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Pérez-Burgos, M., García-Romero, I., Jung, J., Schander, E., Valvano, M. A., & Søgaard-Andersen, L. (2020). Characterization of the exopolysaccharide biosynthesis pathway in Myxococcus xanthus. *Journal of Bacteriology*, Article JB00335-20R1 . Advance online publication. https://doi.org/10.1128/jb.00335-20

**Published in:**
Journal of Bacteriology

**Document Version:**
Peer reviewed version

**Queen's University Belfast - Research Portal:**
Link to publication record in Queen's University Belfast Research Portal

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Download date:25. Jan. 2025

# Characterization of the exopolysaccharide biosynthesis pathway in *Myxococcus xanthus*

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Running title: Exopolysaccharide biosynthesis in *M. xanthus*

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## Abstract

*Myxococcus xanthus* arranges into two morphologically distinct biofilms depending on its nutritional status, i.e. coordinately spreading colonies in the presence of nutrients and spore-filled fruiting bodies in the absence of nutrients. Exopolysaccharide (EPS) is a structural component of both biofilms and is also important for type IV pili-dependent motility and fruiting body formation. Here, we characterize the biosynthetic machinery responsible for EPS biosynthesis using bioinformatics, genetics, heterologous expression, and biochemical experiments. We show that this machinery constitutes a Wzx/Wzy-dependent pathway dedicated to EPS biosynthesis. Our data support that EpsZ (MXAN_7415) is the polyisoprenyl-phosphate hexose-1-phosphate transferase responsible for initiation of the repeat unit synthesis. Heterologous expression experiments support that EpsZ has galactose-1-P transferase activity. Moreover, MXAN_7416, renamed $\mathrm{Wzx}_{\mathrm{EPS}}$, and MXAN_7442, renamed $\mathrm{Wzy}_{\mathrm{EPS}}$, are the Wzx flippase and Wzy polymerase responsible for translocation and polymerization of the EPS repeat unit, respectively. Also, in this pathway, EpsV (MXAN_7421) is the polysaccharide co-polymerase and EpsY (MXAN_7417) the outer membrane polysaccharide export (OPX) protein. Mutants with single in-frame deletions in the five corresponding genes had defects in type IV pili-dependent motility and a conditional defect in fruiting body formation. Furthermore, all five mutants were deficient in type IV pili formation and genetic analyses suggest that EPS and/or the EPS biosynthetic machinery stimulates type IV pili extension. Additionally, we identify a polysaccharide biosynthesis gene cluster, which together with an orphan gene encoding an OPX protein make up a complete Wzx/Wzy-dependent pathway for synthesis of a polysaccharide.


## Importance

Exopolysaccharide (EPS) has important functions in the social life cycle of *M. xanthus*; however, little is known about how EPS is synthesized. Here, we characterized the EPS biosynthetic machinery and show that it makes up a Wzx/Wzy-dependent pathway for polysaccharide biosynthesis. Mutants lacking a component of this pathway had reduced type IV pili-dependent motility and a conditional defect in development. Also, these analysis suggest that EPS and/or the EPS biosynthetic machinery is important for type IV pili formation.

50 **Introduction**

51 Bacteria often exist in biofilms, which are surface-associated communities where cells are
52 embedded in a self-produced extracellular matrix (ECM) (Stoodley *et al.*, 2002). Typically, the
53 ECM is composed of proteins, extracellular DNA (eDNA) and exopolysaccharide (EPS)
54 (Flemming *et al.*, 2016). EPS serve several functions in a biofilm including structural roles,
55 hydration, adhesion to substrates, cohesion between cells and protection against
56 antibacterials, grazing and bacteriophages (Flemming *et al.*, 2007, Flemming & Wingender,
57 2010, Flemming *et al.*, 2016).

58 The Gram-negative delta-proteobacterium *Myxococcus xanthus* is a model organism to study
59 social behaviors in bacteria. Depending on their nutritional status, *M. xanthus* cells organize
60 into two morphologically distinct biofilms (Konovalova *et al.*, 2010, Zhang *et al.*, 2012). In the
61 presence of nutrients, cells grow, divide and move across surfaces by means of two motility
62 systems to generate EPS-embedded colonies in which cells at the colony edge spread
63 outwards in a highly coordinated fashion (Schumacher & Søgaard-Andersen, 2017, Zhang *et*
64 *al.*, 2012, Hu *et al.*, 2013). Under nutrient limitations, growth ceases and cells alter their
65 motility behavior and begin to aggregate. The aggregation culminates in the formation of
66 mounds of cells inside which the rod-shaped cells differentiate into environmentally resistant
67 spores leading to the formation of mature fruiting bodies (Konovalova *et al.*, 2010, Zhang *et*
68 *al.*, 2012). EPS also makes up a substantial part of individual fruiting bodies (Lu *et al.*, 2005,
69 Shimkets, 1986, Lux *et al.*, 2004).

70 The two motility systems of *M. xanthus* are important for formation of both biofilms (Hodgkin
71 & Kaiser, 1979). One motility system depends on type IV pili (T4P), which are highly dynamic
72 filaments that undergo cycles of extension, surface adhesion and retraction. Retractions
73 generate a force sufficient to pull a cell forward (Craig *et al.*, 2019). The second system is for
74 gliding motility and depends on the Agl/Glt complexes (Zhang *et al.*, 2012, Schumacher &
75 Søgaard-Andersen, 2017). Generally, T4P-dependent motility involves movement of groups
76 of cells while gliding motility involves the movement of individual cells (Shi & Zusman, 1993,
77 Hodgkin & Kaiser, 1979).

78 Besides its role as a structural component of spreading colonies and fruiting bodies, EPS in
79 *M. xanthus* is also important for T4P-dependent motility (Li *et al.*, 2003, Lu *et al.*, 2005) and
80 fruiting body formation (Lu *et al.*, 2005, Shimkets, 1986, Arnold & Shimkets, 1988, Yang *et*
81 *al.*, 1998, Bellenger *et al.*, 2002). It has been proposed that EPS stimulates T4P-dependent
82 motility by stimulating retraction of T4P (Li *et al.*, 2003, Hu *et al.*, 2011). Most insights into the
83 function of EPS in *M. xanthus* have been obtained from analyses of regulatory mutants with
84 altered levels of EPS synthesis. Among these mutants, the best studied include those of the
85 Dif chemosensory system and the SgmT/DigR two component system. The Dif system is a

86 key regulator of EPS synthesis; analyses of *dif* (previously *dsp* (Lancero *et al.*, 2002))
87 mutants have shown that decreased EPS accumulation (Yang *et al.*, 2000, Bellenger *et al.*,
88 2002, Black *et al.*, 2015) causes defects in T4P-dependent motility and fruiting body
89 formation (Yang *et al.*, 1998, Bellenger *et al.*, 2002). While the phosphotransfer reactions
90 within the Dif system have been described in detail (Black *et al.*, 2015, Black *et al.*, 2010), it
91 is unknown how the Dif system stimulates EPS synthesis. Similarly, mutants of the
92 SgmT/DigR system in which DigR is a DNA-binding response regulator have increased EPS
93 accumulation, reduced T4P-dependent motility and a defect in fruiting body formation
94 (Overgaard *et al.*, 2006, Petters *et al.*, 2012). Transcriptome analyses support that this
95 inhibitory effect is not caused by a direct effect on the expression of genes for EPS synthesis
96 (Petters *et al.*, 2012). Compared to the several identified regulators of EPS synthesis,
97 relatively little is known about EPS biosynthesis. Here, we focused on the identification of
98 proteins directly involved in EPS biosynthesis.

99 Synthesis of bacterial cell surface polysaccharides can occur via three different pathways,
100 the Wzx/Wzy, the ABC-transporter or the synthase-dependent pathway (Schmid *et al.*, 2015,
101 Whitfield & Trent, 2014) (Fig. 1A). In the Wzx/Wzy and ABC-transporter dependent
102 pathways, synthesis generally starts with the transfer of a sugar-1-P from a UDP-sugar to an
103 undecaprenyl phosphate (Und-P) molecule in the inner leaflet of the inner membrane (IM) to
104 form an Und-PP-sugar molecule (Valvano *et al.*, 2011). The priming enzymes are broadly
105 classified in two groups, polyisoprenyl-phosphate hexose-1-phosphate transferases (PHPTs)
106 or polyisoprenyl-phosphate *N*-acetylhexosamine-1-phosphate transferases (PNPTs)
107 (Valvano, 2011). Subsequently, the polysaccharide chain is elongated by the action of
108 specific glycosyltransferases (GTs) and this depends on the specific pathway. In the
109 Wzx/Wzy-dependent pathway, GTs synthesize the repeat unit of the polysaccharide on the
110 cytoplasmic side of the IM; each unit is then translocated across the IM by the Wzx flippase
111 and polymerized by the Wzy polymerase into a longer chain. Chain length is generally
112 controlled by a Wzz protein, which belongs to the polysaccharide co-polymerase (PCP)
113 family and results in the formation of polysaccharides with a range of lengths (Reid &
114 Szymanski, 2010, Morona *et al.*, 2000). By contrast, in the ABC-transporter dependent
115 pathway, the full-length polysaccharide chain is synthetized on the cytoplasmic side of the
116 IM, and is then translocated across the IM by an ABC transporter (Cuthbertson *et al.*, 2010).
117 In the synthase-dependent pathway, synthesis and translocation across the IM take place
118 simultaneously by a multifunctional synthase protein complex in the IM (Whitney & Howell,
119 2013). In the Wzx/Wzy and ABC-transporter dependent pathways, the polysaccharide
120 molecule reaches the cell surface by translocation through an outer membrane (OM)
121 polysaccharide export (OPX) protein and in the synthase-dependent pathway translocation

122 occurs via an outer membrane β-barrel protein (Schmid *et al.*, 2015, Whitney & Howell,
123 2013).

124 The *eps* locus in *M. xanthus* was identified by transposon mutagenesis and shown to encode
125 homologs of proteins involved in polysaccharide synthesis (Lu *et al.*, 2005). Moreover,
126 several *eps* genes were identified as essential for EPS biosynthesis (Lu *et al.*, 2005,
127 Berleman *et al.*, 2011, Zhou & Nan, 2017, Black *et al.*, 2015, Lancero *et al.*, 2004). Here, we
128 searched the re-annotated *eps* locus and the remaining *M. xanthus* genome for homologs of
129 proteins for polysaccharide biosynthesis. We report that the *eps* locus encodes a complete
130 Wzx/Wzy-dependent pathway for EPS synthesis. In-frame deletions in the corresponding
131 genes specifically resulted in EPS biosynthesis defects without interfering with LPS O-
132 antigen and spore coat polysaccharide biosynthesis. Phenotypic analysis of these mutants
133 including complementation experiments demonstrated that they have a defect in T4P-
134 dependent motility and conditional defects in development. In addition, we identify a
135 polysaccharide biosynthesis gene cluster of unknown function that together with an orphan
136 gene encoding an OPX protein also encode a complete Wzx/Wzy-dependent pathway for
137 synthesis of a polysaccharide of unknown function.

138

139 **Results**

140 Identification of homologs of proteins of Wzx/Wzy-dependent pathways for polysaccharide
141 biosynthesis and export

142 To identify genes for EPS biosynthesis, we searched the *M. xanthus* genome for homologs
143 (*see Materials and Methods*) of the membrane components of the three biosynthesis
144 pathways (Fig. 1A). We identified homologs encoding predicted proteins of the Wzx/Wzy and
145 ABC-transporter pathways but none corresponding to a synthase-dependent pathway (Fig.
146 1B). Several of these homologs were previously shown to be important for LPS synthesis or
147 spore coat polysaccharide biosynthesis (Pérez-Burgos *et al.*, 2019, Guo *et al.*, 1996,
148 Holkenbrink *et al.*, 2014, Müller *et al.*, 2012, Pérez-Burgos *et al.*, 2020) (Fig. 1B). Notably,
149 none of these proteins are required for EPS biosynthesis. The MraY homolog (MXAN_5607),
150 which belongs to the PNPT family and is involved in PG synthesis, was not considered here.

151 The reannotated *eps* locus consists of two gene clusters (*MXAN_7515-_7422* and
152 *MXAN_7441-_7451*) that encode all the proteins of a complete Wzx/Wzy-dependent pathway
153 (Fig. 2A, Table S1). Specifically, these two gene clusters encode homologs of a PHPT
154 (EpsZ/MXAN_7415), a Wzx flippase (MXAN_7416), a Wzy polymerase (MXAN_7442,
155 previously SgnF (Youderian & Hartzell, 2006)), a PCP protein (EpsV/MXAN_7421) and an
156 OPX protein (EpsY/MXAN_7417) as well as five GTs (EpsU/MXAN_7422,
157 EpsH/MXAN_7441, EpsE/MXAN_7445, EpsD/MXAN_7448, EpsA/MXAN_7451) and a
158 serine O-acetyltransferase (EpsC/MXAN_7449). Previous genetic analyses using transposon
159 insertions, plasmid insertions or in-frame deletion mutants demonstrated that genes in both
160 clusters are important for EPS synthesis (Black *et al.*, 2015, Lu *et al.*, 2005, Berleman *et al.*,
161 2011, Zhou & Nan, 2017) (Fig. 2A). Also, genes in both clusters were previously shown to be
162 important for T4P-dependent motility without directly testing for EPS synthesis (Youderian &
163 Hartzell, 2006) (Fig. 2A). The two gene clusters are separated by 13 genes encoding
164 proteins predicted not to be directly involved in polysaccharide synthesis (Fig. 2A and Table
165 S1). Consistently, genetic analyses for some of these genes confirmed that they are not
166 important for EPS synthesis (Lu *et al.*, 2005) except for *MXAN_7440* (Nla24/EpsI), which
167 encodes a c-di-GMP binding NtrC-like transcriptional regulator (Lancero *et al.*, 2004,
168 Skotnicka *et al.*, 2016) that is phosphorylated by the histidine kinase MXAN_7439 (Willett *et*
169 *al.*, 2013).

170 In a bioinformatics approach searching for orthologs of the proteins encoded by the entire
171 *eps* locus in all fully sequenced Myxococcales genomes using a reciprocal best BlastP hit
172 method as in (Pérez-Burgos *et al.*, 2020), we found that the two gene clusters encoding
173 proteins for polysaccharide synthesis are largely conserved in closely related
174 Cystobacterineae (Fig. 2B). Importantly, in several of these genomes, the two clusters are

175 present in a single uninterrupted gene cluster (Fig. 2B). Interestingly, in *M. macrosporus* and
176 *M. fulvus,* the two gene clusters are separated by a set of genes that are conserved between
177 these two organisms but not homologous to the genes separating the two clusters in *M.*
178 *xanthus*. Together, based on previous gene genetic analyses and because genes for
179 polysaccharide biosynthesis are often clustered (Rehm, 2010), our data support that the two
180 separated gene clusters in the *M. xanthus eps* locus encode for a Wzx/Wzy-dependent
181 pathway for EPS synthesis.

182 We also identified a second locus encoding homologs of a Wzx/Wzy pathway (Fig. 3A; Table
183 S2). Specifically, this locus encodes homologs of a PNPT (MXAN_1043), a Wzx flippase
184 (MXAN_1035), a Wzy polymerase (MXAN_1052), a Wzc chain length regulator (MXAN_1025
185 or BtkB (Kimura *et al.*, 2012)) of the PCP-2 family as well as 10 GTs (MXAN_1026,
186 MXAN_1027, MXAN_1029, MXAN_1030, MXAN_1031, MXAN_1032, MXAN_1033,
187 MXAN_1036, MXAN_1037, MXAN_1042) and two acetyltransferases (MXAN_1041 and
188 MXAN_1049). Finally, we identified a gene encoding an OPX protein (MXAN_1915) that is
189 not part of a gene cluster encoding proteins involved in polysaccharide synthesis (Fig. 3B,
190 Table S2). Using bioinformatics, as described above, we found that the large gene cluster as
191 well as *MXAN_1915* are conserved in closely related Cystobacterineae (Fig. 3C).
192 Importantly, the *MXAN_1915* ortholog of *Sandaracinus amylolyticus* is encoded in a cluster
193 with homologs of *MXAN_1025*, *MXAN_1043* and *MXAN_1052*. Because the *MXAN_1025-*
194 *_1052* locus does not encode an OPX homolog, these observation support that MXAN_1915
195 could function together with the proteins encoded by this locus and together they would
196 make up a complete Wzx/Wzy-dependent pathway for biosynthesis of a polysaccharide.
197 Based on these analyses, we hypothesized that the proteins encoded by the *eps* locus and
198 the proteins encoded by the *MXAN_1025-_1052/_1915* loci make up two independent and
199 dedicated pathways for polysaccharide synthesis.

200 The *eps* locus is essential for EPS biosynthesis
201 To test for the importance of genes of the *eps* locus and the *MXAN_1025-_1052/_1915* loci
202 for EPS synthesis, we generated ten in-frame deletions in genes encoding the five conserved
203 core components of Wzx/Wzy-dependent pathways (i.e. the genes for the PH/NPT, Wzx,
204 Wzy, PCP and OPX). Subsequently, we used plate-based colorimetric assays with either
205 Congo red or Trypan blue to assess EPS biosynthesis. As a negative control, we used a
206 Ω*difE* mutant, which has a defect in EPS synthesis (Yang *et al.*, 1998).

207 All five mutations in the *eps* locus abolished EPS synthesis (Fig. 2C). Importantly, the EPS
208 synthesis defects of these five Δ*eps* mutants were complemented by ectopic expression of
209 the relevant full-length gene from a plasmid integrated in a single copy at the Mx8 *attB* site

210 (Fig. 2C). By contrast, in case of the five in-frame deletions in the genes of the *MXAN_1025-*
211 *_1052*/*_1915* loci, only the Δ*MXAN_1035* mutant, which lacks a Wzx flippase homolog (Fig.
212 3A-C) caused a significant decrease in EPS synthesis. Based on several arguments, we do
213 not think that MXAN_1035 is directly involved in EPS synthesis but rather that the
214 Δ*MXAN_1035* mutation results in titration of Und-P. First, mutation of *MXAN_7416,* which
215 encodes a Wzx flippase homolog in the *eps* locus, completely blocked EPS synthesis (Fig.
216 2A-C) supporting that MXAN_7416 is the flippase involved in EPS biosynthesis. Second, as
217 mentioned, enzymes of the same polysaccharide biosynthesis and export pathway are
218 typically encoded in the same locus (Rehm, 2010); however, the three other mutations in the
219 *MXAN_1025-_1052* locus did not have a significant effect on EPS biosynthesis (Fig. 3D).
220 Third, blocking translocation of a specific sugar unit across the IM can cause sequestration of
221 Und-P and, thereby, result in pleiotropic effects on the synthesis of other polysaccharides
222 (Valvano, 2008, Burrows & Lam, 1999, Jorgenson & Young, 2016, Jorgenson *et al.*, 2016,
223 Ranjit & Young, 2016). Consistently, a Δ*MXAN_1035* mutation was previously shown to
224 cause a reduction in glycerol-induced sporulation (see below) likely by interfering with spore
225 coat polysaccharide biosynthesis (Holkenbrink *et al.*, 2014); however, MXAN_3260 (ExoM)
226 was recently shown to be the flippase involved in spore coat polysaccharide synthesis
227 (Pérez-Burgos *et al.*, 2020). Together, these considerations support that it is unlikely that
228 MXAN_1035 is part of the EPS biosynthesis machinery. In total, our results suggest that the
229 *eps* locus encodes homologs of a Wzx/Wzy-dependent pathway for EPS biosynthesis.
230 Therefore, we renamed MXAN_7416 and MXAN_7442 to $Wzx_{EPS}$ and $Wzy_{EPS}$. From here on,
231 we focused on the five core components of the Wzx/Wzy-dependent pathway for EPS
232 synthesis.

233 Lack of EPS biosynthetic proteins does not affect spore coat polysaccharide, LPS synthesis
234 or cell morphology

235 In addition to EPS, *M. xanthus* synthetizes O-antigen LPS (Fink & Zissler, 1989) and a spore
236 coat polysaccharide (Kottel *et al.*, 1975). As mentioned, because blocking synthesis of one
237 polysaccharide can affect synthesis of other polysaccharide including peptidoglycan (PG) by
238 sequestration of Und-P through accumulation of Und-PP intermediates, we determined
239 whether lack of the EPS biosynthetic proteins affects spore coat polysaccharide, LPS or PG
240 synthesis.

241 Synthesis of the spore coat polysaccharide is essential for sporulation in *M. xanthus* (Licking
242 *et al.*, 2000, Müller *et al.*, 2012). To evaluate whether the Δ*eps* mutants synthetized spore
243 coat polysaccharide, we analyzed sporulation independently of starvation. For this, we
244 profited from an assay in which sporulation occurs rapidly and synchronously and is induced
245 chemically by the addition of glycerol at a high concentration (0.5 M) to cells growing in

246 nutrient-rich broth (Dworkin & Gibson, 1964). In response to adding glycerol, cells of WT and
247 all *eps* five in-frame deletion mutants rounded up during the first 4 h and turned into phase-
248 bright resistant spores by 24 h (Fig. 4A). Cells of the Δ*exoE* mutant, which lacks the PHPT
249 for initiating spore coat polysaccharide biosynthesis and were used as a negative control
250 (Holkenbrink *et al.*, 2014, Pérez-Burgos *et al.*, 2020), remained rod-shaped and did not form
251 phase-bright spores. Interestingly, the sporulation efficiency of all five *Δeps* mutants was
252 increased compared to WT (Fig. 4A). Because the spores formed by WT under high
253 concentrations of glycerol adhere to glass surfaces and each other forming large aggregates
254 while the spores formed by the Δ*eps* mutants do not, we speculate that the ease of
255 harvesting the $EPS^-$ spores rather than the *eps* mutations *per se* results in an apparent
256 increase in the overall sporulation efficiency. We conclude that lack of the EPS biosynthetic
257 proteins does not cause a sporulation defect, in agreement with previous observations that
258 mutation of *epsV* did not affect glycerol-induced sporulation (Holkenbrink *et al.*, 2014).

259 LPS in total cell extracts was detected by Emerald staining and the Δ*wbaP* mutant, which
260 lacks the PHPT for O-antigen biosynthesis, served as a negative control (Pérez-Burgos *et*
261 *al.*, 2019). A fast running lipid-A core band and polymeric LPS O-antigen bands were
262 detected in LPS preparations of WT and the five Δ*eps* mutants, while only the lipid-A core
263 band was detected in the Δ*wbaP* mutant (Fig. 4B). The $\Delta wzx_{\mathrm{EPS}}$ mutant accumulated lower
264 levels of LPS O-antigen (Fig. 4B). O-antigen in *M. xanthus* is synthesized via an ABC
265 transporter-dependent pathway and lack of the Wzm/Wzt ABC transporter blocks LPS O-
266 antigen synthesis (Pérez-Burgos *et al.*, 2019, Guo *et al.*, 1996), suggesting that the $\mathrm{Wzx}_{\mathrm{EPS}}$ is
267 not directly involved in O-antigen synthesis. Therefore, we speculate that the reduced O-
268 antigen level in the $\Delta wzx_{\mathrm{EPS}}$ mutant could be caused by sequestration of Und-PP-linked EPS
269 polymers unable to be translocated across the membrane, which reduced the available pool
270 of Und-P for O-antigen synthesis.

271 Interference with PG synthesis during growth in *M. xanthus* causes morphological defects
272 (Treuner-Lange *et al.*, 2015, Treuner-Lange *et al.*, 2013, Schumacher *et al.*, 2017).
273 Therefore, we used cell morphology as a proxy for PG synthesis to test whether lack of the
274 EPS biosynthetic proteins interferes with PG synthesis during growth. Because, cell
275 morphology and cell length of the five Δ*eps* mutants were similar to that of WT cells, we
276 conclude that PG synthesis was not affected in the Δ*eps* mutants (Fig.4A (0 h) and 4C).
277 Therefore, the proteins for EPS biosynthesis are not important for spore coat polysaccharide,
278 LPS synthesis and PG synthesis, supporting that these proteins make up a pathway
279 dedicated to EPS synthesis.

280

281 <u>MXAN_7415 has Gal-1-P transferase activity</u>

282 EpsZ is the predicted PHPT of the EPS biosynthesis pathway. Similar to $WcaJ_{Ec}$ from *E. coli*
283 and $WbaP_{Se}$ from *Salmonella enterica* (Saldías *et al.*, 2008, Furlong *et al.*, 2015, Pérez-
284 Burgos *et al.*, 2020), we identified a PF13727 (CoA_binding _3) domain, a C-terminal
285 PF02397 (Bac_transf) domain and five transmembrane regions in EpsZ (Fig. 5A), all features
286 of PHPT proteins. The fifth TMH of $WcaJ_{Ec}$ does not fully span the IM and this results in the
287 cytoplasmic localization of the C-terminal catalytic domain. This depends on residue P291
288 that causes a helix-break-helix in the structure and forms part of a $DX_{12}P$ motif conserved
289 among PHPTs (Furlong *et al.*, 2015). Because EpsZ contains the $DX_{12}P$ motif and all the
290 conserved essential residues important for catalytic activity that have been identified in the
291 C-terminal catalytic region of WbaP (Patel *et al.*, 2010) (Fig. 5B, Fig S1), we suggest that
292 EpsZ is a PHPT with a membrane topology similar to that of WcaJ.

293 PHPTs generally utilize UDP-glucose (UDP-Glc) or UDP-galactose (UDP-Gal) as substrates
294 to transfer Glc-1-P or Gal-1-P, respectively to Und-P (Lukose *et al.*, 2017, Valvano, 2011).
295 Therefore, following the same strategy as previously reported (Patel *et al.*, 2012, Pérez-
296 Burgos *et al.*, 2019, Pérez-Burgos *et al.*, 2020), we tested whether EpsZ could functionally
297 replace $WcaJ_{Ec}$ or $WbaP_{Se}$, which catalyse the transfer of Glc-1-P and Gal-1-P to Und-P,
298 respectively. To this end, *epsZ* was cloned into pBADNTF resulting in plasmid pMP146,
299 which encodes EpsZ with an N-terminal FLAG-tag ($_{FLAG}$EpsZ) to facilitate detection by
300 immunoblot and under the control of an arabinose inducible promoter.

301 $WcaJ_{Ec}$ initiates colanic acid biosynthesis, which results in a strong glossy and mucoid
302 phenotype of *wcaJ*$_{Ec}^{+}$ cells containing the plasmid pWQ499 encoding the positive regulator
303 RcsA (Furlong *et al.*, 2015). An *E. coli* Δ*wcaJ*$_{Ec}$ (pWQ499) mutant is complemented with the
304 plasmid pLA3 in the presence of arabinose (Furlong *et al.*, 2015), which encodes $_{FLAG}WcaJ_{Ec}$
305 under the control of the arabinose inducible promoter (Fig. 5C). By contrast, no
306 complementation was observed by $_{FLAG}$EpsZ or the empty pBADNTF vector in the presence
307 of arabinose (Fig. 5C), suggesting that EpsZ does not have Glc-1-P transferase activity.

308 $WbaP_{Se}$ initiates O-antigen synthesis in *S. enterica* and the O-antigen synthesis defect of a
309 Δ*wbaP*$_{Se}$ mutant can be partially corrected by complementation with the plasmid pJD132,
310 which encodes the *E. coli* O9:K30 $WbaP_{Se}$ homolog ($WbaP_{Ec\ O9:K30}$), and with the plasmid
311 pSM13, which encodes $WbaP_{Se}$ (Saldías *et al.*, 2008) (Fig. 5D, left panel). The differences in
312 the O-antigen profile between the different complementation strains are likely due to different
313 processing of the O-antigen as previously reported (Saldías *et al.*, 2008). Expression of
314 $_{FLAG}$EpsZ in the Δ*wbaP*$_{Se}$ mutant in the presence of arabinose provoked a change of the LPS
315 profile (Fig. 5D, left panel), while the empty pBADNTF vector did not affect the LPS profile.
316 Because the effect of $_{FLAG}$EpsZ on the O-antigen profile of the Δ*wbaP*$_{Se}$ mutant was relatively

317 modest by silver staining, we repeated these experiments using *Salmonella* O-antigen rabbit
318 antibodies. As shown in Fig. 5D, right panel, also in this analysis, $_{FLAG}$EpsZ complemented
319 the Δ*wbaP*$_{Se}$ mutant in the presence of arabinose. To test for accumulation of $_{FLAG}$EpsZ in the
320 *E. coli* and *S. enterica* strains when grown in the presence of arabinose, we performed
321 immunoblots using α-FLAG antibodies (Fig. 5E). EpsZ accumulated in both strains
322 predominantly in the monomeric form. By contrast, $_{FLAG}$WcaJ$_{Ec}$ showed the characteristic
323 oligomeric and monomeric bands as previously reported for PHPTs (Saldías *et al.*, 2008).
324 We conclude from these experiments that WbaP$_{Mx}$ can transfer Gal-1-P onto Und-P.

325

326 EPS or EPS biosynthetic proteins is important for T4P-dependent motility and T4P formation

327 Next, we tested the five Δ*eps* mutants for motility defects. To this end, cells were spotted on
328 0.5% and 1.5% agar, respectively (Shi & Zusman, 1993). On 0.5% agar, WT cells formed the
329 long flares characteristic of T4P-dependent motility while on 1.5% agar, WT displayed the
330 single cells at the colony edge characteristic of gliding motility. The Δ*pilA* mutant, which lacks
331 the major pilin subunit and does not assemble T4P (Wu & Kaiser, 1997), and the Δ*aglQ*
332 mutant, which lacks a component of the gliding motility machinery (Sun *et al.*, 2011, Nan *et*
333 *al.*, 2013), were used as negative controls for T4P-dependent and gliding motility,
334 respectively. As expected, the Δ*eps* mutants had a T4P-dependent motility defect forming
335 colonies with shorter flares than WT as did the Δ*aglQ* mutant (Fig. 6A). The motility defects of
336 the Δ*eps* mutants were complemented by ectopic expression of the relevant genes (Fig. 6A).
337 On 1.5% agar, the Δ*eps* mutants displayed the single cell motility characteristic of gliding
338 motility but the Δ*aglQ* mutant did not (Fig. 6A). Also, the total colony expansion was reduced
339 similarly to the Δ*pilA* mutant. The parental colony expansion was restored in the five
340 complementation strains (Fig. 6A). Because the Δ*aglQ* mutant made shorter flares on 0.5%
341 agar and had no single cell motility on 1.5% agar, the Δ*pilA* mutant made no flares on 0.5%
342 agar and had reduced colony expansion on 1.5% agar, while the five Δ*eps* mutants
343 generated shorter flares on 0.5% agar and still had single cell motility on 1.5% agar, we
344 conclude that lack of any single one of the five EPS biosynthetic proteins cause a defect in
345 T4P-dependent motility but not in gliding motility.

346 To understand the mechanism underlying the defect in T4P-dependent motility in the Δ*eps*
347 mutants, we determined the level of T4P formation using a shear-off assay in which T4P are
348 sheared off the cell surface and then the level of PilA assessed by immunoblotting. The PilA
349 level in the sheared fraction was strongly reduced in all five Δ*eps* mutants while the total
350 cellular level of PilA was generally similar to that in WT suggesting that these mutants have
351 fewer T4P than WT cells (Fig. 6B). Because a reduced level of T4P can result from an
352 extension defect or hyper-retraction, we deleted the *pilT* gene encoding the PilT retraction

353 ATPase (Jakovljevic *et al.*, 2008) in the five Δ*eps* mutants and then repeated the shearing
354 assay. All five strains with the additional Δ*pilT* mutation assembled T4P at a higher level than
355 the *pilT*+ strains, but at a significantly lower level than the Δ*pilT* strain (Fig. 6C). Thus, the five
356 Δ*eps* mutants have a defect in T4P extension. Of note, the observation that the Δ*eps pilT*+
357 strains make fewer T4P than the Δ*eps* Δ*pilT* strains support that T4P still retract in the
358 absence of the EPS biosynthetic machinery and/or EPS. These observations are in stark
359 contrast to the observations for the Δ*difA* mutant, which lacks the MCP component of the Dif
360 system and is strongly reduced in EPS synthesis (Yang *et al.*, 2000). This mutant was
361 reported to make T4P at WT levels (Yang *et al.*, 2000) or to be hyper-piliated (Li *et al.*, 2003)
362 and EPS was reported to stimulate T4P retractions in this mutant (Li *et al.*, 2003, Hu *et al.*,
363 2011). We conclude that lack of an EPS biosynthetic protein and/or EPS causes a reduction
364 in T4P extension but the fewer T4P made can still retract.

365 To analyze whether the reduced T4P formation in the Δ*eps* mutants was caused by reduced
366 synthesis of one or more of the 10 core proteins of the T4P machine (Craig *et al.*, 2019,
367 Chang *et al.*, 2016) or the Tgl pilotin (Nudleman *et al.*, 2006), we determined their
368 accumulation level in the five *eps* mutants. All 11 proteins were detected at WT levels in the
369 Δ*eps* mutants (Fig. 6D). We conclude that the EPS biosynthetic machinery and/or EPS is
370 important for T4P extension and, therefore, T4P-dependent motility.

371 Cell-cell cohesion has been suggested to depend on EPS (Shimkets, 1986, Dana &
372 Shimkets, 1993, Arnold & Shimkets, 1988). To evaluate whether the Δ*eps* mutants were
373 affected in cell-cell cohesion and agglutination, we transferred exponentially growing cells to
374 a cuvette and measured the change in cell density over time. WT cells agglutinated and
375 sedimented during the course of the experiment, causing a decrease in the absorbance (Fig.
376 7). The Ω*difE* and mutant was used as a negative control and did not agglutinate over time
377 (Yang *et al.*, 2000). None of the five Δ*eps* in-frame deletion strains agglutinated (Fig. 7) and
378 the agglutination defect was complemented in the complementation strains (Fig. 7).

379 <u>EPS or the Eps biosynthetic machinery is conditionally important for fruiting body formation</u>
380 Next, we tested the five Δ*eps* mutants for development. On TPM agar and in submerged
381 culture, WT cells had aggregated to form darkened mounds at 24 h of starvation. (Fig. 8). On
382 TPM agar, the Δ*eps* mutants showed a delay in aggregation but eventually formed larger and
383 less compact fruiting bodies and sporulated with an efficiency similar to that of WT (Fig. 8).
384 Under submerged conditions, the Δ*eps* mutants did not aggregate to form fruiting bodies
385 sporulation as expected from the cell-cell cohesion and agglutination defects and were
386 significantly reduced in sporulation. The developmental defects of the five Δ*eps* mutants were
387 restored by ectopic expression of the corresponding gene (Fig. 8).

388 **Discussion**

389 Here, we focused on elucidating key steps of EPS biosynthesis, and determined functional
390 consequences of loss of EPS and/or the EPS biosynthetic machinery. The EPS structure is
391 unknown; however, chemical analyses support that it contains at least *N*-acetyl-glucosamine
392 (GlcNAc), Glc and Gal while data for other monosaccharides vary depending on the analysis
393 (Behmlander & Dworkin, 1994, Sutherland & Thomson, 1975).

394 Using bioinformatics, we identified the genes for all the components of a Wzx/Wzy pathway
395 in the *eps* locus. Our experimental results support a model in which these genes encode the
396 EPS biosynthesis machinery (Fig. 9A) and that synthesis of the EPS repeat unit is initiated
397 by the PHPT homolog EpsZ (MXAN_7415). We demonstrate in heterologous expression
398 experiments that EpsZ is functionally similar to the Gal-1-P transferase $WbaP_{Se}$, suggesting
399 that Gal is the first sugar of the EPS repeat unit. The *eps* locus encodes five GTs and
400 inactivation of each of these five genes (Lu *et al.*, 2005, Berleman *et al.*, 2011, Youderian &
401 Hartzell, 2006) causes a loss of EPS synthesis or T4P-dependent motility (Fig. 2A).
402 Therefore, we suggest that these five GTs add monosaccharides to build the repeat unit,
403 which is then translocated across the IM by the $Wzx_{EPS}$ flippase (MXAN_7416). The repeat
404 units are polymerized by the $Wzy_{EPS}$ polymerase (MXAN_7442) with the help of the PCP
405 protein EpsV (MXAN_7421) to make the EPS polysaccharide. In the last step, the EPS
406 polymer is transported to the surface through the OPX protein EpsY (MXAN_7417). EpsC
407 (MXAN_7449) is a serine O-acetyltransferase homolog, which is important but not essential
408 for EPS synthesis (Lu *et al.*, 2005). As previously suggested for a paralog encoded by *exoN*
409 (Pérez-Burgos et al., 2020), which is important for spore coat polysaccharide synthesis,
410 MXAN_7449 could be involved in O-acetylation of precursors for EPS synthesis. Finally, the
411 predicted glycosyl hydrolase EpsB (MXAN_7450) is also important but not essential for EPS
412 synthesis (Lu *et al.*, 2005) and its biochemical function remains to be characterized. Overall,
413 our genetic and functional analyses support that the EPS biosynthesis machinery is
414 exclusively dedicated to EPS biosynthesis and not involved in LPS O-antigen or spore coat
415 polysaccharide biosynthesis.

416 We also identified two additional loci, which together encode a complete Wzx/Wzy-
417 dependent pathway (Fig. 9B). Our genetic analysis suggests the proteins of this pathway are
418 not involved in EPS biosynthesis, spore coat polysaccharide and LPS-O-antigen synthesis
419 (unpublished data), indicating a novel function. Islam et al. (Islam *et al.*, 2020) have
420 suggested these gene clusters encode a biosynthetic machinery for synthesis of a
421 biosurfactant but further studies are required for its complete characterization.

422 Genetic analyses of the five core components of the EPS biosynthesis machinery showed
423 that lack of any of these proteins caused a defect not only in EPS synthesis but also in T4P-

424 dependent motility, cell-cell cohesion, and a conditional defect in fruiting body formation.
425 Superficially, these defects are similar to those reported for *dif* mutants with an EPS$^-$
426 phenotype, which are the best studied mutants with decreased EPS synthesis. However,
427 more detailed comparisons reveal important differences. First, *dif* mutants with an EPS$^-$
428 phenotype have a defect in T4P-dependent motility (Yang *et al.*, 1998, Bellenger *et al.*,
429 2002); however, a *difA* mutant makes T4P at WT levels (Yang *et al.*, 2000) or is hyper-
430 piliated (Li *et al.*, 2003). Moreover, it was suggested that EPS stimulates T4P retractions in
431 this mutant because addition of EPS causes reduced piliation (Li *et al.*, 2003, Hu *et al.*,
432 2011). By contrast, we observed that the five Δ*eps* mutants analyzed here are hypo-pilated.
433 Also, further deletion of the gene for the PilT retraction ATPase resulted in an increased level
434 of surface piliation suggesting that T4P in the five Δ*eps pilT*$^+$ mutants can still be retracted.
435 Consistently, T4P-dependent motility was not completely abolished in the five Δ*eps* mutants.
436 These observation suggest that that EPS, or alternatively components of the EPS
437 biosynthetic machinery, is important for T4P formation. Altogether, these comparisons
438 support that the *dif* EPS$^-$ mutations, which are regulatory mutants, and the Δ*eps* mutations
439 described here, which are biosynthetic mutants, both interfere with T4P-dependent motility
440 but the underlying mechanisms are different.

441 Second, *dif* mutants with an EPS$^-$ phenotype neither develop to form spore-filled fruiting
442 bodies on TPM or CF agar nor under submerged conditions (Yang *et al.*, 1998, Bellenger *et*
443 *al.*, 2002, Skotnicka *et al.*, 2016). By contrast, the five Δ*eps* mutants described here develop
444 with only a slight delay on TPM agar but not under submerged conditions. We speculate that
445 this developmental defect is caused by lack of cell-cell cohesion and agglutination in the five
446 Δ*eps* mutants. Whether these phenotypic differences are caused by the differences in T4P
447 levels and functionality in the two types of mutants remains to be investigated.

448 Previously, it was reported that the T4P machinery functions upstream of the Dif pathway to
449 stimulate EPS synthesis (Black *et al.*, 2006, Moak *et al.*, 2015, Wallace *et al.*, 2014, Black *et*
450 *al.*, 2017). How the T4P machinery interfaces with the Dif system is unknown. Similarly, it is
451 unknown how the Dif system stimulates EPS biosynthesis. Here, we show that mutations in
452 the Wzx/Wzy-dependent pathway for EPS synthesis cause a defect in T4P extension. It will
453 be an important future goal to disentangle how *dif* and *eps* mutants at the molecular level
454 affect T4P formation, function and development, as well as how T4P function affects EPS
455 synthesis.

**Acknowledgement**

The authors thank A. Treuner-Lange for construction of pMAT150. This work was supported by Deutsche Forschungsgemeinschaft (DFG, German Research Council) within the framework of the SFB987 “Microbial Diversity in Environmental Signal Response” as well as by the Max Planck Society.

**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability**

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

469 **Materials and Methods**

470 Strains and cell growth. All *M. xanthus* strains are derivatives of the wild type DK1622
471 (Kaiser, 1979). Strains, plasmids and oligonucleotides used in this work are listed in Table 1,
472 Table 2, and Table S3, respectively. *M. xanthus* was grown at 32°C in 1% CTT (1% (w/v)
473 Bacto Casitone, 10 mM Tris-HCl pH 8.0, 1 mM $K_2HPO_4/KH_2PO_4$ pH 7.6 and 8 mM $MgSO_4$)
474 liquid medium or on 1.5% agar supplemented with 1% CTT and kanamycin (50 µg $ml^{-1}$) or
475 oxytetracycline (10 µg $ml^{-1}$), as appropriate (Hodgkin & Kaiser, 1977). In-frame deletions
476 were generated as described (Shi *et al.*, 2008), and plasmids for complementation
477 experiments were integrated in a single copy by site specific recombination into the Mx8 *attB*
478 site. In-frame deletions and plasmid integrations were verified by PCR. Plasmids were
479 propagated in *E. coli* Mach1 and DH5α.

480 *E. coli* and *S. enterica* serovar Typhimurium strains were grown at 37°C in Luria-Bertani (LB)
481 medium (10 mg tryptone $ml^{-1}$, 5 mg yeast extract $ml^{-1}$; 5 mg NaCl $ml^{-1}$) supplemented, when
482 required, with ampicillin, tetracycline, kanamycin or chloramphenicol at final concentrations of
483 100, 20, 40 and 30 µg $ml^{-1}$, respectively. Plasmids for heterologous complementation were
484 introduced into MSS2 and XBF1 strains (Table 1) by electroporation (Dower *et al.*, 1988).

485 Detection of EPS accumulation. Exponentially growing cells were harvested, (3 min, 6000 *g*
486 at RT), and resuspended in 1% CTT to a calculated density of $7 \times 10^9$ cells $ml^{-1}$. 20 µl
487 aliquots of the cell suspensions were placed on 0.5% agar plates supplemented with 0.5%
488 CTT and 10 or 20 µg $ml^{-1}$ of Trypan blue or Congo red, respectively. Plates were incubated
489 at 32°C and documented at 24 h.

490 Glycerol-induced sporulation assay. Sporulation in response to 0.5 M glycerol was performed
491 as described (Müller *et al.*, 2010) with a slightly modified protocol. Briefly, cells were
492 cultivated in 10 ml of CTT medium, at a cell density of $3 \times 10^8$ cells $ml^{-1}$, glycerol was added
493 to a final concentration of 0.5 M. At 0, 4 and 24 h after glycerol addition, cell morphology was
494 observed by placing 5 µl of cells on a 1.5% agar TPM pad on a slide. Cells were immediately
495 covered with a coverslip and imaged with DMi6000B microscope and a Hamamatsu Flash
496 4.0 Camera (Leica). To determine the resistance to heat and sonication of spores formed,
497 cells from 5 ml of the culture after 24 h incubation were harvested (10 min, 4150 *g*, RT),
498 resuspended in 1 ml sterile water, incubated at 50°C for 2 h, and then sonicated with 30
499 pulses, pulse 50%, amplitude 75% with a UP200St sonifier and microtip (Hielscher).
500 Sporulation levels were determined as the number of sonication- and heat-resistant spores
501 relative to WT using a Helber bacterial counting chamber (Hawksley, UK).5 µl of the treated
502 samples were placed on a 1.5 % agar TPM pad on a slide, covered with a coverslip and
503 imaged.

504 LPS extraction and detection. LPS was extracted from *M. xanthus* and visualized by Emerald
505 staining as described (Pérez-Burgos *et al.*, 2019). LPS from *S. enterica* and *E. coli* was
506 extracted and visualized by silver staining as described (Marolda *et al.*, 2006, Pérez-Burgos
507 *et al.*, 2019). For *S. enterica*, O-antigen was detected by immunoblot using rabbit *Salmonella*
508 O antiserum group B (Difco, Beckton Dickinson ref. number 229481) (1:500) and the
509 secondary antibody IRDye 800CW goat α-rabbit immunoglobulin G (1:10000) (LI-COR)
510 (Pérez-Burgos *et al.*, 2019).

511 Cell length determination. 5 μl aliquots of exponentially growing cell suspensions were
512 spotted on glass placed on a metal frame, covered with 1.5% agar supplemented with TPM
513 and imaged using a DMi8 Inverted microscope and DFC9000 GT camera (Leica)
514 (Schumacher & Søgaard-Andersen, 2018). Cell length was determined and visualized as
515 described (Pérez-Burgos *et al.*, 2019). Statistical analyses were performed using SigmaPlot
516 v14. All data sets were tested for a normal distribution using a Shapiro-Wilk test and for all
517 data sets without a normal distribution, the Mann-Whitney test was applied to test for
518 significant differences.

519 Motility assays. Exponentially growing cultures of *M. xanthus* were harvested (6000 *g*, room
520 temperature (RT)) and resuspended in 1% CTT to a calculated density of 7 × $10^9$ cells $ml^{-1}$. 5
521 μl aliquots of cell suspensions were spotted on 0.5% and 1.5% agar supplemented with 0.5%
522 CTT. The plates were incubated at 32°C for 24 h and cells were visualized using a M205FA
523 Stereomicroscope (Leica) and imaged using a Hamamatsu ORCA-flash V2 Digital CMOS
524 camera (Hamamatsu Photonics). Pictures were analyzed using Metamorph® v 7.5
525 (Molecular Devices).

526 Detection of colanic acid biosynthesis. *E. coli* Δ*wcaJ* strains were grown on LB plates with
527 antibiotics and with or without 0.2 % (w/v) arabinose at 37°C overnight. Incubation was
528 extended to 24-48 h at RT to visualize the mucoid phenotype (Furlong et al 2015).

529 Immunoblot analysis. Immunoblots were carried out as described (Sambrook & Russell,
530 2001). For *M. xanthus* immunoblots, rabbit polyclonal α-PilA (dilution: 1:2000), α-PilB
531 (dilution: 1:2000) (Jakovljevic *et al.*, 2008), α-PilC (dilution: 1:2000) (Bulyha *et al.*, 2009), α-
532 PilM (dilution: 1:3000) (Bulyha *et al.*, 2009), α-PilN (dilution: 1:2000) (Friedrich *et al.*, 2014),
533 α-PilO (dilution: 1:2000) (Friedrich *et al.*, 2014), α-PilP (dilution: 1:2000) (Friedrich *et al.*,
534 2014), α-PilT (dilution: 1:3000) (Jakovljevic *et al.*, 2008), α-Tgl (dilution: 1:2000) (Friedrich *et*
535 *al.*, 2014), α-TsaP (dilution: 1:2000) (Siewering *et al.*, 2014), α-PilQ (dilution: 1:5000) (Bulyha
536 *et al.*, 2009) were used together with a horseradish-conjugated goat anti-rabbit
537 immunoglobulin G (Sigma) as secondary antibody. Blots were developed using Luminata
538 crescendo Western HRP Substrate (Millipore) on a LAS-4000 imager (Fujifilm).

539 For *E. coli* and *S. enterica* strains, FLAG-tagged membrane proteins were isolated and
540 detected by immunoblot analysis, as previously described, using α-FLAG M2 monoclonal
541 antibody (Sigma) (1:10000) and a secondary antibody, IRDye 800CW Goat α-Mouse IgG
542 (H+L), 0.5 mg (LI-COR) (1:10000) (Pérez-Burgos *et al.*, 2019).

543 T4P shear off assay. T4P were sheared from cells that had been grown for three days on
544 1.5% agar plates supplemented with 1% CTT at 32°C as described, except that precipitation
545 of sheared T4P was done using TCA as described (Koontz, 2014), and analyzed by
546 immunoblotting with α-PilA antibodies as described previously (Wu & Kaiser, 1997). Blots
547 were developed as indicated.

548 Cell agglutination assay. Cell agglutination was performed as described previously (Wu *et*
549 *al.*, 1997) with a slightly modified protocol. Briefly, 1 ml of exponentially growing cells in 1%
550 CTT was transferred to a cuvette and cell density was measured at the indicated time points.

551 Development. Exponentially growing *M. xanthus* cultures were harvested (3 min, 6000 *g* at
552 RT), and resuspended in MC7 buffer (10 mM MOPS pH 7.0, 1 mM CaCl2) to a calculated
553 density of $7 \times 10^9$ cells $ml^{-1}$. 10 µl aliquots of cells were placed on TPM agar (10 mM Tris-HCl
554 pH 7.6, 1 mM $K_2HPO_4/KH_2PO_4$ pH 7.6, 8 mM $MgSO_4$), and 50 µl aliquots were mixed with
555 350 µl of MC7 buffer and placed in a 24-well polystyrene plate (Falcon) for development in
556 submerged culture. Cells were visualized at the indicated time points using a M205FA
557 Stereomicroscope (Leica) and imaged using a Hamamatsu ORCA-flash V2 Digital CMOS
558 camera (Hamamatsu Photonics), and a DMi8 Inverted microscope and DFC9000 GT camera
559 (Leica). Images were analyzed as previously described. After 120 h, cells were collected and
560 incubated at 50°C for 2 h, and then sonicated as described. Sporulation levels were
561 determined as the number of sonication- and heat-resistant spores relative to WT.

562 Bioinformatics. The KEGG SSDB (Sequence Similarity Database) (Kanehisa & Goto, 2000)
563 database was used to identify homologs of PHPT (PF02397- Bacterial Sugar Transferase),
564 PNPT (PF00953- Glycosyl transferase family 4) (Lehrman, 1994), Wzx (PF01943-
565 Polysacc_synt and PF13440- Polysacc_synt_3), Wzy_C (PF04932- Wzy_C), PCP
566 (PF02706- Wzz) and OPX (PF02563- Poly_export) as in (Pérez-Burgos *et al.*, 2020, Beczała
567 *et al.*, 2015, Dong *et al.*, 2006). For the ABC-transporter dependent pathway we used
568 (PF01061- ABC2_membrane) for the permease and, (PF00005- ABC_tran) and (PF14524-
569 Wzt_C) for the ATPase as in (Pérez-Burgos *et al.*, 2019) together with an analysis of the
570 genetic neighborhood to search for glycan related proteins. BlastP was used to identify
571 homologs of the synthase dependent pathway using previously identified components
572 (Whitney & Howell, 2013). KEGG SSDB was also used to identify EPS homolog proteins in
573 other Myxococcales using a reciprocal best BlastP hit method. UniProt (The-UniProt-
574 Consortium, 2019), KEGG (Kanehisa & Goto, 2000) and the Carbohydrate Active Enzymes

575 (CAZy) (http://www.cazy.org/) (Lombard *et al.*, 2014) databases were used to assign
576 functions to proteins (Fig. 1B, 2A, 3A-B; Table S1 and S2).SMART (smart.embl-
577 heidelberg.de) (Letunic *et al.*, 2015) and Pfam v31.0 and v32.0 (pfam.xfam.org) (Finn *et al.*,
578 2016) were used to identify protein domains. Membrane topology was assessed by TMHMM
579 v2.0 (Sonnhammer *et al.*, 1998) and two-dimensional topology was graphically shown using
580 TOPO2 (Johns). Clustal Omega (Chojnacki *et al.*, 2017) was used to align protein
581 sequences. The phylogenetic tree was prepared as in (Pérez-Burgos *et al.*, 2020) in MEGA7
582 (Kumar *et al.*, 2016) using the Neighbor-Joining method (Saitou & Nei, 1987). Bootstrap
583 values (500 replicates) are shown next to the branches (Felsenstein, 1985).

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878

879

880 **Figures legends**

881

882 **Figure 1.** Identification of homologs of polysaccharide biosynthesis proteins in *M. xanthus.*
883 (A) Schematic of the three pathways for polysaccharide biosynthesis in Gram-negative
884 bacteria.
885 (B) Bioinformatics-based identification of homologs of polysaccharide biosynthesis proteins
886 in *M. xanthus.* Color code as in (A). Note that WaaL is the LPS O-antigen ligase (Pérez-
887 Burgos *et al.*, 2019) while the remaining three proteins with a Wzy_C domain are predicted
888 polymerases.

889

890 **Figure 2**. Bioinformatics and genetic analysis of the *eps* locus.
891 (A) *eps* locus in *M. xanthus*. Genes are drawn to scale and MXAN number or gene name is
892 indicated (Table S1). The color code indicates predicted functions as indicated in the key and
893 are used throughout. Black, grey and white arrow heads indicate mutations previously
894 reported to cause a defect in EPS synthesis (black) (Black *et al.*, 2015, Lu *et al.*, 2005,
895 Lancero *et al.*, 2004, Berleman *et al.*, 2011), a defect in T4P-dependent motility but with no
896 test of EPS synthesis (Youderian & Hartzell, 2006)(grey) and no effect on EPS synthesis (Lu
897 *et al.*, 2005).
898 (B) Taxonomic distribution and synteny of *eps* genes in Myxococcales with fully sequenced
899 genomes. A reciprocal best BlastP hit method was used to identify orthologs. 16S rRNA tree
900 of Myxococcales with fully sequenced genomes (left). Genome size, family and suborder
901 classification are indicated (right). To evaluate gene proximity and cluster conservation, 10
902 genes were considered as the maximum distance for a gene to be in a cluster. Genes found
903 in the same cluster (within a distance of <10 genes) are marked with the same color (i.e.
904 blue, green and dark grey). Light grey indicates a conserved gene that is found somewhere
905 else on the genome (>10 genes away from a cluster); a cross indicates no homolog found.
906 (C) Determination of EPS synthesis. 20 µl aliquots of cell suspensions of strains of the
907 indicated genotypes at 7 × $10^9$ cells $ml^{-1}$ were spotted on 0.5% agar supplemented with 0.5%
908 CTT and Congo red or Trypan blue and incubated 24 h. In the complementation strains, the
909 complementing gene was expressed ectopically from the native (*epsZ*, $wzx_{EPS}$ and $wzy_{EPS}$) or
910 the *pilA* promoter on a plasmid integrated in a single copy at the Mx8 *attB* site. The Ω*difE*
911 mutant served as a negative control.

912 **Figure 3.** Bioinformatics and genetic analysis of the *MXAN_1025-1052/_1915* loci.
913 (A, B) *MXAN_1025-1052 and _1915* loci in *M. xanthus*. Genes are drawn to scale and MXAN
914 number or gene name is indicated (Table S2). The color code indicates predicted functions

915 as indicated in the key and are used throughout.
916 (C) Taxonomic distribution and synteny of genes in the *MXAN_1025-1052/_1915* loci in
917 Myxococcales with fully sequenced genomes. A reciprocal best BlastP hit method was used
918 to identify orthologs. 16S rRNA tree of Myxococcales with fully sequenced genomes (left).
919 Genome size, family and suborder classification are indicated (right). To evaluate gene
920 proximity and cluster conservation, 10 genes were considered as the maximum distance for
921 a gene to be in a cluster. Genes found in the same cluster (within a distance of <10 genes)
922 are marked with the same color (i.e. cyan, magenta, and dark and middle dark grey). Light
923 grey indicates a conserved gene that is found somewhere else on the genome (>10 genes
924 away from a cluster); a cross indicates no homolog found.
925 (C) Determination of EPS synthesis. 20 µl aliquots of cell suspensions of strains of the
926 indicated genotypes at $7 \times 10^9$ cells $ml^{-1}$ were spotted on 0.5% agar supplemented with 0.5%
927 CTT and Congo red or Trypan blue and incubated 24 h. The Ω*difE* mutant served as a
928 negative control. Scale bars, 3 µm.

929 **Figure 4.** Phenotypic characterization of the Δ*eps* mutants.
930 (A) Chemically induced sporulation. Sporulation was induced by addition of glycerol to a final
931 concentration of 0.5 M. At 0, 4 and 24 h after glycerol addition, cell morphology was
932 documented. In images labelled resistant spores, cells were exposed to sonic and heat
933 treatment before microscopy. Sporulation frequency after sonic and heat treatment is
934 indicated as the mean of three biological replicates relative to WT ± standard deviation. Scale
935 bars, 5 µm. (B) Extracted LPS from the same number of cells was separated by SDS-PAGE
936 and detected with Pro-Q Emerald 300. (C) Cell length measurements of the Δ*eps* mutants.
937 Cell length is shown in a violin plot, which indicates the probability density of the data at
938 different cell length values. $n$ = 500 combined from two biological replicates and mean and
939 median values are represented by a continuous and dashed line, respectively. Samples were
940 compared using a Mann-Whitney test; ns, not significant.

941 **Figure 5.** PHPT activity of MXAN_7415.
942 (A) Domain and TMH prediction of EpsZ (MXAN_7415) and WcaJ of *E. coli* ($WcaJ_{Ec}$). Grey
943 rectangles indicate TMH. Numbers indicate domain borders. (B) Topology predictions for
944 EpsZ (MXAN_7415). Domains are indicated in blue and conserved amino acids important for
945 structure or activity of the protein are marked with orange and red, respectively. Sequence
946 alignment of EpsZ (MXAN_7415) with $WbaP_{Se}$, is shown in Fig. S1. (C-E) Complementation
947 of colanic acid synthesis and LPS O-antigen in *E. coli* K-12 W3110 ($\Delta wcaJ_{Ec}$) and *S. enterica*
948 LT2 ($\Delta wbaP_{Se}$) mutants, respectively by plasmids encoding the indicated PHPT proteins. (C)
949 The *E. coli* $\Delta wcaJ_{Ec}$ mutant XBF1 containing pWQ499 ($RcsA^+$) and the indicated
950 complementing plasmids or vector control on LB plates was incubated overnight at 37°C with

951 10 μg $ml^{-1}$ tetracycline (to maintain pWQ499) and with or without arabinose (Ara) to induce
952 gene expression. Incubation was extended to 24-48 h at room temperature to further
953 increase colanic polysaccharide synthesis. (D) Complementation of *S. enterica* Typhimurium
954 LT2 Δ*wbaP*$_{Se}$ mutant containing the indicated plasmids. LPS samples were extracted,
955 separated by electrophoresis on SDS–14% polyacrylamide gels and silver stained (left
956 panel) or examined by immunoblotting using rabbit *Salmonella* O antiserum group B (right
957 panel). Each lane corresponds to LPS extracted from $10^8$ cells. Cultures included addition of
958 arabinose as indicated. (E) Immunoblot using α-FLAG monoclonal antibody to confirm
959 expression of $_{FLAG}$MXAN_7415 and $_{FLAG}$WcaJ in the Δ*wcaJ* mutant, and the expression of
960 $_{FLAG}$MXAN_7415 in *S. enterica*. Note that WbaP expressed from pSM13 was not tested since
961 it is not fused to a FLAG tag. * and ** denote the monomeric and oligomeric forms of the
962 PHPT proteins, usually present under the gel conditions required to ensure their
963 visualization.

964

965 **Figure 6.** Motility analyses of Δ*eps* mutants. (A) Colony-based motility assay of Δ*eps*
966 mutants. T4P-dependent motility and gliding motility were tested on 0.5% and 1.5% agar,
967 respectively. Images were recorded after 24 h. (B-C) T4P shear off assay. Immunoblot
968 detection of the major pilin PilA in sheared T4P (top) and in total cell extract (middle), where
969 the same number of cells grown on 1% CTT 1.5% agar was loaded per lane. The top and
970 middle blots were probed with antibodies against PilA (calculated molecular mass 23.4 kDa).
971 The middle blot was stripped and probed with antibodies against PilC (calculated molecular
972 mass 45.2 kDa), as a loading control. (D) Immunoblot detection of proteins of the T4P
973 machinery using α-PilA, -B, -C, -M, -N, -O, -P, -Q, -T, Tgl and TsaP antibodies. The same
974 number of cells coming from exponentially growing liquid cultures was loaded per lane. As a
975 negative control, cells containing a single in-frame deletion mutation in the relevant gene
976 were used.

977

978 **Figure 7.** Analysis of Δ*eps* mutants for cell-cell cohesion and agglutination.
979 Cell agglutination assay. 1 ml of exponentially growing cells were transferred to a cuvette.
980 Agglutination was monitored by measuring the decrease in absorbance at 550 nm at 3 h
981 relative to the initial absorbance for each strain. The graph shows data from three biological
982 replicates as mean ± standard deviation.

983

984 **Figure 8.** Development of Δ*eps* mutants. Cells on TPM agar and under submerged
985 conditions were followed during development. Images were recorded at the indicated time

986 points. Sporulation efficiency after heat and sonic treatment is indicated as the mean ±
987 standard deviation from at three biological replicates relative to WT. Scale bars: 1mm (left),
988 200 μm (right).

989 **Figure 9**. Wzx/Wzy-dependent pathways for EPS biosynthesis (A) and synthesis of an
990 unknown polysaccharide (B). See text in Discussion for details.

991

992 **Table 1.** Strains used in this work

| **Strain** | **Genotype** | **Reference** |
|---|---|---|
| ***M. xanthus*** | | |
| DK1622 | WT | (Kaiser, 1979) |
| DK8615 | Δ*pilQ* | (Wall *et al.*, 1999) |
| DK10405 | Δ*tgl* | (Rodriguez-Soto & Kaiser, 1997, Wall *et al.*, 1998) |
| DK10409 | Δ*pilT* | (Jakovljevic *et al.*, 2008, Wu *et al.*, 1997) |
| DK10410 | Δ*pilA* | (Wu *et al.*, 1997) |
| DK10416 | Δ*pilB* | (Jakovljevic *et al.*, 2008, Wu *et al.*, 1997) |
| DK10417 | Δ*pilC* | (Wu *et al.*, 1997) |
| SW501 | *difE*::$Km^r$ | (Yang *et al.*, 1998) |
| SA3001 | Δ*pilO* | (Friedrich *et al.*, 2014) |
| SA3002 | Δ*pilM* | (Bulyha *et al.*, 2009) |
| SA3005 | Δ*pilP* | (Friedrich *et al.*, 2014) |
| SA3044 | Δ*pilN* | (Friedrich *et al.*, 2014) |
| SA5923 | Δ*aglQ* | (Jakobczak *et al.*, 2015) |
| SA6011 | Δ*tsaP* | (Siewering *et al.*, 2014) |
| SA7450 | Δ$wbaP_{Mx}$ | (Pérez-Burgos *et al.*, 2019) |
| SA7495 | Δ*exoE* | (Pérez-Burgos *et al.*, 2019) |
| SA7400 | Δ*MXAN_7415* | This study |
| SA7405 | Δ*MXAN_7416* | This study |
| SA7406 | Δ*MXAN_7421* | This study |
| SA7407 | Δ*MXAN_7442* | This study |
| SA7408 | Δ*MXAN_7417* | This study |
| SA7410 | Δ*MXAN_7416 attB*::pMP024 ($P_{nat}$ *MXAN_7416*) | This study |
| SA7411 | Δ*MXAN_7415 attB::*pMP021 ($P_{nat}$ *MXAN_7415*) | This study |
| SA7412 | Δ*MXAN_7417 attB*::pMP030 ($P_{pilA}$ *MXAN_7417*) | This study |
| SA7413 | Δ*MXAN_7421 attB*::pMP032 ($P_{pilA}$ *MXAN_7421*) | This study |
| SA7427 | Δ*MXAN_7416* Δ*pilT* | This study |
| SA7433 | Δ*MXAN_7415* Δ*pilT* | This study |
| SA7435 | Δ*MXAN_7442* Δ*pilT* | This study |
| SA7444 | Δ*MXAN_7417* Δ*pilT* | This study |
| SA7445 | Δ*MXAN_7421* Δ*pilT* | This study |
| SA7451 | Δ*MXAN_1025* | This study |
| SA7452 | Δ*MXAN_1035* | This study |

| SA7456 | Δ*MXAN_1052* | This study |
|---|---|---|
| SA7454 | Δ*MXAN_1915* | This study |
| SA7477 | Δ*MXAN_7442 attB::*pMP091 ($P_{nat}$ *MXAN_7442*) | This study |
| SA8515 | Δ*MXAN_1043* | This study |
| ***E. coli*** | | |
| DH5α | F⁻ φ80*lacZ*ΔM15 *endA recA hsdR*($r_K^-$ $m_K^-$) *nupG thi glnV deoR gyrA relA1* Δ(*lacZYA-argF*)U169 | Lab stock |
| Mach1 | Δ*recA*1398 *endA*1 *tonA* Φ80Δ*lacM*15 Δ*lacX*74 *hsdR*($r_K^-$ $m_K^+$) | Invitrogen |
| XBF1 | W3110*, ΔwcaJ::aph*, $Km^r$ | (Patel *et al.*, 2012) |
| ***Salmonella*** | | |
| LT2 | WT, *S. enterica* serovar Typhimurium | S. Maloy |
| MSS2 | LT2, Δ*wbaP*::*cat* $Cm^r$ | (Saldías *et al.*, 2008) |

993

994 **Table 2.** Plasmids used in this work

| Plasmid | Description | Reference |
|---|---|---|
| pBJ114 | $Km^r$ *galK* | (Julien *et al.*, 2000) |
| pSWU30 | $Tet^r$ | (Wu & Kaiser, 1997) |
| pSW105 | $Km^r$, PpilA | (Jakovljevic *et al.*, 2008) |
| pBADNTF | pBAD24 for N-terminal FLAG fusion and with arabinose inducible promoter, $Amp^r$ | (Marolda *et al.*, 2004) |
| pLA3 | pBADNTF, *wcaJ*, $Amp^r$ | (Furlong *et al.*, 2015) |
| pSM13 | pUC18, *wbaP* from *S. enterica* Ty2 containing a 1 bp deletion at position 583 and a 2 bp deletion at position 645. This causes a frame shift at WbaP I194 and frame restoration at Y215, $Amp^r$ | (Saldías *et al.*, 2008) |
| pJD132 | pBluescript SK, *wbaP* and flanking sequences from *E. coli* O9:K30, $Amp^r$ | (Schäffer *et al.*, 2002) |
| pWQ499 | pKV102 containing *rcsAK30*, $Tet^r$ | C. Whitfield |
| pMAT150 | pBJ114, in-frame deletion construct for *pilT* $Km^r$ | Anke Treuner-Lange |
| pMP001 | pBJ114, in-frame deletion construct for *MXAN_7415* $Km^r$ | This study |
| pMP012 | pBJ114, in-frame deletion construct for *MXAN_7421* $Km^r$ | This study |
| pMP015 | pBJ114, in-frame deletion construct for *MXAN_7442* $Km^r$ | This study |
| pMP016 | pBJ114, in-frame deletion construct for *MXAN_7416* $Km^r$ | This study |
| pMP018 | pBJ114, in-frame deletion construct for *MXAN_7417* $Km^r$ | This study |
| pMP021 | pSWU30, $P_{nat}$ *MXAN_7415* $Tet^r$ | This study |
| pMP024 | pSWU30, $P_{nat}$ *MXAN_7416* $Tet^r$ | This study |
| pMP030 | pSW105, *MXAN_7417* $Km^r$ | This study |

| pMP032 | pSW105, *MXAN_7421* Km$^{r}$ | This study |
|---|---|---|
| pMP091 | pSWU30, P$_{nat}$ *MXAN_7442* Tet$^{r}$ | This study |
| pMP124 | pBJ114, in-frame deletion construct for *MXAN_1043* Km$^{r}$ | This study |
| pMP146 | pBADNTF, MXAN_7415 Amp$^{r}$ | This study |
| pJJ1 | pBJ114, in-frame deletion construct for *MXAN_1035* Km$^{r}$ | This study |
| pJJ2 | pBJ114, in-frame deletion construct for *MXAN_1025* Km$^{r}$ | This study |
| pJJ3 | pBJ114, in-frame deletion construct for *MXAN_1052* Km$^{r}$ | This study |
| pJJ4 | pBJ114, in-frame deletion construct for *MXAN_1915* Km$^{r}$ | This study |

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