Loss of a Cardiolipin Synthase in *Helicobacter pylori* G27 Blocks Flagellum Assembly

Joshua K. Chu,^a Shiwei Zhu,^b Carmen M. Herrera,^c Jeremy C. Henderson,^{c*} Jun Liu,^b M. Stephen Trent,^c Timothy R. Hoover^a

^aDepartment of Microbiology, University of Georgia, Athens, Georgia, USA

^bDepartment of Microbial Pathogenesis, Microbial Sciences Institute, Yale University, West Haven, Connecticut, USA

^cDepartment of Infectious Diseases, University of Georgia, Athens, Georgia, USA

ABSTRACT *Helicobacter pylori* uses a cluster of polar, sheathed flagella for motility, which it requires for colonization of the gastric epithelium in humans. As part of a study to identify factors that contribute to localization of the flagella to the cell pole, we disrupted a gene encoding a cardiolipin synthase (*clsC*) in *H. pylori* strains G27 and B128. Flagellum biosynthesis was abolished in the *H. pylori* G27 *clsC* mutant but not in the B128 *clsC* mutant. Transcriptome sequencing analysis showed that flagellar genes encoding proteins needed early in flagellum assembly were expressed at wild-type levels in the G27 *clsC* mutant. Examination of the G27 *clsC* mutant by cryo-electron tomography indicated the mutant assembled nascent flagella that contained the MS ring, C ring, flagellar protein export apparatus, and proximal rod. Motile variants of the G27 *clsC* mutant were isolated after allelic exchange mutagenesis using genomic DNA from the B128 *clsC* mutant as the donor. Genome resequencing of seven motile G27 *clsC* recipients revealed that each isolate contained the *flgI* (encodes the P-ring protein) allele from B128. Replacing the *flgI* allele in the G27 *clsC* mutant with the B128 *flgI* allele rescued flagellum biosynthesis. We postulate that *H. pylori* G27 FlgI fails to form the P ring when cardiolipin levels in the cell envelope are low, which blocks flagellum assembly at this point. In contrast, *H. pylori* B128 FlgI can form the P ring when cardiolipin levels are low and allows for the biosynthesis of mature flagella.

IMPORTANCE *H. pylori* colonizes the epithelial layer of the human stomach, where it can cause a variety of diseases, including chronic gastritis, peptic ulcer disease, and gastric cancer. To colonize the stomach, *H. pylori* must penetrate the viscous mucous layer lining the stomach, which it accomplishes using its flagella. The significance of our research is identifying factors that affect the biosynthesis and assembly of the *H. pylori* flagellum, which will contribute to our understanding of motility in *H. pylori*, as well as other bacterial pathogens that use their flagella for host colonization.

KEYWORDS *Helicobacter pylori*, cardiolipin, cardiolipin synthase, flagellum

Helicobacter pylori is a member of the subphylum *Epsilonproteobacteria* that colonizes the stomach of about half the human population worldwide (1, 2). Infection of the gastric mucosa by *H. pylori* can lead to a variety of diseases, including chronic gastritis, peptic ulcer disease, gastric cancer, and mucosa-associated lymphoid tissue lymphoma (3–5). *H. pylori* cells have a cluster of flagella at one cell pole that they use for motility, which is required for colonization of the gastric mucosa by the bacterium (6, 7).

The bacterial flagellum is composed of three basic parts, namely, the basal body, hook and filament (8–10). The basal body is the most complex of these structures and includes the flagellar motor. The flagellar motor consists of the MS ring (base for the motor), C ring (switch complex regulating motor rotation), rod (connects MS ring to

Citation Chu JK, Zhu S, Herrera CM, Henderson JC, Liu J, Trent MS, Hoover TR. 2019. Loss of a cardiolipin synthase in *Helicobacter pylori* G27 blocks flagellum assembly. *J Bacteriol* 201:e00372-19. <https://doi.org/10.1128/JB.00372-19>.

Editor Yves V. Brun, Université de Montréal

Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Address correspondence to Timothy R. Hoover, trhoover@uga.edu.

* Present address: Jeremy C. Henderson, Department of Microbiology, Center for RNA Biology, The Ohio State University, Columbus, Ohio, USA.

Received 30 May 2019

Accepted 28 July 2019

Accepted manuscript posted online 19 August 2019

Published 4 October 2019

hook), and stator, which is a membrane protein complex of MotA and MotB. The stator is the torque generator for the flagellar motor and is powered by the proton motive force (11). In addition to regulating motor rotation, the C ring has a role in flagellum assembly (12, 13). Additional components of the basal body include a type III secretion system (T3SS) that transports rod, hook, and filament proteins across the cell membrane, and ring structures (P ring and L ring) that act as bushings embedded in the cell envelope. The hook and filament are located outside the cell envelope. The filament functions as a helical propeller, while the hook serves as a flexible joint that transmits torque from the motor to the flagellar filament over a wide range of angles between the motor axis and filament. Structural analysis of the *H. pylori* flagellar motor using cryo-electron tomography (cryo-ET) revealed that it is one of the largest flagellar motors in bacteria, accommodating up to 18 torque generators (14). The large *H. pylori* flagellar motor may provide the higher torque required for the bacterium to swim in high-viscosity medium.

Flagellum biosynthesis is a complex process that involves the coordinated expression of dozens of flagellar genes with the assembly of the flagellum. Expression of flagellar genes in *H. pylori* is controlled by a transcriptional hierarchy that is organized according to the sigma factor (σ) required for transcription of specific sets of genes (15, 16). These sigma factors are RpoD (σ^{80}), RpoN (σ^{54}), and FliA (σ^{28}). Genes encoding proteins required early in flagellum assembly (i.e., basal body formation) and some regulatory proteins rely on σ^{80} for their transcription (15). A master regulator that initiates the transcriptional hierarchy has been identified for many bacteria (17) but not for *H. pylori*. Transcription of genes whose products are required midway in flagellum assembly (e.g., hook and hook-associated proteins) requires σ^{54} and a two-component system consisting of the sensor kinase FlgS and the response regulator FlgR (16, 18, 19). FlgS may recognize an assembly checkpoint associated with the T3SS to initiate the signaling cascade that results in transcriptional activation of the σ^{54} -dependent regulon, as disrupting genes encoding components of the T3SS interferes with expression of σ^{54} -dependent genes (15, 20–24). Consistent with this hypothesis, FlgS binds a peptide that corresponds to the N terminus of the T3SS component FlhA with high affinity (25). Transcription of the late flagellar genes in *H. pylori* requires σ^{28} and is negatively regulated by the anti- σ^{28} factor FlgM (26, 27). In *Salmonella enterica* serovar Typhimurium, inhibition of σ^{28} activity by FlgM is alleviated by transport of FlgM by the T3SS (28). FlgM does not appear to be exported by the T3SS in *H. pylori*, but rather, its inhibition on σ^{28} may be relieved by binding to FlhA (29).

As part of a study to identify factors involved in localization of flagella to the *H. pylori* cell pole, we examined the potential role of cardiolipin (CL) since it is required for polar localization of certain proteins in *Escherichia coli* (30). CL is an anionic glycerophospholipid that has a small glycerol head group and a large hydrophobic tail consisting of four acyl chains, which gives the molecule a conical shape that results in an intrinsic curvature (31). The shape of the CL molecule favors its localization in negatively curved regions of bacterial membranes, such as the cell poles and septa in rod-shaped bacteria (32–34). As in other bacteria, CL species in *H. pylori* vary with respect to acyl chain composition, i.e., length and degrees of unsaturation and cyclopropanation of the acyl chains. The major fatty acids reported for CL from *H. pylori* NCTC 11638 include the saturated fatty acids myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), the unsaturated fatty acids oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$), and cyclopropane nonadecanoic acid ($C_{19:0}$ cyc), with $C_{14:0}$ and $C_{19:0}$ cyc accounting for about 70% of the fatty acid content (35). Zhou et al. analyzed CL from *H. pylori* NCTC 11637 (ATCC 43504) and proposed structures for the main CL and monolysocardiolipin (MLCL; a glycerophospholipid with three fatty acid chains) species (36).

E. coli possesses three cardiolipin synthases (ClsA, ClsB, and ClsC), which catalyze the formation of CL by transferring phosphatidic acid from a phosphatidylglycerol (PG) or phosphatidylethanolamine (PE) molecule to a PG molecule. ClsA and ClsB use PG as the phosphatidic acid donor for CL biosynthesis, while ClsC uses PE as the phosphatidic acid donor (37, 38). A potential CL synthase in *H. pylori* G27 shares homology with *E. coli*

ClsC. We found that the *H. pylori* *clsC* homolog is required for wild-type levels of CL, and so we designated it *clsC*. Disrupting *clsC* abolished flagellum biosynthesis in *H. pylori* G27, but not in *H. pylori* B128, even though CL levels in the two *clsC* mutants were similarly reduced. Transcriptome sequencing (RNA-seq) analysis revealed that the *H. pylori* G27 *clsC* mutant expressed many of the genes in the σ^{54} regulon at wild-type levels or higher, suggesting the mutant expressed a functional T3SS but was blocked in flagellum assembly. Consistent with this hypothesis, cryo-ET revealed nascent flagellar structures arrested at an early stage in the assembly process in the *H. pylori* G27 *clsC* mutant. Introduction of the *H. pylori* B128 *flgI* allele (encodes the flagellar P-ring protein) into the *H. pylori* G27 *clsC* mutant restored flagellum biogenesis. We infer from these data that assembly of the P ring in *H. pylori* G27, but not *H. pylori* B128, requires normal levels or specific species of CL in the cell envelope.

RESULTS

Identification of a CL synthase in *H. pylori*. The proposed *clsC* homolog in *H. pylori* G27 (locus tag HPG27_174) encodes a protein belonging to the phospholipase D (PLD) superfamily, members of which catalyze the hydrolysis of phosphodiester bonds. A large subset of enzymes belonging to the PLD superfamily possess a conserved HxKx₄Dx₆GSxN motif (HKD motif) (39). The HKD motif is found in all known bacterial CL synthases, including the *H. pylori* G27 homolog, which contains two HKD motifs and shares 32% amino acid identity with *E. coli* ClsC and 21 to 24% identity with the other two *E. coli* CL synthases, ClsA and ClsB. The predicted amino acid sequence of the B128 homolog in both the NCBI and the JGI Integrated Microbial Genomes and Microbiomes databases indicates that its coding sequence consists of two open reading frames (locus tags HPB128_3g68 and HPB128_3g69) that overlap by 59 bp. Resequencing this region, however, revealed a single open reading frame the same length as that of the *H. pylori* G27 *clsC* allele and with a sequence identical to a homologous open reading frame in *H. pylori* B8, a gerbil-adaptive strain derived from *H. pylori* B128 (40).

Since CL is required for polar localization of certain membrane proteins in *Escherichia coli* (30), we hypothesized that interrupting the putative *clsC* homologs would eliminate CL levels and negatively impact the localization of flagella to the cell pole in the *H. pylori* *clsC* mutants. We disrupted the *clsC* homologs in the *H. pylori* G27 and B128 strains with a chloramphenicol resistance (*cat*) cassette to assess their role in CL biosynthesis and examined motility of the resulting mutants in soft agar medium. *H. pylori* G27 *clsC* failed to swim from the stab-inoculation point, indicating a severe defect in motility or chemotaxis (Fig. 1A). Reintroducing the *clsC* homolog from *H. pylori* G27 into the mutant on plasmid pHel3 (plasmid pCIs) rescued motility (Fig. 1A). In contrast, motility in the *H. pylori* B128 *clsC* mutant was only modestly impaired (<20%) (Fig. 1A). Examination of the *H. pylori* G27 *clsC* mutant by transmission electron microscopy (TEM) revealed the cells lacked flagella, while more than 85% of the wild-type cells were flagellated, and most of the cells possessed multiple flagella (Fig. 1B). Plasmid pCIs restored the wild-type flagellation pattern in the *clsC* mutant (Fig. 1B), indicating that ClsC is required for flagellum biosynthesis in *H. pylori* G27. Unlike the *H. pylori* G27 *clsC* mutant, we did not observe a reduction in flagellated cells in the *H. pylori* B128 *clsC* mutant (Fig. 1C).

Analysis of the ³²P-labeled glycerophospholipids from the wild-type and mutant strains revealed that CL levels were reduced in the putative CL synthase mutants in *H. pylori* G27 and B128 (Fig. 2). The glycerophospholipid composition of the wild-type G27 and B128 strains was similar to that of *H. pylori* NCTC 11638 and ATCC 43504 (35), but there were some differences in the glycerophospholipid compositions of the two strains we analyzed (Table 1). Most notably, CL levels appeared to be slightly higher in G27 compared to B128. Introducing plasmid pCIs into both G27 and B128 mutants, but not the empty pHel3 shuttle vector, restored CL to wild-type levels (Fig. 2 and Table 1). These data provide compelling evidence that the *clsC* gene from *H. pylori* G27 encodes a CL synthase.

FIG 1 Effects of *clsC* knockouts on motility and number of flagella per cell in *H. pylori* G27 and B128. (A) Motility was assessed on soft agar medium. Measurements indicate the halo diameters surrounding the point of inoculation after 7 days. Asterisks (single for G27 and double for B128) indicate values that were significantly different ($P < 0.01$) from that of the parental strain. Statistical significance was determined using the two-sample *t* test. (B and C) The numbers of flagella per cell were determined by TEM after negative staining ($n = 100$ per strain). The asterisk indicates a value significantly different from that of the parental strain ($P < 0.05$). Statistical significance was determined using the Mann-Whitney U test.

Matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry of the glycerophospholipids of the *H. pylori* strains showed several molecular ions in the CL range (approximately m/z 1,150 to 1,400) (Fig. 3A). Zhou et al. proposed structures for the main CL and MLCL species from *H. pylori* NCTC 11637 (ATCC 43504)

FIG 2 *ClsC* from *H. pylori* strains G27 and B128 is involved in CL synthesis. Radiolabeled glycerophospholipids from *E. coli* W3310 (lane 1; standard) and various *H. pylori* strains (lanes 2 through 9) were isolated and separated by TLC. Glycerophospholipids were separated in the following order (bottom to top): phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). Wild-type strains of *H. pylori* G27 and B128 (lanes 2 and 6) displayed similar patterns for glycerophospholipid species with two unique spots for CL. The CL-deficient strains (lanes 3 and 7) show a loss of the top CL spot in both strains. Introducing the complementing plasmid (pCIs), but not the empty shuttle vector (pHel3; lanes 4 and 8), in both mutants (lanes 5 and 9) recovers CL levels, indicated by the presence of the top CL spot for these strains.

TABLE 1 Glycerophospholipid distribution of *H. pylori* strains^a

Glycerophospholipid	% distribution							
	Wild type		<i>clsC</i> mutant		<i>clsC</i> pHel3 mutant		<i>clsC</i> pHel3 <i>clsC</i> mutant	
	G27	B128	G27	B128	G27	B128	G27	B128
PE	68	80	87	92	87	92	69	77
CL	29	17	10	4	10	4	27	20
PG	2	3	3	3	3	3	3	2

^aThe glycerophospholipid composition was determined using the Amersham Typhoon scanner control software 2.0. The percentages represent the distribution for each glycerophospholipid isolated from whole cells.

(36). Proposed MLCL species included $C_{14:0}/C_{14:0}/C_{14:0}$ (m/z 1,030), $C_{19:0}$ cyc/ $C_{14:0}/C_{14:0}$ (m/z 1,098), and $C_{19:0}$ cyc/ $C_{14:0}/C_{19:0}$ cyc (m/z 1,166), whereas proposed CL species included $C_{14:0}/C_{14:0}/C_{14:0}/C_{14:0}$ (m/z 1,240), $C_{14:0}/C_{14:0}/C_{14:0}/C_{19:0}$ cyc (m/z 1,308), and $C_{19:0}$ cyc/ $C_{14:0}/C_{14:0}/C_{19:0}$ cyc (m/z 1,376) (36). Peaks with approximate m/z values of 1,261 and 1,329 were absent in the *H. pylori* G27 *clsC* mutant, whereas 1,261, 1,329, and 1,397 were absent in the *H. pylori* B128 *clsC* mutant and likely correspond to CL species missing in the thin-layer chromatography (TLC) analysis (Fig. 2). The peak with the approximate m/z value of 1,189 was observed in all strains, and likely represents the slower-migrating CL species in the TLC (Fig. 2). Proposed structures for the CL species identified in the mass spectrometry, with the addition of a sodium adduct, are consistent with the previously proposed structures mentioned above (Fig. 3B). The fact that residual amounts of CL were present in the *clsC* mutants (Fig. 2 and 3A) suggests both *H. pylori* strains possess an alternative method to synthesize CL. At present, we do not know the identity of the enzyme(s) responsible for the synthesis of the residual amounts of CL in the *clsC* mutants.

Flagellum assembly is blocked at an early stage in the *H. pylori* G27 *clsC* mutant. We reasoned that the lesion in flagellum biosynthesis in the *H. pylori* G27 *clsC* mutant was due to the inhibition of expression of specific flagellar genes or resulted from a block in flagellum assembly. To distinguish between these two hypotheses, we compared global gene expression in the *H. pylori* G27 and *H. pylori* G27 *clsC* mutant strains by RNA-seq. Comparing the *clsC* mutant to the wild-type strain, we identified 26 genes that were differentially regulated and exhibited a $\log_2$ fold change of ≥ 1.5 in the *clsC* mutant (Fig. 4). Seventeen genes were downregulated in the *clsC* mutant, most of which are pseudogenes or encode hypothetical proteins of unknown function. One of the downregulated genes, *cagB*, is located within the *cag* pathogenicity island, while HPG27_RS03555 encodes a protein that belongs to the 50S ribosome-binding GTPase protein family (pfam01926). Nine genes were upregulated in the *clsC* mutant, five of which (*flgE*, *flaB*, *flgK*, *flgL*, and *flgJ*) are dependent on σ^{54} for their transcription (15).

To confirm that flagellar genes were differentially regulated in the *H. pylori* G27 *clsC* mutant, we assessed transcript levels of representative genes from the three flagellar regulons in the mutant by quantitative reverse transcription-PCR (RT-qPCR). *flhA* (encodes a component of the T3SS) and *flhF* (encodes MS ring protein) are dependent on the housekeeping σ factor of *H. pylori* (σ^{80}) for their transcription and encode proteins that are required at early stages in flagellum assembly. *flgE* (encodes hook protein) and *flaB* (encodes a minor flagellin) are σ^{54} -dependent genes, and their products are required at later steps in flagellum assembly. The final gene we examined, *flaA* (encodes the major flagellin), is dependent on σ^{28} for its transcription and encodes a protein that is required near the end of flagellum assembly. Transcript levels of *flhA* and *flhF* in the *clsC* mutant were indistinguishable from those in wild type (both were $\sim 90\%$ of the wild type; $P = 0.57$ and 0.38 , respectively) (Fig. 5). Consistent with the RNA-seq data, transcript levels of *flaB* and *flgE* in the G27 *clsC* mutant were higher (~ 1.9 -fold [$P = 0.0021$] and ~ 2.3 -fold [$P = 0.0017$], respectively) than those in the wild type (Fig. 5). In contrast to the σ^{80} -dependent and σ^{54} -dependent flagellar genes, transcript levels of

FIG 3 MALDI-TOF mass spectrometry of *clsC* mutants in *H. pylori* G27 and B128. (A) The wild-type and complemented strains produced peaks in the CL ranges of 1,150 and 1,400 m/z as reported for *H. pylori* (36). Samples from the wild-type and complemented strains displayed peaks with m/z values of 1,189.9, 1,261.8, 1,329.9, and 1,397.9. In contrast, the sample from the B128 *clsC* mutant only displayed a peak with an m/z value of 1,189.9, while the G27 *clsC* mutant showed peaks with m/z values of 1,189.9 and 1,397.9. (B) Predicted structures for the CL species corresponding to the peaks from the mass spectrometry analysis. MALDI-TOF was performed in the negative mode, resulting in negatively charged ions. The error of the instrument is ± 1 , and so the observed m/z values are consistent with the calculated masses for the Na adducts.

flaA in the G27 *clsC* mutant were slightly lower ($\sim 60\%$; $P = 0.0017$) than those in the wild type (Fig. 5).

Since the T3SS is intimately linked with expression of the σ^{54} -dependent flagellar genes in *H. pylori* (15, 20–25, 41), the upregulation of σ^{54} -dependent flagellar genes in the G27 *clsC* mutant suggested the mutant assembles a nascent flagellum structure that includes the T3SS. Examining the ultrastructure of the *H. pylori* G27 *clsC* mutant by

FIG 4 Genes identified in RNA-seq to be differentially regulated. Each bar represents a gene with a $\log_2$ fold change of ± 1.5 in the G27 *clsC* mutant. Functions for the genes indicated are as follows: *flgE*, flagellar hook protein; *flaB*, minor flagellin B; *crdA*, copper resistance determinant; *flgJ*, predicted muramidase; *flgK*, flagellar hook-associated protein; *flgL*, flagellar hook-associated protein; *sabA*, outer membrane protein; and *cagB*, cag pathogenicity island protein B. The remaining genes either encode proteins of unknown function or are pseudogenes.

cryo-ET revealed nascent flagellum structures in the cell envelope (Fig. 6). The MS ring, C ring, T3SS, and proximal rod were clearly visible within the nascent flagellum structures, while other basal body and flagellar motor components are not apparent, including the P ring, L ring, distal rod, and stator (Fig. 6). The *H. pylori* flagellum has a unique cage-like structure that surrounds the motor (14), and this structure was also absent in the nascent flagellum structures of the *H. pylori* G27 *clsC* mutant (Fig. 6). Taken together with the RNA-seq and RT-qPCR data, the results of the cryo-ET study

FIG 5 Expression levels of select flagellar genes as determined by RT-qPCR. Each bar indicates the mean fold change for three biological replicates in a comparison of transcript levels of the target genes from the G27 *clsC* and the G27 wild-type strain. Fold change values for all target genes were calculated using the $\Delta\Delta C_T$ method (63) using *rpoA* and *rpoD* as reference genes. A statistically significant difference between the mutant and wild-type strains for the middle and late genes was observed using the two-sample *t* test ($P < 0.01$).

FIG 6 *In situ* structure of the nascent flagellum from the *H. pylori* G27 *clsC* mutant. (A) Representative slice of a three-dimensional reconstruction of *H. pylori* G27 *clsC*. (B) Zoom-in view showing the MS/C-ring together with the partial rod density. (C) Central section slice of a subtomogram-averaged structure from *H. pylori* G27 *clsC*. (D) Central section slice from wild-type motor-averaged structure. The scale bars in panels A and B are 50 nm in length, while the scale bars in panels C and D are 20 nm in length. OM, outer membrane; IM, inner membrane.

indicate that reduced CL levels in the cell envelope of the *H. pylori* G27 *clsC* mutant interfere with an early step in flagellum assembly.

The P-ring protein from *H. pylori* B128 rescues flagellum biosynthesis in the *H. pylori* G27 *clsC* mutant. To determine the genetic basis for the disruption in flagellum assembly in the *H. pylori* G27 *clsC* mutant, we isolated seven motile variants (designated mv49, mv50, mv51, mv65, mv70, mv76, and mv77) of the *H. pylori* G27 *clsC* mutant after two independent transformations of the strain with genomic DNA (gDNA) from the *H. pylori* B128 *clsC* mutant (Fig. 7A). TEM analysis revealed that the motile variants were flagellated but varied significantly in their flagellation patterns (Fig. 7B). The motile variant mv49 was highly flagellated, with about 98% of the cells possessing multiple flagella, which was higher than that observed for the other motile variants and the wild type (Fig. 7B). The highly flagellated nature of mv49 corresponded with the enhanced motility of the strain in soft agar medium relative to that of the wild type and the other motile variants (Fig. 7A). In contrast, the motilities of mv50, mv51, mv65, mv70, mv76, and mv77 were similar to that of the wild type (Fig. 7A), and the flagellation patterns of the motile variants were similar to the wild type, although the motile variants had a higher proportion of cells that lacked flagella or had a single flagellum compared to the wild type (Fig. 7B).

Genomes of the motile variants were resequenced to identify B128 alleles that may have rescued flagellum biosynthesis in the G27 *clsC* mutant. DNA sequences derived from the B128 donor were identified as regions with clusters of single nucleotide polymorphisms (SNPs), which ranged in number from 438 to 2,664 in the motile G27 *clsC* recipients. The number of B128 alleles identified in the G27 *clsC* recipients ranged from 23 to 104. Motile variant mv49 was very different from the other motile variants in its genetic makeup since it contained 98 B128 alleles (2,492 SNPs) that were not present in the other motile variants. In contrast, the other motile variants only possessed between four and six unique B128 alleles.

Only three B128 alleles (*kdsA*, *icfA*, and *flgI*) were present in all seven motile variants. *kdsA* and *icfA* are adjacent genes located near the origin of replication. KdsA (3-deoxy-

FIG 7 Motility and numbers of flagella per cell for the G27 *c/sC* motile variants isolated after allelic exchange mutagenesis. (A) Motility was assessed on soft agar medium. Measurements indicate the halo diameters surrounding the point of inoculation after 7 days. The statistical significance was determined using the two-sample *t* test. (B) The number of flagella per cell was assessed by TEM after negative staining ($n = 100$ per strain). The statistical significance was determined using a Mann-Whitney U test. For both panels, one asterisk indicates that a strain is statistically different compared to the *H. pylori* G27 *c/sC* parental strain ($P < 0.01$) and two asterisks indicate that a strain is statistically different compared to the motile variant mv49 ($P < 0.01$).

D-manno-octulosonic acid 8-phosphate synthetase) is part of the CMP-KDO biosynthetic pathway required for lipopolysaccharide biosynthesis, while *IcfA* is a β -carbonic anhydrase that catalyzes the reversible hydration of CO_2 to carbonic acid. *flgI* encodes the flagellar P-ring protein and is located ~ 250 kb from *icfA*.

The coding regions of *kdsA*, *icfA*, and *flgI* in the G27 *c/sC* mutant were replaced with the corresponding regions from B128 to determine whether any of the alleles restored flagellum biosynthesis in the G27 *c/sC* mutant. Substitution of *kdsA* and *icfA* in the G27 *c/sC* mutant with the B128 alleles failed to rescue motility (Fig. 8A) or flagellar biosynthesis (data not shown) in the G27 *c/sC* mutant. The *icfA* *kdsA* locus is near the origin of replication and may be a recombination hot spot, which might have accounted for the presence of the B128 *kdsA* and *icfA* alleles in all seven of the motile variants. In contrast to *kdsA* and *icfA*, introducing the B128 *flgI* allele into the G27 *c/sC* mutant restored motility (Fig. 8A) and flagellum biosynthesis (Fig. 8B). In the RNA-seq assays, the *flgI* transcript levels for the *H. pylori* G27 wild type and *c/sC* mutant did not differ significantly ($\log_2$ fold change of -0.31 [$P = 0.27$], mutant versus wild type), suggesting *flgI* expression is normal in the *c/sC* mutant. Taken together, the results of the RNA-seq, cryo-ET, and complementation studies suggest that the lesion in flagellum biosynthesis in the *H. pylori* G27 *c/sC* mutant results, at least in part, from the failure of FlgI to assemble into the flagellar P ring.

FIG 8 Motility and flagellum count results for the G27 *clsC* mutant strains in which specific B128 alleles were introduced. (A) Motility was assessed on soft agar medium. Measurements indicate the halo size surrounding the point of inoculation after 7 days. The G27 wild-type and G27 *clsC* strains are used as references. Three isolates of the G27 *clsC* mutant in which the B128 *icfA* and *kdsA* alleles (iso146, iso147, and iso148) or the B128 *flgI* allele (iso164, iso168, and iso169) had been introduced were examined in the motility assay. Statistical significance was determined by using a two-sample *t* test. (B) The number of flagella per cell was assessed by TEM after negative staining ($n = 100$ per strain). The statistical significance was determined by using a Mann-Whitney U test. The asterisks indicate a statistically significant difference in the number of flagella per cell for the G27 *clsC* mutants bearing the B128 *flgI* allele (iso164, iso168, and iso169) compared to the G27 *clsC* parental strain ($P < 0.01$).

DISCUSSION

We show here that disrupting *clsC* in *H. pylori* G27 interferes with flagellum assembly, which is relieved by introducing the *flgI* allele from *H. pylori* B128. Disrupting *clsC* in *H. pylori* strains G27 and B128 resulted in reduced amount of total CL, as well as the apparent loss of specific CL species (Fig. 2 and 3A). The *clsC* mutants contains a single, predominant CL species, which we predict to be the monolysocardiolipin $C_{18:0}/C_{16:0}/C_{18:1}$ (Fig. 3B). Our data suggest that assembly of the flagellar P ring in *H. pylori* G27 requires either specific CL species or high levels of CL, but the *H. pylori* B128 P ring protein is not constrained by these limitations. An intriguing question is what accounts for the differences between the G27 and B128 FlgI proteins that allows one to form the P ring in the *H. pylori* G27 *clsC* mutant but not the other? The G27 and B128 FlgI proteins differ at five amino acid positions, but only three of these differences (T140V, N151H, and F216L) were identified in the *H. pylori* G27 *clsC* motile variants from the genome resequencing. One or more of these amino acid differences may affect the stability or transport of FlgI or may influence interactions of the protein with itself or

other proteins, which could account for the failure of the G27 FlgI protein to assemble the P ring in the absence of ClsC. A comparison of FlgI sequences from 64 different *H. pylori* strains revealed that Thr-140, Asn-151, and Phe-216 are the most common amino acids at these positions (frequency ranges from 72 to 80%), but the amino acids occurring at these positions in B128 FlgI (Val-120, His-151, and Leu-216) are fairly common (see Fig. S1 in the supplemental material).

CL plays roles in the localization, oligomeric state, and function of various membrane proteins (42–45), and it is possible that the lesion in flagellum assembly in the *H. pylori* G27 *clsC* mutant is due to interference in one or more of these roles. CL has a small glycerol head group and a large hydrophobic tail consisting of four acyl chains, which gives the molecule a conical shape that results in an intrinsic curvature (31). The shape of the CL molecule favors its localization in negatively curved regions of bacterial membranes, such as the cell poles and septa in rod-shaped bacteria (32–34). The preferential localization of CL to the cell pole presumably accounts for the polar localization of some proteins in *E. coli*, such as the osmotic stress protein ProP (45) and the CL synthase ClsA (44). While ProP and ClsA are inner membrane proteins, CL also has an apparent role in the polar localization of the outer membrane protein IcsA in *Shigella flexneri* (46) and is hypothesized to play a role in the localization or display of adhesion proteins in the outer membrane of *Moraxella catarrhalis* (47). It is possible that FlgI fails to localize to the cell pole in the absence of normal levels of CL or specific CL species in *H. pylori* G27. It is unlikely, though, that FlgI interacts directly with CL, since FlgI is not associated with the inner or outer membranes. Thus, if CL does affect localization of FlgI to the cell pole, then it is likely mediated through another protein that interacts directly with CL.

In *S. Typhimurium*, the Sec machinery transports FlgI into the periplasmic space where it assembles into the P ring surrounding the flagellar rod (48, 49). The bacterial Sec system consists of the SecYEG protein channel complex and SecA ATPase, which work together to secrete preproteins across the cell membrane in a process that is powered by ATP hydrolysis and the proton motive force. The Sec machinery in *E. coli* requires CL for the efficient translocation of preproteins across the cell membrane (50). CL binds the SecYEG complex to stabilize formation of dimers, which creates a binding platform for SecA and stimulates ATP hydrolysis (51). Corey et al. identified two specific CL binding sites in SecYEG that potentiate the role of CL in ATPase and protein transport activity (42). It is possible that the G27 FlgI is not transported by the Sec machinery as efficiently as the B128 protein due to the reduced levels of CL or absence of specific CL species. The amino acid differences in B128 FlgI may overcome the CL deficiency in the *clsC* mutant by stabilizing the protein translocation complex to facilitate assembly of the P ring and restore motility.

The first 19 amino acids of *E. coli* FlgI constitute a signal peptide that is cleaved by the Sec machinery as the protein is translocated across the cell membrane. Signal peptide prediction programs (e.g., SignalP-5.0 or Phobius) identify the first 19 amino acids of *H. pylori* FlgI as a signal peptide. The 120 amino acid residues at the N terminus of the processed *E. coli* FlgI protein form a conserved region that is rich in glycine and proline residues and has been suggested to have roles in stabilizing the protein or interacting with other FlgI monomers or other flagellar proteins (52). The corresponding region of *H. pylori* FlgI shares 55% identity and 75% similarity with that of *E. coli* FlgI and is similarly rich in glycine and proline residues. Two of the amino acid positions where the G27 and B128 FlgI proteins differ (positions 140 and 151) are adjacent to the glycine-/proline-rich region, which could impact the function of this region. For example, the affinity of the G27 and B128 FlgI proteins for themselves or other interaction partners may differ as a result of the amino acid differences at position 140 or position 151. In *Salmonella*, 26 copies of FlgI are predicted to assemble to form the P ring (53). In addition to interacting with itself, FlgI interacts strongly with FlgH, which forms the L ring. As is the case with FlgI, FlgH is transported into the periplasmic space by the Sec machinery (48, 49). FlgI may also interact with peptidoglycan, the flagellar rod proteins, the MotA/B stator complex, or other flagellar components found in the periplasmic

space (52). If transport of FlgI and/or any of its interaction partners in the periplasmic space is impaired in the *clsC* mutant, the reduced levels of these proteins combined with the lower affinity of the G27 FlgI for itself or its interaction partners may account for its failure to assemble the P ring in the *clsC* mutant.

Another FlgI interaction partner is FlgA, which is predicted to be a chaperone that aids in P-ring assembly by facilitating polymerization of FlgI (54). Although *flgA* is required for motility in *S. Typhimurium* under normal conditions, overproducing FlgI in a *flgA* mutant restores flagellum synthesis, indicating that FlgA plays an auxiliary role in assembly of the P ring (54). *H. pylori* strains possess an *flgA* homolog, although we are unaware of any report demonstrating the requirement of this gene for flagellum biosynthesis in *H. pylori*. *S. Typhimurium* FlgA is transported by the Sec machinery (54). Sec-dependent transport of FlgA may be inhibited in the *H. pylori* G27 *clsC* mutant, which could account for the failure of the P ring to assemble in this mutant. If the B128 FlgI protein has a higher affinity for itself and is less dependent on FlgA for assembling the P ring or has a higher affinity for FlgA, this might account for the ability of this protein to form the P ring in the presence of reduced amounts of FlgA. Future work in our lab will attempt to understand the molecular basis for how the B128 FlgI protein is able to assemble the P ring in the *clsC* mutant.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5 α was used for cloning and plasmid construction. *E. coli* strains were grown in Luria-Bertani broth or agar medium. Medium was supplemented with ampicillin (100 μ g/ml), chloramphenicol (30 μ g/ml) or kanamycin (30 μ g/ml) when appropriate. For routine growth of *H. pylori* strains, the cultures were grown microaerobically under an atmosphere consisting of 10% CO₂, 4% O₂, and 86% N₂ at 37°C on tryptic soy agar (TSA) supplemented with 5% horse serum or in tryptic soy broth (TSB) supplemented with 5% heat-inactivated horse serum with shaking under an atmosphere consisting of 5% CO₂, 10% H₂, 10% O₂, and 75% N₂. Growth of *H. pylori* strains for glycerophospholipid extraction was done at 37°C in Mueller-Hinton broth (MHB) with 10% horse serum and 20 mM 2-(4-morpholino)-ethanesulfonic acid (MES; pH 6.0) under an atmosphere consisting of 10% CO₂, 5% O₂ and 85% N₂. Kanamycin (30 μ g/ml) or chloramphenicol (30 μ g/ml) was added to the medium used to culture *H. pylori* when appropriate.

Strain construction. All primers used for PCR in the construction of *H. pylori* mutants are listed in Table S1 in the supplemental material. Genomic DNA from *H. pylori* B128 was purified using the Wizard genomic DNA purification kit (Promega) and used as the PCR template to construct the *clsC* knockout. DNA was amplified from *H. pylori* B128 gDNA using Phusion polymerase (New England Biolabs), and the resulting amplicons were incubated with *Taq* polymerase (Promega) at 72°C for 10 min to add 3'-A overhangs for T/A cloning with pGEM-T Easy plasmid (Promega). To disrupt *clsC* in *H. pylori* G27 and B128, a 634-bp upstream DNA fragment was amplified that included 493 bp of *frdB* (the gene immediately upstream of *clsC*; it encodes succinate dehydrogenase subunit B), an intergenic region of 21 bp, and 99 bp of the 5' end of *clsC* that includes a 21-bp sequence complementary to a chloramphenicol resistance (*cat*) cassette using the primers M54 and M55. A 604-bp downstream DNA fragment that included 508 bp downstream of *clsC* and 75 bp of the 3' end of *clsC*, together with a 21-bp sequence complementary to the *cat* cassette, was amplified using the primers M56 and M57. A *cat* cassette was PCR amplified from pUC20 *cat* using the primers cat5 and cat6, generating a 742-bp fragment (55). PCR-based gene splicing by overlap extension (PCR SOEing) was used to join the upstream and downstream regions to the *cat* cassette, and the resulting amplicon was cloned into pGEM-T Easy to generate the suicide plasmid pJC030, which was introduced into *H. pylori* strains G27 and B128 by natural transformation. Chloramphenicol-resistant transformants were selected on TSA supplemented with horse serum and chloramphenicol. Replacement of *clsC* with the *cat* cassette in the *H. pylori* G27 and B128 chromosome was confirmed by PCR using primers that flanked the *clsC* locus. The resulting amplicons were sequenced (Eton Bioscience) to verify that *clsC* had been replaced with the *cat* cassette.

Complementation of the *clsC* mutation. Plasmid pJC032 is a derivative of the shuttle vector pHel3 (56) that carries *H. pylori* G27 *clsC* under the control of its native promoter located upstream of *frdA*. To construct pJC032, the *frdA* promoter and *clsC* coding regions were amplified using the primer pairs P069/P070 and P071/P072, respectively, and *H. pylori* G27 gDNA as the template. The resulting amplicons were joined by PCR SOEing. Primers that contained 25 bp of homologous sequence with pHel3 were used to amplify the fusion product, and the resulting amplicon was cloned into pHel3 cut with *Sph*I using the sequence- and ligation-independent cloning (SLIC) method (57) to generate pJC032. For complementation assays, pJC032 was introduced into the *H. pylori* *clsC* mutants by natural transformation, which were then examined for CL, motility, and the presence of flagella, as described below.

Motility assay. Motility was assessed using a soft-agar medium comprised of MHB, 10% heat-inactivated horse serum, 20 mM MES (pH 6.0), 5 μ M FeSO₄, and 0.4% Noble agar. Plates were stab inoculated with cells from TSA plates grown for 5 days using wooden sticks and incubated at 37°C under an atmospheric condition consisting of 10% CO₂, 4% O₂, and 86% N₂. Diameters of swim halos were measured after 7 days of incubation. The two-sample *t* test was used to determine statistical significance.

Transmission electron microscopy. *H. pylori* strains were grown to late log phase in TSB supplemented with 10% heat-inactivated horse serum to an optical density at 600 nm (OD_{600}) of ~ 1.0 . One milliliter of culture was collected by centrifugation ($550 \times g$) and then resuspended in 125 μ l of 0.1 M phosphate-buffered saline (PBS). Cells were fixed by adding 50 μ l of 16% EM-grade formaldehyde and 25 μ l of 8% EM-grade glutaraldehyde to the cell resuspension. After incubation at room temperature for 5 min, 10 μ l of the cell suspension was added to 300-mesh, Formvar-coated copper grids, followed by incubation at room temperature for 5 min. The cell suspension was wicked off the grids using a filter paper, and the grids were washed three times with 10 μ l of water. Cells were stained by applying 10 μ l of 1% uranyl acetate to the grids for 30 s. After the stain was removed with filter paper, the grids were washed three times with 10 μ l of water and then air dried. Cells were visualized using a JEOL JEM 1011 transmission electron microscope. Flagellum counts were determined for at least 100 cells for each strain. The Mann-Whitney U test was used to determine whether there were statistically significant differences in number of flagella per cell for the various *H. pylori* strains.

RNA extraction and cDNA synthesis. *H. pylori* cultures were grown in TSB supplemented with 5% horse serum for 24 h, diluted in fresh medium to a cell density of 5 Klett units, and then allowed to grow to a cell density of 90 Klett units. RNA was harvested from the *H. pylori* cultures using the Zymo Direct-zol RNA MiniPrep Plus kit and quantified using a NanoDrop Lite instrument (Thermo Scientific). RNA quality was assessed using a 1% bleach gel (58). RNA preparations were treated with Turbo DNA-free kit (Ambion) according to the manufacturer's protocol to eliminate contaminating gDNA, and the absence of gDNA was confirmed by PCR. RNA samples were analyzed using a Bioanalyzer 2100 instrument (Agilent), and each sample had a minimum RNA integrity number of 9.5. Single-strand cDNA was synthesized from 200 ng of RNA using an iScript cDNA synthesis kit (Bio-Rad).

RNA-seq assay. Ribosomal RNAs were depleted from RNA preparations and libraries were prepared using 5 μ g of total RNA. Illumina sequencing was performed using a NextSeq500 instrument at the University of Georgia Genomics Facility. Quality of the reads were assessed using FastQC and trimmed using Trimmomatic, and the resulting reads were assembled to the *H. pylori* G27 genome (accession no. [NC_011333.1](#)) using Bowtie2 and visualized using Geneious (59, 60). The DESeq2 package in RStudio was used to analyze raw fragment counts and differentially regulated genes were identified with a $\log_2$ fold cutoff of 1.5 (61).

RT-qPCR. The transcript levels of *flhA*, *flf*, *flgE*, *flaB*, and *flaA* were monitored by RT-qPCR as previously described (25, 62). The primers used for this experiment are listed in Table S1 in the supplemental material. RT-qPCR was performed on serially diluted cDNA samples, and concentrations for all samples were determined using a standard curve of serially diluted gDNA. Each RT-qPCR mixture totaled 20 μ l, consisting of 10 μ l of Luna universal qPCR mix (New England BioLabs), 5 μ l of 100-fold diluted cDNA, and 5 μ l of the primers (final concentrations of 250 nM final for each primer). Evaluation of single products in all reactions was performed after each experiment by a melt curve analysis. The Applied Biosystems StepOnePlus real-time PCR system was used to perform all experiments in technical triplicates for three biological replicates of each condition. The $2^{-\Delta\Delta C_q}$ method (63) was used to quantify gene expression levels, and statistical significance was determined using a two-sample *t* test.

Allelic exchange mutagenesis. gDNA was isolated from the *H. pylori* B128 *clsC* mutant using a Wizard genomic DNA purification kit (Promega) and used to transform the *H. pylori* G27 *clsC* mutant as follows. The recipient strain was patched onto fresh TSA plates and incubated for 4 days. The cells were collected, transferred to a fresh plate, and then transformed with 3 μ g of gDNA. After 24 h of incubation, the cells were collected and resuspended in 1 ml of TSB and spot plated onto the soft agar medium used for motility assays. After 7 days of incubation, cells from the edge of the swim halo were used to inoculate fresh soft agar medium, and the inoculated plates were incubated for 7 days. Cells from the edge of the swim halo were streaked onto TSA supplemented with 5% horse serum and chloramphenicol, and the resulting isolated colonies were screened for motility in the soft agar medium.

DNA sequencing and analysis. Following the instructions supplied with the Illumina iTruSeq adaptor kit, genomic libraries were prepared using 500 ng of gDNA from various *H. pylori* strains. The resulting genomic libraries were sequenced at the University of Georgia Genomics Facility by Illumina sequencing. The quality of the reads was assessed using FastQC, and the reads were trimmed using Trimmomatic (59, 60). The reads for *H. pylori* G27 gDNA sequences were mapped using Bowtie2 using the published NCBI genome for *H. pylori* G27 (accession no. [NC_011333.1](#)) and visualized using Geneious. The alignment generated was used to align the gDNA sequence of the *H. pylori* G27 *clsC* mutant, which served as the backbone for aligning the gDNA sequences of the suppressor mutant strains. SNPs were identified in the genome sequences of the motile recipient strains using Geneious. Candidate genes were identified by using RStudio to cross-reference SNPs that were common to all the motile G27 *clsC* recipients.

Replacement of specific alleles in the *H. pylori* G27 *clsC* mutant. For the following PCR and cloning steps, target DNA was amplified using Phusion polymerase, and the resulting amplicons were incubated with *Taq* polymerase at 72°C for 10 min to add 3'-A overhangs to facilitate T/A cloning into plasmid pGEM-T Easy. Primer pair P079 and P080 were used to amplify 558 bp of the *ureA* promoter from *H. pylori* G27. Using the pKSF plasmid (64) as a template, a 2,978-bp DNA fragment was amplified that included the kanamycin cassette and a promoterless *sacB* gene. The two amplicons were joined by PCR SOEing, which generated a 3,536-bp product and was cloned into pGEM-T Easy that resulted in the plasmid pJC038. Regions upstream (568 bp) and downstream (393 bp) of *H. pylori* B128 *icfA* were amplified using the primer pairs P165/P166 and P167/P168, respectively. The resulting amplicons were joined by PCR SOEing and cloned into pGEM-T Easy to generate plasmid pJC068. The *kan-sacB* cassette was introduced into unique *NheI* and *XhoI* sites in plasmid pJC068 to generate a suicide vector (pJC069) that was used to replace the *icfA*

and *kdsA* alleles in the *H. pylori* G27 *clsC* mutant with the corresponding alleles from *H. pylori* B128. The primers P165 and P189 were used to amplify 495 bp of DNA upstream of *H. pylori* G27 *icfA*. The primers P190 and P168 were used to amplify a region of *H. pylori* B128 gDNA corresponding to the entire coding region of *icfA* and the first 164 bp of the *kdsA* coding region. The resulting amplicons were joined using PCR SOEing and cloned into pGEM-T Easy to generate plasmid pJC075. Plasmid pJC075 was introduced by natural transformation into the *H. pylori* G27 *clsC* strain that carried the *kan-sacB* cassette in the *icfA* locus. Transformants in which the *kan-sacB* cassette was replaced with the *icfA-kdsA* alleles carried on pJC075 were isolated following a sucrose-based counterselection as described (64). The *icfA-kdsA* regions for several kanamycin-sensitive, sucrose-resistant isolates were amplified by PCR, and the resulting amplicons were sequenced to confirm that the *H. pylori* G27 *clsC* mutant contained the B128 *icfA-kdsA* alleles.

The primer pairs P169/P170 and P187/P188 were used to amplify 616 and 503 bp, respectively, of DNA sequence upstream and downstream of *H. pylori* G27 *flgI*. The two amplicons were joined using PCR SOEing, and the resulting amplicon was cloned into pGEM-T Easy to generate plasmid pJC074. A *kan-sacB* cassette was inserted in unique *NheI* and *XhoI* sites in pJC074 to generate the suicide vector pJC077 that was used to replace *flgI* coding sequence in the *H. pylori* G27 *clsC* mutant with the *kan-sacB* cassette. The primer pair P169/P191 was used to amplify 552 bp of DNA upstream of *H. pylori* G27 *flgI*, the primer pair P192/P172 was used to amplify 552 bp of DNA downstream of *H. pylori* G27 *flgI*, and the primer pair P193/P194 was used to amplify *H. pylori* B128 *flgI* (1,029 bp of DNA sequence). The three amplicons were joined using PCR SOEing, and the resulting amplicon was cloned into pGEM-T Easy to generate plasmid pJC078. Plasmid pJC078 was transformed into the *H. pylori* G27 *clsC* strain where *flgI* had been replaced with the *kan-sacB* cassette to introduce the *H. pylori* B128 *flgI* allele into the strain using a sucrose-based counterselection.

Glycerophospholipid extraction. Cells were harvested, and pellets were washed once with 5 ml of PBS. Isolation of glycerophospholipids was carried out using a modified method of Bligh and Dyer, as described previously (65). Briefly, cell pellets were resuspended in 5 ml of single-phase Bligh-Dyer mixture consisting of chloroform-methanol-water (1:2:0.8, vol/vol/vol) and then incubated for 20 min at room temperature. Samples were centrifuged at $1,800 \times g$ for 10 min. The resulting supernatants were transferred to clean glass tubes and converted into a two-phase Bligh-Dyer mixture consisting of chloroform-methanol-water (2:2:1.8, vol/vol/vol). This mixture was vortexed and centrifuged to separate the organic and aqueous phases. The lower organic phase was removed and placed in a clean glass tube. A second extraction was performed by adding 2.6 ml of chloroform back to the tube containing the upper phase. The mixture was vortexed and centrifuged. The resulting lower phase was pooled with the previous lower phase and converted again into a two-phase Bligh-Dyer mixture by adding 5.2 ml of methanol and 4.7 ml of water. The resulting mixture was vortexed and centrifuged. Finally, the lower phase containing glycerophospholipids was removed and placed into a new tube, which was subsequently dried under a stream of nitrogen.

Analysis of ^{32}P -labeled glycerophospholipids. Bacterial glycerophospholipids were uniformly radiolabeled with ^{32}P (20 $\mu\text{Ci/ml}$) following a dilution to an OD_{600} of ~ 0.1 using an overnight liquid culture and then incubated for 24 h. Bacteria were transferred from growth bottles into Teflon screw-cap glass tubes (16 by 125 mm) and pelleted by centrifugation at $1,800 \times g$ for 15 min. The cells were resuspended in PBS and then repelleted by centrifugation at $1,800 \times g$ for 15 min. ^{32}P -labeled glycerophospholipids were extracted, spotted on a Silica Gel 60 TLC plate (20,000 cpm), and separated in a glass solvent chamber using a mobile phase consisting of chloroform, methanol, and acetic acid (65:25:5, vol/vol/vol). Upon migration of the solvent front to the top of the TLC plate (~ 2 h), the plates were dried, covered in plastic wrap, and exposed overnight to a phosphor screen. Visualization of the ^{32}P -labeled glycerophospholipids was performed using an Amersham Typhoon imager.

Mass spectrometry. All solvents were high-pressure liquid chromatography grade. Glycerophospholipid samples from unlabeled cells were resuspended in 100 μl of chloroform-methanol (4:1, vol/vol). A solution of 9-aminoacridine matrix (Sigma) was prepared at a concentration of 15 mg/ml in chloroform-methanol (2:1, vol/vol). Sample solutions were mixed with matrix solution (1:1, vol/vol), and 1 μl of the mixture was loaded onto the target plate. A peptide standard mixture (s6104-10ug; ProteoChem, Inc.) was prepared according to manufacturer's recommendations and used as an external calibrant. MALDI-TOF mass spectra of glycerophospholipid extracts were acquired in reflector mode on Autoflex speed mass spectrometer (Bruker Daltonics). A total of 500 single laser shots were averaged from each mass spectrum. Data were acquired in negative ion mode and processed using FlexControl 3.4 and FlexAnalysis 3.4 software (Bruker Daltonics).

Sample preparation for cryo-EM observation. *H. pylori* strains were cultured on plates for 4 days. The cells on the agar surface were collected and then washed with PBS buffer. Colloidal gold solution (10 nm in diameter) was added to the diluted *H. pylori* samples to yield a 10-fold dilution and then deposited on a freshly glow-discharged, holey carbon grid for 30 s. The grid was blotted with filter paper and rapidly plunge-frozen in liquid ethane in a homemade plunger apparatus, as described previously (66).

Cryo-ET data collection and image processing. The frozen-hydrated specimens of *H. pylori* G27 *clsC* mutant were transferred to Titan Krios electron microscope (FEI) equipped with a 300-kV field emission gun and a direct electron detector (Gatan K2 Summit). The images were collected at a defocus near to 0 μm using Volta Phase Plate and the energy filter with 20 eV slit. The data were acquired automatically with SerialEM software (67). During the data collection, the phase shift is monitored at the range of $\pi/3$ to $\sim \pi/2/3$; when the phase shift is out of the above range, next spot of phase plate will be switched to be charged for the use. A total dose of 50 $\text{e}^-/\text{\AA}^2$ is distributed among 35 tilt images covering angles from

−51° to +51° at tilt steps of 3°. The starting tilting angle is +36° instead of 0°. For every single tilt series collection, the dose-fractionated mode was used to generate 8 to 10 frames per projection image. Collected dose-fractionated data were first subjected to the motion correction program to generate drift-corrected stack files (68–70). The stack files were aligned using gold fiducial markers and volumes reconstructed by the weighted back-projection method using IMOD and Tomo3d software to generate tomograms (71, 72).

Subtomogram analysis with i3 packages. Bacterial flagellar motors were detected manually using the i3 program (73, 74). The orientation and geographic coordinates of selected particles were then estimated. In total, six subtomograms of *H. pylori* G27 *clsC* flagellar motors were used to perform subtomogram averaging analysis. The i3 tomographic package was used on the basis of the “alignment by classification” method with missing wedge compensation for generating the averaged structure of the motor, as described previously (66).

Data availability. The raw data used for analysis is available in NCBI Short Read Archive (SRA database) under accession numbers PRJNA545123 (DNA sequencing; <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA545123>) and PRJNA545152 (RNA-seq; <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA545152>).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00372-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

ACKNOWLEDGMENTS

We thank Abigail Courtney and Zachary Lewis for advice on analyzing the genome resequencing data.

This study was supported by National Institutes of Health (NIH) grant AI140444 to T.R.H., NIH grants AI138576 and AI064184 to M.S.T., and NIH grants GM107629 and AI087946 to J.L.

REFERENCES

- Amieva MR, El-Omar EM. 2008. Host-bacterial interactions in *Helicobacter pylori* infection. *Gastroenterology* 134:306–323. <https://doi.org/10.1053/j.gastro.2007.11.009>.
- Atherton JC, Blaser MJ. 2009. Coadaptation of *Helicobacter pylori* and humans: ancient history, modern implications. *J Clin Invest* 119:2475–2487. <https://doi.org/10.1172/JCI38605>.
- Blaser MJ. 1993. *Helicobacter pylori*: microbiology of a “slow” bacterial infection. *Trends Microbiol* 1:255–260. [https://doi.org/10.1016/0966-842X\(93\)90047-U](https://doi.org/10.1016/0966-842X(93)90047-U).
- Cover TL, Blaser MJ. 1992. *Helicobacter pylori* and gastroduodenal disease. *Annu Rev Med* 43:135–145. <https://doi.org/10.1146/annurev.me.43.020192.001031>.
- Kuipers EJ. 1997. *Helicobacter pylori* and the risk and management of associated diseases: gastritis, ulcer disease, atrophic gastritis and gastric cancer. *Aliment Pharmacol Ther* 11(Suppl 1):71–88. <https://doi.org/10.1046/j.1365-2036.11.s1.5.x>.
- Eaton KA, Morgan DR, Krakowka S. 1992. Motility as a factor in the colonization of gnotobiotic piglets by *Helicobacter pylori*. *J Med Microbiol* 37:123–127. <https://doi.org/10.1099/00222615-37-2-123>.
- Ottmann KM, Lowenthal AC. 2002. *Helicobacter pylori* uses motility for initial colonization and to attain robust infection. *Infect Immun* 70:1984–1990. <https://doi.org/10.1128/iai.70.4.1984-1990.2002>.
- Berg HC. 2003. The rotary motor of bacterial flagella. *Annu Rev Biochem* 72:19–54. <https://doi.org/10.1146/annurev.biochem.72.121801.161737>.
- Cheavance FF, Hughes KT. 2008. Coordinating assembly of a bacterial macromolecular machine. *Nat Rev Microbiol* 6:455–465. <https://doi.org/10.1038/nrmicro1887>.
- Macnab RM. 2003. How bacteria assemble flagella. *Annu Rev Microbiol* 57:77–100. <https://doi.org/10.1146/annurev.micro.57.030502.090832>.
- Manson MD, Tedesco P, Berg HC, Harold FM, Van der Drift C. 1977. A protonmotive force drives bacterial flagella. *Proc Natl Acad Sci U S A* 74:3060–3064. <https://doi.org/10.1073/pnas.74.7.3060>.
- Irikura VM, Kihara M, Yamaguchi S, Sockett H, Macnab RM. 1993. *Salmonella typhimurium* *flgG* and *flgN* mutations causing defects in assembly, rotation, and switching of the flagellar motor. *J Bacteriol* 175:802–810. <https://doi.org/10.1128/jb.175.3.802-810.1993>.
- Yamaguchi S, Aizawa S, Kihara M, Isomura M, Jones CJ, Macnab RM. 1986. Genetic evidence for a switching and energy-transducing complex in the flagellar motor of *Salmonella typhimurium*. *J Bacteriol* 168:1172–1179. <https://doi.org/10.1128/jb.168.3.1172-1179.1986>.
- Qin Z, Lin WT, Zhu S, Franco AT, Liu J. 2017. Imaging the motility and chemotaxis machineries in *Helicobacter pylori* by cryo-electron tomography. *J Bacteriol* 199:e00695. <https://doi.org/10.1128/JB.00695-16>.
- Niehus E, Gressmann H, Ye F, Schlapbach R, Dehio M, Dehio C, Stack A, Meyer TF, Suerbaum S, Josenhans C. 2004. Genome-wide analysis of transcriptional hierarchy and feedback regulation in the flagellar system of *Helicobacter pylori*. *Mol Microbiol* 52:947–961. <https://doi.org/10.1111/j.1365-2958.2004.04006.x>.
- Spohn G, Scarlato V. 1999. Motility of *Helicobacter pylori* is coordinately regulated by the transcriptional activator FlgR, an NtrC homolog. *J Bacteriol* 181:593–599.
- Smith TG, Hoover TR. 2009. Deciphering bacterial flagellar gene regulatory networks in the genomic era. *Adv Appl Microbiol* 67:257–295. [https://doi.org/10.1016/S0065-2164\(08\)01008-3](https://doi.org/10.1016/S0065-2164(08)01008-3).
- Beier D, Frank R. 2000. Molecular characterization of two-component systems of *Helicobacter pylori*. *J Bacteriol* 182:2068–2076. <https://doi.org/10.1128/jb.182.8.2068-2076.2000>.
- Brahmachary P, Dashti MG, Olson JW, Hoover TR. 2004. *Helicobacter pylori* FlgR is an enhancer-independent activator of σ^{54} -RNA polymerase holoenzyme. *J Bacteriol* 186:4535–4542. <https://doi.org/10.1128/JB.186.14.4535-4542.2004>.
- Allan E, Dorrell N, Foynes S, Anyim M, Wren BW. 2000. Mutational analysis of genes encoding the early flagellar components of *Helicobacter pylori*: evidence for transcriptional regulation of flagellin A biosynthesis. *J Bacteriol* 182:5274–5277. <https://doi.org/10.1128/jb.182.18.5274-5277.2000>.
- Schmitz A, Josenhans C, Suerbaum S. 1997. Cloning and characterization of the *Helicobacter pylori* *flbA* gene, which codes for a membrane protein involved in coordinated expression of flagellar genes. *J Bacteriol* 179:987–997. <https://doi.org/10.1128/jb.179.4.987-997.1997>.
- Smith TG, Pereira L, Hoover TR. 2009. *Helicobacter pylori* FlhB processing-deficient variants affect flagellar assembly but not flagellar gene expression. *Microbiology* 155:1170–1180. <https://doi.org/10.1099/mic.0.022806-0>.
- Tsang J, Hoover TR. 2014. Requirement of the flagellar protein export apparatus component FliO for optimal expression of flagellar genes in *Helicobacter pylori*. *J Bacteriol* 196:2709–2717. <https://doi.org/10.1128/JB.01332-13>.

24. Tsang J, Smith TG, Pereira LE, Hoover TR. 2013. Insertion mutations in *Helicobacter pylori* *flhA* reveal strain differences in RpoN-dependent gene expression. *Microbiology* 159:58–67. <https://doi.org/10.1099/mic.0.059063-0>.
25. Tsang J, Hirano T, Hoover TR, McMurtry JL. 2015. *Helicobacter pylori* FlhA binds the sensor kinase and flagellar gene regulatory protein FlgS with high affinity. *J Bacteriol* 197:1886–1892. <https://doi.org/10.1128/JB.02610-14>.
26. Colland F, Rain JC, Gounon P, Labigne A, Legrain P, De Reuse H. 2001. Identification of the *Helicobacter pylori* anti- σ^{28} factor. *Mol Microbiol* 41:477–487. <https://doi.org/10.1046/j.1365-2958.2001.02537.x>.
27. Josenhans C, Niehus E, Amersbach S, Horster A, Betz C, Drescher B, Hughes KT, Suerbaum S. 2002. Functional characterization of the antagonistic flagellar late regulators FlhA and FlgM of *Helicobacter pylori* and their effects on the *H. pylori* transcriptome. *Mol Microbiol* 43:307–322. <https://doi.org/10.1046/j.1365-2958.2002.02765.x>.
28. Hughes KT, Gillen KL, Semon MJ, Karlinsey JE. 1993. Sensing structural intermediates in bacterial flagellar assembly by export of a negative regulator. *Science* 262:1277–1280. <https://doi.org/10.1126/science.8235660>.
29. Rust M, Borchert S, Niehus E, Kuehne SA, Gripp E, Bajceta A, McMurtry JL, Suerbaum S, Hughes KT, Josenhans C. 2009. The *Helicobacter pylori* anti-sigma factor FlgM is predominantly cytoplasmic and cooperates with the flagellar basal body protein FlhA. *J Bacteriol* 191:4824–4834. <https://doi.org/10.1128/JB.00018-09>.
30. Romantsov T, Battle AR, Hendel JL, Martinac B, Wood JM. 2010. Protein localization in *Escherichia coli* cells: comparison of the cytoplasmic membrane proteins ProP, LacY, ProW, AqpZ, MscS, and MscL. *J Bacteriol* 192:912–924. <https://doi.org/10.1128/JB.00967-09>.
31. Huang KC, Ramamurthi KS. 2010. Macromolecules that prefer their membranes curvy. *Mol Microbiol* 76:822–832. <https://doi.org/10.1111/j.1365-2958.2010.07168.x>.
32. Bernal P, Munoz-Rojas J, Hurtado A, Ramos JL, Segura A. 2007. A *Pseudomonas putida* cardiolipin synthesis mutant exhibits increased sensitivity to drugs related to transport functionality. *Environ Microbiol* 9:1135–1145. <https://doi.org/10.1111/j.1462-2920.2006.01236.x>.
33. Kawai F, Shoda M, Harashima R, Sadaie Y, Hara H, Matsumoto K. 2004. Cardiolipin domains in *Bacillus subtilis* Marburg membranes. *J Bacteriol* 186:1475–1483. <https://doi.org/10.1128/jb.186.5.1475-1483.2004>.
34. Mileykovskaya E, Dowhan W. 2000. Visualization of phospholipid domains in *Escherichia coli* by using the cardiolipin-specific fluorescent dye 10-N-nonyl acridine orange. *J Bacteriol* 182:1172–1175. <https://doi.org/10.1128/jb.182.4.1172-1175.2000>.
35. Hirai Y, Haque M, Yoshida T, Yokota K, Yasuda T, Oguma K. 1995. Unique cholesteryl glucosides in *Helicobacter pylori*: composition and structural analysis. *J Bacteriol* 177:5327–5333. <https://doi.org/10.1128/jb.177.18.5327-5333.1995>.
36. Zhou P, Hu R, Chandan V, Kuolee R, Liu X, Chen W, Liu B, Altman E, Li J. 2012. Simultaneous analysis of cardiolipin and lipid A from *Helicobacter pylori* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Mol Biosyst* 8:720–725. <https://doi.org/10.1039/c2mb005475b>.
37. Raetz CR, Dowhan W. 1990. Biosynthesis and function of phospholipids in *Escherichia coli*. *J Biol Chem* 265:1235–1238.
38. Tan BK, Bogdanov M, Zhao J, Dowhan W, Raetz CR, Guan Z. 2012. Discovery of a cardiolipin synthase utilizing phosphatidylethanolamine and phosphatidylglycerol as the substrates. *Proc Natl Acad Sci U S A* 109:16504–16509. <https://doi.org/10.1073/pnas.1212797109>.
39. Selvy PE, Lavieri RR, Lindsley CW, Brown HA. 2011. Phospholipase D: enzymology, functionality, and chemical modulation. *Chem Rev* 111:6064–6119. <https://doi.org/10.1021/cr200296t>.
40. Farnbacher M, Jahns T, Willrodt D, Daniel R, Haas R, Goesmann A, Kurtz S, Rieder G. 2010. Sequencing, annotation, and comparative genome analysis of the gerbil-adapted *Helicobacter pylori* strain B8. *BMC Genomics* 11:335. <https://doi.org/10.1186/1471-2164-11-335>.
41. Porwollik S, Noonan B, O'Toole PW. 1999. Molecular characterization of a flagellar export locus of *Helicobacter pylori*. *Infect Immun* 67:2060–2070.
42. Corey RA, Pyle E, Allen WJ, Watkins DW, Casiraghi M, Miroux B, Archaga I, Politis A, Collinson I. 2018. Specific cardiolipin-SecY interactions are required for proton-motive force stimulation of protein secretion. *Proc Natl Acad Sci U S A* 115:7967–7972. <https://doi.org/10.1073/pnas.1721536115>.
43. Gupta K, Donlan JA, Hopper JT, Uzdavinyas P, Landreh M, Struwe WB, Drew D, Baldwin AJ, Stansfeld PJ, Robinson CV. 2017. The role of interfacial lipids in stabilizing membrane protein oligomers. *Nature* 541:421–424. <https://doi.org/10.1038/nature20820>.
44. Romantsov T, Gonzalez K, Sahtout N, Culham DE, Coumoundouros C, Garner J, Kerr CH, Chang L, Turner RJ, Wood JM. 2018. Cardiolipin synthase A colocalizes with cardiolipin and osmosensing transporter ProP at the poles of *Escherichia coli* cells. *Mol Microbiol* 107:623–638. <https://doi.org/10.1111/mmi.13904>.
45. Romantsov T, Helbig S, Culham DE, Gill C, Stalker L, Wood JM. 2007. Cardiolipin promotes polar localization of osmosensing transporter ProP in *Escherichia coli*. *Mol Microbiol* 64:1455–1465. <https://doi.org/10.1111/j.1365-2958.2007.05727.x>.
46. Rossi RM, Yum L, Agaisse H, Payne SM. 2017. Cardiolipin synthesis and outer membrane localization are required for *Shigella flexneri* virulence. *mBio* 8. <https://doi.org/10.1128/mBio.01199-17>.
47. Buskirk SW, Lafontaine ER. 2014. *Moraxella catarrhalis* expresses a cardiolipin synthase that impacts adherence to human epithelial cells. *J Bacteriol* 196:107–120. <https://doi.org/10.1128/JB.00298-13>.
48. Homma M, Komeda Y, Iino T, Macnab RM. 1987. The *flaF* gene product of *Salmonella typhimurium* is a flagellar basal body component with a signal peptide for export. *J Bacteriol* 169:1493–1498. <https://doi.org/10.1128/jb.169.4.1493-1498.1987>.
49. Jones CJ, Homma M, Macnab RM. 1989. L-, P-, and M-ring proteins of the flagellar basal body of *Salmonella typhimurium*: gene sequences and deduced protein sequences. *J Bacteriol* 171:3890–3900. <https://doi.org/10.1128/jb.171.7.3890-3900.1989>.
50. de Vrije T, de Swart RL, Dowhan W, Tommassen J, de Kruijff B. 1988. Phosphatidylglycerol is involved in protein translocation across *Escherichia coli* inner membranes. *Nature* 334:173–175. <https://doi.org/10.1038/334173a0>.
51. Gold VA, Robson A, Bao H, Romantsov T, Duong F, Collinson I. 2010. The action of cardiolipin on the bacterial translocon. *Proc Natl Acad Sci U S A* 107:10044–10049. <https://doi.org/10.1073/pnas.0914680107>.
52. Hizukuri Y, Kojima S, Yakushi T, Kawagishi I, Homma M. 2008. Systematic Cys mutagenesis of FlgI, the flagellar P-ring component of *Escherichia coli*. *Microbiology* 154:810–817. <https://doi.org/10.1099/mic.0.2007/013854-0>.
53. Jones CJ, Macnab RM, Okino H, Aizawa S. 1990. Stoichiometric analysis of the flagellar hook-(basal-body) complex of *Salmonella typhimurium*. *J Mol Biol* 212:377–387. [https://doi.org/10.1016/0022-2836\(90\)90132-6](https://doi.org/10.1016/0022-2836(90)90132-6).
54. Nambu T, Kutsukake K. 2000. The *Salmonella* FlgA protein, a putative periplasmic chaperone essential for flagellar P ring formation. *Microbiology* 146:1171–1178. <https://doi.org/10.1099/00221287-146-5-1171>.
55. Wang Y, Taylor DE. 1990. Chloramphenicol resistance in *Campylobacter coli*: nucleotide sequence, expression, and cloning vector. *Gene* 94:23–28. [https://doi.org/10.1016/0378-1119\(90\)90463-2](https://doi.org/10.1016/0378-1119(90)90463-2).
56. Heuermann D, Haas R. 1998. A stable shuttle vector system for efficient genetic complementation of *Helicobacter pylori* strains by transformation and conjugation. *Mol Gen Genet* 257:519–528. <https://doi.org/10.1007/s004380050677>.
57. Li MZ, Elledge SJ. 2012. SLIC: a method for sequence- and ligation-independent cloning. *Methods Mol Biol* 852:51–59. https://doi.org/10.1007/978-1-61779-564-0_5.
58. Aranda PS, LaJoie DM, Jorczyk CL. 2012. Bleach gel: a simple agarose gel for analyzing RNA quality. *Electrophoresis* 33:366–369. <https://doi.org/10.1002/elps.201100335>.
59. Andrews S. 2014. FastQC: a quality control tool for high-throughput sequencing data. <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>.
60. Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>.
61. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15:50. <https://doi.org/10.1186/s13059-014-0550-8>.
62. Tsang J, Hoover TR. 2015. Basal body structures differentially affect transcription of RpoN- and FlhA-dependent flagellar genes in *Helicobacter pylori*. *J Bacteriol* 197:1921–1930. <https://doi.org/10.1128/JB.02533-14>.
63. Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 3:1101–1108. <https://doi.org/10.1038/nprot.2008.73>.
64. Copass M, Grandi G, Rappuoli R. 1997. Introduction of unmarked mutations in the *Helicobacter pylori* *vacA* gene with a sucrose sensitivity marker. *Infect Immun* 65:1949–1952.
65. Henderson JC, Herrera CM, Trent MS. 2017. AlmG, responsible for polymyxin resistance in pandemic *Vibrio cholerae*, is a glycosyltransferase

- distantly related to lipid A late acyltransferases. *J Biol Chem* 292: 21205–21215. <https://doi.org/10.1074/jbc.RA117.000131>.
66. Zhu S, Qin Z, Wang J, Morado DR, Liu J. 2017. *In situ* structural analysis of the spirochetal flagellar motor by cryo-electron tomography. *Methods Mol Biol* 1593:229–242. https://doi.org/10.1007/978-1-4939-6927-2_18.
67. Mastronarde DN. 2005. Automated electron microscope tomography using robust prediction of specimen movements. *J Struct Biol* 152: 36–51. <https://doi.org/10.1016/j.jsb.2005.07.007>.
68. Li X, Mooney P, Zheng S, Booth CR, Braunfeld MB, Gubbens S, Agard DA, Cheng Y. 2013. Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM. *Nat Methods* 10: 584–590. <https://doi.org/10.1038/nmeth.2472>.
69. Morado DR, Hu B, Liu J. 2016. Using Tomoauto: a protocol for high-throughput automated cryo-electron tomography. *J Vis Exp* 2016: e53608.
70. Zheng SQ, Palovcak E, Armache JP, Verba KA, Cheng Y, Agard DA. 2017. MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nat Methods* 14:331–332. <https://doi.org/10.1038/nmeth.4193>.
71. Agulleiro JI, Fernandez JJ. 2015. Tomo3D 2.0: exploitation of advanced vector extensions (AVX) for 3D reconstruction. *J Struct Biol* 189:147–152. <https://doi.org/10.1016/j.jsb.2014.11.009>.
72. Kremer JR, Mastronarde DN, McIntosh JR. 1996. Computer visualization of three-dimensional image data using IMOD. *J Struct Biol* 116:71–76. <https://doi.org/10.1006/jsbi.1996.0013>.
73. Winkler H. 2007. 3D reconstruction and processing of volumetric data in cryo-electron tomography. *J Struct Biol* 157:126–137. <https://doi.org/10.1016/j.jsb.2006.07.014>.
74. Winkler H, Zhu P, Liu J, Ye F, Roux KH, Taylor KA. 2009. Tomographic subvolume alignment and subvolume classification applied to myosin V and SIV envelope spikes. *J Struct Biol* 165:64–77. <https://doi.org/10.1016/j.jsb.2008.10.004>.