth**

All \( M. \) xanthus strains are derivatives of the wild-type DK1622 (Kaiser, 1979). Strains, plasmids and oligonucleotides used in this work are listed in Tables 1, 2, and S2 respectively. \( M. \) xanthus was grown at 32°C in 1% CTT (1% (w/v) Bacto Casitone, 10 mM Tris-HCl [pH 8.0], 1 mM \( K_2HPO_4/KH_2PO_4 \) [pH 7.6] and 8 mM MgSO_4) liquid medium or on 1.5% of agar supplemented with 1% of CTT and kanamycin (50 µg ml\(^{-1}\)) or oxytetracycline (10 µg ml\(^{-1}\)), as appropriate (Hodgkin and Kaiser, 1977). In-frame deletions were generated as described (Shi et al., 2008), and plasmids for complementation experiments were integrated in a single copy by site specific recombination into the Mx8 attB site. In-frame deletions and plasmid integrations were verified by PCR. Plasmids were propagated in \( E. \) coli Mach1 and DH5α.

\( E. \) coli and \( S. \) enterica serovar Typhimurium strains were grown at 37°C in Luria-Bertani (LB) medium (10 mg tryptone ml\(^{-1}\), 5 mg yeast extract ml\(^{-1}\), 5 mg NaCl ml\(^{-1}\)) supplemented,
when required, with ampicillin, tetracycline, kanamycin or chloramphenicol at final concentrations of 100, 20, 40 and 30 µg ml\(^{-1}\) respectively. Plasmids for heterologous complementation were introduced into MSS2 and XBF1 strains (Table 1) by electroporation (Dower et al., 1988).

### Motility assays

For population-based motility assays, exponentially growing cultures of \textit{M. xanthus} were harvested (6000 g, room temperature (RT)) and resuspended in 1% CTT to a calculated density of \(7 \times 10^7\) cells ml\(^{-1}\). 5 µl aliquots of cell suspensions were spotted on 0.5% and 1.5% agar supplemented with 0.5% CTT and incubated at 32°C. Cells were visualized after 24 h using a M205FA Stereomicroscope (Leica) and imaged using a Hamamatsu ORCA-flash V2 Digital CMOS camera (Hamamatsu Photonics). Gliding and T4P-dependent motility were quantified by determining the increase in colony diameter over three technical replicates. To quantify the movement of single cells, cultures were imaged using a DMi8 Inverted microscope and DFC9000 GT camera (Leica). For

### Table 1. Strains used in this work.

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<th>Strain</th>
<th>Genotype</th>
<th>References</th>
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<tr>
<td>\textit{M. xanthus}</td>
<td>WT</td>
<td>Kaiser (1979)</td>
</tr>
<tr>
<td>DK1622</td>
<td>(\Delta pilA)</td>
<td>Wu and Kaiser (1997)</td>
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<td>DK10410</td>
<td>(\Delta pilQ)</td>
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<td>(\Delta pilE)</td>
<td>This study</td>
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<td>\textit{E. coli}</td>
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<td>Lab stock</td>
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<td>(\Delta\text{recA1998\Delta endA\Delta (\text{lacZYA-argF}^{-})})</td>
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### Table 2. Plasmids used in this work.

<table>
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<td>pBJ114</td>
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<td>pSWU30</td>
<td>Tet(^{r})</td>
<td>Wu and Kaiser (1997)</td>
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<td>pBADNTF</td>
<td>pBAD24 for N-terminal FLAG fusion and with arabinose inducible promoter, Amp(^{r})</td>
<td>Marolda et al. (2004)</td>
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<td>pBADNTF, wcaJ, Amp(^{r})</td>
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<td>Saldías et al. (2008)</td>
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<td>C. Whitfield</td>
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<td>This study</td>
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</table>
the visualization of single cells moving by T4P-dependent motility, exponentially growing cultures were diluted to \(3 \times 10^6\) and 5 \(\mu\)l cell suspension were placed in a 24-well polystyrene plate (Falcon) and incubated 10 min in the dark at RT. Then, 500 \(\mu\)l of 1% methylcellulose in MMC buffer (10 mM MOPS, 4 mM MgSO\(_4\), 2 mM CaCl\(_2\), pH 7.6) were added and cells incubated for 30 min in the dark at RT. Cells were imaged for 10 min with 10 s intervals. To visualize individual cells moving by gliding motility, exponentially growing cultures were diluted to \(3 \times 10^6\) and 5 \(\mu\)l were spotted on 1.5% of agar plates supplemented with 0.5% of CTT and immediately covered by a cover slide. Cells were incubated 4 h at 32°C and then visualized for 10 min with 20 s intervals at 32°C. Pictures were analyzed using Metamorph® v 7.5 (Molecular Devices) and ImageJ (Schindelin et al., 2012).

**Cell length and width determination**

About 5 \(\mu\)l aliquots of exponentially growing cell suspensions were spotted on 1.5% of agar supplemented with 0.2% of CTT, immediately covered with a cover slide and imaged as indicated above. Images were analyzed with Oufi (Paintdakhi et al., 2016) and Matlab R2018a (The MathWorks) to determine the cell length. Violin plots were prepared using Matlab R2018a and the script violin.m (Hoffmann, 2015).

**Development**

Exponentially growing *M. xanthus* cultures were harvested (6000 g at RT), and resuspended in MC7 buffer (10 mM MOPS, pH 7.0, 1 mM CaCl\(_2\)) to a calculated density of \(7 \times 10^8\) cells \(\text{ml}^{-1}\). 10 \(\mu\)l aliquots of cells were placed on TPM agar (10 mM Tris-HCl pH 7.6, 1 mM K\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\) pH 7.6, 8 mM MgSO\(_4\)). Cells were visualized at the indicated time points using a M205FA Stereomicroscope (Leica) and imaged using a Hamamatsu ORCA-flash V2 Digital CMOS camera (Hamamatsu Photonics). After 120 h, cells were collected and incubated at 50°C for 2 h, and then sonicated with 30 pulses, pulse 50% and amplitude 75% with UP200St sonifier and microtip (Hielscher). Sporulation levels were determined as the number of sonication- and heat-resistant spores formed, cells from 5 ml of the culture after 24 h incubation were harvested by centrifugation at 4150 g at RT, resuspended in 1 ml of sterile water, incubated at 50°C for 2 h, and then sonicated as described. 5 \(\mu\)l of the treated samples were placed on a 1.5% of agar pad supplemented with TPM, covered with a coverslip and imaged. To quantify sporulation efficiency, 5 \(\mu\)l of the treated samples were counted using a Helber bacterial counting chamber (Hawksley, UK). Image processing and data analysis were performed using Metamorph® v 7.5 (Molecular Devices).

**Detection of EPS accumulation**

EPS accumulation was detected using a slightly modified protocol from (Skotnicka et al., 2015). Cells were grown in CTT medium to a density of \(7 \times 10^9\) cells \(\text{ml}^{-1}\), harvested by centrifugation (6000 g at RT) and resuspended in 1% CTT to a calculated density of \(7 \times 10^9\) cells \(\text{ml}^{-1}\). About 20 \(\mu\)l aliquots of the cell suspensions were placed on 0.5% of agar plates supplemented with 0.5% of CTT and 40 \(\mu\)g ml\(^{-1}\) Congo red. Plates were incubated at 32°C and documented at 24 h.

**LPS detection by immunoblot**

*M. xanthus* cells growing exponentially in 1% CTT were harvested by centrifugation (6000 g, RT) and resuspended to a calculated density of \(7 \times 10^8\) cells \(\text{ml}^{-1}\) by addition of SDS buffer (0.1 M DTT, 2% SDS, 10% glycerol, 5 mM EDTA, 60 mM Tris-HCl (pH 6.8) and bromophenol blue) to generate whole cell lysates. 15 \(\mu\)l were loaded and separated by SDS-PAGE on a 14% and 10% gel to detect the O-antigen and core respectively. LPS, O-antigen and lipid A-core and proteins were transferred to a nitrocellulose membrane. Immunoblots were performed as described (Sambrook and Russell, 2001) using MAb783 against O-antigen (dilution: 1:2000), MAb2254 against lipid A-core (dilution: 1:2000) (Fink and Zissler, 1989a) and polyclonal rabbit α-PilC antibodies (dilution: 1:2000) (Bulyha et al., 2009) together with horseradish peroxidase-conjugated sheep α-rabbit immunoglobulin G (dilution: 1:2000) (GE Healthcare) and horse radish peroxidase-conjugated goat α-rabbit immunoglobulin G (dilution: 1:15,000) (Sigma) as secondary antibody. Blots were developed using Luminata Forte chemiluminescence reagent (Millipore) on a LAS-4000 imager (FujiFilm). For detection of *Salmonella* O-antigen, immunoblotting was carried out with rabbit *Salmonella* O antiserum group B (Difco, Beckton Dickinson ref. number 229481) (dilution: 1:500) together with IRDye 800CW goat α-rabbit immunoglobulin G (dilution: 1:10,000) (LI-COR) as secondary antibody and detected with a LI-COR Odyssey infrared imaging system.

**LPS purification and detection**

LPS was purified from *M. xanthus* strains and detected as described (Davis and Goldberg, 2012) with some modifications. Briefly, 10 ml of overnight *M. xanthus* cultures grown in 1% of CTT were harvested by centrifugation (4150 g, RT) and resuspended in 200 \(\mu\)l of LPS/SDS buffer (2% β-mercaptoethanol, 2% SDS, 10% glycerol in 0.05 M Tris-HCl (pH 7.6, 8 mM MgSO\(_4\)). Cells were immediately covered with a coverslip and imaged with DMi6000B microscope and a Hamamatsu Flash 4.0 Camera (Leica). To determine the resistance to heat and sonication of spores formed, cells from 5 ml of the culture after 24 h
boiled for 10 min. Then, 10 µl of Proteinase K (10 mg ml⁻¹) (Epicenter) were added, and samples incubated at 37°C for 30 min. 10 µl of Proteinase K (10 mg ml⁻¹) (Epicenter) were added and samples incubated 3 h at 59°C. To extract LPS, 200 µl of ice-cold water saturated phenol was added, samples vortexed and incubated 15 min at 65°C. After addition of 1 ml of diethyl ether, samples were centrifuged for 10 min (16,000 g, RT). Extraction with phenol was repeated until the samples did not appear cloudy. Fifteen µl (1:1; extracted sample: SDS buffer) samples containing the same number of cells were loaded per lane on an Any kD™ Mini-PROTEAN® Gel (Bio-Rad) and separated by SDS-PAGE. Gels were stained with Pro-Q Emerald 300 Lipopolysaccharide Gel Stain Kit (Invitrogen) as described (Marolda et al., 2006). Briefly, gels were fixed overnight with 50 ml fixing solution (60% methanol, 10% acetic acid), and washed twice with 50 ml of 3% acetic acid for 20 min. After incubation with 25 ml of Oxidizing Solution containing periodate, gels were washed three times with 3% acetic acid for 20 min each. Gels were stained with Stain solution containing Pro-Q Emerald 300 in the dark for 2 h and then washed twice for 20 min in the dark with 3% of acetic acid. Stained LPS was detected and imaged using GelStick Imager (Intas).

For complementation experiments with S. enterica serovar Typhimurium, LPS was extracted and visualized by silver staining as described (Marolda et al., 2006). Briefly, bacteria were grown at 37°C overnight on LB plates supplemented with antibiotics and 0.2% arabinose, when needed. Biomass was collected from each plate, resuspended in 5 ml PBS, pH 7.2 and the OD 600nm adjusted to 4. 1 ml of the normalized suspension was transferred to a microcentrifuge tube and centrifuged at 10,000 g for 10 min. Finally, 50 µl of the supernatant was added and extracts incubated at 70°C for 15 min. Samples were centrifuged at 10,000 g for 10 min. Then, 10 µl of Proteinase K (10 mg ml⁻¹) was added and samples were incubated at 60°C for 2 h. To remove proteins, 150 µl of 90% phenol solution (90% phenol, 0.1%, β-mercaptoethanol and 0.2% 8-hydroxyquinoline) was added and extracts incubated at 70°C for 15 min. Samples were centrifuged at 10,000 g for 10 min. Finally, 50 µl of the clear aqueous phase was transferred to a clean microcentrifuge tube and loading buffer was added. Eight microliter of LPS samples were separated on 14% of acrylamide gel under the same conditions. Cultures were centrifuged at 1000 g for 10 min at 4°C. Bacterial pellets were resuspended in 10 ml of 50 mM Tris-HCl pH 8, with protease inhibitor cocktail (Roche) and lysed at 12,000 PSI with a cell disruptor (Constant Systems, Kennesaw, GA). Cell debris was pelleted at 10,000 g for 15 min at 4°C. Total membranes were isolated by centrifugation in microcentrifuge tubes at 42,220 g for 1 h at 4°C and resuspended in 50 µl of 50 mM Tris-HCl pH 8. Protein concentration was determined by the Bradford protein assay (Bio-Rad) and 10 µg of each membrane preparation was separated by 15% SDS-PAGE. Proteins were transferred to nitrocellulose membrane by Trans-Blot Turbo Transfer System (Bio-Rad) and blocking overnight in Blocker™ Casein in TBS (Thermo Fisher Scientific). The primary antibody, α-FLAG M2 monoclonal antibody (Sigma), was diluted 1:10,000 in TBS pH 7.5 (20 mM Tris-HCl, 150 mM NaCl) and incubated for 2 h. Membrane was washed for 1 h with TTBS (TBS supplemented with 0.1% Tween 20 (Sigma), changing the washing solution each 15 min. Secondary antibody, IRDye 800CW Goat α-Mouse IgG (H + L), 0.5 mg (LI-COR) was diluted 1:10,000 in TBS pH 7.5 (20 mM Tris-HCl, 150 mM NaCl) and incubated for 2 h. Membrane was washed for 1 h with TTBS (changing the washing solution each 15 min) and developed using LI-COR Odyssey infrared imaging system. Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards (Bio-Rad) were used as protein mass standards.

**T4P shear off assay**

T4P were sheared from cells that had been grown for 3 days on 1.5% agar plates supplemented with 1% of CTT at 32°C as described except that precipitation of sheared T4P was done using TCA as described (Koontz, 2014) and analyzed by immunoblotting with α-PilA antibodies as described previously (Wu and Kaiser, 1997). Blots were developed using Luminata Forte chemiluminescence reagent (Millipore) on a LAS-4000 imager (Fujifilm). PilA levels were quantified using ImageJ (Schindelin et al., 2012) based on two biological replicates.

**Bioinformatics**

UniProt (The-UniProt-Consortium, 2019) and KEGG (Kanehisa and Goto, 2000) databases were used to assign functions to proteins encoded by LPS gene clusters I and II (Fig. 1A; Table S1). The Carbohydrate Active Enzymes (CAZY) database (http://www.cazy.org/) (Lombard et al., 2014). Pfam v31.0 and v32.0 (pfam.xfam.org) (Finn et al., 2016) and the Conserved Domain tool from NCBI (Marchler-Bauer et al., 2017) were used to identify protein domains. Membrane topology was assessed by TMHMM v2.0 (Sonnhammer et al., 1998) and DAS (Cserzö et al., 1997) and two-dimensional topology was graphically shown using TOPO2 (Johns). BlastP (Boratyn et al., 2013) and the KEGG

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database were used to identify Lpt homologs in *M. xanthus*. *E. coli* and *S. enterica* proteins used for comparison with *M. xanthus* proteins are listed in Table S3. Clustal Omega (Chojnacki et al., 2017) was used to align protein sequences.

**Statistics**

Statistical analyses were performed using SigmaPlot v14. All data sets were tested for a normal distribution using a Shapiro–Wilk test. For all data sets without a normal distribution, the Mann–Whitney test was applied to test for significant differences.

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**Conflict of interest**

The authors declare no conflict of interest.

**Data availability statement**

The data that support the findings of this study are available from the corresponding author upon request.

**References**


**Supporting Information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.