Allosteric activation of CwlD amidase activity by the GerS lipoprotein during *Clostridioides difficile* spore formation

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Abstract

Spore-forming pathogens like *Clostridioides difficile* depend on germination to initiate infection. Spore germination depends on the degradation of the protective spore peptidoglycan layer known as the spore cortex. Cortex degradation is mediated by enzymes that recognize the spore-specific peptidoglycan modification, muramic- δ -lactam (MAL). In *C. difficile*, MAL synthesis depends on the activity of the CwID amidase and the GerS lipoprotein, which directly binds CwID. To gain insight into how GerS regulates CwID activity, we solved the crystal structure of the CwID:GerS complex. In this structure, a GerS homodimer is bound to two CwID monomers such that the CwID active sites are exposed. Although CwID structurally resembles amidase_3 family members, we found that CwID does not bind zinc stably on its own, unlike previously characterized amidase_3 enzymes. Instead, GerS binding to CwID promotes CwID binding to zinc, which is required for its catalytic mechanism. Thus, in determining the first structure of an amidase bound to its regulator, we reveal stabilization of zinc co-factor binding as a novel mechanism for regulating bacterial amidase activity. Our results further suggest that allosteric regulation by binding partners may be a more widespread mode for regulating bacterial amidase activity than previously thought.

Introduction

Many spore-forming pathogens, such as *Clostridioides difficile*, rely on germination to initiate infection. *C. difficile* is a toxin-producing bacterium that is the leading cause of healthcare-associated infections in many developed countries (1, 2). As with many spore-forming bacteria, successful germination depends on the degradation of the thick protective layer of modified peptidoglycan known as the cortex. The cortex functions to maintain spore metabolic dormancy (3) by keeping spores in a partially dehydrated state (3), so its removal is critical for metabolism to resume during germination. Cortex degradation is mediated by cortex lytic enzymes that recognize a cortex-specific peptidoglycan modification known as muramic- δ -lactam (MAL). This strict substrate specificity ensures that cortex lytic enzymes degrade the cortex and not the germ cell wall (3, 4).

MAL residues are generated once the cortex layer is synthesized during sporulation (5). The sequential action of two conserved enzymes, the CwlD amidase and PdaA deacetylase, produce MAL. CwlD removes the peptide side chain from N-acetylmuramic acid (NAM) (6-8) so that PdaA can deacetylate NAM and catalyze lactam ring formation to form MAL (6, 9, 10). Mutants that lack MAL due to loss of either of these cortex-modifying enzymes exhibit severe germination defects in both *Bacillus subtilis* and *C. difficile* (6, 8-11). In the case of *C. difficile*, MAL-deficient mutants also exhibit virulence defects in animal models of infection (9, 12), highlighting the critical importance of this process.

While the functions of CwlD and PdaA are conserved between *B. subtilis* and *C. difficile* (6, 8-10), we recently showed that *C. difficile* CwlD amidase activity depends on the GerS

lipoprotein (6, 12). Notably, GerS is unique to the Peptostreptococcaceae family, suggesting that CwID amidase activity in this family may be differentially regulated relative to other spore-forming organisms (6, 12). Indeed, *B. subtilis* CwID does not appear to require additional partners for its function (7). These observations raise the question as to why *C. difficile* CwID function depends on GerS. Given that GerS and CwID directly interact (6), GerS binding to CwID presumably regulates CwID activity.

CwID is a member of the amidase_3 family (PF01520), which play critical roles during cell division in many bacteria (e.g AmiC) (13). Amidase_3 family members are zinc-dependent N-acetylmuramoyl amidases that use a Zn^{2+} ion as part of their catalytic mechanism (14). The activity of these amidases is tightly regulated in many bacteria because their aberrant cleavage of the peptidoglycan layer can result in cell lysis (15, 16). Studies in the past ~10 years have revealed novel mechanisms for regulating amidase activity, with novel binding partners appearing to allosterically regulate amidase function (17). In many Gram-negative bacteria, the highly conserved amidases, AmiA, AmiB, and AmiC, are activated upon binding regulators like EnvC, NlpD, and the recently discovered ActS (16, 18-20). These regulators likely displace an auto-inhibitory loop unique to this subset of amidases from the amidase active site (21, 22). In addition, the activity of the *Staphylococcus aureus* LytH amidase was recently shown to depend on the transmembrane protein, ActH (15). While the mechanism by which ActH licenses LytH's ability to denude peptidoglycan remains unclear, LytH's ability to cleave stem peptides may be restricted to uncrosslinked peptidoglycan (15). Taken together, these data suggest that amidase activation by binding partners may be a more widespread phenomenon than initially thought.

To gain insight into the molecular mechanism by which GerS regulates CwID amidase activity, we solved the crystal structure of the CwID:GerS complex. This is the first structure of

an amidase bound to its regulator, and it reveals that GerS forms a homodimer that binds two CwID monomers. The two proteins interact extensively at an interface opposite from CwID's active site. Since *C. difficile* CwID adopts a typical amidase_3 fold in the complex, GerS binding likely impacts CwID amidase activity at a distance. Indeed, our biochemical analyses indicate that *C. difficile* CwID does not bind zinc stably on its own in contrast with previously characterized amidase_3 family members. Instead, GerS binding to CwID appears to promote CwID binding to zinc, suggesting a novel mechanism for allosterically regulating amidase_3 family member function. Thus, our analyses expand our understanding of the diverse mechanisms used by bacteria to regulate the activity of peptidoglycan amidases via protein-protein interactions.

Results

Analyzing the dimerization of CwID and GerS and the stoichiometry of the CwID:GerS complex.

To investigate why CwID activity depends on GerS, we sought to biochemically characterize the CwID:GerS complex and identify residues required to mediate this interaction using X-ray crystallography. Constructs encoding the soluble domains of CwID and GerS were over-expressed in *Escherichia coli* and a co-affinity purification strategy was used to purify untagged GerS bound to His-tagged CwID_{Δ1-25} as previously described (6). These domains correspond to CwID and GerS lacking their predicted transmembrane (residues 1-25) and signal peptide/lipobox (residues 1-22) domains, respectively. The stability of this complex was analyzed using size exclusion chromatography (SEC) by comparing the elution profile of the complex

relative to its individual CwID and GerS proteins, which were both purified as His-tagged variants. CwID $_{\Delta 25}$ -His₆ alone primarily eluted as a monomer in solution, with a major peak corresponding to an apparent molecular weight (MW) of 33 kDa (**Figure 1A**) and a second less prominent peak corresponding to an apparent MW of 67 kDa, suggesting that CwID can dimerize. Coomassie staining of the elution fractions confirmed that CwID was the major protein detected in both peaks (**Figure 1A**). GerS $_{\Delta 22}$ -His₆ alone eluted in 3 separate peaks, with apparent MWs of 23, 45 and 81 kDa (**Figure 1B**), strongly suggesting that GerS has the capacity to multimerize. The 23 kDa peak likely represents the monomeric form of the protein, since it corresponds to the expected MW; the 45 kDa peak likely corresponds to a dimer; and the 81 kDa peak likely represents a trimer or tetramer of GerS (**Figure 1B**). Coomassie staining of the three peaks confirmed that they contained predominantly GerS. Together, these results show that CwID and GerS are mostly monomeric in solution but that GerS readily forms higher order complexes.

SEC analysis of the CwID:GerS complex revealed that it primarily eluted as a complex with a calculated MW of 72 kDa (1st peak, **Figure 1C**). This result would be consistent with a ternary complex of a GerS dimer (45 kDa) with a CwID monomer (33 kDa), or vice versa. However, Coomassie staining of the SEC fractions revealed that the two proteins are present at a 1:1 ratio, suggesting that they bind stoichiometrically. In addition to this major peak, a smaller elution peak was observed that corresponds to an apparent MW of 27 kDa. Coomassie staining indicated that this peak primarily contained His-tagged CwID $_{\Delta 25}$ alone (**Figure 1C**). Since the input sample shows more CwID than GerS, this band likely corresponds to free His-tagged CwID $_{\Delta 25}$ that is not bound to GerS $_{\Delta 22}$.

Figure 1: Size Exclusion Chromatography analyses of CwID, GerS, and the CwID:GerS complex. Purified CwID $_{\Delta 25}$ -His₆ (A), GerS $_{\Delta 22}$ -His₆ (B), and the CwID $_{\Delta 25}$ -His₆:GerS $_{\Delta 22}$ complex (C) were analyzed by size exclusion chromatography. mAU corresponds to the UV absorbance measurements (A_{280}) during the protein elution. The estimated molecular weights of the protein species are indicated at the top of each peak. The input sample is derived from the co-affinity purification high imidazole elution fraction. This input sample as well as the fractions (0.5 mL) from the indicated elution volumes were analyzed by SDS-PAGE and Coomassie staining. Dashed grey lines indicate the fractions collected. The data shown are from one biological replicate, which is representative of three biological replicates performed independently.

Crystal structure of the CwID:GerS complex.

Overall complex structure. To gain atomic-level insight into how GerS activates CwID activity, we crystallized the SEC-purified CwID:GerS complex and determined its structure. The asymmetric unit contained one molecule of CwID and one monomer of GerS with the CwID structure being typical of the amidase superfamily. GerS is largely anti-parallel beta sheet with the exception of an N-terminal alpha helix and forms a cupped hand-like shape similar to the LolA family of lipid-associated proteins (23). While the members of the LolA family are typically monomeric (24), completion of the beta fold for GerS required two molecules such that the N-terminal alpha helix through the first 2 beta strands comes from one molecule in the crystal and the remainder of the GerS fold comes from a symmetry mate (**Figure 2A and B**). Notably, both GerS molecules provide contacts to a single CwID. This crossover-dependent assembly of the GerS fold is the same as was observed for the domain swapped assembly structure of the F47E mutant of LolA (PDBID 6FHM) (25). Thus, the likely biological assembly for the CwID:GerS complex contains two molecules of GerS and two CwID and is in agreement with both the DLS data (**Figure S1**) and size exclusion chromatography (**Figure 1**). The combined buried surface area upon binding of CwID to the two GerS molecules is 1415 Å² and contains 10 hydrogen bonds and 6 salt bridges as calculated using PISA (26). This interface is analogous to the interface observed for the C-terminal domain of the anti-anti-sigma factor MucB from *P. aeruginosa* (PDB ID 6IN8) (27) and the stress response factor RseB from *E. coli* (PDB ID 3M4W) (28). Both CwID and GerS have N-terminal sequences used for membrane association that were omitted for the crystallization constructs though neither would be occluded in the complex and would be free to extend to the membrane. For GerS, this would be an extension of the alpha helix on the periphery

of the complex. For CwID, the extension sits surface exposed adjacent to the complex interface. Importantly, the binding orientation for CwID relative to GerS leaves the CwID Zn²⁺-containing active site solvent accessible to substrate rather than at the complex interface.

Figure 2. Structure of the CwID:GerS complex. (A) Shown is the cartoon representation of the CwID:GerS complex within the crystal with CwID in blue tones and GerS in grey. The crystallographic asymmetric unit contains a single protomer of each with the 2 GerS/2 CwID assembly formed by domain swapping of the GerS monomers. (B) The assembly is shown in space filling representation. (C) Least squares superposition of CwID (cyan throughout) and its homologs performed using Superpose (29) in CCP4. (D) Close-up view of the metal binding site for CwID with GerS from a fortuitous crystal packing (grey) providing coordination of the ion. (E) CwID homolog structures (CwID *Paenibacillus polymyxa* PDBID 1JWQ, unpublished; *C. difficile* CD2761 amidase_3 family member, 4RN7, unpublished; *Nostoc punctiforme*, AmiC2, 5EMI (30); and *C. difficile* Cwp6, 5J72 (31)) in the active form have the zinc atom accessible to the solvent. They also have water molecules serving as 1-2 ligands (blue spheres), one of which is coordinated by a conserved glutamate. (F) CwID homolog structures (*Bartonella henselae* AmiB PDBID 3NE8 (22) and *E. coli* AmiC 4BIN (21)) in the inactive forms have residues from their respective inhibitory helices providing zinc ligands.

The CwlD structure. The structure of CwlD is the mixed alpha beta fold expected for the N-acetylmuramoyl-L-alanine amidase superfamily (**Figure 2C**), with 3 conserved glutamate residues and 2 histidines coordinating the zinc ion (**Figure 2D**). His49 (positioned by Glu53), His133, and the carboxylate of Glu65 provide four of the ligands of the near octahedral ion coordination with distances of 2.2 Å. Zinc coordination falls into two classes for the amidase family: (i) the active form where water molecules provide the 2 additional ion ligands, one of which coordinated by the conserved Glu199 position in CwlD (**Figure 2E**) and (ii) the autoinhibited form where a glutamate or glutamine from the inhibitory helix conserved in Ami family amidases (21, 22) is liganded (**Figure 2F**). While CwlD does not have an autoinhibitory helix, the N-terminus of a GerS symmetry molecule provides the additional liganding for the Zn^{2+} . As this symmetry molecule is not one from the 2 GerS monomers used to bind CwlD, the interaction is not physiologically relevant. Instead, it is a fortuitous crystal packing event resulting in the displacement of the water molecules expected to coordinate the zinc in the active form of CwlD in solution.

The GerS structure and CwlD interface. The heavy atom phased solutions revealed that the functional folding of GerS for binding to CwlD requires two molecules of GerS. The LolA-like conserved anti-parallel beta sheet of GerS folds into a pseudo half beta barrel utilizing 11 strands where CwlD sits. The topology for the strands of the beta fold is strand numbers 6-5-4-3-2-1-11-10-9-8-7 where strands 1 and 2 at the core of the fold come from the second molecule of GerS. The interaction of the N-terminal alpha helix and the embedding of beta strands 1 and 2 into the symmetry mate generates an extensive buried surface area of 3766 Å² and is composed of 57 hydrogen bonds and 2 salt bridges involving residues 22 through 71 at which point crossover occurs. Alpha helix 1 (residues 65-82) and the C-terminal alpha helix (residues 217-235) of CwlD

constitute the most significant interface with GerS contacting strands 2 through 6. A series of hydrophobic residues from strands 4-6 provide complementary packing for Trp233, Ile227, Tyr226 and Leu79 of CwID. Numerous other interactions of interest occur at the CwID:GerS interface. Arg169 from CwID forms a salt bridge with Asp106 from strand 6 of GerS while His84 from the loop connecting strands 3 and 4 of GerS creates a communication network via Glu78 of helix 1 of CwID to His61 of strand 2 of GerS.

Zinc stabilizes the CwID:GerS complex.

Our finding that CwID in the co-crystal structure strongly resembles active amidase₃ family members raises the question as to whether GerS binding to CwID induces conformational changes in CwID and whether this binding event licenses CwID activity. Our attempts to gain insight into this former question failed because we were unable to crystallize *C. difficile* CwID_{Δ25-His₆} on its own (**Figure 1A**). We did not attempt to crystallize GerS_{Δ22-His₆} on its own because the SEC-purified protein (**Figure 1B**) aggregated in dynamic light scattering (DLS) analyses and precipitated out of solution within days of SEC purifications (unpublished data, B. Eckenroth, A. Shen & C. Alves Feliciano). In contrast, CwID_{Δ25-His₆} was stably folded in these DLS analyses (**Figure S1**). Interestingly, DLS analyses of the SEC-purified CwID:GerS complex revealed two peaks, one with a predicted radius of 120 kDa (which would correspond to a dimer of dimers of CwID:GerS) and one that appeared to be aggregated protein. This latter peak may represent GerS that has dissociated from the SEC-purified complex.

To identify factors that might affect the stability of GerS binding to CwID, we tested the effect of disrupting (i) CwID binding to Zn²⁺ and (ii) CwID's Zn²⁺-dependent catalytic mechanism

on the CwID:GerS interaction. To disrupt CwID's ability to bind zinc, we generated constructs encoding alanine mutations of CwID's Zn²⁺-binding residues, His49 and Glu65. To disrupt CwID's catalytic mechanism, we mutated the putative catalytic Glu199 residue to alanine. We then co-produced these His-tagged CwID variants in *E. coli* and measured their ability to pull-down untagged GerS using the co-affinity purification assay.

CwID-His₆ variants carrying the zinc-binding mutations (H49A and E65A) reduced the amount of untagged GerS that co-purified, although it was still possible to purify the mutant CwID:GerS complexes (**Figure 3A**). In contrast, wild-type levels of GerS co-purified with the E199A variant. When the co-affinity purifications were analyzed by SEC, the CwID_{H49A}:GerS and CwID_{E65A}:GerS complexes (**Figures 3B and 3C**) eluted later than complexes containing either wild-type CwID or CwID_{E199A} (**Figure 3D**). CwID_{H49A}:GerS and CwID_{E65A}:GerS complexes eluted with a calculated MW of 55 and 60 kDa, respectively, while the wild-type CwID:GerS complex eluted with a predicted MW of 72 kDa (**Figures 3B and C**). Since the elution volume of proteins by SEC depends on both their shape and MW, these observations suggest that the shape and/or stoichiometry of CwID:GerS complexes changes when CwID is able to bind zinc. However, Coomassie staining of the different fractions revealed that both proteins are present at ratios close to 1:1, irrespective of the CwID point mutations (**Figure 3E**). Based on these observations, loss of zinc-binding likely de-stabilizes the CwID:GerS complex.

To directly assess the effect of zinc on the stability of the CwID:GerS complex, co-affinity purified CwID:GerS complex was incubated for one hour in the presence of either a five-fold excess of zinc or 1 mM EDTA and then analyzed by SEC (**Figure 4A**). In the presence of Zn, the wild-type CwID:GerS complex primarily eluted at a higher predicted MW (83 kDa), while incubation with EDTA resulted in three peaks that corresponded to predicted MWs of 65, 45, and

Figure 3. Mutation of CwID zinc-binding residues, His49 and Glu65, decreases the stability of the CwID:GerS complex. (A) Coomassie stain of wild-type CwID-His₆, H49A, E65A or E199A His-tagged CwID variants co-affinity purified with untagged GerS. The indicated proteins were produced in *E. coli* and purified using Ni²⁺-affinity resin. Cleared lysate and eluate fractions were analyzed using Coomassie staining. Comparison of the size exclusion chromatography traces of the wild-type CwID:GerS complex with CwID_{H49A}:GerS (B), CwID_{E65A}:GerS (C) and CwID_{E199A}:GerS (D) complexes. mAU corresponds to the UV absorbance measurements (A₂₈₀) during the protein elution. The 1st peak of each trace corresponds to the protein complex; the estimated molecular weight (MW) in kDa of the complex is given on top of this first peak. (E) Coomassie stain of fractions corresponding to the 1st and 2nd peak of each trace. The numbers at the bottom correspond to the apparent MW of each peak. The data shown are from one biological replicate, which is representative of three biological replicates performed independently.

28 kDa (**Figure 4A**). Although Coomassie staining indicated that the complex was still present in the 65 and 45 kDa peaks, GerS appeared to be present at higher levels in the 45 kDa peak than CwID, suggesting that this peak may comprise GerS dimers along with CwID alone. Since the 28 kDa peak was primarily comprised of His-tagged CwID, these analyses strongly suggest that removing zinc from the CwID:GerS complex via EDTA treatment promotes the dissociation of the complex. Notably, when the CwID_{H49A}:GerS complex was incubated with zinc or EDTA, no changes in the SEC trace were observed (**Figure 4B**). Collectively, these results reinforce the notion that zinc coordination affects the stability of the CwID:GerS complex.

Figure 4. Zinc stabilizes the CwID:GerS complex. Purified wild-type CwID:GerS (A) and CwID_{H49A}:GerS (B) complexes were analyzed by size exclusion chromatography after treatment with a five-fold excess of ZnCl₂ (green traces) or 1 mM EDTA (red traces) for 1 hr. mAU corresponds to the UV absorbance measurements (A_{280}) during the protein elution. Fractions (0.5 mL) corresponding to the indicated elution volumes were analyzed by SDS-PAGE. The numbers at the bottom of the gels correspond to the apparent MW of each peak. The data shown are from one biological replicate, which is representative of three biological replicates performed independently.

To confirm that the CwID zinc-binding residue mutations disrupt binding to zinc, we analyzed the zinc content of SEC-purified wild-type CwID:GerS and CwID_{H49A}:GerS complexes using inductively coupled plasma mass spectroscopy (ICP-MS). An average of about 500 ng Zn/mg protein was detected in the wild-type CwID:GerS, while only background levels of zinc were detected in the CwID_{H49A}:GerS complex (**Figure 5**). The same result was observed for a CwID:GerS complex harboring an alanine mutation in the CwID zinc-binding residue, Glu65 (data not shown). To validate the effects of these mutations on the individual proteins, we purified His-tagged wild-type CwID and CwID_{H49A} on their own (**Figure S3**), as well as His-tagged GerS alone (**Figure 1B**), and quantified the zinc levels in these individual proteins. Intriguingly, no zinc was detected in any of these samples, including with wild-type CwID alone (**Figure 5**). This latter result was unexpected because zinc is present in the crystal structures of many amidase_3 family members even though exogenous zinc was not added to the expression or purification buffers in these studies (21, 22, 30). Because of this surprising result, we purified another *C. difficile* amidase_3 homolog, CD2761, which binds zinc in X-ray crystallography analyses (PDB ID 4RN7, unpublished). Even more zinc was detected bound to CD2761 (~1100 ng Zn/mg protein) than to WT CwID:GerS (~450 ng Zn/mg protein). Thus, if CwID was able to bind zinc stably on its own, we would expect to detect it using this method. Taken together, our results indicate that *C. difficile* CwID behaves differently from all previously characterized amidase_3 family members to our knowledge in that it does not stably bind to zinc. Instead, GerS appears to promote CwID binding to zinc. Conversely, CwID binding to zinc stabilizes the CwID:GerS complex (compare CwID_{H49A} and CwID_{E65A} to CwID_{WT}), **Figures 3 and 5**), suggesting a mutual dependence between CwID, Zn²⁺, and GerS binding at least *in vitro*.

Figure 5. Elemental analyses of CwlD and CwlD:GerS complex. Elemental analyses of zinc in CwlD:GerS complexes corresponding to His-tagged wild-type CwlD:GerS and CwlD_{H49A}:GerS, and individual His-tagged proteins (wild-type CwlD, GerS and CD2761 and CwlD_{H49A}). Elemental analyses were performed on affinity-purified proteins produced in *E. coli* in the absence of zinc supplementation that were further purified using size exclusion chromatography (also in the absence of zinc supplementation). Each dot represents data from a given biological replicate. Three independent protein purifications were analyzed with the exception for CwlD_{H49A} and CD2761, for which two independent protein purifications were performed. Bars represent the mean of the three (or two) independent experiments. Standard deviation is shown. Statistical significance was determined using a one-way ANOVA and Tukey's test. **, $p < 0.005$; ****, $p < 0.0001$.

CwlD binding to zinc is required for amidase activity and stabilizes both CwlD and GerS in mature spores.

We next wanted to confirm that the zinc-binding residues and the presumed catalytic glutamate 199 are required for CwlD function in *C. difficile*. To this end, we designed *cwlD* constructs encoding mutations in the zinc-coordinating residues, His49 and Glu65, and in the putative catalytic Glu199 residue. We then assessed the ability of these constructs to complement the germination defect of a *C. difficile* $\Delta cwlD$ strain. All complementation constructs used in this study were integrated into the ectopic *pyrE* locus using allele-coupled exchange (32) and expressed from their native promoters. To analyze germination, we used an optical density-based assay that measures the decrease in optical density of a population of germinating spores over time due to

cortex hydrolysis and rehydration of the spore cytosol (33). While the optical density of wild-type spores decreased by around 40%, the optical density of the *cwlD* *H49A*, *E65A* and *E199A* complementation strains did not change, similar to the parental Δ *cwlD* strain (**Figure 6A**). Taken together, these results indicate that His49, Glu65 and Glu199 are all required for CwlD function.

Interestingly, the zinc-binding mutations, H49A and E65A, decreased both CwlD and GerS levels in spores, while disruption of the putative catalytic glutamate E199A did not (**Figure 6C**). Notably, the CwlD_{H49A} and CwlD_{E65A} variants were detected at wild-type levels in sporulating cells, indicating that these zinc-binding mutations do not affect the production or stability of CwlD (**Figure 6B**). Instead, they appear to affect CwlD incorporation or stability in mature spores. This result is consistent with our previous observation that loss of CwlD decreases GerS levels in mature spores, whereas loss of GerS does not affect CwlD levels (6). Since the zinc-binding mutations in CwlD did not affect the stability of recombinant His-tagged CwlD in SEC analyses (**Figure S3**), our findings in *C. difficile* reinforce our biochemical data indicating that CwlD binding to zinc stabilizes CwlD:GerS complex formation, which apparently affects CwlD stability or incorporation in mature spores.

Figure 6. Mutations in zinc binding residues disrupt CwID function and affect the stability of the CwID:GerS complex in the spore. (A) Spore germination as measured by the change in the OD₆₀₀ in response to germinant in *cwID* mutants spores relative to wild-type spores. Purified spores were resuspended in BHIS, and germination was induced by adding taurocholate (1% final concentration). The ratio of the OD₆₀₀ of each strain at a given time point relative to the OD₆₀₀ at time zero is plotted. The mean OD₆₀₀ determined from three biological replicates from three independent spore preparations are shown. Shading represents the standard deviation as the area between error bars for each time point measured. Statistical significance relative to wild-type was determined using two-way ANOVA and Tukey's test. **** $p < 0.0001$. (B) Western blot analysis of CwID and GerS levels in sporulating cells from wild type (WT), $\Delta cwID$, and $\Delta cwID$ strains complemented with constructs encoding the CwID variants H49A, E65A and E199A. His49 and Glu65 directly bind Zn^{2+} in the structure, while Glu199 is part of the amidase catalytic mechanism. CwID-c and GerS-c indicate cleaved form of both proteins. $\Delta spo0A$ ($\Delta 0A$) was used as negative control for sporulating cells, and SpoIVA was used as a loading control. (C) Western blot analysis of CwID and GerS levels in spores from the strains used in the experiments described in (A). SleC was used as a loading control.

Identification of a salt bridge critical for GerS binding to CwID.

To directly test whether GerS binding to CwID stabilizes CwID binding to zinc, we sought to identify mutations that disrupt CwID:GerS complex formation separate from the zinc-binding mutations. Guided by the crystal structure of the complex, we identified two potential salt bridges that might promote CwID:GerS binding: (i) CwID arginine 169 (R169)-GerS aspartic acid 106 (D106) and (ii) CwID glutamate 78 (E78)-GerS histidine 61 (H61) (**Figure 7A**). To assess the importance of these salt bridges in mediating binding between CwID and GerS, we generated constructs encoding His-tagged CwID variants carrying the following substitutions: arginine 169 to aspartic acid (R169D) or glutamine (R169Q) and glutamate 78 to glutamine (E78Q). We also generated untagged expression constructs encoding cognate salt bridge mutations in GerS, namely aspartate 106 to arginine (D106R) or alanine (D106A) and histidine 61 to alanine (H61A). We first co-produced the His-tagged CwID variants in *E. coli* and measured their ability to pull-down untagged wild-type GerS using the co-affinity purification assay. CwID variants carrying substitutions in Arg169 reduced GerS co-purification by 90% (**Figure 7B and C; Figure S4**). Similarly, GerS variants carrying substitutions in Asp106 reduced GerS co-purifications with CwID by ~90% also. Swapping the charges on the CwID Arg169: GerS Asp106 interaction by combining the CwID R169D and GerS D106R substitutions partially restored CwID:GerS complex formation (**Figure 7B and C**), further highlighting the importance of this salt bridge in mediating complex formation. In contrast, substitutions in residues comprising the second salt bridge did not strongly affect CwID or GerS binding, since mutation of the CwID_{E78} or GerS_{H61} did not affect the levels of GerS that co-affinity purified with His-tagged CwID (**Figure 7D**). Taken together, these

393 results implicate the CwID_{R169}:GerS_{D106} salt bridge in stabilizing the interaction between
394 recombinant CwID amidase and its binding partner, GerS.

395
396
397 **Figure 7. A salt bridge between CwID_{R169} and GerS_{D106} is important for CwID:GerS binding.**
398 (A) Specific interactions between CwID and GerS observed in the structure. Salt bridge formation
399 between CwID Arg169 (R169) and GerS Asp106 (D106) is highlighted in the solid boxes and
400 between CwID Glu78 (E78) and GerS His61 (H61) in the dotted boxes. The light blue mesh shows
401 the heavy atom phased electron density after density modification and building contoured at 1
402 sigma. The yellow mesh represents the anomalous signal at 3 sigma for the selenomethionine
403 derivative and the maroon represents the sodium iodide derivative after scaling, outlier rejection
404 and phasing within Autosarph (Global Phasing Limited). (B) Coomassie stain of co-affinity
405 purifications of wild-type or R169D CwID-His₆, with wild-type GerS or the D106R variant. The
406 indicated proteins were produced in *E. coli* and purified using Ni²⁺- affinity resin. Cleared lysate
407 (CL) and eluate (E) fractions were analyzed using Coomassie staining. (C) Percentage of GerS
408 pulled down with CwID relative to the amount detected in the wild-type CwID:GerS complex,
409 which was set at 100%. The mean of two assays from two independent co-affinity purifications is
410 shown, and dots represent each of those co-affinity purifications. Statistical significance relative
411 to the wild-type complex was determined using a one-way ANOVA and Tukey's test. **** p <
412 0.0001. (D) Coomassie stain of cleared lysate and elution fractions from co-affinity purifications
413 of His-tagged CwID:GerS, CwID_{E78Q}:GerS, or CwID:GerS_{H61A}.

To confirm the importance of the salt bridge in *C. difficile*, we analyzed CwlD:GerS binding using co-immunoprecipitation analyses that employed FLAG-tagged CwlD as bait. To this end, we generated complementation constructs encoding either wild-type or R169D FLAG-tagged CwlD (CwlD_{FLAG}). We then measured their ability to bind untagged GerS in sporulating *C. difficile* lysates. We also tested the effect of zinc-binding (H49A) and catalytic residue (E199A) mutations on CwlD_{FLAG}:GerS complex formation by generating complementation constructs encoding FLAG-tagged variants carrying these mutations. When wild-type FLAG-tagged CwlD was immunoprecipitated from sporulating cell lysates, untagged GerS readily co-purified. In contrast, immunoprecipitation of CwlD_{R169D} and CwlD_{H49A} resulted in a 5-fold reduction in the amount of GerS pulled-down via FLAG-tagged CwlD (**Figure 8A and B**), consistent with the affinity purification analyses in *E. coli* (**Figure 7B**). CwlD_{E199A} retained the ability to bind GerS, although this interaction decreased ~40% relative to the wild-type CwlD:GerS complex (**Figure 8A and B**). This finding could suggest that decreasing zinc coordination by a water molecule in the active site decreases the affinity of CwlD_{E199A} for GerS in sporulating cells.

Interestingly, the primary form of GerS detected in co-immunoprecipitations with FLAG-tagged CwlD was the cleaved form of GerS (**Figure 8A**). As a lipoprotein, GerS is secreted across the outer membrane by its N-terminal signal peptide in sporulating cells (12). Following secretion, GerS gets lipidated and then its signal peptide is removed through the action of the lipoprotein signal peptidase, Lsp (34). Notably, while full-length GerS and cleaved GerS are detectable in sporulating cells, only cleaved GerS is detected in mature spores (12) consistent with its maturation as a lipoprotein (34). These observations strongly suggest that GerS and

Figure 8. Validation of the CwID:GerS salt bridge by co-immunoprecipitation. (A) Western blot of GerS in co-immunoprecipitation fractions obtained from pulling down FLAG-tagged proteins produced in sporulating cells for wild-type CwID, CwID_{R169D}, CwID_{H49A}, or CwID_{E199A}. CwID was detected using an anti-FLAG antibody, while untagged GerS was detected using an anti-GerS antibody. CwID-c and GerS-c indicate cleaved form of both proteins. (B) Percentage of GerS pulled down with FLAG-tagged CwID relative to the amount detected in pull-downs with FLAG-tagged wild-type CwID. A mean of three independent assays is shown, except for CwID_{E199A}-FLAG, for which two independent biological replicates were performed. Dots represent the % of GerS pulled-down in each biological replicate. Statistical significance relative to the wild-type complex was determined using one-way ANOVA and Tukey's test. **, $p < 0.005$; **** $p < 0.0001$.

CwID interact *after* they are transported across the outer membrane. Altogether, our data indicate that stable CwID:GerS complex formation depends on a salt bridge formed between CwID_{R169} and GerS_{D106} and that stable binding between CwID and GerS likely happens in the outer forespore membrane.

Functional analyses of the salt bridge in *C. difficile*

To test whether the CwID:GerS interaction is critical for the CwID function in *C. difficile*, we analyzed the germination phenotype of the *cwID* complementation strain producing FLAG-tagged CwID_{R169D}. This strain was used in the co-immunoprecipitation assays described above (Figure 8A). We also assessed the effect of a GerS D106R substitution on spore germination by

complementing $\Delta gerS$ with a construct encoding FLAG-tagged GerSD106R. The germination phenotypes of the $\Delta cwID/cwID_{R169D-FLAG}$ and $\Delta gerS/gerS_{D106R-FLAG}$ strains were analyzed using the optical density-based spore germination assay. While the optical density of the parental $\Delta cwID$ and $\Delta gerS$ spores did not significantly decrease over the course of 90 min (6), the optical density of the $\Delta cwID/cwID_{R169D-FLAG}$ and $\Delta gerS/gerS_{D106R-FLAG}$ spores decreased by >40% over this time. $\Delta cwID/cwID_{R169D-FLAG}$ mutant spores germinated like wild-type spores and the wild-type complementation spores, $\Delta cwID/cwID_{FLAG}$ and $\Delta gerS/gerS_{FLAG}$ spores (**Figures 9A and S5A**), whereas $\Delta gerS/gerS_{D106R-FLAG}$ mutant spores germinated more slowly than wild-type and wild-type complementation spores ($p < 0.001$, **Figure 9A**).

To better understand the slower germination observed in this optical density-based assay for $\Delta gerS/gerS_{D106R-FLAG}$ spores, we visually monitored their germination using phase-contrast microscopy. One hour after inducing germination, all wild-type and $\Delta gerS/gerS_{FLAG}$ spores lost their phase-bright appearance (**Figure S6A**) as the cortex is degraded and the core hydrates (35). In contrast, $\Delta gerS/gerS_{D106R-FLAG}$ spores germinated more heterogeneously, with ~30% remaining phase-bright during this time period (**Figure S6A and B**). $\Delta gerS$ spores remained phase-bright during this time consistent with their inability to degrade the cortex, although some spore germination was detectable at the 90' timepoint (12).

We next analyzed the germination efficiency of the $cwID$ and $gerS$ point mutants by plating spores on media containing germinant. While the $\Delta cwID$ and $\Delta gerS$ parental strains exhibited a ~2 log decrease in spore germination efficiency relative to the wild-type strain (**Figure 9B**), no statistically significant defect in spore germination was observed for $cwID_{R169D-FLAG}$ and $gerS_{D106R-FLAG}$ mutant spores relative to wild-type or the $\Delta cwID/cwID_{FLAG}$ and $\Delta gerS/gerS_{FLAG}$ complementation strain spores (**Figure 9B**).

Figure 9. A *GerS*_{D106R} mutation affects the stability of GerS protein and leads to slower germination. (A) Spore germination as measured by the change in OD₆₀₀ in response to germinant in *gerS*_{D106R} and *cwID*_{R169D} mutant spores relative to wild-type spores. Purified spores were resuspended in BHIS, and germination was induced by adding taurocholate (1% final concentration). The ratio of the OD₆₀₀ of each strain at a given time point relative to the OD₆₀₀ at time zero is plotted. The mean of three assays from 3 independent spore preparations are shown. Shading represents the standard deviation as the area between error bars for each time point measured. Statistical significance relative to wild-type was determined using two-way ANOVA and Tukey's test. ***, $p < 0.0005$; ****, $p < 0.0001$. (B) Germination efficiency of *gerS*_{D106R} and *cwID*_{R169D} point mutant spores. The number of colony forming units (CFUs) produced by germinating spores is shown. The mean OD₆₀₀ determined from three independent spore preparations is shown along with the associated standard deviations. Statistical analyses relative to the wild-type were performed using one-way ANOVA and Tukey's test. **, $p < 0.005$; ****, $p < 0.0001$. (C) Western blot analysis of CwID and GerS levels in sporulating cells from the wild-type (WT), $\Delta gerS$, $\Delta gerS$ complemented with either *gerS*, or *gerS* encoding either wild-type or D106R FLAG-tagged GerS variants as well as $\Delta cwID$, $\Delta cwID$ complemented with either *cwID*

encoding either wild-type or R169D CwlD variants. CwlD-c and GerS-c indicate cleaved form of both wild-type proteins; asterisks indicate the cleaved form of FLAG-tagged $\Delta spo0A$ ($\Delta 0A$) was used as negative control for sporulating cells, and SpoIVA was used as a loading control. GerS and CwlD levels were analyzed using GerS, CwlD and FLAG antibodies. (B) Western blot analysis of CwlD and GerS levels in spores from the strains used in the experiments described in (C). SleC was used as a loading control.

Since CwlD enhances the levels of GerS in mature spores (6), and CwlD zinc-binding mutations affect both CwlD and GerS levels in mature spores (**Figure 6**), we analyzed the levels of CwlD and GerS in the *cwlD*_{R169D-FLAG} and *gerS*_{D106R-FLAG} mutant spores. Interestingly, both the *cwlD*_{R169D-FLAG} and *gerS*_{D106R-FLAG} mutations led to lower levels of GerS in spores, while CwlD levels were unaffected by these mutations (**Figure 9D**). The *CwlD*_{R169D-FLAG} variant also ran at a higher apparent MW than wild-type *CwlD*_{FLAG}. To ensure that the point mutations did not affect the stability of these proteins in sporulating cells, we analyzed the levels of these proteins in the different complementation strains using western blotting. *CwlD*_{R169D-FLAG} levels were similar to those detected in the wild-type $\Delta cwlD/cwlD$ _{FLAG} complementation strain. Even though lower levels of *GerS*_{D106R-FLAG} were detected using the GerS antibody in sporulating cells, no differences were found at the level of this protein with the anti-FLAG antibody (**Figure 9C**). This discrepancy suggests that our anti-GerS antibody binds the *GerS*_{D106R} variant less efficiently than wild-type GerS. Consistent with the *GerS*_{D106R} substitution causing a conformational change in GerS, *GerS*_{D106R-FLAG} ran at a different apparent MW than wild-type *GerS*_{FLAG} in sporulating cells. Taken together, these results indicate that disruption of the CwlD:GerS salt bridge decreases the stability of CwlD binding to GerS in both *E. coli* and *C. difficile* (**Figures 7 and 8**). In the case of the *GerS*_{D106R} substitution, decreasing GerS binding to CwlD reduces CwlD function partially in optical density and phase-contrast-based germination assays (**Figures 9A and S6**). However, since the salt bridge mutations did not markedly impair spore germination despite severely decreasing

CwID:GerS binding, our results suggest that low levels of GerS are sufficient to license CwID activity.

Discussion

The activity of cell wall hydrolases like amidases must be tightly regulated during bacterial growth to avoid perturbations that can lead to cell lysis (36). A wide array of mechanisms control cell wall hydrolase activity, including transcription, protein localization, stability, peptidoglycan modification, and protein-protein interactions (17). The regulation of cell wall amidases by protein-protein interactions was first reported only 12 years ago and was previously thought to be largely restricted to the AmiA/B/C amidases of Gram-negative bacteria (16, 22) until the activities of the LytH amidase of *Staphylococcus aureus* (15) and CwID amidase of *C. difficile* (6) were found to depend upon binding partners. However, in the absence of structures of the amidases bound to their protein regulators, it remained unclear whether these amidases were allosterically regulated by their binding partners.

In determining the structure of the *C. difficile* CwID amidase bound to its protein regulator, the GerS lipoprotein, and demonstrating that GerS binds CwID at a site distant from CwID's active site (**Figure 2**), we show in this study that the *C. difficile* CwID amidase is allosterically activated by the Peptostreptococcaceae-specific GerS lipoprotein. Our biochemical data strongly suggest that GerS binding to CwID promotes CwID binding to zinc, which directly participates in the catalytic mechanism of amidase_3 family members. This conclusion is based on the following observations. First, SEC-purified CwID does not bind zinc stably on its own in ICP-MS analyses,

whereas zinc is readily detected in the SEC-purified CwID:GerS complex (**Figure 5**). This stands in contrast with the Amidase_3 family members studied to date to our knowledge, where every structure of an Amidase_3 family member in the PDB contains zinc even though zinc was not added exogenously to the growth media (17, 21, 22, 30), and purified recombinant Amidase_3 proteins have amidase activity (15, 21, 22, 30). Second, we found that the stability of the CwID:GerS complex depends on CwID binding to zinc in both *E. coli* and *C. difficile* (**Figures 3, 4 and 6**), since zinc chelation via EDTA treatment and mutations that disrupt CwID binding to zinc both reduce CwID:GerS complex formation (**Figure 3 and 4**). Notably, loss of CwID zinc binding appears to destabilize CwID in mature *C. difficile* spores despite CwID zinc-binding mutant proteins being present at wild-type levels in sporulating cells (**Figure 5**). Thus, zinc plays a structural role in regulating CwID activity beyond its direct role in catalysis. Third, CwID is bound to zinc in the co-crystal structure and adopts a fold characteristic of active amidase_3 enzymes in the co-crystal structure (**Figure 2**), suggesting that GerS binding to CwID licenses CwID activity. Consistent with this hypothesis, mutation of a salt bridge at the CwID:GerS interface (CwID_{R169}:GerS_{D106}) destabilizes the complex in *E. coli* and *C. difficile* (**Figures 7 and 8**), reduces GerS levels in mature spores, and impairs (albeit subtly) CwID function in germination analyses of Δ gerS/gerS_{D106R} spores (**Figures 9 and S6**). Notably, this salt bridge is conserved across Peptostreptococcaceae family members, suggesting that this interaction plays a similar role in related GerS homologs.

Analyses of salt bridge mutant binding in *C. difficile* using co-immunoprecipitation (**Figure 8**) provided further insight into when and where CwID and GerS interact. While CwID and GerS are produced in the mother cell cytosol during sporulation (37-39), only the cleaved form of GerS binds full-length, FLAG-tagged CwID. Since GerS follows the maturation pathway of

Gram-positive lipoproteins (12, 34, 40) in only undergoing cleavage after it is (i) transported across the mother cell membrane and (ii) lipidated on its conserved cysteine, this observation revealed that CwID only binds GerS after GerS fully matures as a lipoprotein. CwID:GerS complex formation also stabilizes GerS in mature spores, since mutations that disrupt complex formation, e.g. salt bridge mutations, greatly reduce GerS levels in mature spores without affecting GerS levels in sporulating cells (**Figure 9**). This conclusion is consistent with our DLS analyses suggesting that GerS is prone to aggregation in the absence of CwID (**Figure S1**). Taken together, these results imply that the allosteric conduit connecting GerS to CwID's active site functions as a two-way street, with CwID binding to GerS affecting GerS's conformational stability, and GerS binding to CwID controlling CwID's ability to bind Zn^{2+} .

Based on these observations, we propose the following model. GerS binds CwID only after GerS is secreted across mother cell-derived membranes and completes its lipoprotein maturation process. CwID is also transported across mother cell-derived membranes via its predicted N-terminal transmembrane domain (41), but only its full-length, membrane-bound form binds lipidated GerS. This binding event likely triggers a conformational change in CwID that promotes zinc binding, since zinc is only found when CwID is complexed with GerS (**Figure 5**). This interaction may also help GerS orient CwID's active site towards its cortex peptidoglycan substrate close to the membrane, since only the membrane-bound forms of these proteins interact.

Despite these conclusions, our study raises several questions regarding the regulation of CwID amidase activity. How is GerS binding to CwID at the allosteric site communicated to CwID's active site to stabilize its binding to zinc? Does GerS regulate CwID amidase activity outside of GerS's effect on CwID binding to zinc? Does GerS need to remain bound to CwID for CwID to function as an amidase? Why does *C. difficile* CwID require a binding partner when the

highly homologous *B. subtilis* CwID can remodel peptidoglycan on its own when heterologously produced in *E. coli* (7)? Testing whether *B. subtilis* CwID alone can bind zinc and whether the structures of these two CwID homologs differ will provide important insight into these questions.

Addressing the question of whether GerS binding to CwID allosterically activates CwID by inducing a conformational change in CwID, however, depends on the structural characterization of CwID alone. While we were unsuccessful crystallizing only CwID, crystallographic analyses of Ami family amidases prior to their activation by protein regulators indicate that *E. coli* AmiC and *Bartonella henselae* AmiB are auto-inhibited by a regulatory α -helix that sterically hinders access to their active sites (21, 22). Notably, CwID homologs lack this regulatory helix (**Figure 2**), and amidases that lack this helix, such as *Nostoc punctiforme* AmiC2 and *Bacillus polymyxa* CwIV are active on their own (30, 42). While the precise mechanism by which the binding partners of Ami family amidases, EnvC, NlpD, and the recently discovered ActS, activate these amidases (20, 43) has yet to be characterized, the allosteric mechanism that activates *C. difficile* CwID is almost certainly distinct from Ami family amidases.

Accordingly, these observations raise the question of how CwID binding to GerS affects GerS structure and thus stability. In the co-crystal structure, two GerS subunits intimately interact in a hand-shake like manner (**Figure 2**), consistent with GerS's ability to dimerize in solution (**Figure 1**). Given the extensive interactions between the two monomers, the dimerization of GerS is likely critical for GerS to complex with CwID. GerS dimerization may also help position CwID's active site towards its peptidoglycan substrate, since GerS binds close to CwID's N-terminal membrane-associated region in the co-crystal structure (**Figure 2**).

One way to address the importance of GerS dimerization would be by targeting amino acid residues in the cross-over arm. However, this may be challenging because those mutations could affect the stability of GerS itself. Interestingly, when the β -strand exchange between GerS subunits is eliminated by creating a GerS pseudo-monomer, GerS exhibits structural homology to the LolA/LolB family of proteins, which are responsible for the transport of lipoproteins across the periplasm of Gram-negative bacteria during assembly of the outer membrane (23). It is also structurally similar to the N-terminal part of the outer membrane stress response proteins RseB/MucB found in Gram-negative bacteria (44), which regulate the envelope stress response sigma factor, σ^E /MucA (45, 46). The significance of this structural similarity remains unclear, since most LolA superfamily structures are monomeric, and although RseB dimerizes, the formation of dimer is mediated by its C-terminal domain (24). Intriguingly, a single mutation in LolA (F47E) has been reported to convert LolA from a monomer into a domain-swapped homodimer, and the homodimer binds LolA's interacting partner, LolC, more efficiently (25). The equivalent residue in *C. difficile* GerS is an aspartate, raising the possibility that GerS could be converted into a monomer like LolA by mutating to phenylalanine. While future work will address the importance of GerS dimerization to its function, it is interesting to note that both LolA/LolB and RseB/MucB use their structurally similar domains to bind lipids (24, 47). This observation raises the possibility that GerS may also bind lipids through this region, which could impact the localization of GerS in sporulating cells. Regardless, GerS is structurally distinct from previously described activators of amidases, EnvC (43), NlpD, and presumably ActS (20) and ActH (15), indicating that GerS likely represents a new class of regulators that directly bind to and activate cell wall hydrolases. Despite these differences, it is worth noting that all known protein activators of bacterial amidases appear to be membrane-associated (15, 16, 20, 22, 48).

While we cannot rule out the possibility that GerS regulates multiple aspects of CwlD function, e.g. localization, our results collectively suggest a new mechanism for activating amidases via stabilization of zinc co-factor binding and provide novel structural insight into how cell wall hydrolase activity can be regulated by protein binding partners. Indeed, stabilizing co-factor binding to enzymes could be a general strategy used by other peptidoglycan hydrolases. Also, given the diversity of hydrolases and regulators, distinct structures and regulatory mechanisms exist likely remain to be identified (17). Finally, since both GerS lipoprotein and PdaA deacetylase impact *C. difficile* infection (9, 12), our study suggests that inhibiting CwlD:GerS binding during spore formation could impair *C. difficile* spore germination and thus reduce disease transmission and recurrence.

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Conflicts of Interest

AS is a consultant and holds shares in a diagnostic start-up company, BioVector, Inc.

Material and Methods

Bacterial strains and growth conditions. *C. difficile* strain construction was performed using 630 Δ erm Δ cwlD Δ pyrE and 630 Δ erm Δ gerS Δ pyrE as the parental strains via *pyrE*-based allelic-coupled exchange (ACE ((32))). This system allows for single-copy complementation of the Δ cwlD and Δ gerS parental mutants, from an ectopic locus. The *C. difficile* strains used are listed in Table S1. They were grown on brain heart infusion media (BHIS) supplemented with taurocholate (TA, 0.1% wt/vol; 1.9 mM), thiamphenicol (10-15 μ g /mL), kanamycin (50 μ g/ml), cefoxitin (8 μ g/ml) and L-cysteine (0.1% w/v; 8.25 mM) as needed. For ACE, *C. difficile* defined medium (CDDM) (49) was supplemented with 5-fluoroorotic acid (5-FOA) at 2 mg/ml and uracil at 5 μ g/ml. Cultures were grown at 37°C under anaerobic conditions using a gas mixture containing 85% N₂, 5% CO₂ and 10% H₂.

The *Escherichia coli* strains used for BL21(DE3)-based protein production and for HB101/pRK24-based conjugations are listed in Table S1. *E. coli* strains were grown at 37°C with shaking at 225 rpm in Luria-Bertani broth (LB). The media was supplemented with chloramphenicol (20 μ g/ml), ampicillin (50 μ g/ml), or kanamycin (30 μ g/ml) as indicated.

***E. coli* strain construction.** All primers used for cloning are listed in Table S2 in the supplementary material. Details of *E. coli* strain construction are provided in the Supplementary Text S1. Plasmid constructs were confirmed by sequencing using Genewiz and transformed into either the HB101/pRK24 conjugation strain (used with *C. difficile*) or BL21(DE3) expression strain.

***C. difficile* strain construction and complementation.** Allele-couple exchange (ACE) (32) was used to construct all complementation strains. Complementation were performed as previously

described by conjugating HB101/ pRK24 carrying pMTL-YN1C plasmids into $\Delta pyrE$ -based strains (50) using allele-coupled exchange.

Protein purification for Recombinant *E. coli* Analyses. Starter cultures were grown in 20 mL LB broth with 100 μ g/mL ampicillin and, in some cases, 30 μ g/mL of kanamycin. Terrific broth (TB) with ampicillin and kanamycin was inoculated with the starter culture (1:1000) and incubated for ~60 hr at 20°C with 225 rpm shaking. The purifications were carried out in two steps: nickel bead-based affinity purification followed by size exclusion chromatography (SEC). Affinity purification was carried out as stated above: the cells were pelleted, resuspended in lysis buffer (500 mM NaCl, 50 mM Tris [pH 7.5], 15 mM imidazole, 10% [vol/vol] glycerol, 2 mM β -mercaptoethanol), flash frozen in liquid nitrogen, thawed and then sonicated. The insoluble material was pelleted and the soluble fraction was incubated with Ni-NTA agarose beads (Qiagen) for 2 hours to capture the His₆ tagged proteins. Washes were carried out three times with low imidazole buffer (500mM NaCl, 10mM Tris-HCl pH 7.5, 10% (v/v) glycerol, 15 mM imidazole, 2 mM β -mercaptoethanol) to decrease non-specific binding to the beads. Proteins were eluted using high-imidazole buffer (500 mM NaCl, 50 mM Tris [pH 7.5], 200 mM imidazole, 10% [vol/vol] glycerol, 2 mM β -mercaptoethanol) after nutating the sample for 5 to 10 min. Pooled protein elutions were concentrated to 20 mg/ml or less in a gel filtration buffer consisting of 200 mM NaCl, 10 mM Tris HCl pH 7.5, 5% glycerol and 1 mM DTT. SEC was carried out using a Superdex 200 Increase 10/300 GL (GE Healthcare) column. Proteins were resolved on 14% SDS-PAGE gels, and the gels were stained using GelCode Blue according to the manufacturer's instructions (ThermoFisher Scientific). The purified proteins were concentrated, aliquoted, flash frozen, and stored at - 20°C for future analysis.

For EDTA and ZnCl₂ analyses, 50 uM of Ni-affinity purified protein complexes were incubated for 1h at 4°C on gel filtration buffer with either 1 mM of EDTA or fivefold excess of ZnCl₂. Complex samples were then injected on the SEC column primed with gel filtration buffer containing 1 mM of EDTA or 50 uM of ZnCl₂.

For elemental analyses, SEC-purified proteins were concentrated to ~ 6 mg/mL in 50 mM NaCl, 10 mM Tris HCl pH 7.5, 5% glycerol and 1 mM DTT, were diluted 10X in 0.1 % HNO₃ and analyzed by ICP-MS (8900, Agilent, Santa Clara, CA) in helium mode at the Dartmouth Trace Elemental Analyses Core.

Protein purification for Crystallography. *E. coli* B834 (DE3) methionine auxotrophic strain 2593 was used to co-affinity purify the soluble domain of GerS (GerS_{Δ22}) with the His-tagged soluble domain of CwlD (CwlD_{Δ25}). Minimal medium consists of M9 medium (1X M9 salts, 2 mM MgSO₄, 0.1mM CaCl₂ and 5 g/L (0.5%) glucose (dextrose), 40 mg/L of all amino acids except methionine, 40 mg/L Selenomethionine, 2 mg/L Thiamine and 2 mg/L biotin. Briefly, a starter culture was grown in minimal media supplemented with 5% LB overnight at 37 °C. Minimal media was then inoculated with started culture (1:100) and incubated at 37 °C to OD₆₀₀ of 0.6. Cells were induced with 200 uM isopropyl-β- D-1-thiogalactopyranoside (IPTG) for 5h and then harvested by centrifugation at 4000 rpm for 15 min. The complex was purified as described above. Protein purification was performed in the presence of the reducing agent TCEP (1 mM). The protein complex was then purified by SEC using a Superdex 200 Increase 10/300 GL (GE Healthcare) column. Finally, SEC fractions containing the CwlD:GerS complex were pooled, concentrated to 22 mg/mL, and flash frozen in liquid nitrogen.

Crystallization and structure determination. Purified GerS, CwlD, and CwlD:GerS complex were evaluated for crystallization potential using dynamic light scattering using a DynaPro Protein Solutions DLS instrument (**Figure S1**). Purified GerS showed multimodal polydispersity regardless of buffer conditions, in agreement with the gel filtration profile, and was prone to aggregation. Numerous crystallization matrix screens were performed with only phase separation observed for CwlD but several hits using PEG 3350 for the CwlD:GerS complex. The optimized condition for the complex contained 50 mM BisTris at pH 7.5 with PEG 3350 from 12-18 %, 5 % glycerol, and 0.25 M ammonium nitrate. Crystals were grown by hanging drop vapor diffusion at 18°C with 30 mg/ml complex concentration mixed 1:1 with reservoir solution. Crystals were cryocooled in liquid nitrogen after soaking in crystallization reagent increased to 20% PEG 3350 and the addition of 14% ethylene glycol. The native complex and selenomethionine-substituted sample crystalized in the same condition. A derivative containing sodium iodide (NaI) was produced by soaking of native crystals with reagent containing 250 mM NaI for 10 minutes before cooling.

Data were collected at GM/CA Sector 23ID (Advanced Photon Source, Argonne National Laboratory) and data processed with the Proteum3 suite (Bruker AXS), Dials (51), and iMosflm (52). Selenomethionine and NaI derivative data were collected at 12.659 keV while the native data were collected at 12 keV. Initial processing suggested space group P6₁22 or P6₅22 with one complex per asymmetric unit at a resolution of 2.4 Å (**Table S3**). An initial solution was obtained by single anomalous dispersion (SAD) using the selenomethionine (SeMet/Se) incorporated crystal and the SAD routine from Crank2 (53) within CCP4 (29) and showed a preliminary C-alpha trace favoring the core beta sheet for GerS formed via a two-strand exchanged dimer. Data were

subsequently integrated, scaled and merged with multiple programs and phasing trials repeated as Se-SAD using ShelX (54) within Crank2 to produce an initial solution using 6 Se sites (1 Se and 1 Zn from CwID and 4 Se from GerS) CC_{all}/CC_{weak} of 50.1/30.7 and CFOM of 80.9. Additional runs used the native, Se, NaI under multiple isomorphous replacement using Sharp/Autosharp (Global Phasing Limited) and produced near identical solutions to the SAD and summarized in **Table S3**. The R_{merge} relative to the native data for MIR of the SeMet was 31.2% and for the Iodide 43.0 % due to changes in cell edge lengths. Anomalous signal for all three data sets at the conserved zinc site for CwID homologs was observed supporting the presence of the metal (**Figure S2**). Automated iterative build and density modification cycles using Parrot and Buccaneer (29) typically achieved R_{free} values near 30%. Manual building used Coot (55) with model refinement performed using Phenix with a final R_{free} of 24.5%. Structural orthologs were identified and superimposed using the DALI protein structure comparison server (56). All figures were prepared using PyMOL (Schrödinger Inc.).

Plate-based sporulation. *C. difficile* strains were inoculated from glycerol stocks overnight onto BHIS plates containing taurocholate. Colonies arising from these plates were used to inoculate liquid BHIS cultures, which were grown to early stationary phase, back-diluted 1:50 into BHIS, and grown until the cultures reached an OD_{600} between 0.35 and 0.75. Sporulation was induced on 70:30 agar plates as previously described (57) for 18-24 h, after which cells were analyzed by phase-contrast microscopy and harvested for western blot analyses.

Immunoprecipitation analyses. Immunoprecipitations were performed on lysates prepared from cultures induced to sporulate on 70:30 plates for 22 h. The samples were processed as previously described (58). Cultures from three 70:30 plates per strain were scraped into 3 x 1 mL of FLAG

IP buffer (FIB: 50 mM Tris pH 7.5, 150 mM NaCl, 0.02% sodium azide, 1X Halt protease inhibitor (ThermoScientific). The cultures were transferred into tubes containing a ~300 µL of 0.1 mm zirconia/silica beads (BioSpec). The cultures were lysed using a FastPrep-24 (MP Biomedicals) for 4 x 60 s at 5.5 M/s, with 5 min cooling on ice between lysis intervals. The tubes were pelleted at 3,000 x g for 2 min to pellet beads, unbroken cells/spores, and insoluble material. After washing the tubes with additional FIB, the lysates were pooled, and NP-40 detergent was added to a final concentration of 0.1%, to solubilize membrane-associated proteins. Then, the FLAG-conjugated magnetic resin was added. This magnetic resin was generated by incubating Dynabead Protein G (ThermoScientific) with anti-FLAG antibodies (Sigma Aldrich) at room temperature followed by washing. The lysates were incubated with the anti-FLAG resin for 1 hour at room temperature with rotation. After washing the beads with FIB, FLAG-tagged proteins were eluted using FIB containing 0.1 mg/mL FLAG peptide (Sigma Aldrich). After cell lysis, NP-40 detergent was added to the FIB at a concentration of 0.1%. All immunoprecipitations were performed on three independent biological replicates.

Western blot analysis. Samples for immunoblotting were prepared as previously described (59). Briefly, sporulating cell pellets were resuspended in 100 µl of PBS, and 50 µl samples were freeze-thawed for three cycles and then resuspended in 100 µl EBB buffer (8 M urea, 2 M thiourea, 4% [wt/vol] SDS, 2% [vol/vol] β-mercaptoethanol). *C. difficile* spores (~1 x 10⁶) were resuspended in EBB buffer, which can extract proteins in all layers of the spore, including the core. Both sporulating cells and spores were incubated at 95°C for 20 min with vortex mixing. Samples were centrifuged for 5 min at 15,000 rpm, and 4 x sample buffer (40% [vol/vol] glycerol, 1 M Tris [pH 6.8], 20% [vol/vol] β-mercaptoethanol, 8% [wt/vol] SDS, 0.04% [wt/vol] bromophenol blue) was added. Samples were incubated again at 95°C for 5 to 10 min with vortex mixing followed by

centrifugation for 5 min at 15,000 rpm. The samples were resolved by the use of 14% SDS-PAGE gels and transferred to a Millipore Immobilon-FL polyvinylidene difluoride (PVDF) membrane. The membranes were blocked in Odyssey blocking buffer with 0.1% (vol/vol) Tween 20. Polyclonal rabbit anti-GerS and anti-CwlD (6) antibodies were used at a 1:1,000 dilution. Polyclonal mouse anti-SpoIVA (60) and mouse anti-SleC (50) antibodies were used at 1:2,500 dilution and 1:5,000 dilution, respectively. IRDye 680CW and 800CW infrared dye-conjugated secondary antibodies were used at 1:20,000 dilutions. Odyssey LiCor CLx was used to detect secondary antibody infrared fluorescence emissions.

Spore purification. Sporulation was induced on 70:30 agar plates for 2 to 3 days as previously described (57). Spores were washed 6 times in ice-cold water, incubated overnight in water on ice, treated with DNase I (New England Biolabs) at 37°C for 60 min, and purified on a HistoDenz (Sigma- Aldrich) gradient. Phase-contrast microscopy was used to assess spore purity (>95% pure); the optical density of the spore stock was measured at OD₆₀₀; and spores were stored in water at 4°C. Two 70:30 plates were used to induce sporulation for each strain; purified spores were resuspended in 200 µl, and the total optical density of this mixture was determined for each strain. At least three independent spore preparations were performed.

Germination assay. Germination assays were performed as previously described (12). Approximately 1×10^7 (OD₆₀₀ of 0.35) were resuspended in 100 µl of water, and 10 µl of this mixture was removed for 10-fold serial dilutions in PBS. The dilutions were plated on BHIS-TA, and colonies arising from germinated spores were enumerated at 20 h. Germination efficiencies were calculated by averaging the CFU produced by spores for a given strain relative to the number produced by wild-type spores for at least three biological replicates from two independent spore

preparations. Statistical significance was determined by performing a one-way analysis of variance (ANOVA) on natural log-transformed data using Tukey's test.

Germination OD₆₀₀ kinetics assay and phase-contrast microscopy. For OD₆₀₀ kinetics assays with taurocholate in 96-well plates, $\sim 1.6 \times 10^7$ spores (0.55 OD₆₀₀ units) for each condition tested were resuspended in BHIS and 180 μ L were aliquoted into two wells of a 96 well flat bottom tissue culture plate (Falcon) for each condition tested. The spores were exposed to 1% taurocholate, or water (untreated) in a final volume of 200 μ L. The OD₆₀₀ of the samples was measured every 3 minutes in a Synergy H1 microplate reader (Biotek) at 37°C with constant shaking between readings. The change in OD₆₀₀ over time was calculated as the ratio of the OD₆₀₀ at each time point to the OD₆₀₀ at time zero. These assays were performed on three independent spore preparations. Data shown are averages from three replicates.

For phase contrast microscopy, purified spores were resuspended to an OD₆₀₀ of 0.5 in BHIS $\pm$ 1% taurocholate and incubated aerobically for 90 min at 37°C. Samples were spun down (5,000 x g, 10 min, 4°C) washes with PBS and fixed with 3.7% paraformaldehyde for 15 min at RT. Following fixation, samples were washed with PBS and mounted on agarose pads.

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