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Use of a Mariner-Based Transposon Mutagenesis System To Isolate *Clostridium perfringens* Mutants Deficient in Gliding Motility

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***Clostridium perfringens* is an anaerobic Gram-positive pathogen that causes many human and animal diseases, including food poisoning and gas gangrene. *C. perfringens* lacks flagella but possesses type IV pili (TFP). We have previously shown that *C. perfringens* can glide across an agar surface in long filaments composed of individual bacteria attached end to end and that two TFP-associated proteins, PilT and PilC, are needed for this. To discover additional gene products that play a role in gliding, we developed a plasmid-based mariner transposon mutagenesis system that works effectively in *C. perfringens*. More than 10,000 clones were screened for mutants that lacked the ability to move away from the edge of a colony. Twenty-four mutants (0.24%) were identified that fit the criteria. The genes containing insertions that affected gliding motility fell into nine different categories. One gene, *CPE0278*, which encodes a homolog of the SagA cell wall-dependent endopeptidase, acquired distinct transposon insertions in two independent mutants. *sagA* mutants were unable to form filaments due to a complete lack of end-to-end connections essential for gliding motility. Complementation of the *sagA* mutants with a wild-type copy of the gene restored gliding motility. We constructed an in-frame deletion mutation in the *sagA* gene and found that this mutant had a phenotype similar to those of the transposon mutants. We hypothesize that the *sagA* mutant strains are unable to form the molecular complexes which are needed to keep the cells in an end-to-end orientation, leading to separation of daughter cells and the inability to carry out gliding motility.**

Clostridium perfringens is a Gram-positive anaerobic bacteria that causes a wide variety of diseases in humans and animals, including acute food poisoning and gas gangrene (1). We have discovered that *C. perfringens* and other clostridia have the ability to produce type IV pili (TFP), a feature that was previously thought to be confined to Gram-negative bacteria (2). *C. perfringens* lacks flagella and flagellum-mediated swimming but can move across an agar medium with a unique type of gliding motility, in which curvilinear flares of densely packed cells move away from a colony (2). The flares are themselves composed of filaments of individual bacteria lined up in an end-to-end orientation. This orientation is essential for gliding motility; cells that are randomly oriented do not form the filaments and flares seen in motile cells (2; see Video S1 in the supplemental material). The bacteria within a filament can clearly be seen growing and dividing as the filament extends, suggesting that growth and division of individual bacteria provide at least some of the force necessary for movement across the surface of the agar (2; see Video S1). We also noted that the curvilinear flares move away from the colony in the direction of the long axis of the cells lined up within the filaments but eventually, due to their curvilinear nature, collide, leaving a region surrounded by *C. perfringens* cells. This empty space is then quickly filled in by growing bacteria. Thus, gliding motility has the effect of engulfing a local area, leading to consumption of the nutrients available within the engulfed area. We observed that plasmid insertion mutations in the *pilT* and *pilC* genes, which encode a retraction ATPase and an integral membrane protein, respectively, cause a significant defect in gliding motility (2). Therefore, we surmised that TFP appear to play a role in gliding motility (2), although the exact nature of this function is still unknown.

There are three separate loci that include TFP-associated genes in *C. perfringens* strain 13: (i) a monocistronic *pilT* gene; (ii) a putative operon comprised of four genes, *pilB-pilC-CPE1842-CPE1841*; and (iii) a multigene locus extending from *CPE2288* to *CPE2277*, which likely includes more than one operon (data not shown). To determine if any of these gene products, or any other gene products, were needed for gliding motility on agar plates, we sought a random mutagenesis procedure that would function in *C. perfringens* and yield mutations that were easily mappable. So far, three different methods, all using transposons, have been described for carrying out random mutagenesis in this bacterium. The first method utilizes a Tn*916*-based vector system (3–5) but has significant disadvantages; it frequently results in multiple insertions in the same chromosome, has several "hot spots" where the majority of the insertions are located, and results in deletions at the site of insertion (3, 4). The second utilizes a derivative of Mu phage for insertion, and while this resulted in single transposon insertions, ~43% were in rRNA operons and 12% in intergenic regions (6). The phage Mu transposition system was also inefficient, giving just 239 transformants/μg DNA in strain JIR325, a derivative of strain 13 (6). A more efficient EZ-Tn*5*-based random mutagenesis system showed a lower but significant frequency of insertion into rRNA genes (18%) in comparison to the phage Mu-based system (7). Another major limitation of the EZ-Tn*5*

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TABLE 1 Bacterial strains and plasmids used in this study

Strain/plasmid/primer	Relevant characteristics or sequence	Source or reference
Strains		
E. coli DH10B	F$^-$ *mcrA*Δ *mrr-hsdRMS mcrBC* ϕ80d*lacZ*ΔM15 *lacX74 deoR recA1 araD139*Δ *ara leu7697 galU galK*Δ*rpsL endA1 nupG*	Gibco/BRL
C. perfringens HN13	Strain 13, Δ*galKT*	15
HLL50	HN13, Δ*CPE0278* (*sagA*)	
C. difficile JIR8094		
Plasmids		
pGEM-T Easy	PCR cloning vector, ampicillin resistance	Promega
pBADC9		13
pJIR1457		13
pMTL82151		14
pKRAHI	Contains *bgaR*-P_{bgaL}, chloramphenicol resistance	12
pCM-GALK	Contains a *C. beijerinckii galK* gene under the control of a ferredoxin promoter from *C. perfringens*	15
pBSII KS+	Ampicillin resistance	Fermentas
pBL79	Contains a *Himar1* C9 transposase under a *tcdA* promoter, erythromycin and chloramphenicol resistance	This study
pHLL7	Contains P_{bgaL} in pGEM-T Easy	This study
pHLL9	Replaced *tcdA* promoter with P_{bgaL} in pBL79	This study
pHLL23	*galK* with its ferredoxin promoter in pGEM-T Easy	This study
pHLL24	Contains a *Himar1* C9 transposase under P_{bgaL} and the *galK* gene from pCM-GALK, erythromycin and chloramphenicol resistance	This study
pHLL42	The *cpe0278* gene under the control of the lactose-inducible promoter in pKRAH1	This study
pHLL49	Contains the 5′ and 3′ flanking regions of *CPE0278* (*sagA*)	This study

and phage Mu systems is that both require that the recipient strain be highly transformable by electroporation (6, 7).

Given the lack of a truly random mutagenesis procedure that shows no preference for rRNA genes, does not require expensive reagents, and is flexible in how it is used, we sought to develop a mariner transposon-based system for mutagenesis of *C. perfringens*. Mariner transposon mutagenesis systems have been developed for use in many Gram-negative and Gram-positive bacteria, including *Clostridium difficile* (8). The advantage of the mariner system is that its preferred target is a TA dinucleotide. Given the low G+C content of *C. perfringens* strains, 27 to 29% (9, 10), a mariner-based transposon would be ideal for mutagenesis due to the preponderance of TA sequences found in the genome. In this report we describe the construction of a plasmid-based mariner transposon system that has three unique features not found in other *C. perfringens* systems: (i) it is carried on a replicating plasmid, so every cell is subject to transposition; (ii) the transposase is under the control of an inducible promoter; and (iii) a negative selection system has been incorporated into the plasmid for efficient elimination of the replicating plasmid once transposition has occurred. This system was used to identify gene products required for *C. perfringens* gliding motility on agar plates, providing valuable insights into the mechanism used for moving across surfaces.

MATERIALS AND METHODS

Bacterial strains, growth conditions and DNA manipulations. Bacterial strains and plasmids used in this study are listed in Table 1, and primers used in this study are listed in Table 2. *Escherichia coli* was grown in Luria-Bertani (LB) medium supplemented with antibiotics as needed: 400 μg/ml erythromycin, 100 μg/ml ampicillin, or 20 μg/ml chloramphenicol. *C. perfringens* was grown in a Coy anaerobic chamber at 37°C. Two different media were used for *C. perfringens*: brain heart infusion (BHI) medium (Difco), and TY medium (3% tryptone, 2% yeast extract, 0.1% sodium thioglycolate), with the appropriate antibiotics as indicated: 30 μg/ml erythromycin or 20 μg/ml chloramphenicol. Galactose (3%) was used as a supplement in TY medium when needed; this medium is referred to as TYGal.

DNA manipulation was performed using standard protocols (11). Transformation of *E. coli* and *C. perfringens* was performed by electroporation as described before (12). All constructs were verified by DNA sequencing.

Construction of plasmids. We created pBL79, a plasmid for delivery of the mariner transposon, in several steps from the following components: (i) a promoterless version of the hyperactive *Himar1* C9 transposase gene (1,045 bp) from pBADC9 (13); (ii) a 519-bp fragment containing the *tcdA* promoter region amplified from *C. difficile* strain JIR8094 with primers OLB94/OLB95; (iii) a 1,264-bp mariner transposable element obtained by cloning the 1,087-bp *ermB* gene of pJIR1457 (amplified using primers OLB97 and OLB98) between the two internal terminal repeats of pMMOrf (13); and (iv) the 5,172-bp EcoRI-HindIII backbone fragment of pMTL82151 (14). Note that during the construction the *Himar1* C9 gene was PCR modified using primers OLB92 and OLB93 to convert an internal NcoI site to NdeI and an NdeI site of pMTL82151 was destroyed. We designed primers, OHL15 and OHL16, to amplify the lactose-inducible promoter P_{bgaL} from pKRAH1 (12). A consensus ribosome binding site and XbaI site were included in primer OHL15, while an NdeI site was included in OHL16. The PCR product was ligated to pGEM-T Easy to create plasmid pHLL7. pBL79 and pHLL7 were digested with XbaI and NdeI, and the *tcdA* promoter was replaced with the PbgaL fragment after ligation, which gave plasmid pHLL9. The *galK* gene with its ferredoxin promoter was amplified from pCM-GALK (15) by PCR using primers OHL41 and OHL42 and ligated to pGEM-T Easy to give plasmid pHLL23. pHLL9 and pHLL23 were digested with KpnI and SacII, and the *galK* gene and its promoter were ligated to pHLL23 and to give plasmid pHLL24.

Mariner transposon mutagenesis. pHLL24 was introduced into *C. perfringens* strain HN13 (15) by electroporation, and transformants were plated on BHI plus erythromycin and chloramphenicol. Cells resistant to both antibiotics were used to inoculate liquid BHI containing erythromycin and chloramphenicol and grown to mid-log phase. The culture was

TABLE 2 Primers used in this study

Primer	Sequence (5′ to 3′)[a]	Restriction site(s)
OLB92	GGAGGAATTC*CATATG*GAAAAAAAGGAATTTCGTGTTTTGATAAAATACTG	EcoRI/NdeI
OLB93	*AAGCTT*GCATGC**CTGCAG**ATTCCGGTCTAACAAAG	PstI/SphI/HindIII
OLB94	GGAATTCCATATGAACCTCCTAGTATTATTATTTTTGATAATAAATCC	NdeI
OLB95	CCCGAA*TTCTAGA*GCATGGTCAGTTGGTAAAATCTATTAAGC	EcoRI/XbaI
OLB96	GCAACTCTCTACTGTTTCTCCATACCCG	
OLB97	TCCCCCGGGACATGTAGCTCACTCATTAGGCACCCCAGG	SmaI
OLB98	TCCCCCGGGCTCTTCCAGGGTTATGCAGCGGAAAAGATCCGTCGA	SmaI
OHL15	GCTTCTAGATTCTACCTCCTAACCTATAAAATTAGCC	XbaI
OHL16	CATATGACCCTCCTCCTTCATTTAAAATAATTATGTATTCATGAAACATGATTG	NdeI
OHL21	GCAATGAAACACGCCAAAGTAAACAATTTAAGTACCG	
OHL22	GTTTTATTATTTGGTTGAGTACTTTTTCACTCG	
OHL41	GGTACCTAGGCTAAATATGCTTAAAAGAG	KpnI
OHL42	CCGCGGCTAGTTTATTTTTTTAGCACCATC	SacII
OHL75	GTCGACATGAAGAAAAAAATAATTTCAACAGTTC	SalI
OHL76	GGATCCTTATAATATTCTTCTTGCTGATGAG	BamHI
OHL105	GTCGACGGATGTTTTATATGGCTTTATTTACTGC	SalI
OHL106	CTTCTTGCTGATGAGAAATTATATATAGGCTTCATATCTACTTGCTCCCATTTAATG	
OHL107	CATTAAATGGGAGCAAGTAGATATGAAGCCTATATATAATTTCTCATCAGCAAGAAG	
OHL108	GGATCCCTCATTTTTATTTACTCTCCCTTCC	BamHI
OHL109	GAGTTATACTTTTTATTCATAATAAATCCTCC	
OHL110	CTTGTATCCTCAGCAGCTATTAAATCAACAC	

[a] Underlining indicates the locations of restriction sites used for subsequent cloning. Bold type and italics are used to separate the sites from each other when there are multiple sites in the same primer.

then washed twice with Dulbecco's modified phosphate-buffered saline (DPBS), resuspended in the original culture volume of BHI plus 1 mM lactose, and incubated for 2 h. The cell culture was then washed three times with DPBS and diluted 100-fold in liquid BHI and incubated for 4 h at 37°C. The culture was again diluted 100-fold into liquid TY medium containing 3% galactose and erythromycin and incubated for 4 h. In principle, only those bacteria in which transposition had occurred and the plasmid had been lost would be able to grow. Cells were then plated on TYGal plates plus erythromycin, after which all the colonies were harvested and stored at −80°C to generate a transposon mutant pool.

To check for the randomness of insertion, Southern blot analysis was done with the *ermBP* gene as a probe on 17 randomly chosen mutants from the pool. Chromosomal DNA was extracted using the Quickextract chromosomal DNA extraction kit (Epicentre Biotechnologies) and digested overnight with EcoRI, and a Southern blot assay was performed as previously described (11).

Screen for motility mutants. The *C. perfringens* HN13 mariner transposon mutant library was plated from a frozen stock culture onto BHI plates plus 30 μg/ml erythromycin and incubated overnight at 37°C. Since colonies of the parent strain HN13 have an irregular border due to migration on agar plates, mutagenized bacteria that formed colonies with smooth edges were picked and saved as potential motility mutants.

Sequencing analysis to locate the insertion sites of mutants. The chromosomal DNA of motility mutants was extracted using the Quickextract chromosomal DNA extraction kit (Epicentre Biotechnologies), digested overnight with HindIII, and then ligated to HindIII-digested pBSII KS+. The ligation products were introduced into *E. coli* strain DH10B by electroporation and plated on LB agar plus ampicillin and erythromycin. Purified recombinant plasmids were sequenced using primers OHL21 and OHL22, which anneal inside the *ermBP* gene but are directed toward the DNA flanking the *ermBP* and transposon sequences. To identify the genomic location of transposon insertions, sequence data were analyzed using BLAST and compared to the genome sequence of *C. perfringens* strain 13, the parent of strain HN13 (http://www.xbase.ac.uk/genome/clostridium-perfringens-str-13).

Complementation of the *CPE0278* mutations in strain HN13. The coding sequence of *CPE0278* was amplified with primers OHL75 and OHL76, using HN13 chromosomal DNA as the template, and cloned in pKRAH1 at the unique SalI and BamHI sites (12). This placed the *CPE0278* gene under the control of the lactose-inducible promoter (P_{bgaL}) in pKRAH1 (12). This plasmid, pHLL42, was introduced into the *CPE0278* mutant strains HLL9 and HLL28 by electroporation.

Construction of a *CPE0278* in-frame deletion mutant. An in-frame deletion mutant of the *CPE0278* gene was constructed as previously described (15). The 5′ (1,003 bp) and 3′ (991 bp) flanking regions of gene *CPE0278* were amplified using *C. perfringens* HN13 chromosomal DNA as the template, with primers OHL105 and OHL106 (N terminal) and OHL107 and OHL108 (C terminal), respectively. A second round of overlapping PCR was performed using the flanking region PCR products as the template and OHL105 and OHL108 as primers. This PCR product was ligated into pGEM-T Easy and then subcloned into pCM-GALK (15) using SalI and BamHI as restriction sites. This plasmid, pHLL49, was transformed into *C. perfringens* HN13 by electroporation. Mutants were screened (15) and confirmed by PCR using primers OHL109 and OHL110. The mutant retained, in frame, the first 2 and last 12 codons of the *CPE0278* gene (data not shown).

Expression of *CPE0278* in *C. perfringens* HN13. pHLL42, which has the *CPE0278* gene under the control of the lactose-inducible promoter P_{bgaL}, was transformed into *C. perfringens* HN13 by electroporation. Expression was induced for 2 h by adding lactose to a final concentration of 10 mM in liquid BHI medium to a culture in mid-log phase.

Microscopy and video imaging. Between 194 and 587 individual bacteria from each sample were used to measure cell length and width using MicrobeTracker software (16) linked to a MATLAB (Mathworks) platform. Frozen stocks of cells were streaked on BHI agar plates with 0.5 mM lactose and incubated in the anaerobic chamber for 6 h. Cells were scraped from the plate, suspended in PBS and examined in an Olympus IX81 upright microscope linked to a Hamamatsu model C4742 CCD camera. Slidebook 5.1 Intelligent Imaging Innovations imaging software was used to compile motility videos. Images of colonies were obtained using a Bio-Rad Gel Doc XR imager with Applied One 4.6.5 version software. Colony areas were measured using ImageJ software (http://rsbweb.nih.gov/ij/).

RESULTS

Construction of a mariner transposon system for *C. perfringens*. Our goal was to design a self-replicating plasmid that would

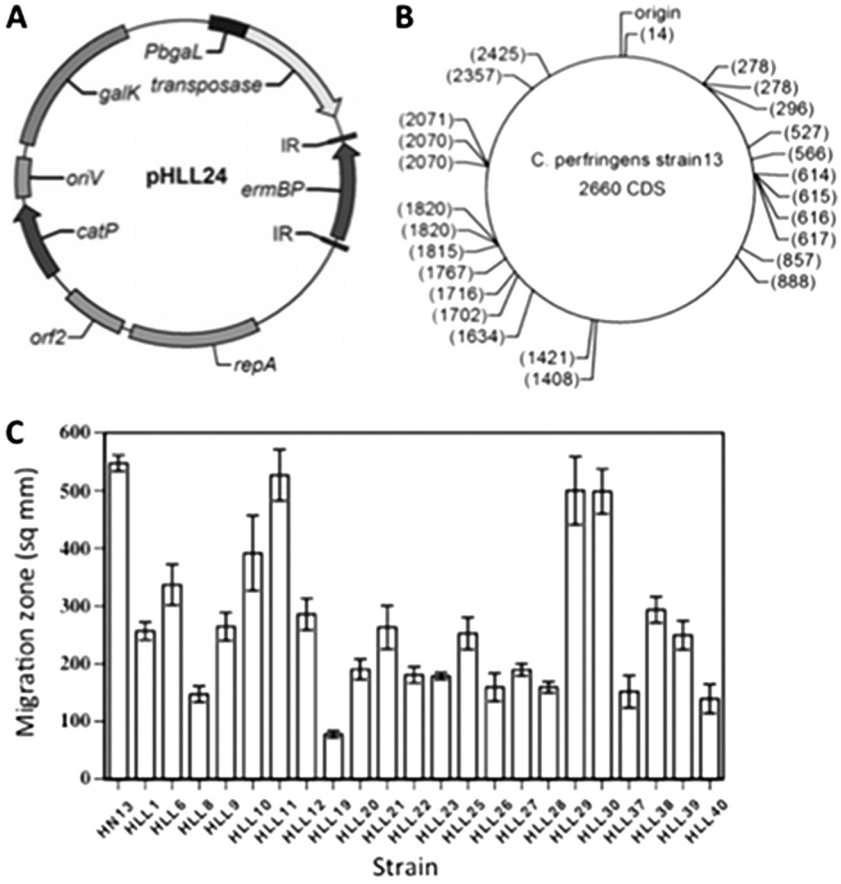

FIG 1 (A) Schematic diagram of plasmid pHLL24 showing important genetic elements. (B) Chromosomal location of mariner transposon insertions that disrupted gliding motility in strain *C. perfringens* HN13. Numbers in parentheses indicate gene assignments. (C) Area covered by migration of bacteria away from the initial site of inoculation on BHI agar plates. Values show the means and standard deviations of results from quadruplicate samples.

deliver the mariner transposon to the *C. perfringens* chromosome under the control of an inducible promoter. As the backbone for the vector, we used pMTL82151, a plasmid developed by Heap et al. (14) that contains a Gram-positive origin of replication from pBP1 and a chloramphenicol resistance gene. The *C. difficile tcdA* promoter was initially used to drive expression of the Himar transposase. While the constructed plasmid, pBL79, could replicate in *C. perfringens*, it did not provide efficient delivery of the mariner transposon to the chromosome (data not shown). To provide regulated, higher-level expression of the transposase, we substituted the *C. perfringens bgaL* promoter for the *tcdA* promoter. We have shown previously that the *bgaL* promoter is inducible in *C. perfringens* by the addition of lactose and mediates high levels of expression of genes under its control (12). The modified plasmid, pHLL9, was able to deliver the transposon to the chromosome of *C. perfringens* strain 13, as indicated by the presence of a few clones that were erythromycin resistant and chloramphenicol sensitive. However, even after 24 h of growth in nonselective medium, 80 to 90% of all clones were still chloramphenicol resistant, indicating that the plasmid was still present. Therefore, we added a counterselectable marker originally used by Nariya et al. (15). We added the *galK* gene from pCM-GALK (15) to pHLL9 to create pHLL24 (Fig. 1A) and showed that the introduction of pHLL24 into strain HN13, a derivative of *C. perfringens* strain 13, in which the *galK* and *galT* genes have been deleted (15), causes the cells to be unable to grow in the presence of a high concentration of galactose (see Fig. S1 in the supplemental material). The presumed mechanism of lethality is that expression of *galK* in the absence of *galT* leads to accumulation of toxic levels of galactose-1-phosphate. In addition, after induction of the transposase by lactose followed by 24 h of growth in galactose-containing medium, ~90% of the clones were erythromycin resistant and chloramphenicol sensitive (data not shown), indicating that *galK*-mediated negative selection was effective at removing the pHLL24 plasmid backbone after transposition had occurred.

To see if the mariner transposon had hopped randomly, chromosomal DNA was isolated from 17 mutants, digested with the restriction enzyme EcoRI, and analyzed by Southern blotting using an *ermBP* gene-specific probe. The banding pattern indicated that each of the mutants had an insertion in a different location and only 2 (~12%) of the clones had multiple insertions (data not shown). These are higher rates of multiple insertions than those reported for the Mu transposition system (0 out of 30 tested [6]) and the EZ-Tn5-based system (0 out of 8 tested [7]), but this rate may be lowered by changing the level of induction of the transposase gene. The chromosomal DNA flanking the site of insertion in 10 of these clones was sequenced (see Materials and Methods); the sites of insertion were randomly distributed around the chromosome, and none of the insertions were in rRNA operons (see

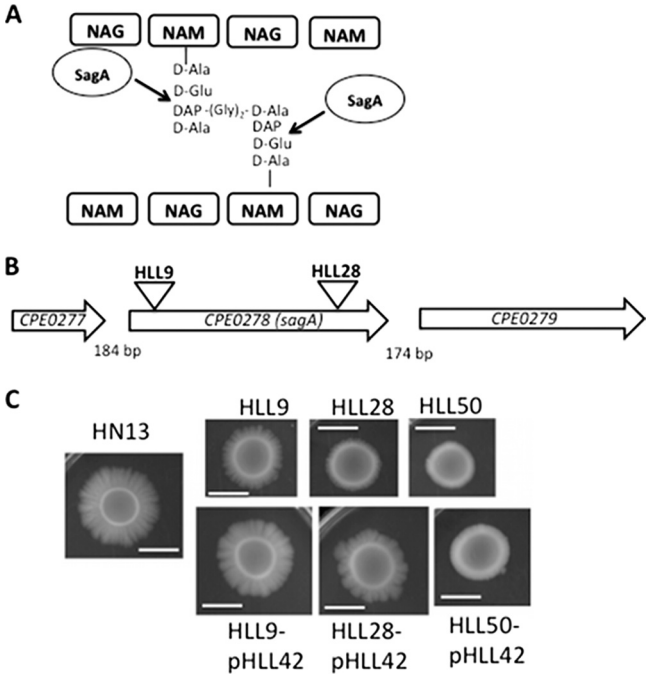

FIG 2 (A) Schematic diagram showing the structure of peptidoglycan in *C. perfringens* and the sites of activity of the SagA endopeptidase. NAG, *N*-acetylglucosamine; NAM, *N*-acetyl muramic acid; DAP, meso-diaminopimelic acid. (B) Diagram of the genetic locus containing the *CPE0278* (*sagA*) gene in strain HN13. Triangles indicate the positions of mariner transposon insertions in mutant strains HLL9 and HLL28. The lengths of the intergenic regions are shown below the arrows representing the genes. (C) Colony morphology of wild-type and mutant strains as indicated above and below the images. All images are set to the same scale.

Table S1 in the supplemental material). The point of insertion of each mutant corresponded to a TA dinucleotide, which was duplicated, as is typical for mariner transposition.

Identification of mutants lacking gliding motility on agar plates. We had observed that cells of strain HN13, similar to those of strain 13, exhibit a distinctive ability to migrate away from the colony when grown on agar plates containing a low-glucose medium such as BHI (see Fig. 2C for an example). Since we have shown in previous work that migration across agar surfaces is dependent on type IV pilus-related functions (2), we screened a transposon-mutagenized culture for mutants lacking the ability to migrate on BHI plates. Colonies that showed a smooth, round appearance were chosen and streaked on plates containing either chloramphenicol and erythromycin or erythromycin alone to identify mutants with transposon insertions but lacking the pHLL24 plasmid backbone.

Of more than 10,000 mutant colonies screened, 24 transposon mutants (0.24%) were identified that fit the criteria described above. The site of insertion in each mutant was determined by sequencing the flanking chromosomal DNA. The genes with transposon insertions that affected gliding motility are listed in Table 3. A schematic diagram showing the locations of the affected genes on a map of the *C. perfringens* chromosome is shown in Fig. 1B. Two features of the transposon insertion sites are apparent: (i) they appear at many locations around the chromosome, and (ii) there are specific clusters of genes in operons that were affected. Three different genes, *CPE0278*, *CPE1820*, and *CPE2070*, each had two transposon insertions but at different locations in the same gene, indicating they are not siblings but rather independent mutants. Given the small percentage of transposon mutants that were lacking gliding motility and clustering of insertions in the same gene or genes in the same operon (see below), it appeared that a small but specific subset of gene products is required for gliding motility.

Characterization of gliding motility mutants. Gliding motility on agar plates gives colonies an irregular pattern, with multiple flares coming out of the edge of a colony. To determine the extent of the deficiency in gliding motility of the transposon mutants listed in Table 3, four replicates of a 15-μl suspension of cells of each mutant at an optical density of 600 nm (OD_{600}) of 10 were placed on a BHI plate, giving a zone of ~1 cm of dense growth (see Fig. 2C for an example). Movement away from this zone (i.e., gliding motility) was estimated by measuring the area covered by the entire colony and then subtracting the central zone where bacteria were plated using ImageJ software. The results are shown in Fig. 1C. In the original screen, in which several hundred colonies each with an ~2-mm diameter were on each plate, all of the nonmotile colonies had smooth edges. For a few of the mutants, HLL29 and HLL30 for example, in the quantitative migration assay (where the inoculation zone was ~1 cm), a few flares spread out rapidly from the edge of the colony and covered an area similar to that seen with the parent strain, HN13. We hypothesize

TABLE 3 Mariner transposon mutants with altered gliding motility

Gene	Gene locus tag (if designated), description	Strain
None	Parent strain, wild type	HN13
CPE0013-CPE0014	Intergenic between CPE0013 and SerS seryl-tRNA synthetase, *serS*	HLL38
CPE0278	SagA homolog	HLL28
CPE0278	SagA homolog	HLL9
CPE0296	*tktN*, transketolase	HLL20
CPE0527	ABC transporter	HLL37
CPE0566	*ribB*, riboflavin biosynthesis protein	HLL19
CPE0614	*rfbP*, undecaprenyl phosphate galactosephosphotransferase	HLL40
CPE0615	*rfbN*, rhamnosyl transferase	HLL26
CPE0616	*rfbA*, glucose-1-phosphate thymidylyltransferase	HLL22
CPE0617	*rfbC* dTDP-4-dehydrorhamnose 3,5-epimerase	HLL36
CPE1408	Chloride channel protein	HLL39
CPE1421	Putative membrane protein	HLL11
CPE1634	*tagO*, undecaprenyl-phosphate *N*-acetylglucosaminyltransferase	HLL10
CPE1702	*topA*, DNA topoisomerase I	HLL6
CPE1716	*smc*, chromosome partition protein SMC	HLL1
CPE1767	*pilT*, twitching motility protein	HLL29
CPE1815	*recN*, DNA repair protein	HLL27
CPE1820	Geranyltranstransferase	HLL23
CPE1820	Geranyltranstransferase	HLL30
CPE2070	tRNA 2-selenouridine synthase	HLL25
CPE2070	tRNA 2-selenouridine synthase	HLL21
CPE2071	Glycosyl transferase	HLL8
CPE2357	Phosphoenolpyruvate-protein phosphotransferase	HLL12
CPE2425	*thyX*, FAD-dependent thymidylate synthase	HLL14

these flares are due to reversion of the original mutation or secondary compensating mutations that permit the bacteria to glide on the plates. Because a single bacterium, if it can glide, will quickly multiply and move across the plate, these flares show up as visually obvious features, despite the frequency of reversion being quite low within the large population at the center of the colony.

The genes found to have transposon insertions that affected gliding motility fell into the following extremely diverse categories: (i) cell wall biosynthesis/maintenance (*CPE0278*, *CPE1634*), (ii) chromosomal DNA replication/segregation (*CPE1702*, *CPE1716*, *CPE1815*), (iii) transport across the cytoplasmic membrane (*CPE0527*, *CPE1408*, *CPE1421*), (iv) carbohydrate metabolism (*CPE0296*, *CPE0614-CPE0617*, *CPE1634*, *CPE2071*), (v) vitamin biosynthesis (*CPE0566*), (vi) lipid metabolism (*CPE1820*), (vii) enzymes that modify tRNA (*CPE0014*, *CPE2070*), and (viii) type IV pilus functions (*CPE1767*). These assignments were made with the caveat that the effects on gliding motility may be due to polar effects on downstream genes in the same operon and not to inactivation of the gene.

Characterization and complementation of mutant strains HLL9 and HLL28. We chose the transposon mutants HLL9 and HLL28 for further characterization and complementation of the motility phenotype. Both strains have insertions in the gene *CPE0278*: the insertion in HLL9 is at the 5′ end of the gene, while the insertion in HLL28 is at the 3′ end of the gene (Fig. 2B). *CPE0278* encodes a homolog of the SagA (secreted antigen) protein family that has been found in many Gram-positive species. Therefore, we designated the *sagA* alleles in HLL9 and HLL28 *sagA2* and *sagA3*, respectively. In other Gram-positive bacteria, the SagA protein has been shown to function as a peptidoglycan endopeptidase that plays a role in septum formation and maintaining cell shape (17–20). It cleaves the bond between D-Glu and meso-diaminopimelic acid in the peptide cross-link between the polysaccharide chains (Fig. 2A). The *sagA* gene in *C. perfringens* is predicted to be monocistronic by the operon prediction software available in MicrobesOnline (http://www.microbesonline.org/cgi-bin/fetchLocus.cgi?locus=184876&disp=1). A rho-independent transcription terminator is predicted to lie in the 174-bp intergenic region between *sagA* and *CPE0279* (Fig. 2B) using the ARNold software (http://rna.igmors.u-psud.fr/toolbox/arnold/index.php).

Since it was possible that failure to migrate could be a consequence of a lower growth rate, we cultured strains HN13, HLL9 (*sagA2*), and HLL28 (*sagA3*) in liquid BHI medium and observed no difference in growth rate under these conditions (see Fig. S2 in the supplemental material).

Since the SagA protein is likely a peptidoglycan-specific endopeptidase, it could be toxic or have significant phenotypic effects if expressed at high levels on a multicopy plasmid under the control of its own promoter. Therefore, to complement the *sagA* mutants, the wild-type *sagA* gene was cloned in the vector pKRAH1, and a plasmid we developed for regulating gene expression in *C. perfringens* using the lactose-inducible promoter P_{bgaL} (12), creating pHLL42. After 43 h of growth on BHI with 0.5 mM lactose, strain HN13 exhibited the dense flare-like pattern of migration away from the initial site of inoculation, while the *sagA2* and *sagA3* mutant strains failed to migrate to any significant extent (Fig. 2C). Both strains, when carrying the complementing plasmid pHLL42, showed similar and significant levels of migration, although somewhat less than that seen with the parental strain HN13 (Fig. 2B), suggesting that the mutations play a significant role in the mutant phenotypes. The failure of the complementing plasmid to provide complete restoration of gliding motility is likely due to the *sagA* gene being expressed at a somewhat lower level than that of the wild-type strain. The addition of higher lactose levels leads to increased expression of the *sagA* gene when under the control of P_{bgaL}, but high levels of lactose also inhibit gliding motility in *C. perfringens* strains (2, 21), which is the phenotype we are studying here.

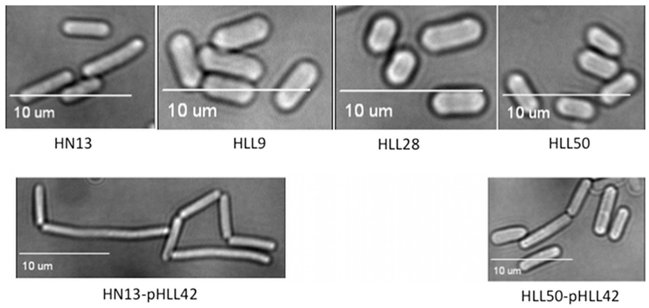

FIG 3 Representative images of wild-type and *sagA* mutant cells of *C. perfringens*. HN13, wild type; HLL9, *sagA2* allele; HLL28, *sagA3* allele; HLL50, *sagA1* allele (in-frame deletion).

We next examined the morphology of individual cells of the *sagA2* and *sagA3* mutants by phase-contrast microscopy (Fig. 3). In comparison to strain HN13, cells of the *sagA2* mutant were 32% and the *sagA3* mutant 22% shorter in length (5.24 ± 1.45 versus 3.95 ± 0.75 and 4.28 ± 0.83 μm, respectively; $P < 0.001$ for both). In contrast, the cells of strain HLL28 were 15% wider than those of strain HN13 (1.2 ± 0.068 versus 1.01 ± 0.081 μm [$P < 0.001$]). The complemented mutant strains exhibited cell lengths and widths closer to those seen in strain HN13 than the mutants, but normal cell dimensions were not fully restored (Fig. 3). Since the mutants did exhibit changes in cell morphology, we placed pHLL42, which has the *sagA* gene under the control of the lactose-inducible promoter P_{bgaL}, into wild-type strain HN13 and induced its expression by adding 10 mM lactose to cells in the mid-log phase of growth. Two hours after induction the cells were examined by light microscopy for changes in morphology, but no significant differences were detected (data not shown).

Using mutants lacking the type IV pilus-related proteins PilT and PilC we have demonstrated that gliding motility seen at the colony level is related to the ability of *C. perfringens* strains to form long chains with end-to-end connections between individual cells (2). We made time-lapse videos of strain HN13 and the *sagA2* and *sagA3* mutants on BHI agar to see if the mutants were defective in any aspect of gliding motility at the microscopic level. Similar to what we had observed with strain 13 (2), strain HN13 forms extensive and stable long chains of cells (i.e., filaments) that migrate over the agar surface (see Video S1 in the supplemental material). In contrast, the *sagA2* and *sagA3* mutant strains failed to form stable end-to-end connections, did not form chains of any length, and had no organized migration pattern detectable in the videos (see Videos S2 and S3, respectively, in the supplemental material). The complemented mutant strains carrying pHLL42 showed an intermediate phenotype between the wild-type and mutant strains; they formed end-to-end connections between the cells and had filaments, but the filaments were not as long or as stable as

the filaments seen with strain HN13 (see Videos S4 and S5 in the supplemental material for HLL9 [*sagA2*] and HLL28 [*sagA3*], respectively).

To confirm that the mutations in the *sagA* gene were solely responsible for the phenotypes we observed, we constructed an in-frame deletion mutation in the *sagA* gene using a homologous recombination/negative selection method reported by Nariya et al. (15). This mutant allele, called *sagA1*, retained, in-frame, only the first 2 and last 12 codons of the *sagA* gene (see Materials and Methods). The migration pattern on BHI agar plates was similar to those of the *sagA2* and *sagA3* mutants (Fig. 2C) and the extent of migration (228 ± 26 mm^2) lay between those seen with the other *sagA* mutant strains, HLL9 and HLL28 (compare to Fig. 1C). The cell dimensions of the *sagA1* mutant were 3.10 ± 0.58 μm long and 1.21 ± 0.08 μm wide. While similar to the wild-type strain in length, the *sagA1* mutant was 33% wider than the wild-type strain (Fig. 3). Video microscopy of the *sagA1* mutant showed the same lack of end-to-end connections seen in the other *sagA* mutants (compare Video S6 to Videos S2 and S3 in the supplemental material). Complementation of the *sagA1* mutant using pHLL42 partially restored gliding motility on agar plates (Fig. 2C) and the end-to-end connections seen in video microscopy on BHI agar plates (see Video S7 in the supplemental material). Therefore, the in-frame deletion mutation, *sagA1*, gave phenotypes very similar to those of the transposon-generated mutants in all the assays we tested, suggesting that the *sagA* gene product is responsible for the effects we observed.

DISCUSSION

We constructed a random mutagenesis system for *C. perfringens* using a mariner transposon carried on a multicopy plasmid. We showed that the transposon hops in a random fashion and shows no detectable hot spots in rRNA operons or other locations. Because the transposon was delivered on a replicating plasmid in *C. perfringens*, it was possible to create and screen very large mutant libraries quickly and at low cost with no detectable bias toward insertions in rRNA genes. This construct seems to provide a versatile and useful mutagenesis system for use in *C. perfringens*. One potential limitation is the need to use as the host a strain lacking the *galK* and *galT* genes in order to make the selection against plasmid retention efficient. Since strain HN13 is a derivative of strain 13 and appears to harbor all of the known secreted virulence factors found in strain 13 (15), for most experiments this would not be a significant issue. To broaden the usefulness of this method, we have constructed a *galK galT* mutant of strain SM101 (data not shown), which is used as a model for sporulation and enterotoxin synthesis and regulation in *C. perfringens* (22). A similar approach can be used in any strain that is transformable enough to allow integration of a nonreplicating (suicide) plasmid into the chromosome, as demonstrated by Nariya et al. (15).

The mariner transposon mutagenesis scheme reported here allowed us to generate novel mutants defective in gliding motility on agar plates. Of the 24 gliding motility-defective mutants isolated, only one had a mutation in a type IV-pilus-associated gene (*pilT*) that had been identified previously as being involved in gliding motility. This may be due to the fact that the number of clones that we screened, ~10,000, did not reach that required for saturation mutagenesis (>30,000 [23]).

We chose to characterize the two mutations in the *CPE0278* gene in more detail because this gene is likely monocistronic and its presumed product, SagA, has known biological activities. In *S. mutans* the homologous protein is secreted *in vivo* to such an extent that it induces a significant antibody response in human patients (18). In *Enterococcus faecium*, the SagA protein binds to host extracellular matrix proteins and is essential for growth but does not have cell wall hydrolase activity (20). The *C. perfringens* SagA protein has a signal sequence compatible for secretion via the Sec pathway (not shown). The SagA homolog in *C. perfringens* strain ATCC 13124 was identified as the most abundant secreted protein, even more so than alpha toxin and collagenase, two well-studied secreted toxins (24). This is consistent with our observation that even maximum expression from the P_{bgaL} promoter on a multicopy plasmid did not fully complement the *sagA* mutants. The *C. perfringens* SagA protein displayed three different isoforms in two-dimensional gel electrophoresis, which suggests posttranslational modification of the protein (24).

The FUGUE structural prediction software (http://tardis.nibio.go.jp/fugue/prfsearch.html) found that the closest structural homolog available is the C-terminal domain of the rv1477 protein (also designated RipA) from *Mycobacterium tuberculosis* (17). Originally identified as a gene product necessary for invasion and intracellular replication (19), rv1477 was later found to be a cell wall hydrolase similar in function to SagA homologs in the *Firmicutes* (25). This is consistent with the *CPE0278* gene product playing a role in maintaining cell shape, which supports its assignment as a SagA homolog. The homology to rv1477 extends over the C-terminal half of the *C. perfringens* 432-residue SagA protein from Arg247 to Ser427 (see Fig. S3 in the supplemental material). This region contains the NLP/P60 cell wall hydrolase domain which catalyzes cleavage of the peptide bond between meso-DAP and D-glutamate in the peptide cross-link between glycan strands (17). In *Mycobacterium smegmatis*, a *ripA* mutant forms long chains and branches (25). In *C. perfringens*, the three *sagA* mutants showed some defects in cell length and/or width and failed to form end-to-end connections important for gliding motility (Fig. 3; see also Videos S2, S3, and S6 in the supplemental material). These different phenotypes suggest that the SagA homologs in these species have different effects on maintenance of shape and morphology mediated by the cell wall.

Since our screening procedure identified only mutants that can grow on agar plates but not spread, it is clear that gliding motility requires more than ability to grow. The videos in the supplemental material show that end-to-end connections are essential for gliding motility. We theorize that most of our mutants, for one reason or another, have a defect in placing the cell organelle responsible for cell-cell adherence on the surface of the bacteria, where it is needed. The nature of the structures responsible for the end-to-end connections has not yet been identified, but it is a major focus of our current research.

The wide variety of gene categories that emerged from the screen indicated that gliding motility depends upon multiple factors. The *CPE0614-0617* gene cluster had four individual insertions in four adjacent genes (Table 3). The dTDP-L-rhamnose biosynthesis pathway I, which converts glucose-1 phosphate and TTP to dTDP-L-rhamnose requires, in consecutive order, the activity of the RfbABCD enzymes. In *C. perfringens* strain 13, the *rfbABCD* genes are distributed in two separate operons, *CPE0613-rfbP-rfbN-rfbA* and *rfbC-rfbD-rfbB* (http://www.microbesonline.org/cgi-bin/fetchLocus.cgi?locus=185212&disp=1). The function of CPE0613 is unknown but it contains a haloacid

dehydrogenase (HAD) hydrolase domain (COG0561) which is often associated with hydrolysis of sugar phosphates (http://www.ncbi.nlm.nih.gov/COG/grace/wiew.cgi?COG561). RfbP and RfbN are associated with transport of rhamnose across the cytoplasmic membrane and transfer of rhamnose to a growing polysaccharide, respectively. These gene functions and their association with gliding motility suggest that they are involved in synthesis of a polysaccharide that is exposed on the surface of *C. perfringens*. Since there are no reports in the literature regarding the nature of surface polymers such as teichoic or lipoteichoic acid in *C. perfringens*, the role these genes play in gliding motility remains an open question.

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REFERENCES

1. **Rood JI.** 1998. Virulence genes of *Clostridium perfringens*. Annu. Rev. Microbiol. **52**:333–360.
2. **Varga JJ, Nguyen V, O'Brien DK, Rodgers K, Walker RA, Melville SB.** 2006. Type IV pili-dependent gliding motility in the Gram-positive pathogen *Clostridium perfringens* and other clostridia. Mol. Microbiol. **62**:680–694.
3. **Awad MM, Rood JI.** 1997. Isolation of alpha-toxin, theta-toxin and kappa-toxin mutants of *Clostridium perfringens* by Tn916 mutagenesis. Microb. Pathog. **22**:275–284.
4. **Briolat V, Reysset G.** 2002. Identification of the *Clostridium perfringens* genes involved in the adaptive response to oxidative stress. J. Bacteriol. **184**:2333–2343.
5. **Lyristis M, Bryant AE, Sloan J, Awad MM, Nisbet IT, Stevens DL, Rood JI.** 1994. Identification and molecular analysis of a locus that regulates extracellular toxin production in *Clostridium perfringens*. Mol. Microbiol. **12**:761–777.
6. **Lanckriet A, Timbermont L, Happonen LJ, Pajunen MI, Pasmans F, Haesebrouck F, Ducatelle R, Savilahti H, Van Immerseel F.** 2009. Generation of single-copy transposon insertions in *Clostridium perfringens* by electroporation of phage mu DNA transposition complexes. Appl. Environ. Microbiol. **75**:2638–2642.
7. **Vidal JE, Chen J, Li J, Mcclane BA.** 2009. Use of an EZ-Tn5-based random mutagenesis system to identify a novel toxin regulatory locus in *Clostridium perfringens* strain 13. PLoS One **4**:e6232. doi:10.1371/journal.pone.0006232.
8. **Cartman ST, Minton NP.** 2010. A mariner-based transposon system for in vivo random mutagenesis of *Clostridium difficile*. Appl. Environ. Microbiol. **76**:1103–1109.
9. **Myers GS, Rasko DA, Cheung JK, Ravel J, Seshadri R, Deboy RT, Ren Q, Varga J, Awad MM, Brinkac LM, Daugherty SC, Haft DH, Dodson RJ, Madupu R, Nelson WC, Rosovitz MJ, Sullivan SA, Khouri H, Dimitrov GI, Watkins KL, Mulligan S, Benton J, Radune D, Fisher DJ, Atkins HS, Hiscox T, Jost BH, Billington SJ, Songer JG, Mcclane BA, Titball RW, Rood JI, Melville SB, Paulsen IT.** 2006. Skewed genomic variability in strains of the toxigenic bacterial pathogen, *Clostridium perfringens*. Genome Res. **16**:1031–1040.
10. **Shimizu T, Ohtani K, Hirakawa H, Ohshima K, Yamashita A, Shiba T, Ogasawara N, Hattori M, Kuhara S, Hayashi H.** 2002. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc. Natl. Acad. Sci. U. S. A. **99**:996–1001.
11. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
12. **Hartman AH, Liu H, Melville SB.** 2011. Construction and characterization of a lactose-inducible promoter system for controlled gene expression in *Clostridium perfringens*. Appl. Environ. Microbiol. **77**:471–478.
13. **Lampe DJ, Churchill ME, Robertson HM.** 1996. A purified mariner transposase is sufficient to mediate transposition in vitro. EMBO J. **15**:5470–5479.
14. **Heap JT, Pennington OJ, Cartman ST, Minton NP.** 2009. A modular system for *Clostridium* shuttle plasmids. J. Microbiol. Methods **78**:79–85.
15. **Nariya H, Miyata S, Suzuki M, Tamai E, Okabe A.** 2011. Development and application of a method for counterselectable in-frame deletion in *Clostridium perfringens*. Appl. Environ. Microbiol. **77**:1375–1382.
16. **Sliusarenko O, Heinritz J, Emonet T, Jacobs-Wagner C.** 2011. High-throughput, subpixel precision analysis of bacterial morphogenesis and intracellular spatio-temporal dynamics. Mol. Microbiol. **80**:612–627.
17. **Both D, Schneider G, Schnell R.** 2011. Peptidoglycan remodeling in *Mycobacterium tuberculosis*: comparison of structures and catalytic activities of RipA and RipB. J. Mol. Biol. **413**:247–260.
18. **Chia JS, Chang LY, Shun CT, Chang YY, Tsay YG, Chen JY.** 2001. A 60-kilodalton immunodominant glycoprotein is essential for cell wall integrity and the maintenance of cell shape in *Streptococcus mutans*. Infect. Immun. **69**:6987–6998.
19. **Gao LY, Pak M, Kish R, Kajihara K, Brown EJ.** 2006. A mycobacterial operon essential for virulence in vivo and invasion and intracellular persistence in macrophages. Infect. Immun. **74**:1757–1767.
20. **Teng F, Kawalec M, Weinstock GM, Hryniewicz W, Murray BE.** 2003. An *Enterococcus faecium* secreted antigen, SagA, exhibits broad-spectrum binding to extracellular matrix proteins and appears essential for *E. faecium* growth. Infect. Immun. **71**:5033–5041.
21. **Mendez M, Huang IH, Ohtani K, Grau R, Shimizu T, Sarker MR.** 2008. Carbon catabolite repression of type IV pilus-dependent gliding motility in the anaerobic pathogen *Clostridium perfringens*. J. Bacteriol. **190**:48–60.
22. **Zhao Y, Melville SB.** 1998. Identification and characterization of sporulation-dependent promoters upstream of the enterotoxin gene (*cpe*) of *Clostridium perfringens*. J. Bacteriol. **180**:136–142.
23. **Cameron DE, Urbach JM, Mekalanos JJ.** 2008. A defined transposon mutant library and its use in identifying motility genes in *Vibrio cholerae*. Proc. Natl. Acad. Sci. U. S. A. **105**:8736–8741.
24. **Sengupta N, Alam SI, Kumar B, Kumar RB, Gautam V, Kumar S, Singh L.** 2010. Comparative proteomic analysis of extracellular proteins of *Clostridium perfringens* type A and type C strains. Infect. Immun. **78**:3957–3968.
25. **Hett EC, Chao MC, Deng LL, Rubin EJ.** 2008. A mycobacterial enzyme essential for cell division synergizes with resuscitation-promoting factor. PLoS Pathog. **4**:e1000001. doi:10.1371/journal.ppat.1000001.