INTRODUCTION

Bacteria have evolved different strategies that enable their survival in response to environmental changes. Often these strategies include alterations in gene expression, motility behaviour and/or metabolism without evident changes in cell morphology. However, as an alternative strategy, some bacteria undergo cellular differentiation resulting in the formation of cell types with altered functions and environmental resistance.

Identification of the Wzx flippase, Wzy polymerase and sugar-modifying enzymes for spore coat polysaccharide biosynthesis in *Myxococcus xanthus*

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Abstract
The rod-shaped cells of *Myxococcus xanthus*, a Gram-negative deltaproteobacterium, differentiate to environmentally resistant spores upon starvation or chemical stress. The environmental resistance depends on a spore coat polysaccharide that is synthesised by the ExoA-I proteins, some of which are part of a Wzx/Wzy-dependent pathway for polysaccharide synthesis and export; however, key components of this pathway have remained unidentified. Here, we identify and characterise two additional loci encoding proteins with homology to enzymes involved in polysaccharide synthesis and export, as well as sugar modification and show that six of the proteins encoded by these loci are essential for the formation of environmentally resistant spores. Our data support that MXAN_3260, renamed ExoM and MXAN_3026, renamed ExoJ, are the Wzx flippase and Wzy polymerase, respectively, responsible for translocation and polymerisation of the repeat unit of the spore coat polysaccharide. Moreover, we provide evidence that three glycosyltransferases (MXAN_3027/ExoK, MXAN_3262/ExoO and MXAN_3263/ExoP) and a polysaccharide deacetylase (MXAN_3259/ExoL) are important for formation of the intact spore coat, while ExoE is the polyisoprenyl-phosphate hexose-1-phosphate transferase responsible for initiating repeat unit synthesis, likely by transferring N-acetylgalactosamine-1-P to undecaprenyl-phosphate. Together, our data generate a more complete model of the Exo pathway for spore coat polysaccharide biosynthesis and export.

KEYWORDS
development, Exo, Nfs, O-antigen, polysaccharide, sporulation
morphology and novel characteristics. The best-studied examples of environmentally induced bacterial differentiation include spore formation in three phylogenetically widely separated species *Bacillus subtilis* (Tan & Ramamurthi, 2014), *Streptomyces coelicolor* (Flär dh & Buttner, 2009) and *Myxococcus xanthus* (Konovalova, Petters, & Segarra-Andersen, 2010). While the spore formation process varies among these three species, the resulting spores have in common that they have a spore coat that confers resistance to environmental stress.

In *B. subtilis*, sporulation is initiated in response to starvation and depends on an unusual cell division event in which the division septum is placed asymmetrically close to a cell pole, resulting in the formation of a large mother cell and a smaller forespore. Next, the mother cell engulfs the forespore and lysis of the mother cell finally releases the mature spore (Tan & Ramamurthi, 2014). The spore envelope, partly generated by the mother cell and partly by the forespore, consists of a multilayered structure comprising from inside to outside: the cytoplasmic membrane, peptidoglycan (PG), an outer membrane, which is originally the cytoplasmic membrane of the mother cell and a proteinaceous coat (Driks & Eichenberger, 2016; McKenney, Driks, & Eichenberger, 2013). In response to nutrient depletion, *S. coelicolor* generate aerial hyphae, and here, multiple synchronous cell divisions give rise to the spores (Flär dh & Buttner, 2009; Sigle, Ladwig, Wohlleben, & Muth, 2015). The spore envelope of *S. coelicolor* is less well-studied but contains PG, a proteinaceous sheath made of chaplins and rodlins and spore wall teichoic acids (Flär dh & Buttner, 2009; Sigle et al., 2015). In the Gram-negative deltaproteobacterium *M. xanthus*, sporulation is also typically induced by starvation (Konovalova et al., 2010). However, in this bacterium, spores are formed independently of a cell division event and during the sporulation process, PG is replaced by a spore coat consisting mainly of polysaccharide. Here, we focus on the identification of proteins important for formation of the spore coat polysaccharide in *M. xanthus*.

In response to nutrient limitation, the rod-shaped cells of *M. xanthus* initiate a developmental programme resulting in the formation of multicellular spore-filled fruiting bodies (Konovalova et al., 2010). Fruiting bodies are formed as cells aggregate to form mounds during the first 24 hr of starvation. These mounds eventually convert into fruiting bodies as the rod-shaped cells that have accumulated inside the mounds begin to differentiate into spherically convert into fruiting bodies as the rod-shaped cells that have

none of these proteins are essential for spore formation (Curtis, Atwood, Orlando, & Shimkets, 2007; Inouye, Inouye, & Zusman, 1979b; Komano, Furuichi, Teintze, Inouye, & Inouye, 1984; Lee et al., 2011; Leng et al., 2011), lack of the spore coat polysaccharide causes a sporulation defect (Holk enbrink et al., 2014; Müller et al., 2012). Because, only cells inside fruiting bodies differentiate to spores, starvation-dependent sporulation depends on the processes that are important for aggregation of cells into mounds including intracellular and intercellular signalling cascades, exopolysaccharide (EPS), lipopolysaccharide (LPS) and motility (Konovalova et al., 2010). Interestingly, spore formation can also occur independently of starvation, that is, in the presence of nutrients, in response to addition of glyceral (Dworkin & Gibson, 1964), other alcohols (e.g., isopropanol and ethylene glycol) (Sadler & Dworkin, 1966), dimethyl sulphoxide (Komano, Inouye, & Inouye, 1980) or β-lactams and D-amino acids (O’Connor & Zusman, 1997). Spore formation by this process, often referred to as chemically induced sporulation, occurs rapidly and synchronously within 4–8 hr; these spores are not identical to the spores formed in response to starvation since the spore coat polysaccharide is thinner and several proteins that are present in starvation-induced spores are absent (Downard & Zusman, 1985; Inouye et al., 1979a, 1979b; Komano et al., 1980; Mc Cleary et al., 1991; Müller et al., 2012; Otani et al., 1998). However, the morphogenesis process associated with chemically induced sporulation involves a similar cellular remodelling process as for starvation-induced spores; the composition of the spore coat polysaccharide appears to be similar in both (Kottel, Bacon, Clutter, & White, 1975; Sutherland & Mackenzie, 1977) and formation of the spore coat polysaccharide is essential for formation of both types of spores (Licking, Gorski, & Kaiser, 2000; Müller et al., 2012).

Synthesis of the *M. xanthus* spore coat polysaccharide involves the ExoA-I proteins and the NfsA-H/AglQRS systems (Holk enbrink et al., 2014; Licking et al., 2000; Müller et al., 2012; Ueki & Inouye, 2005; War tel et al., 2013). The ExoA-I proteins, encoded by the exoA-I locus, were suggested to be components of an incomplete Wzx/Wzy-dependent pathway for polysaccharide synthesis and export (Table S1 and Figure 1a) (Holk enbrink et al., 2014; Müller et al., 2012; Schmid et al., 2015; Valvano, 2011, Valvano, Furlong, Mackenzie, 1977) and formation of the spore coat polysaccharide is essential for formation of both types of spores (Licking, Gorski, & Kaiser, 2000; Müller et al., 2012).

In response to nutrient limitation, the rod-shaped cells of *M. xanthus* initiate a developmental programme resulting in the formation of multicellular spore-filled fruiting bodies (Konovalova et al., 2010). Fruiting bodies are formed as cells aggregate to form mounds during the first 24 hr of starvation. These mounds eventually convert into fruiting bodies as the rod-shaped cells that have accumulated inside the mounds begin to differentiate into spherical desiccation-, heat- and sonication-resistant spores. Spore morphogenesis occurs from ~24 hr and over the next 48 hr; in this process, the ~7 μm × ~0.5 μm rod-shaped cells are remodelled to become shorter and wider, ultimately forming spherical spores with a diameter of 1–2 μm (Dworkin & Gibson, 1964; Dworkin & Voelz, 1962). The PG cell wall is removed during this cellular remodelling process; in parallel, the spore coat is synthesised (Bui et al., 2009; Holkenbrink, Hoiczky, Kahtn, & Higgs, 2014; Müller, Schink, Hoiczky, Cserti, & Higgs, 2012). The spore coat consists of a thick layer of polysaccharide and several proteins outside of the outer membrane (OM) (Inouye, Inouye, & Zusman, 1979a; Leng, Zhu, Jin, & Mao, 2011; McCleary, Esmon, & Zusman, 1991). While
by a polyisoprenyl-phosphate hexose-1-phosphate transferase (PHPT) or a polyisoprenyl-phosphate N-acetylhexosamine-1-phosphate transferase (PNPT). Next, specific glycosyltransferases (GTs) transfer the additional sugar building blocks from nucleotide-sugar donors to the Und-PP-sugar primer molecule to generate the Und-PP-repeat unit, which can be further modified by additional enzymes. Individual repeat units are transported across the inner membrane (IM) by the Wzx flippase, assembled into the polysaccharide by the Wzy polymerase together with a polysaccharide co-polymerase (PCP) protein and transported across the OM by a Wza OM polysaccharide export (OPX) protein (Valvano et al., 2011). In the Exo pathway (Figure 1a), ExoE is a predicted PHPT responsible for priming synthesis of individual repeat units (Holkenbrink et al., 2014). The integral membrane protein ExoC together with the cytoplasmic ExoD tyrosine kinase form part of a bipartite Wzc protein of the PCP-2 family, in which ExoD (formerly...
BtkA (Kimura, Yamashita, Mori, Kitajima, & Takegawa, 2011) is thought to participate in regulating ExoC activity (Holkenbrink et al., 2014; Kimura et al., 2011). ExoA (formerly FdgA [Ueki & Inouye, 2005]) is a homolog of Wza OPX proteins (Holkenbrink et al., 2014). ExoG and Exol are N-acetyltransferase homologs that could be involved in modifying sugars before or after incorporation into the Und-PP-repeat units before export; ExoH is homologous to aminotransferases. ExoF is a putative gluconeogenesis factor and ExoB is an OM β-barrel protein of unknown function (Holkenbrink et al., 2014). All Exo proteins except for ExoF are essential for sporulation and synthesis of an intact spore coat polysaccharide (Holkenbrink et al., 2014; Licking et al., 2000; Ueki & Inouye, 2005). Generally, Wzc proteins of the PCP-2 family are components of Wzx/Wzy-dependent pathways for polysaccharide synthesis and export (Morona, Purins, Tocilj, Matte, & Cygler, 2009) supporting the notion that the ExoA-I proteins are part of a Wzx/Wzy pathway. Notably, such an Exo pathway is incomplete and lacks several key enzymes including the GTs that add sugars from nucleotide-sugar donors to the Und-PP-sugar primer molecule, the Wzx flippase and the Wzy polymerase (Figure 1a).

Here, we report the identification of two additional gene clusters encoding seven proteins that have homology to enzymes involved in polysaccharide synthesis and/or modification, and show that they are essential for sporulation and by implication for synthesis of the spore coat polysaccharide. We identify MXAN_3260 as the Wzx flippase (renamed ExoM) and MXAN_3026 (renamed to ExoJ) as the Wzy polymerase. We also identify five additional proteins important for spore coat polysaccharide synthesis including three GTs and determine the nucleotide sugar specificity of the ExoE priming enzyme, thus, generating a more complete model of the Exo pathway for spore coat polysaccharide biosynthesis.

2 RESULTS

2.1 Identification of two loci encoding a Wzx flippase, a Wzy polymerase and other proteins involved in polysaccharide synthesis

To identify missing components for spore coat polysaccharide biosynthesis, we used a two-pronged strategy. First, as polysaccharide biosynthesis genes are often clustered (Rehm, 2010), we searched for homologs of the missing components in the M. xanthus genome. Because, the genome encodes at least 66 GTs, we specifically searched for Wzx flippase and Wzy polymerase homologs (see Section 4). A domain search suggested that the M. xanthus genome encodes three Wzx flippase homologs (MXAN_1035, MXAN_3260 and MXAN_7416) and four Wzy_C domain proteins (MXAN_1052, MXAN_2919, MXAN_3026 and MXAN_7442). Second, because expression of the exoA-I and nfsA-H genes is induced in response to starvation and chemical induction of sporulation (Giglio, Zhu, Klunder, Kummer, & Garza, 2015; Kimura et al., 2011; Licking et al., 2000; Müller, Treuner-Lange, Heider, Huntley, & Higgs, 2010; Ueki & Inouye, 2005; Wartel et al., 2013), we identified those candidate genes whose transcription pattern was similar to that of the exoA-I and nfsA-H genes during chemically induced sporulation using published data (Müller et al., 2010). Among the seven candidate genes, only the genes for the Wzx homolog MXAN_3260 and the Wzy_C domain protein MXAN_3026 were upregulated (Figure 1b) suggesting these two proteins could be the missing Wzx flippase and Wzy polymerase, respectively, for spore coat polysaccharide synthesis.

Further, mutation of MXAN_1035 was previously reported to only slightly affect spore formation (Holkenbrink et al., 2014), while MXAN_1052 is in the same polysaccharide biosynthesis gene cluster as MXAN_1035, and therefore, likely also not involved in spore coat synthesis. MXAN_7416 and MXAN_7442 are part of the eps locus, which is important for EPS synthesis (Lu et al., 2005). Finally, MXAN_2919 is the WaaL O-antigen ligase involved in LPS synthesis (Pérez-Burgos, García-Romero, Jung, Valvano, & Søgaard-Andersen, 2019). Therefore, we investigated the Wzx flippase homolog MXAN_3260 and the Wzy polymerase homolog MXAN_3026 for a potential role in spore coat polysaccharide synthesis.

Sequences of Wzx flippases and Wzy polymerases are not well conserved, but both are membrane proteins with a high number of transmembrane helices (TMHs) (Hug & Feldman, 2011; Raetz & Whitfield, 2002). Sequence analysis of MXAN_3260 revealed a PF13440 (Polysacc_synth_3) domain (Figure 1c), similar to the LPS O-antigen Wzx flippase of Yersinia similis serotype O:9 (Beczala et al., 2015) and a PF14667 (Polysacc_synth_C) domain. The protein had also 11 or 12 predicted TMHs according to TMHMM and SPOCSTOPUS, respectively (Figure 1c), as found in other flippases (Valvano, 2011). The Wzy polymerase candidate MXAN_3026
contains a PF04932 (Wzy_C) domain (Figure 1c), which is also found in O-antigen ligases. Wzy polymerases and O-linked oligosaccharidyltransferases (Hug & Feldman, 2011; Schild, Lampeacht, & Reidl, 2005) and multiple TMHs whose topology depended on the prediction programme used (Figure 1c).

MXAN_3260 and MXAN_3026 are encoded by genes in two distinct gene clusters that we renamed to exo gene cluster III and II, while we renamed the exoA-I gene cluster to Exo gene cluster I (Figure 1d). Analysis of the genetic neighbourhood of MXAN_3260 and MXAN_3026 (Figure 1d; Table S1) showed that MXAN_3262, MXAN_3263 and MXAN_3027 are putative GTs, each containing a single GT4 domain according to the CAZy database. MXAN_3259 is a polysaccharide deacetylase homolog while MXAN_3261 is a serine O-acetyltransferase homolog. In the three exo gene clusters, all genes for which published microarray data are available are upregulated during chemically induced sporulation with 0.5 M glycerol (Figure 1e) (Müller et al., 2010). As discussed in details below, exo gene cluster I and III make up one cluster in Vulgatibacter incomptus and all three clusters are present as one cluster in Anaeromyxobacteraceae supporting the idea that the gene products of the three clusters may function together.

As shown below, the genes of exo gene cluster II and III are important for sporulation and our data support that the encoded proteins form part of the same machinery. For simplicity and to facilitate identification of the genes throughout this study, we renamed MXAN_3026, MXAN_3027 and MXAN_3259-MXAN_3263 to ExoJ-P following the Exo nomenclature (Holkenbrink et al., 2014; Müller et al., 2012) (Figure 1d).

### 2.2 ExoJ-ExoP are important for chemically induced sporulation

To determine the importance of the seven exoJ-P genes in sporulation, we generated in-frame deletion mutations in each of the genes separately and determined their importance for sporulation using chemical induction (Figure 2a,b). After addition of glycerol to a final concentration of 0.5 M, wild-type (WT) cells rounded up during the first 4 hr and had turned into phase-bright resistant spores by 24 hr. Cells of the ΔexoE mutant, which cannot produce spore coat polysaccharide (Holkenbrink et al., 2014), served as a negative control. As previously described (Holkenbrink et al., 2014), ΔexoE cells initially shortened becoming ovoid by 4 hr; by 24 hr, most ΔexoE cells had reverted to a non-phase-bright rod-shape while a few remained non-phase-bright ovoid-shaped or were branched and non-phase-bright. ΔexoE cells were not resistant to heat and sonic treatment. The ΔexoM and ΔexoJ mutants formed large round cells by 4 hr; by 24 hr, many cells had reverted to rod-shape, however, a significant fraction were ovoid and a few were branched or had turned into large spheres. None of these cells were phase-bright or resistant to heat and sonic treatment. Finally, the ΔexoN mutant formed phase-bright spores that were resistant to heat and sonic treatment but at a two-fold reduced level compared to WT; moreover, a significant fraction of cells at 24 hr were non-phase-bright rod-shaped or ovoid while a small fraction were branched or formed large spheres. Sporulation of all eight in-frame deletion mutants was restored by ectopic expression of the corresponding full-length gene under the control of the native promoter (Pnuc) on a plasmid integrated in a single copy at the Mx8 attB site (Figures 1d and 2).

We conclude that all seven ExoJ-P proteins, except ExoN, are essential for chemically induced sporulation. These data agree with the idea that ExoM is the Wzx flippase, ExoJ the Wzy polymerase, ExoK/O/-P GTs and ExoL a polysaccharide deacetylase homolog while MXAN_3261 is a serine O-acetyltransferase homolog. In the three exo gene clusters, all genes for which published microarray data are available are upregulated during chemically induced sporulation with 0.5 M glycerol (Figure 1e) (Müller et al., 2010). As discussed in details below, exo gene cluster I and III make up one cluster in Vulgatibacter incomptus and all three clusters are present as one cluster in Anaeromyxobacteraceae supporting the idea that the gene products of the three clusters may function together.

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#### 2.3 Loss of ExoE and ExoJ-ExoP neither affects EPS and LPS synthesis, cell morphology nor motility

In addition to the spore coat polysaccharide, M. xanthus synthesises two additional polysaccharides, that is, EPS and LPS, both of which are important for fruiting body formation. Because, blocking synthesis of one glycan polymer can affect synthesis of other polymers including PG by sequestration of Und-P through accumulation of Und-PP intermediates (Burrows & Lam, 1999; Jorgenson, Kannan, Laubacher, & Young, 2016; Jorgenson & Young, 2016; Ranjit & Young, 2016; Valvano, 2008), we determined whether lack of Exo proteins interferes with EPS, LPS or PG synthesis during growth.

EPS synthesis was tested using nutrient-rich agar supplemented with Congo red. As a result of binding of the dye to EPS, WT colonies acquired a red colour while the negative control, a ΔωfE mutant (Yang, Geng, Xu, Kaplan, & Shi, 1998), did not (Figure 3a). All exo mutants tested accumulated EPS at WT level. LPS was extracted from cell extracts from growing cells and detected by Emerald green staining. For WT as well as all tested exo mutants, a fast running lipid A-core band and polymeric O-antigen bands were detected while only the lipid A-core band was detected in the Δωbap negative control strain, which is impaired in O-antigen synthesis (Pérez-Burgos et al., 2019) (Figure 3b). Because, interference with PG synthesis causes the formation of abnormally shaped cells including filamentous cells in the presence of nutrients (Schumacher et al., 2017; Treuner-Lange et al., 2013, 2015), we used cell morphology as a proxy for PG synthesis to test whether lack of the Exo proteins
interferes with PG synthesis during growth. Lack of the tested components of the Exo machinery did not affect cell morphology (Figure 2 (0 hr) and 3c), with the exception of ΔexoL cells, which were marginally but significantly ($p = .029$) shorter than WT. Therefore, loss of spore coat polysaccharide synthesis does not interfere with EPS, LPS or PG synthesis during growth in agreement with the observation that exo gene expression is induced during sporulation. Moreover, these observations support that the Exo machinery is dedicated to spore coat synthesis and that the EPS, LPS and PG machineries function independently of the Exo proteins during growth.

*M. xanthus* possesses two distinct motility systems that are important for fruiting body formation; one of them depends on type IV pili (T4P) and the other one depends on the Agl/Glt gliding motility complexes (Schumacher & Søgaard-Andersen, 2017; Zhang, Ducret, Shaevitz, & Mignot, 2012). EPS and LPS are important for motility (Lu et al., 2005; Pérez-Burgos et al., 2019). On 0.5% agar, which favours T4P-dependent motility, WT displayed the flares characteristic of this type of motility; by contrast, the negative control ΔpilA strain, which lacks the major pilin of T4P (Wu & Kaiser, 1996), did not. On 1.5% agar, which favours gliding motility, single cells were

**FIGURE 2** Chemically induced sporulation in Δexo mutants. (a) Sporulation was induced by addition of glycerol to a final concentration of 0.5 M. At 0, 4 and 24 hr, cell morphology was observed by phase contrast microscopy. In images labelled resistant spores, cells were exposed to sonic and heat treatment before microscopy. Sporulation frequency after sonic and heat treatment is indicated as the mean ± SD from three biological experiments relative to WT. P native (Pnat) is short for the native promoter and is used throughout the study. Scale bars, 5 µm. (b) Quantification of cell morphology at 24 hr before sonic and heat treatment relative to WT (100%); $n = 300$ combined from three biological replicates.
observed at the colony edge of WT, while the ΔaglQ mutant, which lacks an essential component of the gliding machinery motor (Nan et al., 2013; Sun, Wartel, Cascales, Shaevitz, & Mignot, 2011), had a smooth edge. By contrast, all tested exo mutants were indistinguishable from WT (Figure S1; Table 1). Together, these results indicate that loss of the Exo machinery does not interfere with motility during growth.

### 2.4 ExoJ-M and ExoO-P are important for starvation-induced sporulation

Having shown that the Exo proteins are neither important for EPS, LPS or PG biosynthesis nor for motility during growth, we asked whether the exo mutants are able to generate starvation-induced spores during fruiting body formation. Previous analyses showed
that ΔexoA, ΔexoC, ΔexoE, ΔexoD and ΔexoJ mutants are generally able to form fruiting bodies but have a reduced sporulation efficiency (Kimura et al., 2011; Licking et al., 2000; Müller et al., 2012; Ueki & Inouye, 2005). Because, these experiments were performed under different conditions, we tested the developmental proficiency of eight exo mutants on TPM 1.5% agar and under submerged conditions.

Similar to WT, all eight exo mutants had aggregated to form fruiting bodies by 24 hr under both conditions (Figure S2). However, all exo mutants with the exception of the ΔexoN mutant had a strong sporulation defect (Figure S2; Table 1). Importantly, the sporulation defects were partially or completely complemented by ectopic expression of the relevant full-length gene from the native promoter (Figures 1d and S2; Table 1). Because, the sporulation defects of the exo mutants during chemically induced sporulation were fully complemented, we speculate that the partial complementation observed for some of the exo mutants is caused by insufficient expression of the relevant gene during starvation. As in the case of chemically induced sporulation, we conclude that ExoJ-P proteins, with the exception of ExoN, are essential for starvation-induced sporulation and by implication in formation of an intact spore coat.

2.5 | ExoE has GalNAc-1-P transferase activity

ExoE was suggested to be the PHPT homolog that initiates repeat unit biosynthesis for spore coat polysaccharide biosynthesis (Holkenbrink et al., 2014); however, it is unknown which sugar ExoE transfers to Und-P. Similarly to the Escherichia coli WcaJ Ec, which transfers Glc-1-P to Und-P and the Salmonella enterica WbaP Se, which transfers Gal-1-P to Und-P (Furlong, Ford, Albarnez-Rodriguez, & Valvano, 2015; Saldías et al., 2008), ExoE contains a PF02397 (Bacterial Sugar Transferase) domain in the C-terminus and five putative transmembrane domains (Figure 4a). By contrast, PNPTs including the E. coli WecA Ec, which transfers GlcN-1-P to Und-P, contain a PF00953 (Glycosyl transferase family 4) domain (Figure 4a) (Lehrman, 1994). In WcaJ Ec, the fifth TMH forms a helix-break-helix structure and does not fully span the IM resulting in the cytoplasmic localisation of the C-terminal catalytic domain. This depends on the residue P291 that forms part of a DX(2)P motif highly conserved among PHPTs (Furlong et al., 2015). ExoE also carries the DX(2)P motif and contains all the other conserved essential residues important for catalytic activity that have been identified in the C-terminal catalytic domain of WbaP Se (Patel, Furlong, & Valvano, 2010) (Figures 4b and S3). Thus, ExoE is a PHPT with a predicted topology similar to that described for WcaJ Ec and WbaP Se.

Compositional analysis of the spore coat polysaccharide showed that it is composed of 1-3-, 1-4-linked GalNAc, 1-4-linked Glc (GalNAc:Glc ratio 17:1) and glycine (Holkenbrink et al., 2014). These findings suggest that ExoE could use either UDP-Glc or UDP-GalNAc as a substrate. To test the activity of ExoE, we performed heterologous expression experiments in E. coli and S. enterica. To this end, we generated the plasmids pMP158 and pMP147, which encode native, untagged ExoE and a FLAG-tagged ExoE variant (FLAG-ExoE), respectively, from an arabinose-inducible promoter in plasmid pBAD24. We used native ExoE to test for ExoE activity and FLAG-ExoE to test for protein accumulation using immunoblotting.

To determine whether ExoE can use UDP-Glc, we carried out heterologous complementation experiments in a ΔwcaJEc E. coli strain, which lacks the ability to produce colanic acid as previously reported (Patel et al., 2012; Pérez-Burgos et al., 2019). For this experiment, native ExoE was synthesised in the ΔwcaJEc strain also containing pWQ499, which encodes the RcsA regulator that increases the production of colanic acid (Furlong et al., 2015). Cells growing in the absence and presence of arabinose were examined for a glossy and mucoid colony phenotype characteristic of colanic acid capsule production (Figure 4c). Only cells containing the FLAG-WcaJ Ec-encoding plasmid pLA3 exhibited the distinct mucoid phenotype representing colanic acid production, whereas the strain synthesising native ExoE or containing the pBAD24 vector control did not display this phenotype. As shown in Figure S4a, FLAG-ExoE accumulated although at a slightly lower level than FLAG-WcaJ Ec. Together, these results indicate ExoE is not a Glc-1-P transferase.

PHPT proteins were initially described as hexose-1-phosphate transferases while PNPTs are considered N-acetylhexosamine-1-phosphate transferases; however, there are several examples of proteins of the PHPT family with specificities for N-acetylated nucleotide sugars (Chamot-Rooke et al., 2007; Glover, Weerapana, Chen, & Imperiali, 2006; Merino et al., 2011; Power et al., 2000). Because, ExoE did not transfer Glc-1-P, we next investigated whether ExoE could transfer GalNAc-1-P. To this end, we performed heterologous expression experiments in E. coli in which we first tested for transfer of GalNAc-1-P and subsequently for transfer of GalNAc-1-P. GlcNAc-1-P transferase activity can be tested using the E. coli strain MV501, which has a transposon insertion in wecA Ec. As described, WecA Ec is a PNPT that uses UDP-GlcNAc for initiating synthesis of O7 polysaccharide antigen (Alexander & Valvano, 1994). Native ExoE in MV501 did not restore O7 polysaccharide synthesis (Figure 4d). In immunoblot experiments, we observed that FLAG-ExoE accumulated in MV501, especially in an oligomeric form, although at a low level (Figure S4b). By contrast, pMAV11 carrying the wecA Ec gene complemented the defect in O-antigen synthesis. These observations suggest that ExoE is not a GlcNAc-1-P transferase.

E. coli lacks the Gne epimerase, which interconverts UDP-GlcNAc and UDP-GalNAc. Previously, Merino et al. (Merino et al., 2011) demonstrated that the Aeromonas hydrophila PHPT homolog WecP Ah, in the presence of a plasmid encoding the Gne homolog from A. hydrophila modified the MV501 lipid A-core. This modification is consistent with formation of an O7 repeat containing GalNAc. This modified lipid A-core likely only contains one O7 repeat because the addition of GalNAc to the repeat may interfere with O7 polymerisation (Merino et al., 2011). Similar to Merino et al., we observed formation of this GalNAc containing O7 repeat in MV501 in the presence of plasmids encoding FLAGEcWecP Ah (pSEF88) and the A. hydrophila Gne homolog (pGEMT-Gne Ah) (Figure 4e). More importantly, co-expression of ExoE and Gne Ah in
FIGURE 4  ExoE has GalNAc-1-P transferase activity. (a) Domain and TMH prediction for WbaP_{Se}, WcaJ_{Ec}, WecA_{Ec} and ExoE. Grey rectangles indicate TMH. Numbers indicate domain borders. (b) Topology predictions for ExoE. The catalytic PF02397 domain is indicated in blue and conserved amino acids important for structure or activity are marked with orange and red, respectively. The amino acid sequence alignment of ExoE with WbaP_{Se} is shown in Figure S3. (c–g) Heterologous complementation experiments to characterise ExoE specificity. (c) E. coli XBF1 (ΔwcaJ_{Ec}) containing pWQ499 (rcsA^+) was transformed with the indicated plasmids and plated on LB agar in the absence or presence of 0.2% arabinose (ara) to induce gene expression. Cells were incubated for 24 hr at 37°C, and then, 24 hr at room temperature before scoring the mucoid phenotype. (d,e) Silver-stained polyacrylamide gels of LPS extracted from the E. coli wcaA::Tn10 mutant strain MV501 carrying the indicated plasmids. In (e), MV501 also contained the plasmid pGEM-T-Gne, which encodes the UDP-GlcNAc/UDP-GalNAc epimerase Gne_{Ah}. VW187 is the parental wcaA^{+} strain. Arabinose was added as indicated. (f) Silver-stained polyacrylamide gel and (g) immunoblot with rabbit Salmonella group B O-antigen antiserum of LPS extracted from S. enterica LT2 (WT) and the MSS2 ΔwbaP_{Se} mutant carrying the indicated plasmids. Arabinose was added as indicated.

MV501 resulted in a lipid A-core modified band similar to that observed with co-expression of pLAC_WecP_{Ah} and Gne_{Ah} (Figure 4e). This result supports that ExoE can transfer GalNAc-1-P to Und-P in E. coli.

Finally, we investigated the specificity of ExoE for UDP-Gal, in this case using the S. enterica ΔwbaP_{Se} mutant MSS2 that is blocked in the first step in O-antigen synthesis. ExoE did not restore O-antigen synthesis in MSS2 despite the FLAG-tagged variant of the
protein accumulating (Figures 4f,g and S4a). By contrast, the control plasmids pJD132 and pSM13, which encode WbaP<sub>ES</sub> of E. coli and WbaP<sub>se</sub> of S. enterica, respectively, both restored O-antigen synthesis (Figure 4f,g). Collectively, the heterologous expression experiments support that ExoE has specificity for UDP-GalNAc but lacks specificity for UDP-Glc, UDP-GlcNAc and UDP-Gal. These data, together with the observation that the spore coat polysaccharide contains Glc and GalNAc, suggest that ExoE is a GalNAc-1-P transferase forming Und-PP-GalNAc and that GalNAc is likely the first sugar added to Und-P during the biosynthesis of the spore coat polysaccharide repeat.

2.6 | The exo and nfs gene clusters co-occur only in a subset of sporulating Myxococcales

Because, the majority of the members of the order Myxococcales can sporulate (Reichenbach, 1999), we hypothesised that the Exo and Nfs machineries for formation of the rigid spore coat would be conserved in Myxococcales. We, therefore, searched for orthologs of each Exo and Nfs protein in Myxococcales with fully sequenced genomes using a reciprocal best BlastP hit method (Section 4).

Within the suborder Cystobacterineae, all individual components of the Exo machinery are conserved in F. Myxococcales, F. Archangiaceae and F. Vulgatibacteraceae, while there is somewhat less conservation, especially of cluster III, in the Anaeromyxobacteraceae (Figure 5a). By contrast, in the suborders Nannocystineae and Sorangineae, Exo orthologs were largely missing. Interestingly, in the small genomes of V. incomptus and Anaeromyxobacteraceae that only have approximately half the size of other myxobacterial genomes (Figure 5a, right), exo gene cluster I and III are organised in one cluster (V. incomptus), while all three clusters are present in one in Anaeromyxobacteraceae (Figures 5a and 5S), lending further support to the idea that these proteins function in the same pathway. The taxonomic distribution of the exo genes supports an evolutionary scenario in which the last common ancestor of the Cystobacterineae acquired the exo gene cluster, and then, over time gene organisation diversified. Alternatively, a common ancestor of the myxobacteria contained the exo gene cluster and the exo genes were lost in ancestors of the Nannocystineae or Sorangineae.

The NfsA-H proteins are paralogs of the GltA-H proteins that are important for gliding motility (Agrebi, Wartel, Brochier-Armanet, & Mignot, 2015; Wartel et al., 2013). While NfsA-H are encoded in one gene cluster, GltA-H are encoded in two gene clusters in the M. xanthus genome (Figure 5a,b). In agreement with previous analyses (Agrebi et al., 2015; Luciano et al., 2011), in which conservation of GltA-H/NfsA-H homologs were studied without distinguishing between the two machineries, orthologs of the GltA-H proteins are widely conserved in Myxococcales although with less conservation in the Nannocystineae and Sorangineae. Moreover, the two glt gene clusters are in close proximity outside of the F. Myxococcales, F. Archangiaceae and F. Vulgatibacteraceae (Figures 5a and 5Sb) as previously described for Anaeromyxobacter (Luciano et al., 2011). By contrast, our analysis shows that orthologs of NfsA-H are exclusively found in the F. Myxococcales, F. Archangiaceae and F. Vulgatibacteraceae. The taxonomic distribution of the glt and nfs gene clusters suggests that the primitive gltA-H genes were present in the last common ancestor of the Myxococcales and that the nfs cluster results from a duplication event of the ancestral gltA-H gene cluster shortly after the divergence of the Anaeromyxobacteraceae from the remaining Cystobacterineae. This agrees with a previous suggestion (Agrebi et al., 2015; Luciano et al., 2011), except that our analysis clearly supports that the primitive Glt proteins are ancestral to the Nfs proteins.

Also, our analysis shows that the exo and nfs genes co-occur in the Cystobacterineae except in the Anaeromyxobacteraceae. Interestingly, except for V. incomptus, for which no fruiting body formation and sporulation were observed (Yamamoto, Muramatsu, & Nagai, 2014), all the species containing both the exo and nfs gene clusters have been reported to form phase-bright spores. By contrast, Haliangium ochraceum, Minicytis rosea, Sorangium cellulosum and Chondromyces crocus also form spores despite they generally lack the Exo and Nfs machineries. These observations suggest that sporulation occurs by a different mechanism in the sporulating Cystobacterineae compared to sporulating Nannocystineae and Sorangineae. Consistently, Sorangineae spores have been reported to be less phase-bright than the M. xanthus spores and rod-shaped (Garcia & Müller, 2014b) and M. rosea spores are phase-dark and rod-shaped (Garcia, Gepmperlein, & Müller, 2014).

3 | DISCUSSION

Cells of M. xanthus generate at least three different polysaccharidic cell surface structures, namely LPS, EPS and the spore coat polysaccharide. Here, we focused on identifying the proteins that would function together with the ExoA-I proteins in spore coat polysaccharide biosynthesis and export.

Using bioinformatics and gene co-expression analyses, we identified two loci that encode proteins important for sporulation. One of them, named the exo gene cluster II, encodes a homolog of Wzy polymerases (ExoJ, MXAN_3026) and a predicted GT (ExoK, MXAN_3027), while the other, exo gene cluster III, encodes a predicted polysaccharide deacetylase (Exol, MXAN_3259), a Wzx flippase (ExoM, MXAN_3260), a serine O-acetyltransferases (ExoN, MXAN_3261) and two GTs (ExoQ, MXAN_3262 and ExoP, MXAN_3263). All seven proteins with the exception of ExoN, which is only partially required, are essential for sporulation, and therefore, predicted to function in formation of the intact spore coat polysaccharide. Based on these findings, we propose a revised model for spore coat polysaccharide biosynthesis (Figure 6).

The M. xanthus spore coat polysaccharide is composed of 1–3-, 1–4-linked GalNAc, 1–4-linked Glc and glycine (Holkenbrink et al., 2014) and with the latter proposed to form glycine bridges between polysaccharide chains (Holkenbrink et al., 2014). The spore coat
polysaccharide is also acetylated (Filer, White, Kindler, & Rosenberg, 1977b; Holkenbrink et al., 2014). However, the precise structure of the spore coat polysaccharide is unknown. The data of Holkenbrink et al. (Holkenbrink et al., 2014), together with our results, suggest a model in which ExoE is the PHPT homolog responsible for the first step in repeat unit synthesis by catalysing the transfer of a sugar-1-P donor to Und-P (Holkenbrink et al., 2014). Here, we demonstrate that ExoE is functionally similar to WecP_Av, a GalNAc-1-P transferase from A. hydrophila (Merino et al., 2011) in heterologous expression experiments in E. coli, suggesting that GalNAc is the first sugar of the spore coat repeat unit. Alternatively, because, several of the Exo proteins are sugar-modifying enzymes, it is also possible that ExoE has affinity for GalNAc in E. coli but incorporates a modified GalNAc as the first sugar in the repeat unit in M. xanthus. Subsequently, we

![Diagram of gene clusters in myxobacteria]

**FIGURE 5** Analysis of exo, nfs, and glt gene occurrence and organisation in myxobacteria. (a) Taxonomic distribution, co-occurrence and synteny of the exo, nfs and glt genes in Myxococcales. Left, 16S rRNA tree of Myxococcales with fully sequenced genomes. Family and suborder classification are indicated. Genome size is indicated on the tree. Used strains are listed in Table S2. S. species that form spores; (S), tested for sporulation but with ambiguous results; NT, sporulation not tested; N, sporulation tested and not observed (dos Santos et al., 2014; Fudou, Jojima, Iizuka, & Yamanaka, 2002; Garcia et al., 2014; Garcia & Müller, 2014a, 2014b; Mohr, Garcia, Gerth, Irschik, & Müller, 2012, Sanford, Cole, & Tiedje, 2002; Yamamoto et al., 2014). For the exo, nfs and glt gene clusters, a reciprocal best BlastP hit method was used to identify orthologs. Generally, the exo gene clusters are marked in green (cluster II), blue (cluster I) and red (cluster III) and the nfs and glt gene clusters in orange (nfs), light brown (glt cluster I) and dark brown (glt cluster II). To evaluate gene proximity and cluster conservation, 10 genes were considered as the maximum distance for a gene to be in a cluster. Genes found in the same cluster are marked with the same colour. If two or three gene clusters are within a distance of <10 genes, all genes are marked in the same colour (e.g., two of the exo clusters in *V. incomptus* are marked with the same colour). If two or three gene clusters are within a distance of >10 genes, all genes are marked in the same colour (e.g., two of the exo clusters in *V. incomptus* are marked with the same colour). If two or three gene clusters are within a distance of >10 genes, all genes are marked in the same colour (e.g., two of the exo clusters in *V. incomptus* are marked with the same colour). If two or three gene clusters are within a distance of >10 genes, all genes are marked in the same colour (e.g., two of the exo clusters in *V. incomptus* are marked with the same colour). If two or three gene clusters are within a distance of >10 genes, all genes are marked in the same colour (e.g., two of the exo clusters in *V. incomptus* are marked with the same colour).
predict that the GTs ExoK, ExoO and ExoP transfer sugar building blocks to the repeat unit, which is likely a tetrasaccharide. The $N$-acetyltransferase homologs ExoG and Exol, the aminotransferase homolog ExoH and the polysaccharide deacetylase homolog ExoL presumably modify sugars before or after incorporation into the repeat unit.

Based on the composition of the spore coat polysaccharide (Holkenbrink et al., 2014), we suggest that the GTs ExoK, ExoO and ExoP incorporate GalNAc and Glc into the repeat unit. Acetylation of the spore coat polysaccharide may involve the ExoG and Exol $N$-acetyltransferases (but see also below). ExoL is the first identified potential polysaccharide deacetylase implicated in $M$. xanthus spore coat synthesis. Interestingly, phase-bright spores were not detected in the exoG and exol mutants (Holkenbrink et al., 2014); similarly, the exol mutant did not form phase-bright spores (here) suggesting that proper acetylation of the spore coat polysaccharide is important for its synthesis, stability and/or function. However, it is unknown which residue is modified by ExoG, Exol and ExoL, and whether these proteins function on the same or independent targets. In Caulobacter crescentus, the polysaccharide deacetylase HfsH and the $N$-acetyltransferase HfsK affect acetylation of the holdfast polysaccharide; in the absence of any of these two proteins there is a defect in adhesive and cohesive properties of the holdfast polysaccharide without affecting its synthesis (Sprecher et al., 2017; Wan, Brown, Elliott, & Brun, 2013).

ExoH is predicted to be a pyridoxal phosphate-dependent (PLP) aminotransferase with a DegT/Dnjl/EryC1/StrS family domain (PF01041), which generally catalyses the transfer of an amino group from an amino acid to an amino acceptor (John, 1995). Similarly to the aminotransferase ArnB that transfers an amino group to arabinose in S. enterica (Noland et al., 2002) or the PLP aminotransferase Psec from Helicobacter pylori, which transfers an amino group to a sugar moiety prior to acetylation by PseH (Ud-Din, Liu, & Roujeinikova, 2015), we suggest that ExoH may add an amino group to monosaccharides before their incorporation into the repeat unit or modify sugar(s) in the repeat unit.

The glycine in the spore coat polysaccharide was proposed to form glycine bridges between polysaccharide chains (Holkenbrink et al., 2014). Holkenbrink et al. also suggested that glycine is added to the spore coat polysaccharide in the cytoplasm. Interestingly, a structure-based search with HHpred revealed that the closest homolog of the $N$-acetyltransferases ExoG and Exol is FemX from Staphylococcus aureus, that is, for ExoG and Exol, the probabilities of homology to FemX is 100% with an E-value of $1.9e-31$ and 100% with an E-value of $4.1e-30$. The Fem proteins belong to GCN5-related N-acetyltransferases (GNAT) that generally transfer acetylated molecules to an amino acceptor of different target molecules including sugars (Favrot, Blanchard, & Vergnolle, 2016; Reith & Mayer, 2011; Ud-Din et al., 2015). In S. aureus, the FemA/B/X proteins add five glycine residues to the lysine in the stem peptide of the lipid II PG precursor using glycyl-charged tRNA molecules as substrates (Favrot et al., 2016). The pentaglycine modification crosslinks PG glycan chains (Favrot et al., 2016). Therefore, it is tempting to speculate that one or both of ExoG and Exol rather than being involved in acetylation of the spore coat repeat unit could be involved in glycine addition to amino group(s) in the repeat unit. In this context, we speculate that the amino group added by ExoH could serve as an acceptor for glycine transfer. This is also consistent with the absence of glycine modified sugars after acid hydrolysis of the spore coat polysaccharide (Holkenbrink et al., 2014). Amino acid modified sugars, in this case with serine, have also been identified in the K40 capsular polysaccharide of E. coli O8 and the modification demonstrated to be essential for the polymerisation of the capsular repeat unit (Amor, Yethon, Monteiro, & Whitfield, 1999).

Two Exo proteins are important, but not essential, for formation of phase-bright spores and by implication spore coat synthesis: ExoF (Holkenbrink et al., 2014) and ExoN (here). ExoN is a putative serine O-acetyltransferase, which are commonly involved in the first step
of cysteine synthesis from serine. *M. xanthus* utilises amino acids and lipids as carbon and energy sources and does not grow on carbohydrates because it lacks required catabolic enzymes (Dworkin, 1962; Hemphill & Zahler, 1968; Watson & Dworkin, 1968). During glycerol-induced sporulation, genes for large portions of the tricarboxylic acid cycle are downregulated, whereas genes for the glyoxylate shunt and gluconeogenesis are upregulated (Müller et al., 2010), for example, the activity of at least six enzymes putatively involved in synthesis of the major spore coat component UDP-GalNAc increases in response to glycerol addition prior to shortening of cells (Filer, Kindler, & Rosenberg, 1977a). Given these metabolic changes, we speculate that ExoN may contribute to synthesis of monosaccharides or other metabolites important for spore coat polysaccharide synthesis, and therefore, without ExoN, cells may lack this precursor(s). The *M. xanthus* genome encodes two additional serine O-acetyltransferase homologs (*MXAN_1572* and *MXAN_7449*), which may function redundantly with ExoN, and therefore, the ΔexoN mutant is still able to form some phase-bright spores. Similarly, the partially dispensable ExoF, which is a putative gluconeogenesis factor, has been suggested to be important for biosynthesis of activated sugar precursors (Holkenbrink et al., 2014).

After the repeat unit has been synthesised on the cytoplasmic side of the IM, translocation occurs via the Wzx flippase homolog ExoM. In the periplasm, the Wzy polymerase ExoJ elongates the chain with the help/control of the Wzc homolog formed by the integral membrane protein ExoC and the cytoplasmic ExoD tyrosine kinase, which could regulate ExoC activity (Kimura et al., 2011). Subsequently, the polysaccharide chain is transported to the cell surface via the Wza OPX homolog ExoA. The Nfs machinery modifies the Exo-generated polysaccharide by an unknown mechanism to generate shorter polysaccharide chains and the rigid polysaccharide spore coat. How, where and when the Nfs proteins do this is not known.

Disruption of the synthesis of one polysaccharide can have pleiotropic effects on the synthesis of other polysaccharidic molecules. It was previously shown that *M. xanthus* cells lacking the PHPT WbaP for LPS O-antigen synthesise EPS and spore coat and has a normal cell morphology; similarly, mutants that do not synthesise EPS, synthesise LPS and the spore coat polysaccharide (Holkenbrink et al., 2014; Lu et al., 2005; Pérez-Burgos et al., 2019). Here, we show that during growth mutants that are unable to synthesise the spore coat polysaccharide synthesise WT levels of LPS and EPS and have a normal cell morphology in the absence of glycerol. Together, these observations suggest the existence of dedicated biosynthesis machineries for LPS, EPS and spore coat polysaccharide synthesis.

A fascinating aspect of the sporulation process in *M. xanthus* is that the PG is degraded during spore morphogenesis (Bui et al., 2009). It has been suggested that the spore coat protects cells from bursting due to intracellular turgor in the absence of PG (Bui et al., 2009; Müller et al., 2012). Therefore, we predict that the removal of PG must be closely coordinated with synthesis of the spore coat polysaccharide. Previous research on chemical induction of sporulation in the exoA-I mutants showed that mutant cells initiate the sporulation process with cell shortening and widening. However, at a certain point the sporulation process is aborted and cells regain rod-shape even in the continued presence of glycerol. Of note, after abortion of the sporulation process, many cells display severe morphological defects including branching, formation of spiral-shaped cells and formation of large spherical cells (Holkenbrink et al., 2014; Müller et al., 2012). Here, we observed similar morphological defects in mutants impaired in spore coat polysaccharide synthesis after chemical induction of sporulation. Interestingly, cells lacking the PHPT ExoE have less severe shape defects after 4 and 24 hr of glycerol-induction than mutants lacking enzymes suggested to act downstream of the priming step. These observations have two implications. First, we speculate that the abortion of the sporulation process in the exo mutants (as opposed to cell lysis due to lack of PG as well as spore coat) is caused by a coupling between spore coat polysaccharide synthesis and the PG removal process. Therefore, in the absence of proper spore coat polysaccharide synthesis, PG would not be completely removed and cells regain rod-shape through de novo synthesis of PG. Because, the Exo proteins are not important for PG synthesis during growth, we speculate that the coupling between spore coat polysaccharide synthesis and the PG removal process is regulatory rather than involving shared proteins. Second, we speculate that in the absence of ExoE PHPT activity, Und-P is not sequestered in intermediates for spore coat polysaccharide biosynthesis, and therefore, PG can be resynthesised. By contrast, in the ΔexoJ-M, O-P mutants (here) and the previously described ΔexoA-D, G-I (Holkenbrink et al., 2014) mutants, Und-P would be sequestered in intermediates for spore coat polysaccharide, thus, titrating Und-P away from PG metabolism resulting in more cells with an abnormal shape. A future goal will be to understand how spore coat polysaccharide synthesis and PG removal are coordinated.

Our analysis of the taxonomic distribution of the exo and nfs gene clusters lend support to the notion that the spore coat could be synthesised by a different mechanism in sporulating Cystobacterineae compared to sporulating Nanocystineae and Sorangineae. Based on a comparison of gene content in four fruiting body and sporulating Myxococcales (*M. xanthus*, *Stigmatella aurantiaca*, *H. ochraceum*, *S. cellulosum*), we previously reported that key developmental regulators in *M. xanthus* are not widely conserved outside the Cystobacterineae (Huntley et al., 2011). This finding also suggests that the genetic programmes for fruiting body formation and sporulation in *M. xanthus* and *S. aurantiaca* are highly similar but significantly different from the genetic programme directing fruiting body formation in *S. cellulosum* and *H. ochraceum* (Huntley et al., 2011). The distribution of the exo and nfs genes supports this scenario also at the level of the spore coat formation. Thus, it remains an open question whether fruiting body formation including sporulation in the Myxococcales is the result of convergent evolution or divergent evolution from a shared primordial genetic programme (Huntley et al., 2011).
4 | EXPERIMENTAL PROCEDURES

4.1 | Strains and cell growth

*M. xanthus* cells were grown in 1% CTT (1% w/v Bacto Casitone, 10 mM of Tris-HCl pH 8.0, 1 mM of K₂HPO₄/KH₂PO₄ pH 7.6 and 8 mM of MgSO₄) liquid medium or on 1.5% agar supplemented with 1% CTT at 32°C (Hodgkin & Kaiser, 1977). Oxytetracycline and kanamycin were used at final concentrations of 10 μg/ml and 50 μg/ml, respectively. All *M. xanthus* strains are derivatives of the WT strain DK1622 (Kaiser, 1979). *M. xanthus* strains and plasmids used in this work are listed in Tables 2 and 3, respectively. In-frame deletions were generated as described previously (Shi et al., 2008) and plasmids for complementation experiments were integrated in a single copy by site-specific recombination into the Mx8 attB site. All 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 in Luria-Bertani medium (LB) (10 mg of tryptone ml⁻¹, 5 mg of yeast extract/ml; 5 mg of NaCl/ml) at 37°C. When required, medium was supplemented with ampicillin, tetracycline, kanamycin or chloramphenicol at final concentrations of 100, 20, 40 and 30 μg/ml, respectively. Electroporation was used to introduce plasmids for heterologous complementation into MSS2, XBF1 and MV501 strains (Dower, Miller, & Ragsdale, 1988).

| TABLE 2 | Strains used in this work |
| --- | --- |
| **Strain** | **Genotype** | **Reference** |
| **M. xanthus** |  |  |
| DK1622 | Wildtype | (Kaiser, 1979) |
| DK10410 | ΔpilA | (Wu & Kaiser, 1997) |
| SA5923 | ΔaglQ | (Jakobczak, Keilberg, Wuichet, & Søgaard-Andersen, 2015) |
| SW501 | diff::Km’ | (Yang et al., 1998) |
| SA7450 | ΔwbaP | (Pérez-Burgos et al., 2019) |
| SA7495 | ΔexoE | (Pérez-Burgos et al., 2019) |
| SA8534 | ΔexoE attB::pMP136 (Pnat exoE) | This study |
| SA7455 | ΔMXAN_3026 | This study |
| SA7489 | ΔMXAN_3026 attB::pJJ18 (Pnat MXAN_3026) | This study |
| SA8507 | ΔMXAN_3260 | This study |
| SA8502 | ΔMXAN_3260 attB::pJJ18 (Pnat MXAN_3260) | This study |
| SA8516 | ΔMXAN_3267 | This study |
| SA8523 | ΔMXAN_3270 attB::pMP125 (Pnat MXAN_3270) | This study |
| SA8519 | ΔMXAN_3259 | This study |
| SA8522 | ΔMXAN_3259 attB::pMP126 (Pnat MXAN_3259) | This study |
| SA8527 | ΔMXAN_3261 | This study |
| SA8528 | ΔMXAN_3261 attB::pMP133 (Pnat MXAN_3261) | This study |
| SA8547 | ΔMXAN_3262 | This study |
| SA8548 | ΔMXAN_3262 attB::pMP134 (Pnat MXAN_3262) | This study |
| SA8521 | ΔMXAN_3263 | This study |
| SA8531 | ΔMXAN_3263 attB::pMP135 (Pnat MXAN_3263) | This study |
| **E. coli** |  |  |
| DH5α | F’ phi80lacZΔM15 endA recA hsdR(rK- mK-) nupG thi glnV deoR gyrA relA1 Δ(lacZYA-argF)U169 | Lab stock |
| Mach1 | ΔrecA1398 endA1 tonA Φ80ΔlacM15 ΔlacX74 hsdR(rK- mK-) | Invitrogen |
| XBF1 | W3110, ΔwcaJ::aph, Km’ | (Patel et al., 2012) |
| VW187 | O7:K1, clinical isolate | (Valvano & Crosa, 1984) |
| MV501 | VW187, wecA::Tn10 Tc’ | (Alexander & Valvano, 1994) |
| Salmonella |  |  |
| LT2 | WT, *S. enterica* serovar Typhimurium | S. Maloy |
| MSS2 | LT2, ΔwbaP::cat Cm’ | (Saldías et al., 2008) |
4.2 | Motility assays

Exponentially growing cultures of *M. xanthus* were harvested (6,000 g, room temperature (RT)) and resuspended in 1% CTT to a calculated density of $7 \times 10^9$ cells/ml. About 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 visualised after 24 hr using a M205FA Stereomicroscope (Leica) and imaged using a Hamamatsu ORCA-flash V2 Digital CMOS camera (Hamamatsu Photonics). Pictures were analysed using Metamorph® v 7.5 (Molecular Devices).

4.3 | Glycerol-induced sporulation assay

Assay was performed as described (Müller et al., 2010) with a slightly modified protocol. Briefly, cells were cultivated in 10 ml of CTT and induced at a density of $3 \times 10^8$ cells/ml with glycerol to a final concentration of 0.5 M. At 0, 4 and 24 hr cell morphology was observed by placing 5 µl of cells on a thin 1.5% agar TPM pad on a slide, immediately covered with a coverslip and imaged. To determine the efficiency of glycerol-induced sporulation, 5 ml of the culture were harvested (10 min, 4,150 g, RT) after 24 hr induction, resuspended in 1 ml of sterile water, incubated at 50°C for 2 hr, and then, sonicated with 30 pulses, pulse 50%, amplitude 75% with a UP200St sonifier and microtip (Hielscher). About 5 µl of the treated samples were placed on a glass slide, covered with a coverslip and imaged. Sporulation levels were determined as the number of sonication- and heat-resistant spores relative to WT using a Helber bacterial counting chamber (Hawksley, UK).

4.4 | Development

Exponentially growing *M. xanthus* cultures were harvested (3 min, 6,000 g at RT) and resuspended in MC7 buffer (10 mM of MOPS pH
7.0, 1 mM of CaCl2) to a calculated density of $7 \times 10^5$ cells/ml. About 10 µl of aliquots of cells were placed on TPM agar (10 mM of Tris-HCl pH 7.6, 1 mM of K2HPO4/KH2PO4, pH 7.6, 8 mM of MgSO4), while for development in submerged culture, 50 µl of aliquots were mixed with 350 µl of MC7 buffer and placed in a 24-well polystyrene plate (Falcon). Cells were visualised at the indicated time points using a M205FA Stereomicroscope (Leica) and imaged using a Hamamatsu ORCA-flash V2 Digital CMOS camera (Hamamatsu Photonics) and a DMi8 Inverted microscope and DFC9000 GT camera (Leica). After 120 hr, cells were collected and incubated at 50°C for 2 hr, and then, sonicated as described for chemically induced spores. Sporulation levels were determined as the number of sonication- and heat-resistant spores relative to WT using a Helber bacterial counting chamber (Hawksley, UK).

### 4.5 Detection of EPS accumulation

EPS accumulation was detected as in (Pérez-Burgos et al., 2019). Briefly, exponentially growing cells were harvested, and resuspended in 1% CTT to a calculated density of $7 \times 10^5$ cells/ml. About 20 µl aliquots of cell suspensions were placed on 0.5% CTT 0.5% agar supplemented with 40 µg/ml Congo red. The plates were incubated at 32°C and documented at 24 hr.

### 4.6 LPS extraction and detection

LPS was extracted from M. xanthus and visualised by Emerald staining as previously described (Pérez-Burgos et al., 2019). LPS from S. enterica and E. coli was extracted and visualised by silver staining as previously described (Marolda, Lahiry, Vines, Saldias, & Valvano, 2006; Pérez-Burgos et al., 2019). For S. enterica, O-antigen was detected by immunoblot using rabbit Salmonella O antiserum group B (Difco, Beckton Dickinson ref. number 229481) (1:500) and the secondary antibody IRDye 800CW goat α-rabbit immunoglobulin G (1:10,000) (LI-COR) (Pérez-Burgos et al., 2019).

### 4.7 Cell length determination

About 5 µl aliquots of exponentially growing cultures were spotted on 1.5% agar supplemented with 0.2% CTT, immediately covered with a cover slide, imaged using a DMi8 Inverted microscope and DFC9000 GT camera (Leica) and cell length determined and visualised as described (Pérez-Burgos et al., 2019).

### 4.8 Detection of colanic acid biosynthesis

E. coli strains were grown at 37°C overnight on LB plates with antibiotics plus 0.2% (w/v) arabinose, when needed, to induce protein synthesis. Incubation was prolonged to 24–48 hr at RT to visualise the mucoid phenotype (Furlong et al., 2015).

### 4.9 Immunoblot analysis

Total cell extracts were prepared and FLAG-tagged proteins detected by immunoblot analysis as previously described using α-FLAG M2 monoclonal antibody (Sigma) (1:10,000) and a secondary antibody, IRDye 800CW Goat α-Mouse IgG (H + L), 0.5 mg (LI-COR) (1:10,000) (Pérez-Burgos et al., 2019).

### 4.10 Bioinformatics

The KEGG SSDB (Sequence Similarity DataBase) (Kanehisa & Goto, 2000) database was used to identify Wzx homologs (PF01943-Polysacc_synt and PF13440-Polysacc_synt_3) and Wzy_C (PF04932) domain containing proteins. KEGG SSDB was also used to identify homologs of Exo, Nfs and Glt proteins in other Myxococcales using a reciprocal best BlastP hit method. UniProt (The-UniProt-Consortium, 2019) and the KEGG databases were used to assign functions to proteins (Figure 1d; Table S1). SMART (smart.embl-heidelberg.de) (Letunic, Doerks, & Bork, 2015) and the Carbohydrate Active Enzymes (CAZY) database (http://www.cazy.org/) (Lombard, Golaconda Ramulu, Drula, Coutinho, & Henrissat, 2014) were used to identify protein domains. Membrane topology was assessed by TMHMM v2.0 (Sonhammer, Hejne, & Krogh, 1998) and SPOCTOPUS (Viklund, Bernsel, Skwark, & Elofsson, 2008). Structure-based searches with HHPred were done using the https://toolkit.tuebingen.mpg.de/ (Zimmermann et al., 2018), Clustal Omega (Chojnacki, Cowley, Lee, Fox, & Lopez, 2017) was used to align protein sequences.

### 4.11 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.
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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

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