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Phenotypic and Transcriptomic Characterization of *Bacillus subtilis* Mutants with Grossly Altered Membrane Composition▽†

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The *Bacillus subtilis* membrane contains diacylglycerol-based lipids with at least five distinct headgroups that together help to define the physical and chemical properties of the lipid bilayer. Here, we describe the phenotypic characterization of mutant strains lacking one or more of the following lipids: glycolipids (*ugtP* mutants), phosphatidylethanolamine (*pssA* and *psd* mutants), lysylphosphatidylglycerol (*mprF*), and cardiolipin (*ywnE* and *ywjE*). Alterations of membrane lipid headgroup composition are generally well-tolerated by the cell, and even severe alterations lead to only modest effects on growth proficiency. Mutants with decreased levels of positively charged lipids display an increased sensitivity to cationic antimicrobial compounds, and cells lacking glycolipids are more sensitive to the peptide antibiotic sublancin and are defective in swarming motility. A quadruple mutant strain (*ugtP pssA mprF ywnE*), with a membrane comprised predominantly of phosphatidylglycerol, is viable and grows at near-wild-type rates, although it forms long, coiled filaments. Transcriptome comparisons identified numerous regulons with altered expression in cells of the *ugtP* mutant, the *pssA mprF ywnE* triple mutant, and the *ugtP pssA mprF ywnE* quadruple mutant. These effects included a general decrease in expression of the SigD and FapR regulons and increased expression of cell envelope stress responses mediated by σ^M and the YvrGHb two-component system.

The cytoplasmic membrane forms an essential permeability barrier and is a defining feature of life. In most bacteria, the membrane is a lipid bilayer composed of fatty acid esters with *sn*-glycerol-3-phosphate (46). These complex lipids vary not only in the length and modifications of the acylated fatty acids but also in the composition of their headgroups, which differ significantly in charge and propensity to form nonbilayer structures. The membrane of the soil bacterium *Bacillus subtilis* is a complex structure comprised mainly of the anionic phospholipid phosphatidylglycerol (PG) and the zwitterionic phosphatidylethanolamine (PE). Other components include a relatively large amount (~30%) of neutral glycolipids (GL), a variable amount of positively charged lysylphosphatidylglycerol (LPG), and a small amount of anionic cardiolipin (CL) (Fig. 1).

The complex composition of the membrane is presumed to be important because the physical properties of membrane lipids influence many cell processes, including division (43), DNA replication (22), and protein transport (13). For example, the anionic lipids PG and CL mediate the cycling of the DNA replication protein DnaA from the ADP-DnaA to ATP-DnaA (active) state and inhibit the binding of DnaA to *oriC* via sequestration (7). *Escherichia coli* cells with reduced amounts of anionic phospholipids accumulate outer membrane proteins in the cytoplasm, and some proteins insert incorrectly into the membrane (60). Furthermore, CL and PE facilitate the formation of nonbilayer structures (13) that are important in cell division and sporulation (35). Recent evidence suggests that these various lipid species may assort into spatially distinct lipid microdomains (56), which in turn may affect the localization of membrane proteins (35). In *B. subtilis* CL-rich and PE-rich domains are localized to the septal regions and the poles (26, 41), corresponding with the FtsZ-dependent subcellular localization of the lipid biosynthesis enzymes PssA, YwnE, and PgsA (phosphatidylglycerophosphate synthase) (41). Moreover, recent results suggest that anionic lipids may assemble into a spiral structure along the long axis of the cell (3).

In *B. subtilis*, membrane lipids are synthesized from the common precursor phosphatidic acid (PA) (Fig. 1). In the case of PE, PA is converted to CDP-diacylglycerol (CDP-DAG), which is condensed with serine and then rapidly decarboxylated to generate PE. Condensation of CDP-DAG with glycerol-3-phosphate followed by removal of the phosphate leads to PG, the only essential complex lipid in *B. subtilis*. PG can be further modified to form two minor complex lipids, CL and LPG. CL is formed by the condensation of two PG molecules by cardiolipin synthase (CLS). *B. subtilis* contains two CLS enzymes: the major form (YwnE; also called ClsA [26]) is expressed during vegetative growth, while the minor (YwjE) is involved in sporulation (26). LPG is formed when MprF transfers a lysyl group from lysyl-$tRNA^{Lys}$ to PG. Finally, GL are created by dephosphorylation of PA to diacylglycerol, which is then modified by the transfer of one or two glucose molecules from UDP-glucose by UgtP (25). Fatty acid synthesis and desaturation are controlled by the FapR (52) and DesRK (14) regulatory systems, and both chain length and desaturation may be regulated by various stress conditions (31, 32, 38, 49). In contrast, little is known about how membrane headgroup composition is regulated, although the extracytoplasmic function σ factor σ^X has been shown to contribute to expression of PE biosynthesis genes (11).

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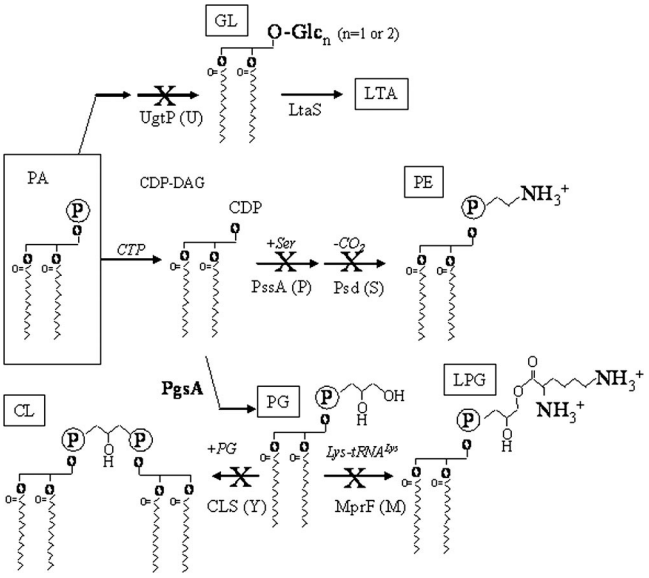

FIG. 1. Pathways for membrane lipid synthesis in *B. subtilis*. Membrane lipid synthesis begins with the common precursor PA and leads to the generation of GL, PG, PE, CL, and LPG. Key enzymes and substrates are indicated, and steps blocked by mutation are denoted with an X. Note that in the absence of UgtP, LTA is still synthesized but the glycerol-phosphate copolymer is linked to the membrane by DAG rather than by diglucosyl-DAG.

Here, we report the characterization of a series of isogenic *B. subtilis* strains with altered membrane composition. Mutant strains were characterized for growth, antibiotic resistance, morphology, and alterations in global gene expression patterns. Our results suggest that the cell can tolerate even large changes in membrane composition. Remarkably, *B. subtilis* retains viability and even rapid growth when the membrane is comprised predominantly, if not exclusively, of PG.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *B. subtilis* strains used were derivatives of either CU1065 (W168 *trpC2 attSP*β) or the related strain NCIB3610 (Table 1). *Escherichia coli* strain DH5α was used for standard cloning procedures. Bacteria were grown in Luria-Bertani (LB) medium, LB supplemented with 25 mM $MgSO_4$, or modified minimal medium (MM) (9) at 37°C with vigorous shaking. Antibiotics were added to the growth medium when appropriate: 100 μg/ml ampicillin for *E. coli*; 1 μg/ml erythromycin plus 25 μg/ml of lincomycin (macrolide-lincomycin-streptogramin B [MLS] resistance), 10 μg/ml chloramphenicol, 10 μg/ml kanamycin, 20 μg/ml tetracycline, and 100 μg/ml spectinomycin for *B. subtilis*. Sensitivity to nisin was measured by growing the different strains in LB and then adding the indicated concentration during early exponential growth (optical density at 600 nm [OD_{600}], ~0.25). Sensitivity to sublancin was tested via spot-on-lawn assay. Lawns were created by inoculating 100 μl of mid-exponential-phase cultures into 2 ml of 0.7% LB agar. This was poured into one well of an eight-well rectangular multidish (26 mm by 33 mm; Nunc). Once set, the plates were dried for 30 min in a laminar flow hood, and 5-μl aliquots of strains JH642 and HB6164 (JH642 *sunA*::*kan* [Table 1]), grown to an OD_{600} of 0.6, were spotted in the center of the well. Plates were incubated overnight at 37°C in an airtight container with moist paper towels to prevent drying.

Construction of null mutants. Chromosomal deletions were created in the CU1065 background by using long-flanking homology PCR (LFH-PCR) as described previously (34) with the following changes: flanking fragments were amplified using *Pfu* DNA polymerase (Stratagene), and the flanking fragments and antibiotic resistance marker were joined using the Expand Long Template PCR system (Roche). The xylose-inducible mutants were created by LFH using a spectinomycin resistance gene fused with the P_{xylA} region amplified from pTn7SX (8). A detailed protocol is available at http://www.micro.cornell.edu/faculty/helmann/supplemental%20index.htm.

Primer sequences used for mutant constructions are detailed in Table 1.

The null mutants in the NCIB3610 background were created by SPP1-mediated transduction from the CU1065 strains harboring the mutation of interest as previously described (28). Transductants were tested for their ability to grow on the appropriate antibiotic and in MM without the addition of tryptophan.

Lipid extraction and thin-layer chromatography. Ten-ml aliquots of mid-exponential-phase cultures were centrifuged for 10 min at 4,500 × *g*, and lipids were extracted from the pellet via a modified Bligh-Dyer method (29). Briefly, the cell pellet was resuspended in 100 μl distilled deionized H_2O with the addition of perchloric acid to a final concentration of 1 M. The cell suspension was then incubated at 0°C for 30 min, after which lipids were extracted by the addition of 1 ml methanol-chloroform-water (12:6:2 [vol/vol]) followed by incubation for 50 min on ice. Phase separation was achieved by the sequential addition of 0.3 ml water and 0.3 ml chloroform, after which suspensions were incubated overnight at −20°C and then centrifuged for 5 min at 720 × *g* at 4°C. The organic phase was then removed and dried under nitrogen. The lipids were resuspended in 20 μl of chloroform-methanol (2:1 [vol/vol]), spotted to silica gel 60 plates (VWR), and separated using the solvent mixture chloroform-methanol-water (65:25:4 [vol/vol]). Phospholipids were detected using molybdenum blue spray reagent (Sigma-Aldrich). PG and PE standards were obtained from Sigma-Aldrich. For two-dimensional thin-layer chromatography (TLC) assays, cells were grown in LB for 4 to 5 h and membrane lipids were labeled with 0.05 μCi of [2-^{14}C]acetic acid per ml. Lipids were extracted and spotted to silica plates as described above. Lipids were first separated (*x* dimension) using the solvent mixture chloroform-methanol-water (65:25:4 [vol/vol]) and then (*y* dimension) using the solvent mixture chloroform-acetic acid-methanol-water (80:15:12:4 [vol/vol]). Spots for ^{14}C-labeled lipids were visualized using a Storm 840 PhosphorImager scanner (Molecular Dynamics) after exposure of a PhosphorImager screen.

Microscopy. Phase-contrast and fluorescence microscopy were performed using an Olympus BX61 epifluorescence microscope, with 40× UPlanFl (numerical aperture, 0.75) and 100× UPlanApo (numerical aperture, 1.35) objectives. The microscope was equipped with filter cubes for viewing fluorescein, 7-aminoactinomycin D/Mitotracker red, and 4′,6-diamidino-2-phenylindole/Hoechst fluorescence. Images were acquired using Cooke SensiCam and Slidebook software (Intelligent Imaging Inc.). Figures were assembled using Adobe Photoshop.

Swarming motility assay. LB plates containing 0.7% agar were dried in a laminar flow hood for 30 min and then spotted in the center with 5 μl of mid-exponential-phase cultures grown in LB medium. The plates were then dried for another 15 min and incubated overnight at 37°C.

Construction and analysis of P_{sigM} transcriptional fusion. The DNA fragment of the *sigM* regulatory region was PCR amplified using primers 2866 and 436. The fragment was digested with HindIII and BamHI and cloned into the vector pDG1661, which contains a promoterless *lacZ* gene (21), resulting in plasmid pLS30. The sequences of the insert were verified by DNA sequencing (Cornell DNA sequencing facility). *B. subtilis* CU1065 and HB5346 (*ugtP*::MLS) were transformed to Cm^r with the ScaI-linearized plasmid, which integrated into the *amyE* locus, creating strains HB5423 and HB5426, respectively. For quantitative measurements of β-galactosidase activity, strains HB5423 and HB5426 were grown in LB at 37°C with vigorous shaking, and samples were collected at different growth stages as determined by the OD_{600}. To test promoter induction, strains HB5423 and HB5426 were grown in LB until the OD_{600} reached ~0.3 and then the cultures were split into aliquots, which were either unchallenged or challenged with vancomycin (2 μg/ml) for 30 min. β-Galactosidase activity was measured according to the method of Miller (37), except that cells were lysed by the addition of lysozyme to a final concentration of 20 μg/ml followed by a 30-min incubation at 37°C.

RNA isolation and microarray analysis. Strains CU1065 (wild type [WT]), HB5346 (*ugtP*, abbreviated as U), HB5437 (*mprF pssA ywnE* triple mutant, abbreviated as T), and HB5391 (*ugtP mprF pssA ywnE* quadruple mutant, abbreviated as Q) were inoculated into LB and grown at 37°C with vigorous shaking until an OD_{600} of ~0.4, and RNA isolation was performed using the RNeasy mini kit (Qiagen). RNA was subsequently DNase treated with Turbo DNA-free (Ambion) and precipitated overnight. The RNA was dissolved in RNase-free water and quantified using a NanoDrop spectrophotometer (Nanodrop Tech. Inc., Wilmington, DE). RNA was isolated from three biological replicates.

cDNA synthesis was performed using the SuperScript Plus indirect cDNA labeling system (Invitrogen) as per the manufacturer's instructions with 20 μg of total RNA and then purified using the Qiagen MinElute kit (Qiagen, Maryland) and quantified with NanoDrop. Total cDNA was labeled overnight with Alexa

TABLE 1. Bacterial strains and primers used in this study

Strain or primer	Genotype or sequence[a]	Source or reference
Bacterial strains		
CU1065	W168 *trpC2 attSP*β	Lab strain
NCIB3610	Wild-type isolate	BGSC 3A1
JH642	*pheA1 trpC2*	BGSC 1A96
HB6164	JH642 *sunA*::*kan*	10
HB5337	CU1065 *mprF*::*kan*	LFH-PCR → CU1065
HB5343	CU1065 *psd*::MLS	LFH-PCR → CU1065
HB5346	CU1065 *ugtP*::MLS	LFH-PCR → CU1065
HB5347	CU1065 *ywnE*::*tet*	LFH-PCR → CU1065
HB5348	CU1065 *ywjE*::*kan*	LFH-PCR → CU1065
HB5361	CU1065 *pssA*::*spc*	LFH-PCR → CU1065
HB5362	CU1065 *ywnE*::*cat*	LFH-PCR → CU1065
HB5445	CU1065 *ywjE*::*cat*	LFH-PCR → CU1065
HB5344	CU1065 *psd*::MLS *mprF*::*kan*	HB5337 chr DNA → HB5343
HB5349	CU1065 *ywjE*::*kan ugtP*::MLS	LFH-PCR → HB5348
HB5350	CU1065 *mprF*::*kan ugtP*::MLS	LFH-PCR → HB5337
HB5387	CU1065 *ywnE*::*cat mprF*::*kan*	LFH-PCR → HB5362
HB5388	CU1065 *mprF*::*kan pssA*::*spc*	LFH-PCR → HB5337
HB5438	CU1065 *ugtP*::MLS *pssA*::*spc*	LFH-PCR → HB5346
HB5439	CU1065 *ugtP*::MLS *ywnE*::*cat*	LFH-PCR → HB5346
HB5440	CU1065 *pssA*::*spc ywnE*::*cat*	LFH-PCR → HB5361
HB5441	CU1065 *ywnE*::*cat ywjE*::*kan*	HB5348 chr DNA → HB5362
HB5442	CU1065 *ywnE*::*cat psd*::MLS	HB5343 chr DNA → HB5362
HB5443	CU1065 *pssA*::*spc ywjE*::*kan*	HB5348 chr DNA → HB5361
HB5389	CU1065 *ugtP*::MLS *mprF*::*kan pssA*::*spc*	LFH-PCR → HB5350
HB5390	CU1065 *ugtP*::MLS *mprF*::*kan ywnE*::*cat*	LFH-PCR → HB5350
HB5436	CU1065 *ugtP*::MLS *pssA*::*spc ywnE*::*cat*	LFH-PCR → HB5438
HB5437	CU1065 *mprF*::*kan pssA*::*spc ywnE*::*cat*	LFH-PCR → HB5388
HB5391	CU1065 *ugtP*::MLS *pssA*::*spc ywnE*::*cat mprF*::*kan*	LFH-PCR → HB5436
HB5481	CU1065 *ugtP*::MLS *pssA*::*spc mprF*::*kan ywnE*::*tet*	LFH-PCR → HB5389
HB5482	CU1065 *ugtP*::MLS *pssA*::*spc mprF*::*kan ywnE*::*tet ywjE*::*cat*	HB55445 DNA → HB5481
HB5406	CU1065 *pssA*::*spc*-P_{xylA}	LFH-PCR → CU1065
HB5407	CU1065 *ybfM*::*spc*-P_{xylA}	LFH-PCR → CU1065
HB5461	NCIB3610 *pssA*::*spc*	Transduction → NCIB3610
HB5462	NCIB3610 *ugtP*::MLS	Transduction → NCIB3610
HB5463	NCIB3610 *mprF*::*kan*	Transduction → NCIB3610
HB5464	NCIB3610 *ywnE*::*cat*	Transduction → NCIB3610
HB5465	NCIB3610 *ywjE*::*cat*	Transduction → NCIB3610
HB5466	NCIB3610 *ugtP*::MLS *pssA*::*spc*	Transduction → HB5462
HB5467	NCIB3610 *ugtP*::MLS *mprF*::*kan*	Transduction → HB5462
HB5468	NCIB3610 *ywnE*::*cat ugtP*::MLS	Transduction → HB5464
HB5469	NCIB3610 *ugtP*::MLS *ywjE*::*cat*	Transduction → HB5462
HB5470	NCIB3610 *pssA*::*spc mprF*::*kan*	Transduction → HB5461
HB5471	NCIB3610 *ywnE*::*cat pssA*::*spc*	Transduction → HB5464
HB5472	NCIB3610 *pssA*::*spc ywjE*::*cat*	Transduction → HB5461
HB5473	NCIB3610 *ywnE*::*cat mprF*::*kan*	Transduction → HB5464
HB5474	NCIB3610 *mprF*::*kan ywjE*::*cat*	Transduction → HB5463
HB5475	NCIB3610 *ugtP*::MLS *mprF*::*kan pssA*::*spc*	Transduction → HB5467
HB5476	NCIB3610 *ugtP*::MLS *pssA*::*spc ywnE*::*cat*	Transduction → HB5466
HB5477	NCIB3610 *ugtP*::MLS *mprF*::*kan ywnE*::*cat*	Transduction → HB5467
HB5478	NCIB3610 *mprF*::*kan pssA*::*spc ywnE*::*cat*	Transduction → HB5470
HB5479	NCIB3610 *ugtP*::MLS *mprF*::*kan pssA*::*spc ywnE*::*cat*	Transduction → HB5475
HB5480	NCIB3610 *ugtP*::MLS *mprF*::*kan ywnE*::*cat pssA*::*spc*	Transduction → HB5476
HB5423	CU1065 *amyE*::P_{sigM}-*lacZ*	pLS30 → CU1065
HB5426	CU1065 *ugtP*::MLS *amyE*::P_{sigM}-*lacZ*	pLS30 → HB5346
Oligonucleotide primer no., name		
1761, yfiW up fwd	tccaagcgcctgaatcagcc	
1762, yfiW up rev (kan)	CCTATCACCTCAAATGGTTCGCTGtgatgaccatgagcgtacg	
1763, yfiX do fwd (kan)	CGAGCGCCTACGAGGAATTTGTATCGccatgttcctcgttacacg	
1764, yfiX do rev	gagccgtaaagtcatggaagg	
1913, psd up fwd	ccgatccttctggcaatcgg	
1914, psd up rev (MLS)	GAGGGTTGCCAGAGTTAAAGGATCgacagacgatgattcgtcagc	
1915, psd do fwd (MLS)	CGATTATGTCTTTTGCGCAGTCGGCcaagtcggagaactgatagg	
1916, psd do rev	gacatcttctgtgtgccatacagcg	
2092, ugtP up fwd	gacgagctatactacgtgcc	
2093, ugtP up rev (MLS)	GAGGGTTGCCAGAGTTAAAGGATCttggctacctgcacatgtcc	
2094, ugtP do fwd (MLS)	CGATTATGTCTTTTGCGCAGTCGGCggaatcagaaatgatgaccg	

Continued on following page

TABLE 1—*Continued*

Strain or primer	Genotype or sequence[a]	Source or reference
2095, ugtP do rev	tgccgcttatccgtaaagcc	
2100, ywnE up fwd	gctcttcctttatttgtcgtgcg	
2101, ywnE up rev (tet)	GAGAACAACCTGCACCATTGCAAGAccaagatgcactggcatcgc	
2102, ywnE do fwd (tet)	GGGATCAACTTTGGGAGAGAGTTCctatgaggagtatctgcagc	
2103, ywnE do rev	tttgtacagtgacgcagacc	
2367, ywnE up rev (cat)	CTTGATAATAAGGGTAACTATTGCCccaagatgcactggcatcgc	
2368, ywnE do fwd (cat)	GGGTAACTAGCCTCGCCGGTCCACGctatgaggagtatctgcagc	
2104, ywjE up fwd	agaaagcccatgctcatgcc	
2105, ywjE up rev (kan)	CCTATCACCTCAAATGGTTCGCTGgatatcaagcagaatcaaggc	
2106, ywjE do fwd (kan)	CGAGCGCCTACGAGGAATTTGTATCGtggaggatttcagca agcg	
2107, ywjE do rev	Agacaaacggaacatcctcc	
3880, ywjE up rev (cat)	CTTGATAATAAGGGTAACTATTGCCgatatcaagcagaatca aggc	
3881, ywjE do fwd (cat)	GGGTAACTAGCCTCGCCGGTCCACGtggaggatttcagcaagcg	
2369, pssA up fwd	cttgagacggcacatctggc	
2370, pssA up rev (spc)	CGTTACGTTATTAGCGAGCCAGTCcctatcgtaatcatacaggg	
2371, pssA do fwd (spc)	CAATAAACCCTTGCCCTCGCTACGcagaaaacctggagtctggg	
2372, pssA do rev	gaaccacaccgtcaacaggg	
2449, pssA up rev (spc xylA)	CAATAAACCCTTGCCCTCGCTACGcctatcgtaatcatacaggg	
2450, pssA do fwd (spc xylA SD)	CCAACCAGATAAGTGCGAGCCAGTCAAGAGGATCTGC atggaattagttcagcagc	
2451, ybfM up rev (spc xylA)	CAATAAACCCTTGCCCTCGCTACGcgtaatccgctatgagctgc	
2866, PsigM fwd (H)	ccgtaagctttgggtactatatagtaatggtg	
436, SigM-r3	cgggatccagtaagtcttcagcaagatg	

[a] Uppercase letters indicate sequence complementary to antibiotic resistance genes. Underlined sequences indicate restriction sites.

Fluor 555 or Alexa Fluor 647 (Invitrogen) and then purified using the Qiagen MinElute kit (Qiagen, Maryland) and quantified with NanoDrop. Equal amounts (100 to 150 pmol) of labeled cDNA (WT/U, WT/T, and WT/Q) were combined to a final volume of 15 μl, and 1 μl salmon sperm DNA (10 mg/ml; Invitrogen) plus 16 μl 2× hybridization buffer (50% formamide, 10× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS]) were added. cDNA mix was denatured at 95°C and hybridized for 16 to 18 h at 42°C to DNA microarray slides which had been prehybridized for at least 30 min at 42°C in 1% bovine serum albumin, 5× SSC, 0.1% SDS, washed in water, and dried. Following hybridization the slides were washed sequentially in 2× SSC plus 0.1% SDS for 5 min at 42°C, 2× SSC plus 0.1% SDS for 5 min at room temperature, 2× SSC for 5 min at room temperature, and 0.2× SSC for 5 min at room temperature and finally dipped in water and spun until dry. Arrays were scanned using a GenePix 4000B array scanner (Axon Instruments, Inc.). Our arrays are based on a *B. subtilis* oligonucleotide library manufactured by Sigma-Genosys consisting of 4,128 oligonucleotides (65-mers) representing 4,106 *B. subtilis* genes, 10 control oligonucleotides (from *E. coli* and Brome mosaic virus), and 12 random oligonucleotides. A single oligonucleotide was designed to represent each of the *B. subtilis* genes as annotated in the genome data, release R16.1 (26 April 2001), at the SubtiList website (http://genolist.pasteur.fr/SubtiList/). The arrays were printed onto poly-L-lysine-coated Corning CMT-Gap slides at the W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University. Each array contains 8,447 features corresponding to duplicates of each open reading frame-specific oligonucleotide, additional oligonucleotides of control genes, and 50% dimethyl sulfoxide blank controls.

Raw data files were produced from the scanned images using the GenePix Pro 4.0 software package (GPR files), and the red/green fluorescence intensity values were normalized such that the ratio of medians of all features was equal to 1. The normalized data were exported to Excel for analysis. The data sets were filtered to remove those genes that were not expressed at levels significantly above background under either condition (sum of mean fluorescence intensity, <30). For analysis, we filtered to identify those genes that were altered at least 1.5-fold in signal intensity in at least two of the three biological replicates (and excluding those genes with an opposing change in the third replicate).

FAME analysis. Two 500-ml biological replicas of mid-exponential-phase cultures of either the WT, *ugtP*::MLS single mutant, *mprF*::*kan pssA*::*spc ywnE*::*cat* triple mutant, or *ugtP*::MLS *mprF*::*kan pssA*::*spc ywnE*::*cat* quadruple mutant grown in LB were combined, and the frozen pellets were submitted for fatty acid methyl ester (FAME) analysis at Microbial ID, Newark, DE (http://www.microbialid.com/). Results of this analysis are summarized in Table S1 of the supplemental material.

Microarray data accession numbers. The microarray data sets and related files are available at NCBI GEO under accession numbers GSE13036.

RESULTS AND DISCUSSION

The *Bacillus subtilis* cytoplasmic membrane can be dramatically simplified. Bacterial cytoplasmic membranes display a great variety of lipid compositions. PE is a dominant inner membrane component in *E. coli* (75% of total [13]), whereas *Staphylococcus aureus* membranes are predominantly formed from PG (~90% of total [40]). In *Mycobacterium tuberculosis*, membranes are dominated by phosphatidylinositol mannosides (~70% [51]). In many other cases, no single lipid type dominates, and in all systems there are at least three or more different types of lipid headgroups represented. These observations are consistent with the hypothesis that membrane function requires lipid components with different physico-chemical properties.

Here, we set out to characterize a set of strains with altered membrane compositions by using *B. subtilis* as a model system. We inactivated genes involved in the biosynthesis of complex lipids, including genes necessary for the synthesis of PE (*pssA*; P), GL (*ugtP*; U), LPG (*mprF*; M), and CL (*ywnE*; Y), by allelic replacement with antibiotic resistance cassettes (Table 1). We also constructed mutants of *psd* (S) and *ywjE* (J). The *psd* mutant accumulates phosphatidylserine but is unable to synthesize PE. The *ywjE* mutant is missing a minor CLS thought to be present during sporulation (26). Allelic replacements were verified by PCR, and the absence of PE and LPG was verified by TLC (Fig. 2 and data not shown, but see also Fig. S1

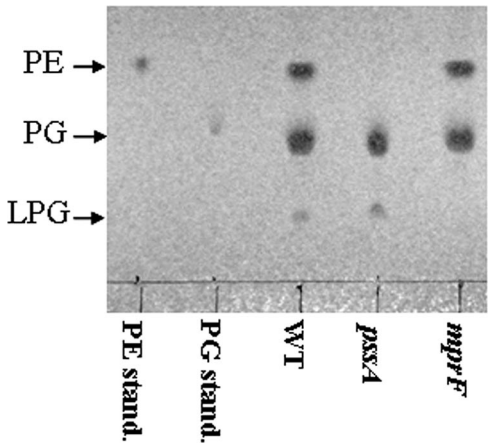

FIG. 2. TLC analysis of membrane mutants. Membrane lipids were extracted from exponential cultures of WT or membrane mutants grown in LB using a modified Bligh-Dyer method (6). Extracted lipids were spotted to silica TLC plates, detected with molybdenum blue (Sigma), and compared to standards as indicated.

in the supplemental material). By additional rounds of transformation we created a suite of mutants comprised of various combinations of these mutations with increasingly simplified membrane compositions. Surprisingly, we were able to create a viable quadruple mutant strain (Q; *ugtP pssA mprF ywnE*) with membranes containing predominantly, if not exclusively, PG (Table 1). The only other DAG-based lipids that might have been present were PA and DAG itself. Previously, DAG was reported to be present in low levels in *B. subtilis* membranes (5). DAG is formed as a precursor to GL and is also generated as an intermediate during the synthesis of lipoteichoic acids (24).

We hypothesized that alterations to membrane headgroup composition might be compensated, to some extent, by alterations in fatty acid composition. To test this idea, the fatty acid compositions of the wild-type, *ugtP* single, MPY triple, and the UPMY quadruple mutant strains were determined by FAME analysis (see Table S1 in the supplemental material). While overall composition was quite similar in these strains, the results were consistent with a slight decrease in C_{15} (15:0 iso) and a corresponding increase in C_{17} lipids in the *ugtP* and Q mutant strains (but not the MPY triple mutant). Thus, perturbation of membrane headgroup composition, and in particular a lack of GL, may be compensated in part by alterations of fatty acid synthesis and/or desaturation.

We monitored growth rates for each of our strains by using a BioScreen automated growth curve (optical density) analysis system. All mutant strains were able to grow in a variety of liquid and solid media, including LB, LB plus 2% glucose, Difco sporulation medium (DSM), and minimal competence medium, as well as a defined MM. However, some of the strains had increased doubling and/or lag times. In LB medium, most of the single mutants had doubling times comparable to wild type, with the exception of the mutant strain lacking the neutral lipid GL (*ugtP* mutant; U) (Table 2). Surprisingly, the quadruple mutant (Q; UPMY) exhibited a doubling time only slightly longer than the wild type in LB, but it was significantly impaired in growth rate in MM (Table 2). In addition, the lag time of the Q strain, as well as all strains

TABLE 2. Effects of membrane headgroup alteration on cell growth

Strain[a]	Membrane lipid[b] (charge)					Doubling time (min)[c] in:	
	PG (−1)	CL (−2)	PE (0)	GL (0)	LPG (+1)	LB	MM
WT	+	+	+	+	+	34 ± 1	48 ± 3
P	+	+	−	+	+	37 ± 2	49 ± 3
U	+	+	+	−	+	50 ± 3	51 ± 4
M	+	+	+	+	−	38 ± 3	45 ± 2
Y	+	−	+	+	+	36 ± 4	50 ± 1
S	+	+	−	+	+	37 ± 2	52 ± 2
J	+	+/−	+	+	+	38 ± 0	49 ± 2
UP	+	+	−	−	+	37 ± 5	50 ± 3
UM	+	+	+	−	−	44 ± 7	62 ± 8
UY	+	−	+	−	+	47 ± 8	51 ± 2
UJ	+	+/−	+	−	+	40 ± 2	51 ± 2
PM	+	+	−	+	−	40 ± 4	44 ± 5
PY	+	−	−	+	+	41 ± 5	50 ± 2
PJ	+	+/−	−	+	+	39 ± 1	55 ± 2
MY	+	−	+	+	−	40 ± 4	50 ± 3
MS	+	+	−	+	−	38 ± 2	47 ± 2
YJ	+	−	+	+	+	41 ± 1	49 ± 2
YS	+	−	−	+	+	39 ± 3	52 ± 4
UPM	+	+	−	−	−	37 ± 2	55 ± 6
UPY	+	−	−	−	+	53 ± 9	50 ± 4
UMY	+	−	+	−	−	42 ± 9	49 ± 5
MPY (T)	+	−	−	+	−	37 ± 3	46 ± 7
UPMY (Q)	+	−	−	−	−	40 ± 7	74 ± 9

[a] P, *pssA*::*spc*; U, *ugtP*::MLS; M, *mprF*::*kan*; Y, *ywnE*::*cat*; S, *psd*::MLS; J, *ywjE*::*kan*; T, *mprF*::*kan pssA*::*spc ywnE*::*cat* triple mutant; Q, *ugtP*::MLS *mprF*::*kan pssA*::*spc ywnE*::*cat* quadruple mutant.

[b] +, lipid is present in the membrane; −, lipid is absent; +/−, CL may be partially lacking, at least under some growth conditions, due to loss of the minor CLS encoded by *ywjE*.

[c] Doubling time calculated for maximum specific growth rate.

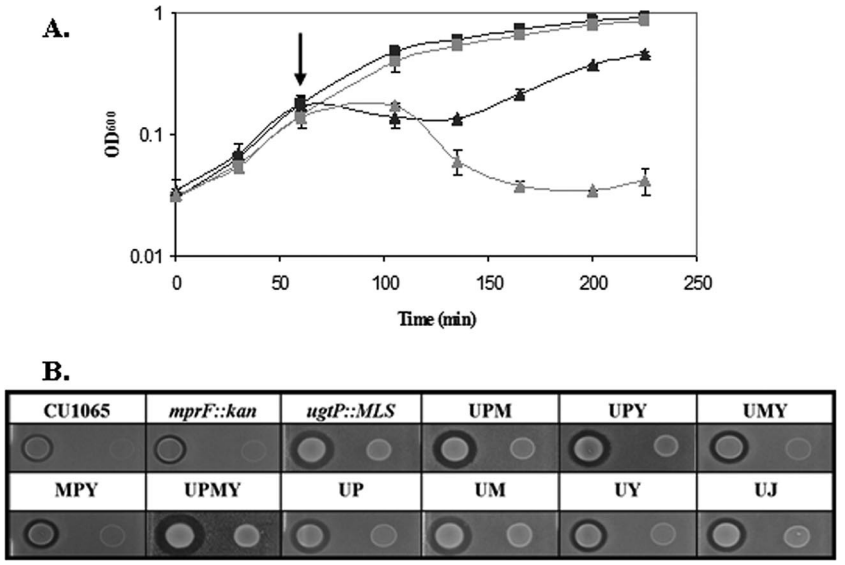

FIG. 3. Variations of cytoplasmic membrane composition result in altered antibiotic sensitivity. A. Deletion of *mprF* leads to increased sensitivity to nisin. WT (black symbols) and *mprF*::*kan* cells (gray symbols) were grown in LB to early log phase and then either left untreated (squares) or treated with 10 μg/ml nisin (triangles). The arrow denotes the time at which nisin was added. B. Strains lacking *ugtP* demonstrated increased sensitivity to sublancin. Lawns of various strains spotted with the sublancin-producing *B. subtilis* JH642 (left spot) or a JH642 strain containing a *sunA* deletion are shown.

lacking *ugtP*, was considerably longer than that of the WT (data not shown). It is important to note, however, that UgtP plays several roles in the cell. In addition to the synthesis of GL, UgtP is involved in generating anchor lipids for lipoteichoic acids (25) and functions as a sensor to couple cell division to the availability of nutrients (61).

In minimal medium, in which cells grow considerably slower, the only strain to demonstrate a significantly increased doubling time was the UPMY quadruple mutant (74 min versus 48 min for WT). Growth rates in DSM were similar to those in LB (data not shown). All strains were capable of sporulating: phase-bright spores were observed after growth in DSM for 24 h, and all strains formed pigmented colonies when streaked on DSM plates.

The viability and robust growth of strains with greatly simplified membrane composition are surprising in light of previous studies of *E. coli* mutants. For example, an *E. coli pssA* null mutant had severe growth defects and requires divalent metal ion supplementation for growth (15), and a mutant lacking both PE and CL was nonviable (42). These defects are thought to result from a requirement for the formation of nonbilayer structures within the membrane (facilitated by PE) as well as the proper localization of membrane proteins (2, 36). These differences may result, in part, from the fact that *B. subtilis* only contains a single membrane, whereas *E. coli* requires both an inner and outer membrane.

***mprF* and *ugtP* mutants exhibit increased sensitivity to cationic antimicrobial peptides.** Modification of the membrane has been proposed as a strategy for protection against cationic antimicrobial peptides (57). We tested our collection of strains for their sensitivity to a variety of cell envelope-active compounds, including vancomycin, nisin, sublancin, and duramycin. In the presence of the glycopeptide antibiotic vancomycin, most strains grew to an OD_{600} comparable to that of WT. However, all *ugtP* mutant strains were vancomycin sensitive (data not shown). Conversely, in the presence of the cationic lantibiotic nisin, *mprF* mutants were sensitive (Fig. 3A). LPG can constitute up to 20% of the *B. subtilis* membrane (16) and has been shown to impart resistance to cationic antimicrobials, including nisin, vancomycin, and human defensins, in *S. aureus* (50, 57).

Next we tested sensitivity to the bacteriocin sublancin produced by *B. subtilis* strains harboring the SPβ prophage. Our mutant strains were constructed in *B. subtilis* CU1065, which lacks the SPβ prophage and is therefore sublancin sensitive. To test sublancin sensitivity, lawns were spotted with *B. subtilis* JH642 (a sublancin producer) or an isogenic strain unable to produce sublancin (*sunA*::*kan* mutant). Among the singly mutant strains, an increased zone of inhibition was only observed for the *ugtP* mutant (Fig. 3B). Similarly, the Q mutant (UPMY) was significantly more sensitive to sublancin than the MPY triple mutant. These results suggest that the presence of GL in the membrane provides some protection against sublancin.

Duramycin is a lantibiotic produced by *Streptoverticillium cinnamoneum*. Previous studies have reported that strains selected for increased resistance to duramycin demonstrate decreased levels of PE and increased levels of CL (17), and these studies have predicted a mode of action in which duramycin binds to PE and then inserts into the membrane, creating pores (23). Consistent with this expectation, *pssA* and *psd* mutant strains exhibited greatly increased resistance to duramycin relative to the WT strain (data not shown), consistent with previous results (8). No other single mutation conferred this resistance, and double and triple mutants lacking either *pssA* or *psd* showed similar resistance levels to that of the single mutants. Unexpectedly, the UPMY quadruple mutant was more sensitive to duramycin than other PE-lacking mutants, but still

more resistant than the WT. This suggests that the requirement for PE in the biological action of duramycin may be bypassed in strains with grossly perturbed membrane structure.

In *B. subtilis*, the PE biosynthetic genes (*pssA* and *psd*) are part of a tricistronic operon, *pssA ybfM psd*. In order to study the possible role of *ybfM* in PE biosynthesis and duramycin sensitivity, we constructed a strain in which *ybfM* was replaced by a spectinomycin resistance cassette and *psd* was placed under the control of a xylose-inducible promoter (*ybfM*::*spc*-P_{xylA}). In the presence of xylose, strain *ybfM*::*spc*-P_{xylA} was as sensitive to duramycin as WT, whereas in the presence of glucose it was as resistant as a *pssA* null mutant (data not shown). This suggests that *ybfM* is not required for the synthesis or localization of PE. In other organisms containing homologs of these proteins, the tricistronic operon structure is not conserved, and often *pssA* and *psd* are even situated in separate operons.

Cells containing simplified cytoplasmic membranes exhibit aberrant morphologies. Microscopic observation indicates that mutants with an altered membrane composition have distinctive changes in cell morphology. During mid-exponential growth, the *ugtP* mutant was the only single mutant to demonstrate a change in cell shape: cells were shorter and tended to curl at the ends (Fig. 4B). Double mutants lacking *ugtP* appeared mostly as curling short chains of cells or clumps of curled cells, while triple mutants demonstrated lengthened filaments of curled cells (data not shown). The most dramatic effect was observed in the quadruple mutant, in which the filamentation and curling was even more pronounced (Fig. 4D). Double mutants that still contain *ugtP* resembled WT cells, with a small amount of cells in longer chains (data not shown), while the MPY triple mutant did not curl but was highly filamentous (Fig. 4C). At later growth stages in LB the filamentation phenotype is lost and cells appear as either single cells or short chains (data not shown). When the MPY and UPMY mutants were grown in LB supplemented with Mg^{2+}, which is known to stabilize the effects of PE loss in *E. coli* (13), the filamentation was reduced. Staining of these filamentous cells with the membrane stain FM 4-64 and fluorescently labeled vancomycin (which targets un-cross-linked peptidoglycan-containing pentapeptide side chains) clearly demonstrated that these are septated filaments (Fig. 4C and D). Fluorescently labeled vancomycin stains the septal regions and also a characteristic helical pattern along the axis of the cell, as also seen for WT cells (59) (Fig. 4 and data not shown), suggesting that peptidoglycan synthesis and incorporation remains mostly unimpaired, even in the quadruple mutant. Staining with FM 4-64 showed a similar accumulation at the septal region, with no obvious aberrant membrane formations, such as involutions or blebs.

In general, the most dramatic morphological changes were observed in those cells lacking GL. *ugtP* codes for a multifunctional UDP-glucose:diacylglycerol glucosyltransferase that is required for (i) formation of GL (25, 48), (ii) synthesis of diglucosyldiacylglycerol, the membrane anchor for lipotechoic acids (25, 30), and (iii) coupling nutrient availability to cell division in *B. subtilis* (61). When nutrients are available, UgtP is expressed at high levels, evenly distributed in the cytoplasm, and concentrated at the septum, colocalizing and interacting with FtsZ (61). UgtP is proposed to inhibit the assembly of FtsZ rings by sequestering the FtsZ subunits, resulting in larger cells at the time of division (55, 61). Upon carbon depletion, localization of UgtP to the septum is abolished resulting in smaller cells (61). This dual role of UgtP in envelope biosynthesis and regulation of cell size could explain the severity of the defects seen in *ugtP* null mutants.

Cells containing simplified cytoplasmic membranes are altered for swarming motility. In order to study the effects of alteration of cytoplasmic membrane composition on motility and biofilm formation, we transferred our mutant collection into an NCIB3610 background which, unlike most *B. subtilis* 168 laboratory strains, exhibits a robust swarming phenotype (28). In this background, the *ugtP* mutant strains exhibited decreased swarming while, curiously, some of the single mutants displayed enhanced swarming (Fig. 5). This enhanced swarming was reduced only in the PY and MY double mutants, as well as in the MPY triple mutant. This does not seem to be due to a specific lack of CL, since the Y single mutant did not show a similar decrease. Surprisingly, all strains tested were able to form characteristic pellicles on the air-liquid interface of MSgg medium (8a). However, the biofilms of strains lacking *ugtP* were somewhat flatter and more matte, while that of the MPY triple mutant resembled the WT (data not shown).

Transcriptome comparisons. We conducted DNA microarray experiments to compare the transcriptional profiles of the *ugtP* single mutant, MPY triple mutant, and UPMY quadruple mutant to that of WT cells in LB medium at mid-log stage. These mutations are highly pleiotropic, and we identified a large number of genes that were reproducibly altered in expression levels relative to WT (detailed in Tables S2 to S4 in the supplemental material). These included genes involved in carbon and protein metabolism, cell wall and membrane biosynthesis, DNA metabolism and repair, motility and chemotaxis, and production of secondary metabolites, as well as a large number of uncharacterized membrane proteins. In addition, the expression levels of many known or putative ABC transporter and other transport permeases were affected, including multidrug efflux pumps and antibiotic resistance proteins. Alterations of membrane composition also affected the levels of expression of numerous known and predicted transcriptional regulators. In general, these changes were most dramatic for the *ugtP* mutant and the UPMY quadruple mutant, with generally more modest changes noted in the MPY triple mutant. These results suggest that cell envelope perturbations caused by the absence of UgtP are potentially the most deleterious to the cell and result in a shift in the transcriptional profile.

To determine the likely origins of these large shifts in transcriptional patterns, we have linked many of the genes affected (either positively or negatively) by membrane alterations to known regulons. As summarized in Fig. 6, large fractions of several known regulons were altered in their expression in one or more of the tested mutant backgrounds. For example, genes regulated by σ^D (involved in motility, chemotaxis, and autolysin synthesis [33]) were expressed at lower levels in all three mutant backgrounds. In contrast, numerous members of the large DegU and σ^B (general stress response) regulons were elevated in expression in these strains. There was also a general increase in the expression of genes under the control of the sporulation-specific sigma factors σ^E, σ^F, σ^G, and σ^K in all

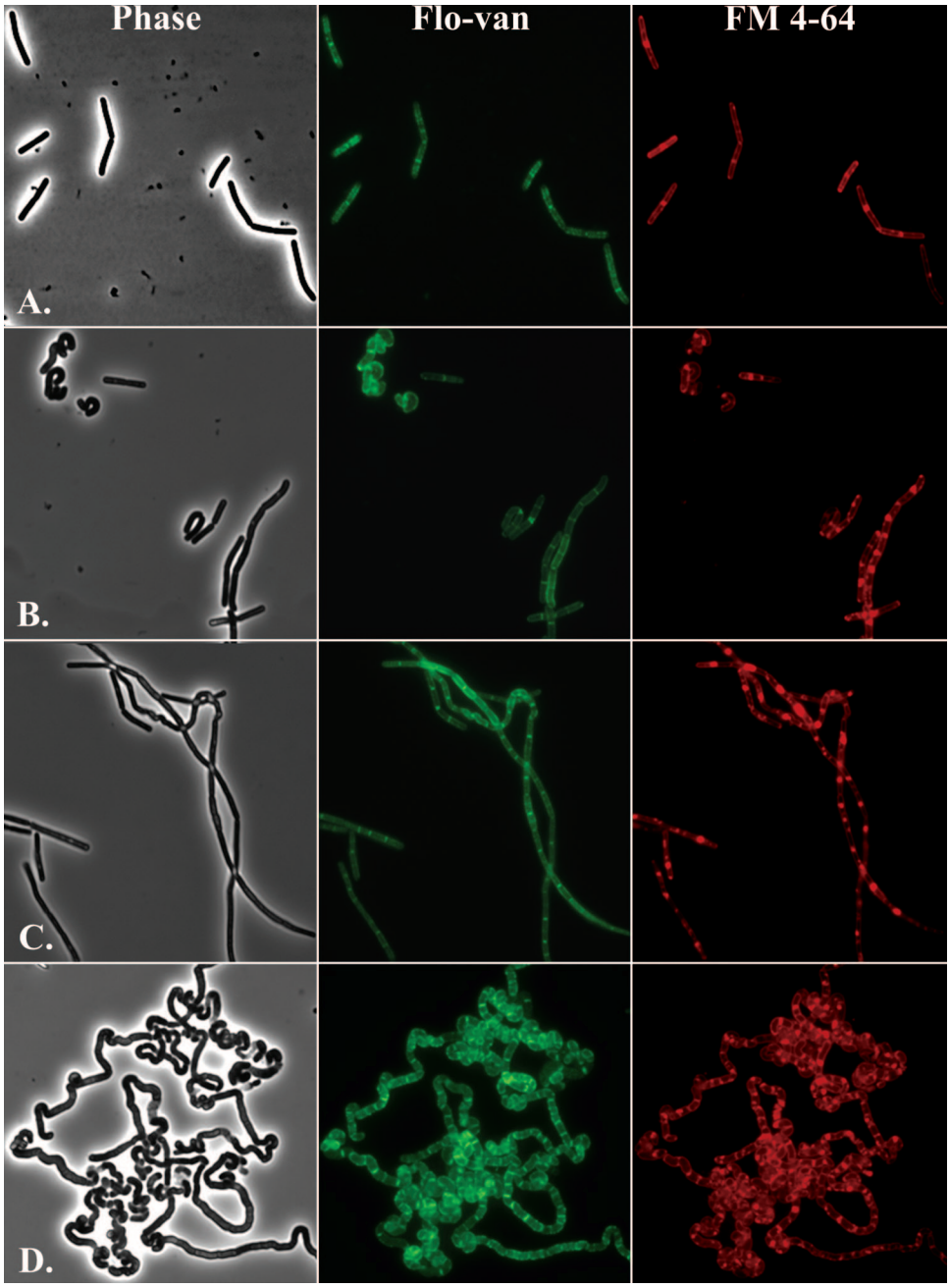

FIG. 4. Alteration of the cytoplasmic membrane leads to aberrant morphology. Exponential-phase cultures grown in LB were treated with both the membrane dye FM 4-64 and fluorescently labeled vancomycin. (A) Wild-type; (B) *ugtP*::MLS single mutant; (C) *mprF*::*kan pssA*::*spc ywnE*::*cat* triple mutant; (D) *ugtP*::MLS *mprF*::*kan pssA*::*spc ywnE*::*cat* quadruple mutant.

backgrounds (Fig. 6). This suggests that sporulation may have initiated earlier and asynchronously within this growing population, whereas sporulation genes are generally not expressed during logarithmic growth in the WT.

In several cases, the role of UgtP appeared to be most important, since the transcriptome changes were most dramatic for the U and UPMY mutations with relatively little effect noted in the MPY triple mutant. For example, genes involved in biosynthesis of fatty acids and under the negative control of FapR were strongly downregulated in both the U and UPMY backgrounds but only slightly altered in the MPY triple mutant. Genes negatively regulated by SinR, involved in the production of exopolysaccharides and biofilm formation (12), were also downregulated only in a U and UPMY background. In addition, the σ^M cell envelope stress regulon (18) was induced only in the U and UPMY backgrounds. This induction was verified by β-galactosidase assays, which showed that in a *ugtP* null background the expression level of the P_{sigM} promoter was higher throughout all growth phases (Fig. 7A), although it could still be additionally induced by treatment

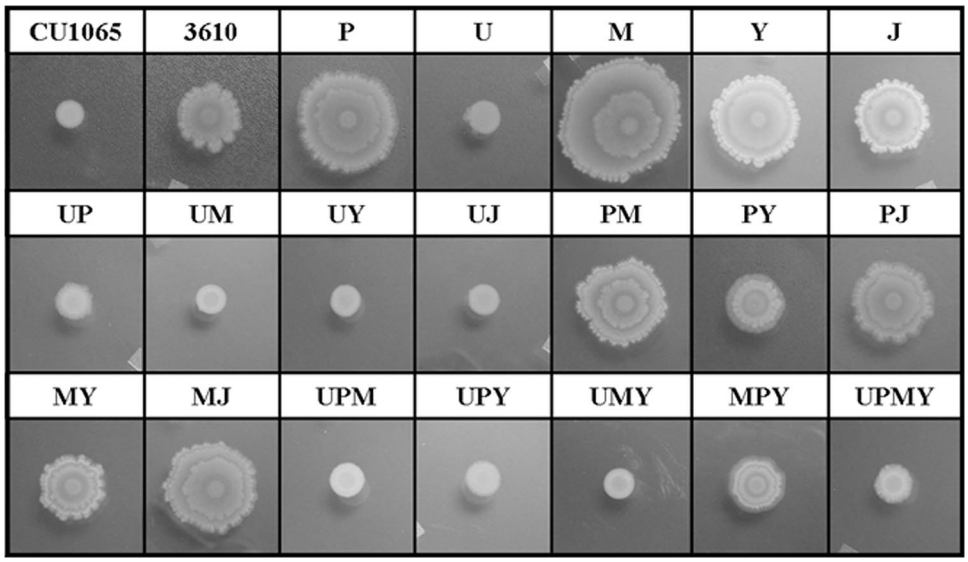

FIG. 5. Deletion of *ugtP* results in a swarming defect. Membrane mutants in the NCIB3610 background were grown to mid-exponential phase in LB and then spotted to 0.7% LB agar plates. Plates were incubated overnight.

with vancomycin (Fig. 7B). Interestingly, the expression of the regulons of extracytoplasmic function sigma factors σ^W (which provides intrinsic resistance to antimicrobial compounds [10]) and σ^X (which regulates modification of the cell envelope and resistance to cationic antimicrobial peptides [11]) was unaltered. A contrasting example is provided by analysis of genes assigned to the YvrGHb TCS regulon. YvrGHb is reported to regulate cell surface homeostatic functions (53). This regulon was expressed at a reduced level in the MPY triple mutant but affected to a lesser extent in the quadruple mutant.

Among the most highly induced genes in the UPMY and MPY mutant backgrounds were the *yrkNM* operon, encoding a GCN5-related N-acetyltransferase and an unknown protein, respectively, and *yrkO*, which encodes a putative transporter. *yrkN* and *yrkO* are thought to be positively regulated by the divergently encoded response regulator YrkP, which was suggested to regulate membrane function (44). However, the expression of the *ykcBC* operon, also thought to be regulated by YrkP (44), remained unchanged in all three backgrounds tested.

Concluding remarks. We report here the construction of a large set of isogenic strains with alterations in membrane lipid composition. Perhaps the most surprising finding from this work is that the membrane of *B. subtilis* can be greatly simpli-

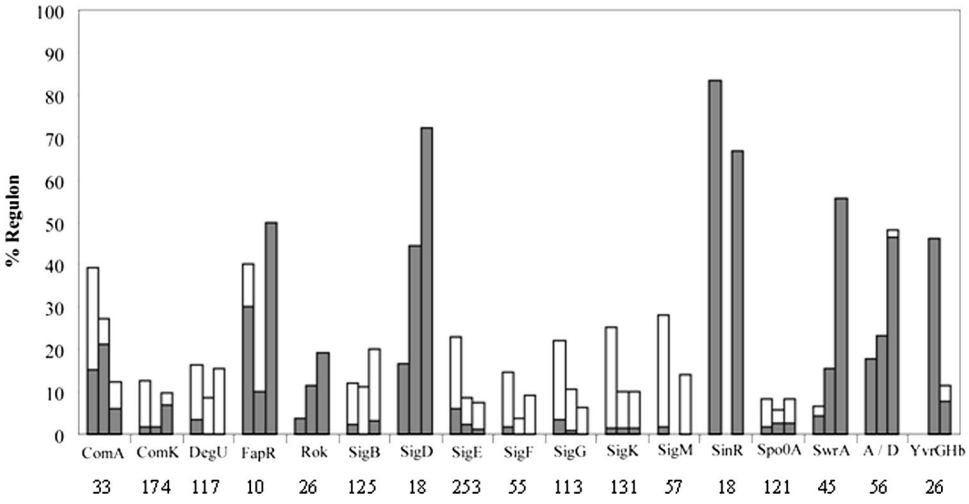

FIG. 6. Analysis of regulons, demonstrating differential expression patterns. Microarray experiments compared WT with either *ugtP*, MPY, or UPMY mutant strains. RNA was extracted from mid-log-phase cells grown in LB. Results represent averages of three separate experiments. Each three bars correspond to a specific regulon; the order of experiments is WT versus *ugtP*, WT versus MPY, and WT versus UPMY. The height of a bar represents the overall percentage of the regulon with altered expression. Gray sections represent the percentage of the regulon that was downregulated at least 1.5-fold, and white sections represent the percentage of the regulon that was upregulated at least 1.5-fold The number of genes in each regulon is beneath the regulon name (adapted from references 1, 4, 12, 18–20, 27, 39, 45, 47, 52 to 54, and 58).

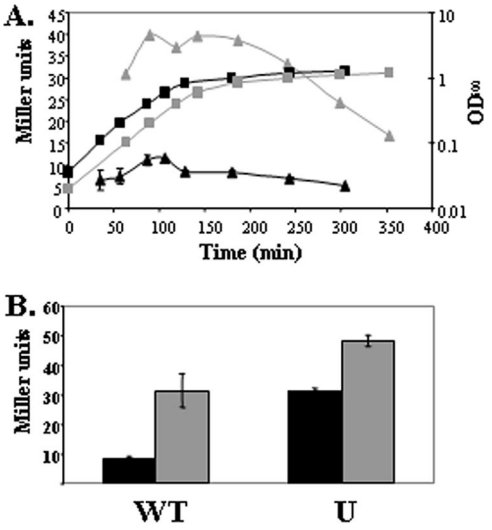

FIG. 7. Activity of the σ^M promoter is increased in a *ugtP*::MLS background. A. WT (black symbols) and *ugtP*::MLS (gray symbols) cells containing a P_{sigM}-*lacZ* promoter fusion were grown in LB. Samples were taken periodically, and the OD_{600} (squares) and LacZ activity (triangles) were monitored. B. P_{sigM} can still be induced in a *ugtP*::MLS background. Mid-log-phase cells were either untreated (black) or treated with 2 μg/ml vancomycin for 30 min.

fied by removal of GL, PE, LPG, and CL, and the cells grow robustly. These cells are morphologically abnormal and exhibit a combination of cell separation and cell shape defects: they grow as long and curled filaments. In this initial survey, we have highlighted the effects of grossly perturbing membrane composition on antibiotic sensitivity, swarming motility, and global transcription patterns. In many of our phenotypic assays, the loss of *ugtP* appears to have a dominant effect, with changes in the *ugtP* single mutant often more dramatic than in the MPY triple mutant. Additional studies are clearly required to further understand these complex phenotypes and to determine if the *ugtP* phenotypes are due to the loss of GL or in addition or instead to the important role of this protein as a metabolic sensor.

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