Streptococcus agalactiae MprF synthesizes a novel cationic glycolipid and contributes to brain entry and meningitis

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Running title: GBS MprF glycolipid synthesis and role in meningitis

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Abstract

Group B *Streptococcus* (GBS) is a major human pathogen, causing meningitis and severe infection in newborns, yet little is known about its lipid membrane. Here, we investigated the GBS lipid membrane and identify a novel cationic glycolipid, lysyl-glucosyl-diacylglycerol (Lys-Glc-DAG). Multiple peptide resistance factor (MprF) is highly conserved in many bacterial pathogens and plays a critical role in resistance against cationic antimicrobial peptides, cationic bacteriocins, and antibiotics. The MprF protein has been shown to catalyze the amino-acylation of the anionic phospholipid phosphatidylglycerol (PG). Most notably, MprF uses L-lysyl-tRNA to esterify PG with a positively charged lysine to produce lysyl-phosphatidylglycerol (Lys-PG). We demonstrate through heterologous host expression and gene deletion that the GBS MprF has an expanded substrate repertoire and is the biosynthetic enzyme responsible for both Lys-Glc-DAG and Lys-PG biosynthesis in GBS. Furthermore, we demonstrate that MprF contributes specifically to meningitis pathogenesis at the blood-brain barrier both *in vitro* and in an *in vivo* hematogenous murine infection model but does not contribute to bloodstream survival. These results greatly expand our knowledge of MprF functions and reveal insights into the survival mechanisms and pathogenesis of meningitis caused by GBS.

Importance

The lipidomes of many important Gram-positive human pathogens remain largely uncharacterized. By investigating the lipid membrane of *Streptococcus agalactiae* (Group B *Streptococcus*; GBS), an etiological agent responsible for meningitis and severe

48 diseases in newborns, we uncovered a novel glycolipid and biochemical activity of
49 Multiple peptide resistance factor (MprF) in GBS. MprF is known to add lysine to
50 phosphatidylglycerol (PG), forming Lys-PG. We show in GBS that it also adds lysine to
51 the glycolipid, glucosyl-diacylglycerol (Glc-DAG), to form Lys-Glc-DAG. We demonstrate
52 the GBS MprF contributes to brain entry and meningitis pathogenesis in mice. These
53 results expand our knowledge of MprF functions and reveal insights into the survival
54 mechanisms and pathogenesis of meningitis caused by GBS.

55

Observation

Streptococcus agalactiae (Group B *Streptococcus*; GBS) is a Gram-positive bacterium that colonizes the lower genital and gastrointestinal tracts of ~30% of healthy women (1, 2). GBS causes sepsis and pneumonia in neonates and is a leading cause of neonatal meningitis, resulting in long-lasting neurological effects in survivors (3-5). New therapeutic and preventative approaches and a more complete understanding of GBS pathogenesis are needed to mitigate the devastating impact of GBS on neonates. Despite the critical role of the bacterial membrane in host-pathogen interactions, little is known about this for GBS. Here, we investigated GBS membrane lipids using normal phase liquid chromatography (NPLC) coupled with electrospray ionization (ESI) high-resolution tandem mass spectrometry (HRMS/MS).

GBS lipidomic profile

The membrane lipids of three GBS clinical isolates of representative serotypes were characterized: COH1 (6), A909 (7), and CNCTC 10/84 (serotypes III, 1a, and V, respectively) (8). Common Gram-positive bacterial lipids were identified by normal phase LC coupled with negative ion ESI/MS/MS, including diacylglyercol (DAG), monohexosyldiacylglycerol (MHDAG), dihexosyldiacylglycerol (DHDAG), phosphatidylglycerol (PG), and lysyl-phosphatidylglycerol (Lys-PG), as shown by the negative total ion chromatogram (TIC) (Fig. 1A).

Surprisingly, the positive TIC (Fig. 1B, Supplemental Figure S1) shows highly abundant peaks of unknown identity at the retention time ~25-29 min. The mass spectra (Fig. 1C) and LC retention times of this lipid do not match with any other bacterial lipids we have

analyzed or exact masses in lipidomic databases. Tandem MS (MS/MS) in the positive ion mode (Fig. 1D), negative ion mode (Fig. 1E), and high-resolution mass measurement (Fig. 1C) allowed us to propose lysyl-glucosyl-diacylglyerol (Lys-Glc-DAG) (Fig. 1F) as the structure of this unknown lipid. Observed and exact masses of Lys-Glc-DAG are shown in Table S1. The assignment of glucose was based on the observation that glucosyl-diacylglycerol (Glc-DAG) is a major membrane component of GBS and other streptococci (9), and results from an isotopic labeling experiment using ^{13}C-labeled glucose (Glucose-$^{13}C_6$). The assignment of lysine modification was supported by an isotopic labeling experiment with deuterated lysine (lysine-*d4*). The expected mass shifts (+4 Da) were observed in both molecular ions and MS/MS product ions (Supplemental Figure S2). Comparison of both MS/MS spectra of labeled (Glucose-$^{13}C_6$) and unlabeled Lys-Glc-DAG indicates the lysine residue is linked to the 6-position of glucose (Supplemental Figure S2). Lys-Glc-DAG consists of several molecular species with different fatty acyl compositions resulting in different retention times and multiple, unresolved TIC peaks (~25-29 min).

GBS MprF synthesizes Lys-PG and Lys-Glc-DAG

The enzyme MprF (multiple peptide resistance factor) catalyzes the aminoacylation of PG with lysine in some Gram-positive pathogens (10, 11). GBS MprF is responsible and sufficient for synthesizing both Lys-Glc-DAG and Lys-PG. Deletion of *mprF* from COH1 abolishes both Lys-Glc-DAG and Lys-PG synthesis, which are restored by complementation (Fig. 1G). Deletion of GBS *mprF* does not confer a growth defect in Todd-Hewitt broth or tissue culture medium. The oral colonizer *Streptococcus mitis* does

not encode *mprF* or synthesize Lys-PG, but synthesizes Glc-DAG and PG (12, 13). Heterologous expression of GBS *mprF* in *S. mitis* results in Lys-Glc-DAG and Lys-PG production (Fig. 1H), while expression of *Enterococcus faecium mprF* results in only Lys-PG production (Fig. 1H), as expected (14). Biosynthetic pathways involving MprF are shown in Fig. 1I.

MprF contributes to GBS pathogenesis

We investigated whether MprF contributes to GBS invasion into brain endothelial cells and development of meningitis. To mimic the human blood-brain barrier (BBB), we utilized the human cerebral microvascular endothelial cell line hCMEC/D3. *In vitro* assays for adhesion and invasion were performed as described previously (9, 15, 16). There was no significant difference in the ability of Δ*mprF* compared to WT and complement cells to attach to hCMEC/D3 cells (Fig. 2A). However, we observed a significant decrease in the amount of Δ*mprF* recovered from the intracellular compartment of hCMEC/D3 cells (Fig. 2A). The reduced invasion phenotype was confirmed in the hypervirulent serotype V strain, CJB111 (17, 18) (Supplemental Figure S3). Intracellular survival requires GBS to survive low pH conditions in lysosomes (pH 4.5 – 5.5) (19), and Δ*mprF* is unable to survive low pH conditions (Fig. 2B). This suggests that MprF promotes GBS invasion into the BBB, and intracellular survival.

We hypothesized that these *in vitro* phenotypes of Δ*mprF* would translate into a diminished ability to penetrate the BBB and produce meningitis *in vivo*. Using our

125 standard model of GBS hematogenous meningitis (9, 15) mice were challenged with
126 either WT GBS or Δ*mprF*. Mice were sacrificed at 72 h to determine bacterial loads in
127 blood and brain tissue. We recovered significantly less CFU in the brains of Δ*mprF*-
128 infected mice compared to the WT-infected mice (Fig. 2C). However, there was no
129 significant difference in CFU recovered from the bloodstream (Fig. 2D), demonstrating
130 that Δ*mprF* does not have a general *in vivo* growth defect. Furthermore, mice challenged
131 with WT GBS had significantly more leukocyte infiltration, meningeal thickening and
132 neutrophil chemokine, KC, in brain homogenates compared to Δ*mprF* mutant-infected
133 animals (Fig. 2E-G). Taken together, *mprF* contributes to GBS penetration into the brain
134 and to the pathogenesis of meningitis *in vivo*.
135
136 **Conclusion**
137 Bacteria use enzymes such as MprF to fine-tune cell envelope properties for stress
138 response and virulence. We discovered that GBS MprF uniquely synthesizes a novel and
139 highly abundant cationic glycolipid, Lys-Glc-DAG, as well as Lys-PG. This establishes
140 that GBS capitalizes on MprF to modulate charges of both glycolipids and phospholipids
141 at the membrane. Deletion of *mprF* impacts GBS virulence. Interestingly, this effect is
142 observed for meningitis, and not for bacteremia, demonstrating that MprF and/or MprF-
143 synthesized lipids play specific roles in BBB penetration but not *in vivo* survival in general.
144 In future studies, generating GBS MprF variants that synthesize only Lys-Glc-DAG or
145 Lys-PG at wild-type levels will allow for identification of the specific roles each lipid plays
146 in meningitis.
147

Our identification of the novel Lys-Glc-DAG glycolipid rationalizes further study of the lipidomes of human pathogens. The decreased *in vivo* pathogenicity of Δ*mprF* identifies GBS MprF as a candidate for targeting by antimicrobial strategies. Moreover, that Lys-Glc-DAG is a major GBS membrane component holds promise that Lys-Glc-DAG could be utilized as a specific molecular biomarker for GBS diagnostics.

Materials and methods

Methods were performed using established techniques; see Supplemental Text S1. Strains listed in Table S2 and primers Table S3. Illumina sequence reads are deposited in the Sequence Read Archive, accession PRJNA675025.

Conflict of interest

The authors have declared that no conflict of interest exists.

Acknowledgements

We thank Kathryn Patras at the University of California San Diego for the CNCTC 10/84 strain and Moutusee Islam in Kelli Palmer's lab at The University of Texas at Dallas for *E. faecium* 1,231,410 DNA.

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Figure legends

Fig 1. Total ion chromatogram (TIC) of LC/MS in A) negative ion mode, B) positive ion mode shows a major unknown lipid eluting at ~25-29 min. C) Positive ESI/MS showing the $[M+H]^+$ ions of the unknown lipid. D) Positive ion MS/MS spectrum of $[M+H]^+$ at *m/z* 885.6 and E) negative ion MS/MS spectrum of $[M-H]^-$ at *m/z* 883.6 of the unknown lipid. F) Lys-Glc-DAG (16:0/18:1) is proposed as the structure of the unknown lipid. G) TIC showing loss of Lys-Glc-DAG and Lys-PG in COH1Δ*mprF* which is present when *mprF* is complemented *in trans*. H) Lys-Glc-DAG and Lys-PG is only present in *S. mitis* when expressing GBS *mprF* compared to Lys-PG only when expressing *E. faecium mprF*. "*" denotes methylcarbamate of Lys-Glc-DAG, an extraction artifact due to the use of chloroform. I) Biosynthetic pathways involving MprF.

Fig 2. A) *In vitro* assays for adherence and invasion of hCMEC cells indicates *mprF* contributes to invasion but not adherence to brain endothelium (mean of 3 replicate experiments with 4 technical replicates, mean and SEM). B) pH-adjusted medium growth indicates Δ*mprF* cannot survive in low pH conditions, mean and SD. Groups of CD-1 mice were injected intravenously with COH1 WT or COH1Δ*mprF* strains and bacterial counts were assessed in the C) brain and D) blood after 72h. Representative data from 2 independent experiments are shown (WT, $n = 20$; Δ*mprF,* $n = 19$). E) Hematoxylin-eosin-stained brain sections from representative mice infected with WT (top) or Δ*mprF* mutant (bottom); arrows indicate meningeal thickening and leukocyte infiltration. F) Quantification of meningeal thickening using ImageJ. G) KC chemokine production measured by ELISA. Panels C,D,F, and G) median indicated. Statistical analyses performed using GraphPad Prism: A) One-way ANOVA with Tukey's multiple comparisons test; C,D,F) unpaired two-tailed t-test; G) Mann-Whitney U test; p-values indicated; ns, no significance (p-value > 0.05).

Supplemental files

Supplemental Text S1: Materials and Methods

Supplemental Figure S1. Detection of Lys-PG and Lys-Glc-DAG in *S. agalactiae* A909 and *S. agalactiae* CNCTC 10/84.

Supplemental Figure S2. Isotopic incorporation of deuterated lysine and ^{13}C-labeled glucose into Lys-Glc-DAG and Lys-PG.

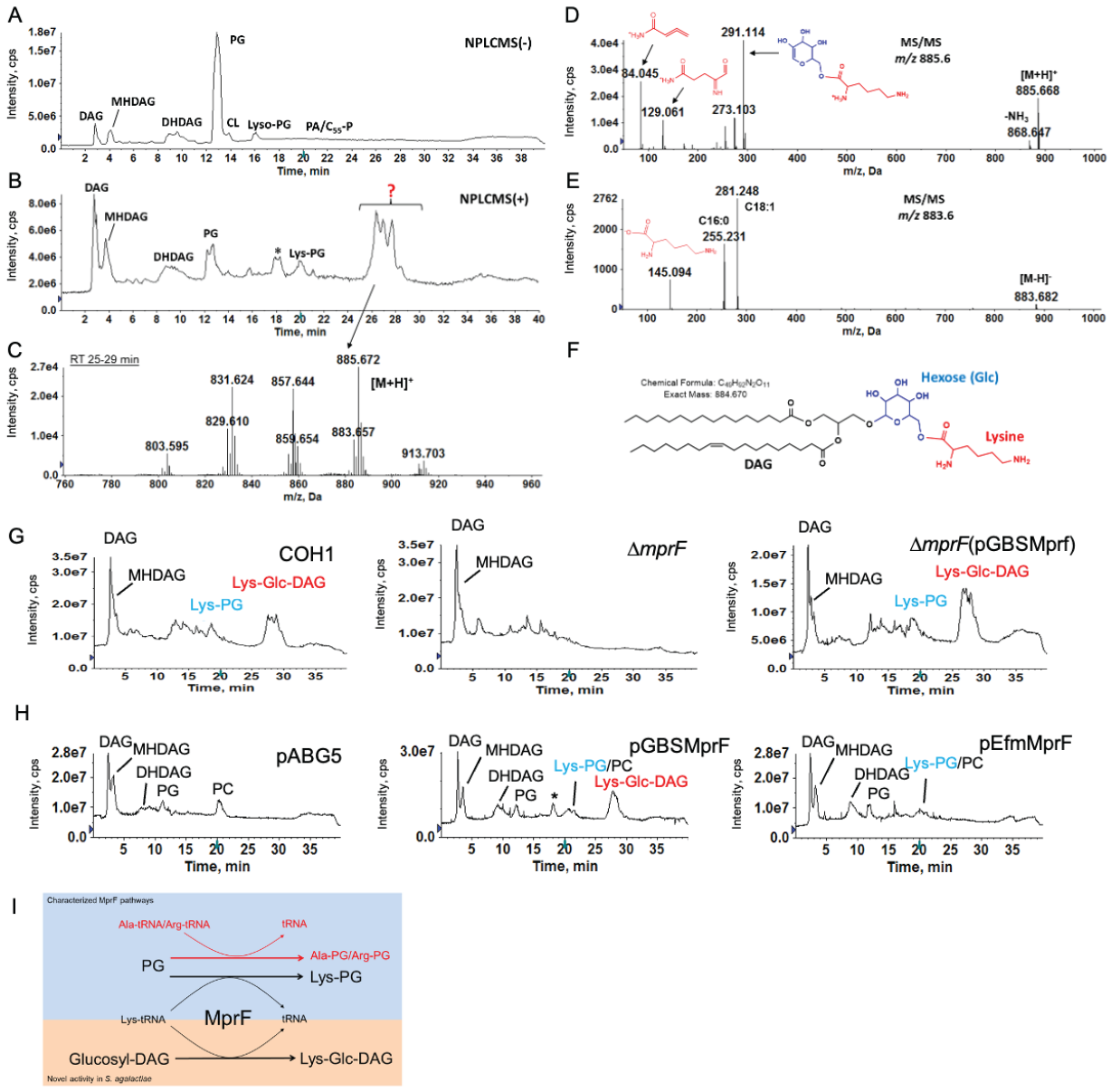
Supplemental Figure S3. *In vitro* hCMEC adhesion and invasion of CJB111 strains.

Supplemental Table S1. Observed and calculated exact masses of the $[M+H]^+$ ions of Lys-Glc-DAG molecular species in *S. agalactiae* COH1.

278 Supplemental Table S2. Strains and plasmids used in this study.

279 Supplemental Table S3. Primers used in this study.

280



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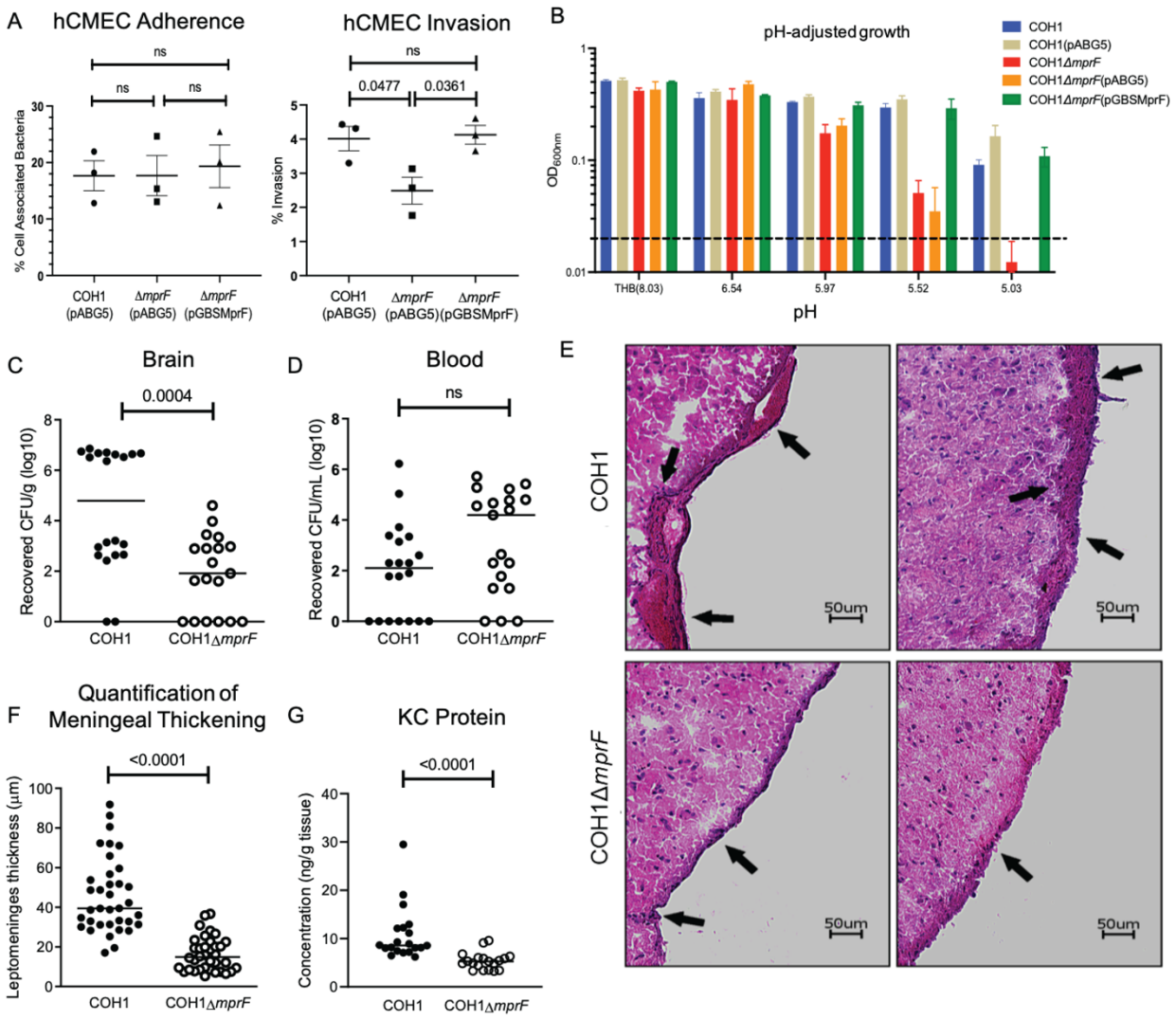

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307 **Supplemental Text, Figures, and Tables**

308 ***Streptococcus agalactiae* MprF synthesizes a novel cationic glycolipid and contributes to**

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Supplemental Text S1: Materials and Methods

Bacterial strains, media, and growth conditions

GBS strains were grown statically at 37°C in Todd-Hewitt Broth (THB) and *S. mitis* strains were grown statically at 37°C and 5% CO_2, unless otherwise stated. Streptococcal chemically defined medium (20) was diluted from stock as described (21) with 1% w/v glucose (referred to as DM), slightly modified from (22), unless otherwise stated. *Escherichia coli* strains were grown in Lysogeny Broth (LB) at 37°C with rotation at 225 rpm. Kanamycin and erythromycin (Sigma-Aldrich) were supplemented to media at 50 µg/mL and 300 µg/mL for *E. coli,* respectively, or 300 µg/mL and 5 µg/mL, respectively, for streptococcal strains.

Routine molecular biology techniques

All PCR reactions utilized Phusion polymerase (Thermo Fisher). PCR products and restriction digest products were purified using GeneJET PCR purification kit (Thermo Fisher) per manufacturer protocols. See Table S3 for primers. Plasmids were extracted using GeneJET plasmid miniprep kit (Thermo Fisher) per manufacturer protocols. Restriction enzyme digests utilized XbaI, XhoI, and PstI (New England Biolabs) for 3 h at 37°C in a water bath. Ligations utilized T4 DNA ligase (New England Biolabs) at 16°C overnight or Gibson Assembly Master Mix (New England Biolabs) per manufacturer protocols where stated. All plasmid constructs were sequence confirmed by Sanger sequencing (Massachusetts General Hospital DNA Core or CU Anschutz Molecular Biology Core).

345 **Deuterated lysine and $^{13}C_6$-D-glucose isotope tracking**

346 A GBS COH1 colony was inoculated into 15 mL of DM containing 450 µM lysine-*d4* (Cambridge
347 Isotopes Laboratories) or a single COH1 colony was inoculated into 10 mL DM supplemented
348 with 0.5% w/v $^{13}C_6$D-glucose (U-13C6, Cambridge Isotopes Laboratories) for overnight growth for
349 lipidomic analysis described below.

350

351 **Construction of MprF expression plasmids**

352 Genomic DNA was isolated using the Qiagen DNeasy Blood and Tissue kit per the manufacturer's
353 protocol with the exception that cells were pre-treated with 180 µL 50 mg/mL lysozyme, 25 µL
354 2500 U/mL mutanolysin, and 15 µL 20 mg/mL pre-boiled RNase A and incubated at 37°C for 2 h.
355 The *mprF* genes from GBS COH1, (GBSCOH1_1931), GBS CJB111 (ID870_10050), and *E.*
356 *faecium* 1,231,410 (EFTG_00601) were amplified and either Gibson ligated into pABG5Δ*phoZ*
357 (23) or ligated into pDCErm (24). Plasmid constructs were transformed into chemical competent
358 *E. coli*. Briefly, chemically competent cells were incubated for 10 min on ice with 5 µL of Gibson
359 reaction before heat shock at 42°C for 70 sec, then placed on ice for 2 min before 900 µL of cold
360 SOC medium was added. Outgrowth was performed at 37°C, with shaking at 225 rpm, for 1 h.
361 Cultures were plated on LB agar plates containing 50 µg/mL kanamycin. Colonies were screened
362 by PCR for presence of the *mprF* insert.

363

364 **Expression of *mprF* in *S. mitis***

365 Natural transformation was performed as previously described (12). Briefly, precultures were
366 thawed at room temperature, diluted in 900 µL of THB, further diluted 1:50 in prewarmed 5 mL
367 THB, and incubated for 45 min at 37°C. 500 µL of culture was then aliquoted with 1 µL of 1 mg/ml

competence-stimulating peptide (EIRQTHNIFFNFFKRR) and 1 µg/mL plasmid. Transformation reaction mixtures were cultured for 2 h at 37°C in microcentrifuge tubes before being plated on THB agar supplemented with 300 µg/mL kanamycin. Single transformant colonies were cultured in 15 mL THB overnight. PCR was used to confirm the presence of the *mprF* insert on the plasmid. Plasmids were extracted and sequence confirmed as described above. Lipidomics was performed as described below in biological triplicate.

Construction of *mprF* deletion plasmid

Regions ~2 kb upstream and downstream of the GBS COH1 *mprF* (GBSCOH1_1931) or CJB111 (ID870_10050) were amplified using PCR. Plasmid, pMBSacB (25), and the PCR products were digested using appropriate restriction enzymes and ligated overnight. 7 µL of the ligation reaction was transformed into chemically competent *E. coli* DH5α as described above, except that outgrowth was performed at 28°C with shaking at 225 rpm for 90 min prior to plating on LB agar supplemented with 300 µg/mL erythromycin. Plates were incubated at 28°C for 72 h. Colonies were screened by PCR for correct plasmid construction. Positive colonies were inoculated into 50 mL LB media containing antibiotic and incubated at 28°C with rotation at 225 rpm for 72 h. Cultures were pelleted using a Sorvall RC6+ centrifuge at 4,280 x *g* for 6 min at room temperature. Plasmid was extracted as described above except the cell pellet was split into 5 columns to prevent overloading and serial eluted into 50 µL. Plasmid construction was confirmed via restriction digest using XhoI and XbaI, and the insert was PCR amplified and sequence-verified.

391 **Generation of electrocompetent GBS cells for *mprF* knockout**

392 Electrocompetent cells were generated as described (25) with minor modifications. Briefly, a GBS
393 COH1 or CJB111 colony was inoculated in 5 mL M17 medium (BD Bacto) with 0.5% glucose and
394 grown overnight at 37°C. The 5 mL was used to inoculate a second overnight culture of 50 mL
395 pre-warmed filter-sterilized M17 medium containing 0.5% glucose, 0.6% glycine, and 25% PEG
396 8000. The second overnight was added to 130 mL of the same medium and grown for 1 h at
397 37°C. Cells were pelleted at 3,200 x *g* in a Sorvall RC6+ at 4°C for 10 min. Cells were washed
398 twice with 25 mL cold filter-sterilized GBS wash buffer containing 25% PEG 8000 and 10%
399 glycerol in water, and pelleted as above. Cell pellets were re-suspended in 1 mL GBS wash buffer
400 and either used immediately for transformation or stored in 100 µL aliquots at -80°C until use.

401

402 **Deletion of GBS COH1 and CJB111 *mprF***

403 Electrocompetent cells were generated as described (25) with minor modifications. The double
404 crossover homologous recombination knockout strategy was performed as described previously
405 (16, 25, 26) with minor modifications. 1 µg of plasmid was added to electrocompetent GBS cells
406 and transferred to a cold 1 mm cuvette (Fisher or BioRad). Electroporation was carried out at 2.5
407 kV on an Eppendorf eporator. 1 mL of THB containing 12.5% PEG 8000, 20 mM $MgCl_2$, and 2
408 mM $CaCl_2$ was immediately added and then the entire reaction was transferred to a glass culture
409 tube. Outgrowth was at 28°C for 2 h followed by plating on THB agar supplemented with 5 µg/mL
410 erythromycin. Plates were incubated for 48 h at 28°C. A single colony was cultured overnight in
411 5 mL THB with 5 µg/mL erythromycin at 28°C. The culture was screened via PCR for the plasmid
412 insert with the initial denaturing step extended to 10 min. The overnight culture was diluted 1:1000
413 THB containing 5 µg/mL erythromycin and incubated overnight at 37°C to promote single cross
414 over events. The culture was then serial diluted and plated on THB agar plates with antibiotic and

incubated at 37°C overnight. Colonies were screened for single crossover events by PCR. Single crossover colonies were inoculated in 5 mL THB at 28°C to promote double crossover events. Overnight cultures were diluted 1:1000 into 5 mL THB containing sterile 0.75 M sucrose and incubated at 37°C. Overnight cultures were serial diluted and plated on THB agar and incubated at 37°C overnight. Colonies were patched onto THB agar with and without 5 µg/mL erythromycin to confirm loss of plasmid. Colonies were also screened by PCR for the loss of *mprF*. Colonies positive for the loss of *mprF* were inoculated into 5 mL THB at 37°C. Cultures were stocked and gDNA extracted as described above, with minor modifications. Sequence confirmation of the *mprF* knockout was done via Sanger sequencing (Massachusetts General Hospital DNA Core or CU Anschutz Molecular Biology Core). The mutant was grown overnight in 15 mL THB at 37°C and pelleted at 6,150 x *g* for 5 min in a Sorvall RC6+ centrifuge at room temperature for lipid extraction as described. Genomic DNA of COH1Δ*mprF* was isolated as described above and whole genome sequencing was performed in paired-end reads (2 by 150 bp) on the Illumina NextSeq 550 platform at the Microbial Genome Sequencing Center (Pittsburgh, PA).

Complementation of *mprF* in COH1Δ*mprF* and CJB111Δ*mprF*

Electrocompetent COH1Δ*mprF* were generated as previously described (27). Briefly, GBSΔ*mprF* was inoculated into 5 mL THB with 0.6% glycine and grown overnight. The culture was expanded to 50 mL in pre-warmed THB with 0.6% glycine and grown to an OD_{600} nm of 0.3 and pelleted for 10 min at 3200 x *g* at 4°C in a Sorvall RC6+ floor centrifuge. The pellet was kept on ice through the remainder of the protocol. The pellet was washed twice with 25 mL and once with 10 mL of cold 0.625 M sucrose and pelleted as above. The cell pellet was resuspended in 400 µL of cold 20% glycerol, aliquoted in 50 µL aliquots, and used immediately or stored at -80°C until use. Electroporation was performed as described above, with recovery in THB supplemented with 0.25 M sucrose, and plated on THB agar with kanamycin at 300 µg/mL.

Acidic Bligh-Dyer extractions

Centrifugation was performed using a Sorvall RC6+ centrifuge. Cultures were pelleted at 4,280 x *g* for 5 min at room temperature unless otherwise stated. The supernatants were removed, and cell pellets were stored at -80°C until acidic Bligh-Dyer lipid extractions were performed as described (12). Briefly, cell pellets were resuspended in 1X PBS (Sigma-Aldrich) and transferred to Coring Pyrex glass tubes with PTFR-lined caps (VWR), followed by 1:2 vol:vol chloroform:methanol addition. Single phase extractions were vortexed periodically and incubated at room temperature for 15 minutes before 500 x *g* centrifugation for 10 min. A two-phase Bligh-Dyer was achieved by addition of 100 µL 37% HCl, 1 mL $CHCl_3$, and 900 µl of 1X PBS, which was then vortexed and centrifuged for 5 min at 500 x *g*. The lower phase was removed to a new tube and dried under nitrogen before being stored at -80°C prior to lipidomic analysis.

Liquid Chromatography/Electrospray Ionization Mass Spectrometry

Normal phase LC was performed on an Agilent 1200 quaternary LC system equipped with an Ascentis silica HPLC column (5 µm; 25 cm by 2.1 mm; Sigma-Aldrich) as described previously (28, 29). Briefly, mobile phase A consisted of chloroform-methanol-aqueous ammonium hydroxide (800:195:5, vol/vol), mobile phase B consisted of chloroform-methanol-water-aqueous ammonium hydroxide (600:340:50:5, vol/vol), and mobile phase C consisted of chloroform-methanol-water-aqueous ammonium hydroxide (450:450:95:5, vol/vol). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min, then linearly increased to 100% mobile phase B over 14 min, and held at 100% mobile phase B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min, held at 100% mobile phase C for 3 min, and, finally, returned to 100% mobile phase A over 0.5 min and held at 100% mobile phase A for 5 min. The LC eluent (with a total flow rate of 300 µl/min) was introduced into

464 the ESI source of a high-resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA).
465 Instrumental settings for negative-ion ESI and MS/MS analysis of lipid species were: IS = -4,500
466 V, CUR = 20 psi, GSI = 20 psi, DP = -55 V, and FP = -150V. Settings for positive-ion ESI and
467 MS/MS analysis were: IS = +5,000 V, CUR = 20 psi, GSI = 20 psi, DP = +55 V, and FP = +50V.
468 The MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using
469 Analyst TF1.5 software (Sciex, Framingham, MA).

470

471 **pH-adjusted THB growth**

472 Approximately 30 mL of fresh THB were adjusted to different pH values, measured using a Mettler
473 Toledo FiveEasy pH/MV meter, and sterile-filtered using 0.22 µM syringe filters. A final volume of
474 200 µL culture medium was aliquoted per well in a flat-bottom 96 well plate (Falcon); culture media
475 were not supplemented with antibiotics. Overnight cultures of GBS strains were used to inoculate
476 the wells to a starting OD_{600nm} 0.02 per well. Plates were incubated for 24 h at 37°C before OD_{600nm}
477 was read using a BioTek MX Synergy 2 plate reader. This experiment was performed in biological
478 triplicate.

479

480 **hCMEC cell adherence and invasion assays**

481 Human Cerebral Microvascular Endothelial cells hCMEC/D3 (obtained from Millipore) were grown
482 in EndoGRO-MV complete media (Millipore, SCME004) supplemented with 5% fetal bovine
483 serum (FBS) and 1 ng/ml fibroblast growth factor-2 (FGF-2; Millipore). Cells were grown in tissue
484 culture treated 24 well plates and 5% CO_2 at 37°C.

485

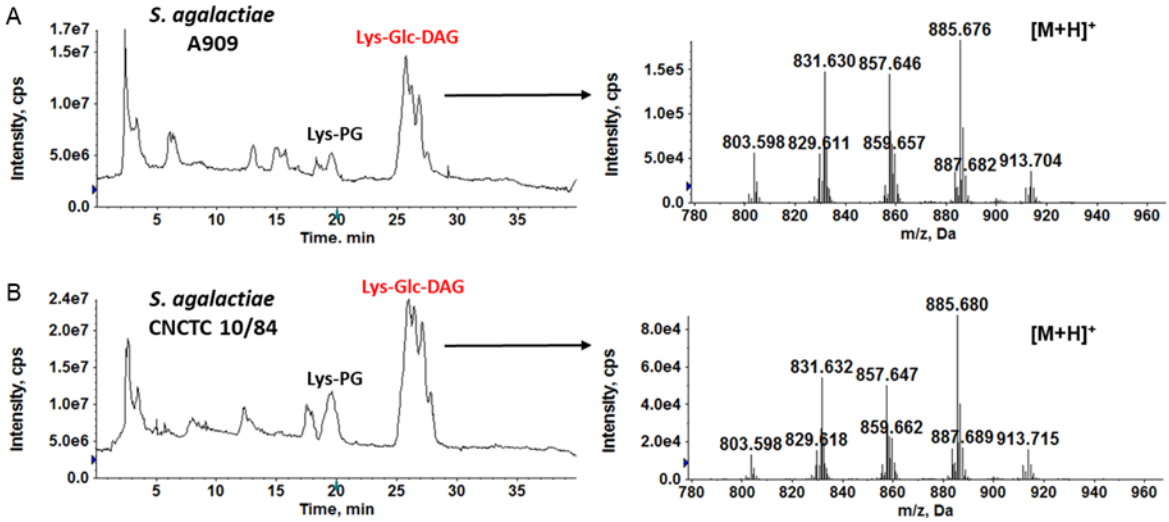
Assays to determine the total number of bacteria adhered to host cells or intracellular bacteria were performed as described previously (15, 16). Briefly, bacteria were grown to mid log phase (OD_{600nm} 0.4-0.5) and normalized to 1 x 10^8 to infect cell monolayers at a multiplicity of infection (MOI) of 1 (1 x 10^5 CFU per well). The total cell-associated GBS were recovered after 30 min incubation. Cells were washed slowly five times with 500 µL 1X PBS (Sigma) and detached by addition of 100 µL of 0.25% trypsin-EDTA solution (Gibco) and incubation for 5 min before lysing the eukaryotic cells with the addition of 400 µL of 0.025% Triton X-100 (Sigma) and vigorous pipetting. The lysates were then serially diluted and plated on THB agar and incubated overnight to determine CFU. Bacterial invasion assays were performed as described above except infection plates were incubated for 2 h before incubation with 100 µg gentamicin (Sigma) and 5 µg penicillin (Sigma) supplemented media for an additional 2 h to kill all extracellular bacteria, prior to being trypsinized, lysed, and plated as described. Experiments were performed in biological triplicate with four technical replicates per experiment.

Murine model of GBS hematogenous meningitis

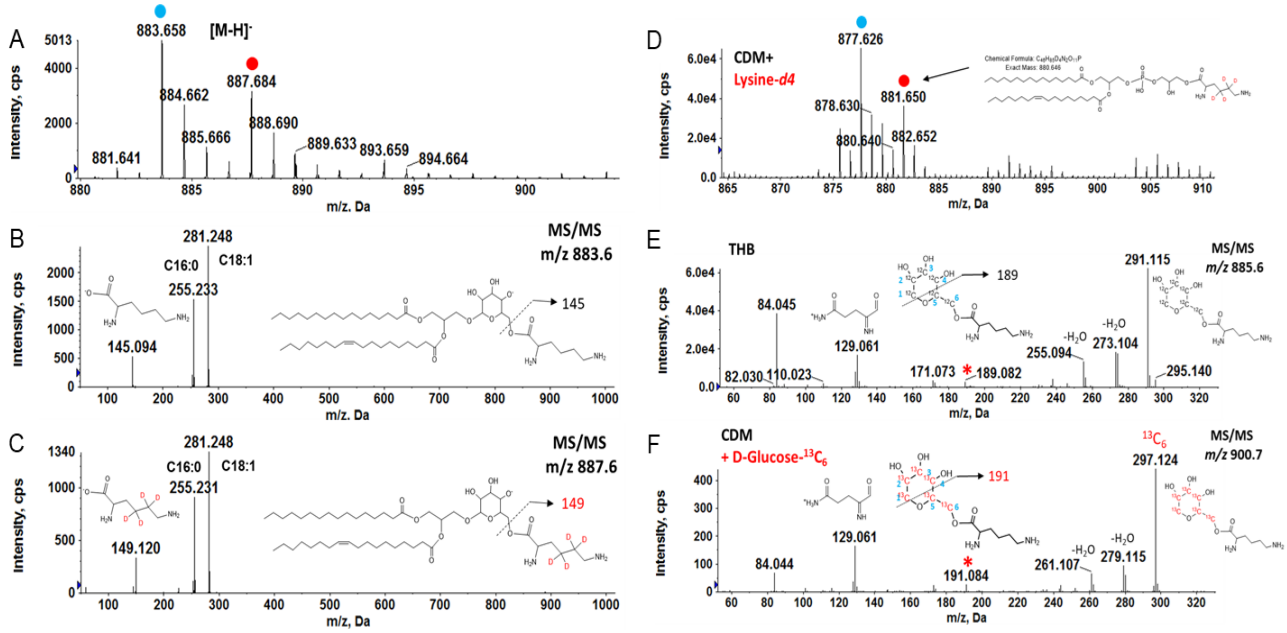
All animal experiments were conducted under the approval of the Institutional Animal Care and Use Committee (#00316) at the University of Colorado Anschutz Medical Campus and performed using accepted veterinary standards. The murine meningitis model was performed as previously described (16, 30, 31). Briefly, 7-week-old male CD1 (Charles River) mice were challenged intravenously with 1 × 10^9 CFU of WT COH1 or the isogenic Δ*mprF* mutant. At 72 h post-infection, mice were euthanized and blood and brain tissue were harvested, homogenized, and serially diluted on THB agar plates to determine bacterial CFU.

Histology and ELISA

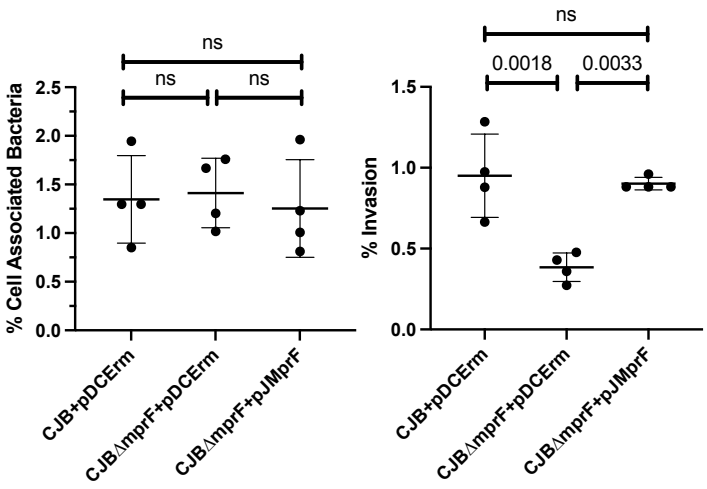
Mouse brain tissue was frozen in OCT compound (Sakura) and sectioned using a CM1950 cryostat (Leica). Sections were stained using hematoxylin and eosin (Sigma) and images were taken using a BZ-X710 microscope (Keyence). Images were analyzed using ImageJ software. Meningeal thickening was quantified from sections taken from three different mice per group, and six images per slide. Meningeal thickening was quantified across two points per image. KC protein from mouse brain homogenates was detected by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D systems).



Supplemental Figure S1. Detection of Lys-PG and Lys-Glc-DAG in *S. agalactiae* A909 and *S. agalactiae* CNCTC 10/84. Positive TICs (left panels) showing the presence of Lys-PG and Lys-Glc-DAG in S. *agalactiae* A909 and S. *agalactiae* CNCTC 10/84. Mass spectra (right panels) show the $[M+H]^+$ ions of Lys-Glc-DAG.



Supplemental Figure S2. Isotopic incorporation of deuterated lysine and ^{13}C-labeled glucose into Lys-Glc-DAG and Lys-PG. The lipid extracts of *S. agalactiae* COH1 cultured in DM, DM supplemented with 450 µM L-lysine-*d4* (4,4,5,5-D4), or in DM containing 0.5% w/v D-Glucose (U-$^{13}C_6$) were analyzed by LC-ESI/MS in the positive ion mode. A) Negative ESI/MS of $[M-H]^-$ ions of major Lys-Glc-DAG species in *S. agalactiae* COH1 when cultured in DM supplemented with lysine-*d4*. The incorporation of lysine-*d4* into Lys-Glc-DAG is evidenced by an upward *m/z* shift of 4 Da of the $[M-H]^-$ ion (from *m/z* 883 to *m/z* 887). B) MS/MS of $[M-H]^-$ at *m/z* 883.6 produces a deprotonated lysine residue at *m/z* 145. C) MS/MS of $[M-H]^-$ at *m/z* 887.6 produces a deprotonated lysine-*d4* residue at *m/z* 149. D) $[M+H]^+$ ions of major Lys-PG species in *S. agalactiae* COH1 cultured in DM supplemented with lysine-*d4.* The incorporation of lysine-*d4* in Lys-PG is evidenced by an upward *m/z* shift of 4 Da from unlabeled Lys-PG (blue dot) to labeled Lys-PG (red dot). E) MS/MS of 885.6. A major product ion at *m/z* 291.1 is derived from glucose-lysine residue. F) MS/MS of *m/z* 900.7 (containing fifteen ^{13}C atoms). The presence of *m/z* 297.1 (with 6 Da shift) is consistent with glucose in Lys-Glc-DAG is replaced with D-Glucose (U-$^{13}C_6$). The other nine ^{13}C atoms are incorporated into the DAG portion of Lys-Glc-DAG. Furthermore, MS/MS data indicate that lysine is linked to the C6 position of glucose by the fragmentation schemes for forming *m/z* 189 ion from the unlabeled Lys-Glc-DAG and *m/z* 191 ion from the ^{13}C-labeled Lys-Glc-DAG.



Supplemental Figure S3. *In vitro* hCMEC adhesion and invasion of CJB111 strains. *In vitro* assays for adherence and invasion of hCMEC cells indicates *mprF* contributes to invasion but not adherence to brain endothelium. Data indicates the percentage of the initial inoculum that was recovered. Experiments were performed three times with each condition in quadruplicate. Data from one representative experiment is shown, mean and standard deviation indicated. One-Way ANOVA with Tukey's multiple comparisons statistical test was used. P-values indicated; ns, not significant.

554 **Supplemental Table S1. Observed and calculated exact masses of the $[M+H]^+$ ions of Lys-**
555 **Glc-DAG molecular species in *S. agalactiae* COH1.**

Lys-Glc-DAG[1]	$[M+H]^+$	
	Observed mass	Exact mass
C28:1	801.575	801.583
C28:0	803.595	803.599
C30:1	829.610	829.615
C30:0	831.624	831.630
C32:2	855.623	855.630
C32:1	857.644	857.646
C34:2	883.657	883.622
C34:1	885.672	885.677
C36:2	911.686	911.693
C36:1	913.703	913.709

556 [1]The numbers before and after colons indicate the total acyl chain carbon atoms and double
557 bonds, respectively.

Supplemental Table S2. Strains and plasmids used in this study.

Organism	Strain	Description	Ref
S. agalactiae	ATCC BAA-1176 (COH1)	Wild-type *S. agalactiae* strain, serotype III	(6)
	COH1Δ*mprF*	*mprF* (GBSCOH1_1931) deletion strain	This work
	COH1Δ*mprF(*pABG5)	Empty vector control strain	This work
	COH1Δ*mprF*(pGBSMprf)	Expresses GBS *mprF* from P_{prtF} in pABG5Δ*phoZ*	This work
	COH1(pABG5)	Empty vector control	This work
	CJB111	Wilt-type *S. agalactiae* strain, serotype V	(17, 18)
	CJB111Δ*mprF*	*mprF* (ID870_10050) deletion strain	This work
	CJB111Δ*mprF*(pDCErm)	Empty vector control strain	This work
	CJB111Δ*mprF*(pJMprF)	Expresses GBS *mprF* in pDCErm	This work
	ATCC BAA-1138 (A909)	Wild-type *S. agalactiae* strain, serotype Ia	(7)
	CNCTC 10/84	Wild-type *S. agalactiae* strain, serotype V. Obtained from Dr. K Patras, UCSD	(8, 32)
S. mitis	ATCC 49456	Wild-type *S. mitis* type strain, also known as NCTC 12261	(33)
	ATCC 49456(pABG5)	Empty vector control	This work
	ATCC 49456(pGBSMprF)	Expresses GBS *mprF* from P_{prtF} in pABG5Δ*phoZ*	This work
	ATCC 49456(pEfmMprF1)	Expresses *E. faecium mprF1* from P_{prtF} in pABG5Δ*phoZ*	This work
	ATCC 49456(pEfmMprF2)	Expresses *E. faecium mprF1* from P_{prtF} in pABG5Δ*phoZ*	This work
E. coli	DH5α	Plasmid cloning host; F^-, φ80*lacZ*ΔM15, *rec*A1, *end*A1, *hsd*R17, *pho*A, *sup*E44, λ^- *thi*-1, *gyr*A96, *rel*A1	(34)
	DH5α(pABG5)	Empty vector control	This work
	MC1061	Plasmid cloning host; F^-, araD139, Δ(araABC-leu)7696, Δ(lac)X74, galU, galK, hsdR2, (r_K^- m_K^+), mcrB1, rpsL, (Str^r)	(35)
	MC1061(pDCErm)	Empty vector control	This work

	DH5α(pGBSMprF)	Expresses COH1 *mprF* (GBSCOH1_1931) from P_{prtF} in pABG5Δ*phoZ*	This work
	DH5α(pEfmMprF1)	Expresses *E. faecium mprF1* from P_{prtF} in pABG5Δ*phoZ*	This work
	DH5α(pEfmMprF2)	Expresses *E. faecium mprF1* from P_{prtF} in pABG5Δ*phoZ*	This work
	DH5α(pMBMprFKO)	Allelic exchange plasmid containing ~2 kb sequence flanking GBSCOH1_1931	This work
	MC1061(pJMprFKO)	Allelic exchange plasmid containing ~2 kb sequence flanking ID870_10050	This work
	MC1061(pJMprF)	Expresses CJB11 *mprF* from $P_{tetM/erm}$ in pDCErm	This work
E. faecium	1,231,410	Wild type *E. faecium* strain	(36)

Plasmid	**Description**	**Ref**
pABG5Δ*phoZ*	Constitutive expression vector for streptococci with the P_{prtF} promoter. Confers kanamycin resistance. Referred to as pABG5 throughout the text	(23)
pGBSMprF	pABG5Δ*phoZ* expressing COH1 *mprF* (GBSCOH1_1931) from P_{prtF}	This work
pEfmMprF1	pABG5Δ*phoZ* expressing *E. faecium* 1,231,410 *mprF1* (EFTG_00601) from P_{prtF}	This work
pMBSacB	Allelic exchange plasmid for *S. agalactiae*. Confers erythromycin resistance and sucrose sensitivity	(25)
pMBMprFKO	Knockout plasmid containing ~2 kb sequence flanking GBSCOH1_1931	This work
pJMprFKO	Knockout plasmid containing ~2 kb sequence flanking ID870_10050	This work
pDCErm	Consitituitive expression vector for streptococcus from $P_{tetM/erm}$	(24)
pJMprF	pDCErm expressing CJB111 *mprF* (ID870_10050)	This work

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561 **Supplemental Table S3. Primers used in this study.**

Primer	5' – 3' sequence	Use
GBS_MprF_F	GAGAGGTCCCTTTCCTTGAAAAAGC TAATTGAAAAAGTC	Amplify GBSCOH1_1931 for Gibson assembly
GBS_MprF_R	ACCAATACCTTTATCTTATTTAACAA TCTTAATTTTACTATC	Amplify GBSCOH1_1931 for Gibson assembly
Faec_MprF1_F	GAGAGGTCCCTTTCCTTGTTAAAAA ATACCATACAATG	Amplify EFTG_00601 for Gibson assembly
Faec_MprF1_R	ACCAATACCTTTATCTTAATACTTTC TTCGTATCC	Amplify EFTG_00601 for Gibson assembly
MpF_SacII	ACGTCACCGCGGTTGAAAAAGCTAA TTGAAAAAGTC	Amplify CJB111 *mprF* ID870_10050 for ligation
MpR_BamHI	ACGTCAGGATCCTTATTTAACAATCT TAATTTTACTATC	Amplify CJB111 *mprF* ID870_10050 for ligation
pABG5-5'	GGAAAGGGACCTCTCTTCCTAAAC	Linearize pABG5Δ*phoZ* for Gibson assembly
pABG5-3'	GATAAAGGTATTGGTAAATAACAAA	Linearize pABG5Δ*phoZ* for Gibson assembly
Expression plasmid sequencing		
GBS_S1	GAATGGAATAATATAGTAGGCT	For sequencing pGBSMprF/pJMprF, amplifies with pABG5_Fup2/ pF
GBS_S2	GATTGTATCCCTTATTCC	For sequencing pGBSMprF/pJMprF, amplifies with GBS_S3
GBS_S3	CGATTCAATAGCTTCAC	For sequencing pGBSMprF/pJMprF, amplifies with GBS_S2
GBS_S4	GATAAAAGGCTCTACTGG	For sequencing pGBSMprF/pJMprF, amplifies with pABG5_FDwn/pR
pABG5_FDwn	CCAATAATAATGACTAGAGAAG	For pABG5 plasmid insert sequencing
pABG5_Fup2	CAAAAGGTTTCGACTTTTCACC	For pABG5 plasmid insert sequencing
EF1_S1	GAATAACGCTGATCAAAAGT	For sequencing pEfmMprF1, amplifies with pABG5_Fup2
EF1_S2	TGCCAAGAGAAATAGTC	For sequencing pEfmMprF1, amplifies with EF1_S3
EF1_S3	ACAATCTCTTCGCTTG	For sequencing pEfmMprF1, amplifies with EF1_S2
EF1_S4	CCAACTGTTCTTCTCCAA	For sequencing pEfmMprF1, amplifies with pABG5_FDwn
pF	AGCGCTAGGAGGAAAC	For pDCErm plasmid insert sequencing
pR	CCCATGCCATCTCCAATC	For pDCErm plasmid insert sequencing
GBSCOH1_1931 knockout plasmid construction, sequencing, and integration screening		
Mp1F_PstI	ACGTCACTGCAGTTCAATTAGCTTTT TCAACAATTTC	Amplifies upstream fragment from within GBSCOH1_1931/ID870_10050 leaving 6 codons, with Mp1R_XhoI
Mp1R_XhoI	ACGTCACTCGAGGCTGTTTATGGTG CTTTG	5' most primer of upstream fragment, amplifies with Mp1F_PstI
Mp2F_XbaI	ACGTCATCTAGAGAAAAGGCTAGAT TACGAAC	3' most primer of downstream fragment, amplifies with Mp2R_PstI
Mp2R_PstI	ACGTCACTGCAGGTTAAATAAGCTTT ATTTGGCA	Amplifies downstream fragment leaving 2 codons and stop codon of GBSCOH1_1931/ID870_10050, with Mp2F_XbaI
T7 promoter	TAATACGACTCACTATAGGG	Amplifies with MpS5F below to sequence plasmid, amplifies with T3 promoter for insert screening and plasmid presence
T3 promoter	AATTAACCCTCACTAAAGGG	Amplifies with MpS3R below, amplifies with T7 promoter for insert screening and plasmid presence
Int_F	GCTAATTGAACTGCAGGTTAAATAA G	Anneals at *mprF* knockout site, amplifies with Out_R for single integration screening

Out_R	GCTATTATATTTAGTGGTTTAATTGG	Anneals outside recombination arms, amplifies with Int_F, for single integration screening
Genomic knockout region sequencing		
MpS3F	CATTAGCTAGTCTTATCGGAG	Anneals outside integration arms, amplifies with MpS3R
MpS3R	ACAGCTACTTGGTAGTTCA	Amplifies with MpS3F
MpS4F	GCTACTAAGGCAAGATACG	Amplifies with MpS4R, knockout screening and plasmid sequencing primer
MpS4R	ATGGTCAGCGATGGTG	Amplifies with MpS4F, knockout screening and plasmid sequencing primer
MpS5F	CATAAGCGAAATAACTTGAG	Amplifies with MpS5R
MpS5R	GTATACAACGGCTTGATTGG	Anneals outside integration arms, amplifies with MpS5F

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