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Conserved Oligopeptide Permeases Modulate Sporulation Initiation in *Clostridium difficile*

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The anaerobic gastrointestinal pathogen *Clostridium difficile* must form a metabolically dormant spore to survive in oxygenic environments and be transmitted from host to host. The regulatory factors by which *C. difficile* initiates and controls the early stages of sporulation in *C. difficile* are not highly conserved in other *Clostridium* or *Bacillus* species. Here, we investigated the role of two conserved oligopeptide permeases, Opp and App, in the regulation of sporulation in *C. difficile*. These permeases are known to positively affect sporulation in *Bacillus* species through the import of sporulation-specific quorum-sensing peptides. In contrast to other spore-forming bacteria, we discovered that inactivating these permeases in *C. difficile* resulted in the earlier expression of early sporulation genes and increased sporulation *in vitro*. Furthermore, disruption of *opp* and *app* resulted in greater virulence and increased the amounts of spores recovered from feces in the hamster model of *C. difficile* infection. Our data suggest that Opp and App indirectly inhibit sporulation, likely through the activities of the transcriptional regulator SinR and its inhibitor, SinI. Taken together, these results indicate that the Opp and App transporters serve a different function in controlling sporulation and virulence in *C. difficile* than in *Bacillus subtilis* and suggest that nutrient availability plays a significant role in pathogenesis and sporulation *in vivo*. This study suggests a link between the nutritional status of the environment and sporulation initiation in *C. difficile*.

Clostridium difficile is an anaerobic, Gram-positive pathogen that causes a significant gastrointestinal disease in humans and other mammals. Because *C. difficile* is an obligate anaerobe, its survival outside the host requires the formation of a metabolically inactive spore, which can withstand harsh environmental conditions. The ability of *C. difficile* to form a spore is critical to the spread and recurrence of the disease (1). Spore formation also contributes to *C. difficile* resistance to traditional antibiotic therapies. Despite the importance of spore formation in the pathogenesis of *C. difficile*, little is understood about how the initiation of sporulation is regulated.

Sporulation is a complex process governed by multiple regulators and feedback processes in bacteria; however, the regulatory components and environmental signals that control the initiation of sporulation in well-studied sporeformers are not well conserved in *C. difficile* (2–7). In all studied spore-forming bacteria, including *C. difficile* (1, 8), the initiation of sporulation is controlled by the activity of the highly conserved transcription factor Spo0A, which functions as the master regulator of sporulation. In *Bacillus* species, Spo0A activity is tightly regulated through a phosphorylation-mediated signal transduction pathway in response to nutrient availability (9). One mechanism that controls Spo0A phosphorylation and, thereby, the activity of Spo0A in *Bacillus subtilis* and other species is the uptake of small, quorum-signaling peptides known as Phr peptides (10). The Phr peptides are imported by the Opp (Spo0K) and App oligopeptide permeases and contribute to the induction of sporulation or genetic competence when bacterial population densities are high (11–13). The Phr peptides are encoded by multiple *phr* genes and are initially secreted as proproteins that are subsequently processed to form pentapeptides (10, 13). Once imported by Opp or App, the Phr peptides directly target the Rap phosphatases, which would otherwise inhibit sporulation by dephosphorylating components of the Spo0A phosphorelay pathway (10, 14).

Opp (also known as Spo0K in spore-forming bacteria and Ami in *Streptococcus* species) and App are membrane-associated five-protein complexes of the ABC transporter family and are found in both Gram-positive and Gram-negative species. These transporters contain an extracellular ligand-binding lipoprotein (OppA/AppA), two transmembrane proteins (OppBC/AppBC) that form a membrane-spanning pore, and two cytoplasmic ATPases (OppDF/AppDF) that drive the transport of the peptide into the cell (15, 16). The Opp and App oligopeptide transport systems influence many cellular processes besides sporulation, including competence in *Bacillus* and *Streptococcus* species (11, 17, 18), plasmid transfer in *Enterococcus faecalis* (19, 20), and the expression of virulence factors in *Bacillus thuringiensis* (21, 22). However, the primary function of Opp and App in bacteria is to import small, heterogeneous peptides varying from 3 to 20 amino acids relatively nonspecifically as nutrient sources (23–27). These imported peptides may be utilized as carbon or nitrogen sources and can also serve as the substrates for the generation of ATP in clostridial species via the Stickland reaction (28–32).

The *C. difficile* genome encodes orthologs of the Opp and App oligopeptide transporter systems and two Rap phosphatase orthologs; however, no clear Phr ortholog is detectable by sequence analysis. In this study, we asked whether the conserved oligopeptide transporter systems have a role in regulating the initiation of

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sporulation of *C. difficile*. By studying *opp*- and *app*-null mutants, we determined that the absence of App or both Opp and App results in increased sporulation *in vitro*, as well as earlier and increased expression of sporulation genes. Our results indicate that the loss of either or both putative transporters results in a hypervirulent phenotype as well as increased spore formation during infection in a hamster model. Further, we observed that the increase in sporulation in *opp app* mutants is associated with the differential expression of the genes encoding SinR, a transcriptional regulator, and the predicted SinI, a putative inhibitor of SinR. Together these data suggest that the *C. difficile* Opp and App transporters indirectly inhibit sporulation by facilitating the uptake of peptides and, thus, the availability of intracellular nutrients.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Clostridium difficile* strains were routinely cultured in brain heart infusion-supplemented (BHIS) broth or on BHIS agar plates (33). Media for the growth of *C. difficile* were supplemented with 2 to 10 μg thiamphenicol ml^{-1}, 5 μg erythromycin ml^{-1}, or 30 μg lincomycin ml^{-1} (Sigma-Aldrich), as needed. Counterselection of *Escherichia coli* after conjugation with *C. difficile* was done using 50 μg kanamycin ml^{-1}, as detailed previously (34). Taurocholate was added to cultures (0.1%) to induce the germination of *C. difficile* spores, as indicated below (Sigma-Aldrich) (35, 36). *C. difficile* strains were cultured in an anaerobic chamber maintained at 37°C (Coy Laboratory Products) with an atmosphere of 10% H_2, 5% CO_2, and 85% N_2, as previously described (37, 38). In minimal-medium growth experiments, oligopeptides composed of 5 or 10 random amino acids (Peptide 2.0) were added to minimal defined medium (39) containing no other amino acid sources. *Escherichia coli* strains were grown at 37°C in LB (40) or BHIS medium and supplemented with 20 μg chloramphenicol ml^{-1} or 100 μg ampicillin ml^{-1} as needed.

Sporulation efficiency assays. Sporulation assays were performed in 70:30 sporulation broth medium (36). *C. difficile* cultures were started in BHIS medium supplemented with 0.1% taurocholate and 0.5% fructose until mid-log phase (active growth) to allow the germination of any spores within the starting inoculum. Cultures were then back-diluted and equilibrated to an optical density at 600 nm (OD_{600}) of 0.1 in 70:30 medium. These cultures were further diluted 1:10 in 70:30 medium, incubated at 37°C, and monitored for the production of spores. Samples were taken at the point of maximum cell density (approximately 2 h after the onset of stationary phase [T_2]), serially diluted, and plated onto BHIS medium with 0.1% taurocholate to determine the total number of CFU from which spores could form. To determine the number of spores present, 500-μl samples were taken from each culture, mixed 1:1 with 95% ethanol, and incubated for 1 h to kill all nonspores (vegetative cells) present. Ethanol-treated samples were then serially diluted, plated onto BHIS medium containing 0.1% taurocholate, and incubated for 24 to 48 h to enumerate the spore CFU. The sporulation frequency was determined by dividing the number of spores by the total number of CFU at T_2 for each culture (spore ratio) and multiplying that value by 100 (to obtain the percentage of spores formed). The sporulation-defective *spo0A* mutant was used as a negative control to ensure that all vegetative cells were killed during ethanol treatment. Cultures were assessed at the time of inoculation and at the start of stationary phase (time zero [T_0]) to ensure that no contaminating spores were present in the starter cultures.

Strain and plasmid construction. The oligonucleotides used in this study are listed in Table 2. Details of DNA cloning and vector construction are outlined in File S1 in the supplemental material. *C. difficile* strain 630 (GenBank accession NC_009089.1) was used as the template for primer design, unless otherwise specified. Strain 630Δ*erm* was used as the template for PCR amplification, except where use of strain R20291 (GenBank accession number NC_013316.1) as the template is specified (41). Isolation of plasmid DNA, PCR, and cloning were performed using standard protocols. Genomic DNA was prepared and genetic manipulation of *C. difficile* was performed as previously described (37, 42). Null mutations in *C. difficile* were created by retargeting the group II intron from pCE240 using the intron-retargeting primers listed in Table 2, as previously described (43–45). The double *opp app* mutant (MC307) was created by using the *oppB*-specific group II intron in MC301 (*app* single mutant) and selecting for the second intron integration event on BHIS medium plates containing lincomycin (30 μg ml^{-1}). To complement the *opp* and *app* disruptions, plasmids containing the *oppBCAD* (pMC213) or *appFDABC* (pMC238) operons and upstream promoter regions were transferred into *C. difficile* mutants from *E. coli* by conjugation as previously described, except that 50 μg kanamycin ml^{-1} was used to counterselect against *E. coli* postconjugation (46). Cloned DNA fragments were verified by sequencing (Eurofins MWG Operon) prior to use.

Phase-contrast microscopy. *C. difficile* strains were grown in 70:30 sporulation medium as described above. At the indicated time points, 1 ml of culture was removed from the anaerobic chamber, centrifuged at full speed for 30 s, and resuspended in ~10 μl of supernatant. Slides were prepared by placing 2 μl of the concentrated culture onto a thin layer of 0.7% agarose applied directly to the surface of the slide. Phase-contrast microscopy was performed using a ×100 Ph3 oil immersion objective on a Nikon Eclipse Ci-L microscope. At least three fields of view for each strain were acquired with a DS-Fi2 camera and used to calculate the percentage of spores (the number of spores divided by the total number of spores, prespores, and vegetative cells) from two independent experiments.

Fluorescence microscopy. *C. difficile* strains were grown in 70:30 sporulation medium as described above. At approximately 4 h after the onset of stationary phase (~T_4), 2 ml of culture was removed from the anaerobic chamber, pelleted, and resuspended in 50 μl of brain heart infusion medium. The membrane-specific dyes FM4-64 and MitoTracker green were added to a final concentration of 165 μM and 100 nM, respectively, and the cultures were incubated for 30 min at room temperature. Slides were prepared by first applying a thin layer of 1.0% agarose to the slide, followed by application of 8 μl of the concentrated culture onto the agarose bed. Fluorescence microscopy was performed using a ×100 oil immersion objective (numerical aperture, 1.49) on a Nikon structured illumination microscope (N-SIM). At least three fields of view for each strain were used to calculate the percentage of cells entering sporulation from two independent experiments. The percentage of sporulating cells was defined as the number of cells possessing polar septa or partially/completely engulfed forespores divided by the total number of cells.

Southern blot analysis. Genomic DNA was isolated from overnight cultures of *C. difficile* strains 630Δ*erm*, MC296, MC301, and MC307 as previously described (42). Six micrograms of DNA from each strain was digested with HindIII (NEB), separated on a 0.7% agarose gel, and transferred onto Hybond-N+ nylon membranes (GE Healthcare). DNA was then fixed to the membranes by UV cross-linking. Southern blot analysis was performed using a DIG High Prime labeling and detection kit (Roche). An intron-specific probe was prepared by PCR using primers OBD522 and OBD523 as previously described (47).

Spore preparation and quantification. To prepare spores for animal studies, strains for spore preparation were grown in BHIS broth overnight and 100 μl was spread onto 70:30 agar plates (36). The plates were then incubated for 48 h to allow spores to form. Following incubation, cells were scraped from the plates, resuspended in 5 ml of phosphate-buffered saline (PBS), pelleted at 3,000 × *g* for 15 min, and resuspended in 5 ml of PBS. The resuspended cells were then combined 1:1 with 95% ethanol and incubated at room temperature for 1 h to kill all nonspores present. The spores were then pelleted, washed twice in PBS, and resuspended in 5 ml of fresh PBS. Spore suspensions were then heated to 70°C for 20 min, followed by addition of 1% bovine serum albumin (BSA) to prevent spores from sticking to each other and plastic or glass surfaces. Spore preparations were serially plated in PBS with 1% BSA to determine the

TABLE 1 Bacterial strains and plasmids

Plasmid or strain	Relevant genotype or features	Source or reference
Strains		
E. coli		
DH5α Max Efficiency	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Invitrogen
MC101	HB101/pRK24	B. Dupuy
MC135	HB101 containing pRK24 and pMC123	48
MC253	HB101 containing pRK24 and pMC199	This study
MC263	HB101 containing pRK24 and pMC213	This study
MC274	HB101 containing pRK24 and pMC217	This study
MC277	HB101 containing pRK24 and pMC211	This study
MC295	HB101 containing pRK24 and pMC230	This study
MC306	HB101 containing pRK24 and pJS107::*spo0A178*::TargeTron	69
MC329	HB101 containing pRK24 and pMC238	This study
MC330	HB101 containing pRK24 and pMC240	This study
C. difficile		
630	Clinical isolate	97
630Δ*erm*	Erm^s derivative of strain 630	N. Minton and 98
R20291	Clinical isolate	41
MC282	630Δ*erm* pMC211	This study
MC283	630Δ*erm* pMC217	This study
MC296	630Δ*erm oppB*::*ermB*	This study
MC301	630Δ*erm appA*::*ermB*	This study
MC307	630Δ*erm oppB*::*ermB appA*::*ermB*	This study
MC310	630Δ*erm spo0A*::*ermB*	This study
MC322	630Δ*erm oppB*::*ermB*/pMC213	This study
MC323	630Δ*erm oppB*::*ermB appA*::*ermB*/pMC213	This study
MC324	630Δ*erm*/pMC123	This study
MC331	630Δ*erm appA*::*ermB*/pMC238	This study
MC332	630 Δ*erm oppB*::*ermB*/pMC240	This study
MC334	630Δ*erm oppB*::*ermB appA*::*ermB*/pMC238	This study
MC337	63 Δ*erm oppB*::*ermB*/pMC123	This study
MC338	630Δ*erm appA*::*ermB*/pMC123	This study
MC339	630Δ*erm oppB*::*ermB appA*::*ermB*/pMC123	This study
Plasmids		
pRK24	Tra$^+$ Mob$^+$ *bla tet*	99
pBL100	Untargeted group II intron-TargeTron vector	29
pCR2.1	*bla kan*	Invitrogen
pCE240	*C. difficile* TargeTron construct based on pJIR750ai (group II intron, *ermB*::RAM *ltrA*) *catP*	C. Ellermeier (44)
pJS107-*spo0A*-178TT	Group II intron targeted to *spo0A*	69
pMC123	*E. coli-C. difficile* shuttle vector, *bla catP*	48
pMC199	pBL100 with group II intron targeted to *oppB*	This study
pMC211	pMC123 with *cprA* promoter	This study
pMC213	pMC123 with P*oppBCAD* operon	This study
pMC217	pMC211 with CD0852	This study
pMC220	pCR2.1 with group II intron targeted to *appA*	This study
pMC226	pCE240 with *appA*-targeted intron	This study
pMC230	pMC123 with *appA*-targeted intron, *ermB*::RAM *ltrA catP*	This study
pMC237	pCR2.1 with P*appFDABC* operon	This study
pMC238	pMC123 with P*appFDABC* operon	This study
pMC239	pCR2.1 with P*oppBCADF* from R20291	This study
pMC240	pMC123 with P*oppBCADF* from R20291	This study

number of CFU present and diluted prior to use. Spores were stored in glass tubes at room temperature.

Quantitative reverse transcription-PCR (qRT-PCR) analysis. Samples of *C. difficile* grown in 70:30 sporulation medium were harvested into 1:1 ethanol-acetone, and RNA was purified as previously described (42, 48). RNA isolated from hamster cecal contents was treated identically, except that cells were mechanically disrupted, using a bead beater, for twice as long (a total of 6 min per sample). cDNA synthesis was performed with random hexamers using a Tetro cDNA synthesis kit (Bioline), and either 50 ng (samples isolated from *in vitro* cultures) or 200 ng (samples isolated from cecal contents) cDNA per reaction mixture was used for quantitative PCR (qPCR) analysis. qPCR analysis was performed using a

TABLE 2 Oligonucleotides

Primer	Sequence[a] (5′ → 3′)	Purpose (operon)	Source or reference
EBS universal	5′-CGAAATTAGAAACTTGCGTTCAGTAAAC-3′		Sigma-Aldrich
oMC44	5′-CTAGCTGCTCCTATGTCTCACATC-3′	*rpoC* qPCR (CD0067)	46
oMC45	5′-CCAGTCTCTCCTGGATCAACTA-3′	*rpoC* qPCR (CD0067)	46
oMC112	5′ -GGCAAATGTAAGATTTCGTACTCA- 3′	*tcdB* qPCR (CD0660)	
oMC113	5′ -TCGACTACAGTATTCTCTGAC- 3′	*tcdB* qPCR (CD0660)	
oMC152	5′-GTTATGGAAGTCAAGGACATGCAC-3′	*ilvC* qPCR (CD1565)	42
oMC153	5′-GCTTCTGCTACACTCTTAACTTCA-3′	*ilvC* qPCR (CD1565)	42
oMC158	5′-CCAGAAGAATCTATGGTAAAGGTTG-3′	*hisZ* qPCR (CD1547)	42
oMC159	5′-ACCATCTTCCCATCTTGGTACA-3′	*hisZ* qPCR (CD1547)	42
oMC215	5′-GCGAATTCGACATGGAAGTAGAAGTTAAGG-3′	P*cprA* cloning	
oMC331	5′-CTCAAAGCGCAATAAATCTAGGAGC-3′	*spo0A* qPCR (CD1214)	
oMC332	5′-TTGAGTCTCTTGAACTGGTCTAGG-3′	*spo0A* qPCR (CD1214)	
oMC339	5′-GGGCAAATATACTTCCTCCTCCAT-3′	*sigE* qPCR (CD2643)	
oMC340	5′-TGACTTTACACTTTCATCTGTTTCTAGC-3′	*sigE* qPCR (CD2643)	
oMC346	5′-GCGGATCCAATTTTCATCCTTTCTTTTGTCAAAC-3′	P*cprA* cloning	
oMC349	5′-CCTTTGTGCTAGCCTTATTGTTAGG-3′	*oppB* qPCR (CD0853)	
oMC350	5′-AAGTATGAGTACTAAGGCAACCCA-3′	*oppB* qPCR (CD0853)	
oMC363	5′-GACTTGCTCTGTGTTAGTTCCATC-3′	*spoIID* qPCR (CD0124)	
oMC364	5′-TGTTTATAGATACTGGGCTCTCTGG-3′	*spoIID* qPCR (CD0124)	
oMC365	5′-GGAAGTAACTGTTGCCAGAGAAGA-3′	*sigF* qPCR (CD0772)	
oMC366	5′-CGCTCCTAACTAGACCTAAATTGC-3′	*sigF* qPCR (CD0772)	
oMC371	5′-AAAAGCTTTTGCAACCCACGTCGATCGTGAA ATATACAGTCAAGTGCGCCCAGATAGGGTG-3′	*oppB* (CD0853) intron retargeting	
oMC372	5′- CAGATTGTACAAATGTGGTGATAACAGATAAGTC AGTCAATATAACTTACCTTTCTTTGT-3′	*oppB* (CD0853) intron retargeting	
oMC373	5′-CGCAAGTTTCTAATTTCGGTTTATATTCGATAGA GGAAAGTGTCT-3′	*oppB* (CD0853) intron retargeting	
oMC427	5′-GTGGTGTTAATACATCAGAACTTCC-3′	*sigG* qPCR (CD2642)	
oMC428	5′-CAAACTGTTGTCTGGCTTCTTC-3′	*sigG* qPCR (CD2642)	
oMC429	5′-GCCTGTGCTTCCAATGATAAAG-3′	*appA* qPCR (CD2672)	
oMC430	5′-ATATCTGGGTCACTTGCCATAG-3′	*appA* qPCR (CD2672)	
oMC431	5′-GTTGGAGAATCAGGATGTGGTA-3′	*appD* qPCR (CD2671)	
oMC432	5′-GCTGGGTTTAAAGATGTCATTGG-3′	*appD* qPCR (CD2671)	
oMC439	5′- GCCGGATCCCGTTTGCTTTTATGTGGTTT-3′	*oppB*::*erm* verification	
oMC440	5′- GCCGGATCCCTGTTGGTGTGTTAATCCATTTTC-3′	*oppBCAD* cloning	
oMC441	5′-GCCTGCAGGCTTTTACAGTAGTACTTTTGTCC-3′	*oppBCAD* cloning	
oMC506	5′-GCCGGATCCAACCATTTAAGAGGTGATTT-3′	CD0852 cloning	
oMC507	5′-GACCTGCAGCTTTCTCCAAATACACCTAT-3′	CD0852 cloning	
oMC527	5′-AGGCAGGTTTACATCCAACATA-3′	*sinR* qPCR (CD2214)	
oMC528	5′-AGTGGTATGTCTAAAGCAGTAGC-3′	*sinR* qPCR (CD2214)	
oMC544	5′-AAAAGCTTTTGCAACCCACGTCGATCGTGAA GTTAACGTAGAAGTGCGCCCAGATAGGGTG-3′	CD2672 (*appA*) intron retargeting	
oMC545	5′-CAGATTGTACAAATGTGGTGATAACAGATAAGTC GTAGAAAATAACTTACCTTTCTTTGT-3′	CD2672 (*appA*) intron retargeting	
oMC546	5′-CGCAAGTTTCTAATTTCGGTTTTAACTCGATAGA GGAAAGTGTCT-3′	CD2672 (*appA*) intron retargeting	
oMC547	5′-TGGATAGGTGGAGAAGTCAGT-3′	*tcdA* qPCR (CD0663)	
oMC548	5′-GCTGTAATGCTTCAGTGGTAGA-3′	*tcdA* qPCR (CD0663)	
oMC553	5′-TGAATGCACCAGAACCAACT-3′	*appA*::*ermB* verification	
oMC587	5′-GTCGGATCCGGCTTTACATAACATGGTAGAGTAGTA-3′	*appFDABC* cloning	
oMC588	5′-AGCGAATTCAACAGCATGAAATCCCATCTTAATC-3′	*appFDABC* cloning	
oMC589	5′-GACACATGAACCAACATACATGG-3′	*oppC* qPCR (CD0854)	
oMC590	5′-AGATGCTGAACCACTTATACTACC-3′	*oppC* qPCR (CD0854)	
oMC591	5′-GATAAGAAGGCAGATACGCCTAAA-3′	*oppA* qPCR (CD0855)	
oMC592	5′-GCTAGACAAGGAACCACCTTATTA-3′	*oppA* qPCR (CD0855)	
oMC593	5′-ATTTCAAGACCCTATGACATCTCTC-3′	*oppD* qPCR (CD0856)	
oMC594	5′-TCTCAGGATTATCAATTCCAACCA-3′	*oppD* qPCR (CD0856)	
oMC595	5′-CATGAATCTACTGGTGGAGAAGTAA-3′	*oppF* qPCR (CD0857)	
oMC596	5′-CTGCAATTGTCATACGTGGATTT-3′	*oppF* qPCR (CD0857)	
oMC597	5′-TGCAGGAAGAACCATTCTTAGAT-3′	*appF* qPCR (CD2670)	
oMC598	5′-ATGTTCTATAACTGCCTCGGATATT-3′	*appF* qPCR (CD2670)	

(Continued on following page)

TABLE 2 (Continued)

Primer	Sequence[a] (5′ → 3′)	Purpose (operon)	Source or reference
oMC599	5′-GGTTAATATGGGACTTGATAAACCAG-3′	*appB* qPCR (CD2673)	
oMC600	5′-CATAATTTGTTCTGTAACGGGCATA-3′	*appB* qPCR (CD2673)	
oMC601	5′-CTTCAAGAACCTAGCTTAAAGCATATC-3′	*appC* qPCR (CD2674)	
oMC602	5′-ATAACACCTACAGCAGTACCAAATA-3′	*appC* qPCR (CD2674)	
oMC612	5′-ACAGAAGAAGGTGTTGAAGGATATG-3′	*appA* (3′) qPCR	
oMC613	5′-TTATGCCCAAATCTACCTGTGTTAT-3′	*appA* (3′) qPCR	
oMC654	5′-GCCTGCAGCCTGTTCAAGTATTTTTATACTAAAC-3′	*oppF* cloning	
oMC683	5′-GTATCTGACAACATCAATTGCCTAAA-3′	CD0341 qPCR	
oMC684	5′-TCAGCTTGAGATTCAATTTCTTCATT-3′	CD0341 qPCR	
oMC733	5′-AGGTTTGGAGCAATCAAGAAAGA-3′	*murG* qPCR (CD2651)	
oMC734	5′-TGCTCATGTATTATGGCTGGTATTT-3′	*murG* qPCR (CD2651)	
oMC735	5′-CCTGGTAATTGGTCTTGGAAATAGA-3′	*gpr* qPCR (CD2470)	
oMC736	5′-CCTCAGTGAAATCATCATCATAGTCTTTA-3′	*gpr* qPCR (CD2470)	
oMC739	5′-AAGTGTGGCATTTGTAGACCATAATA-3′	*acnB* qPCR (CD0833)	
oMC740	5′-ACCATTTCCTGGCTTAGAGAAATAAA-3′	*acnB* qPCR (CD0833)	
oMC741	5′-CTCTGCAAATGTAGGTCATGGTAATAA-3′	*gabT* qPCR (CD2158)	
oMC742	5′-TTCCACAAGCTTCTCAGCTAATTT-3′	*gabT* qPCR (CD2158)	
oMC745/oBD522	5′-ATCTGTAGGAGAACCTATGGGAAC-3′	Intron forward primer for Southern blotting	47
oMC746/oBD523	5′-CACGTAATAAATATCTGGACGTAAAA-3′	Intron reverse primer for Southern blotting	47
oMC747	5′-TCAACGGAAGATCAGGATGATTTA-3′	*sigK* qPCR (CD1230)	
oMC748	5′-CATATGTTGCTAATCGAGTTCCTTTAT-3′	*sigK* qPCR (CD1230)	
oMC756	5′-TGGCAAGTAACAATAACAACAACAG-3′	*sspA* qPCR (CD2688)	
oMC757	5′-CCATTTGTTGTTCAGCCATTTCA-3′	*sspA* qPCR (CD2688)	

[a] Underlining indicates sequence-specific sites within gene of interest for intron retargeting.

SensiMix SYBR & Fluorescein kit (Bioline) and a Bio-Rad CFX96 real-time system as previously described (49). Mock cDNA synthesis reaction mixtures containing no reverse transcriptase were used to control for genomic contamination in subsequent amplifications. The primers used for qPCRs were designed using the PrimerQuest tool by Integrated DNA Technologies, and primer efficiencies were determined for each primer set prior to use. qPCRs were performed in technical triplicate for each cDNA sample and primer pair. qPCR was performed on cDNA isolated from a minimum of three biological replicates. Results are presented as the means and standard errors of the means of the data obtained. Results were calculated by the comparative cycle threshold method (50), with the expression of the amplified target sequence being normalized to that of the internal control transcript, *rpoC*. A two-tailed Student's *t* test was used to compare the transcriptional ratios of the variable to the control sets, as indicated below.

Animal studies. Female Syrian golden hamsters (*Mesocricetus auratus*) weighing between 70 and 120 g were purchased from Charles River Laboratories and maintained in an animal biosafety level 2 facility in the Emory University Division of Animal Resources. Animals were housed individually in sterile cages and were fed a standard rodent diet and provided water *ad libitum*. Five days prior to inoculation with *C. difficile*, the hamsters were administered a single dose of clindamycin (30 mg kg of body weight^{-1}) by oral gavage to perturb the intestinal microbiota and induce susceptibility to infection (51, 52). At 5 days after antibiotic treatment, the hamsters were administered approximately 500 spores of a single strain of *C. difficile* and monitored for symptoms of disease (weight loss, lethargy, diarrhea, and wet tail). The hamsters were weighed at least once per day, and fecal samples were collected daily for enumeration of spores. Cohorts of 5 to 6 animals were tested per strain, and experiments were performed a minimum of two times. Negative-control animals that were treated with clindamycin but not administered *C. difficile* were included in all experiments. Animals were considered moribund if (i) they had lost 15% or more of their starting weight or (ii) if they presented with diarrhea, lethargy, and wet tail. Animals meeting either of these criteria were euthanized to prevent unnecessary suffering. Hamsters were euthanized by CO_2 asphyxiation, followed by thoracotomy as a secondary method of euthanasia. At the time of death, animals infected with either the 630Δ*erm* or MC307 strain were necropsied, and cecal contents were collected and stored in a 1:1 acetone-ethanol solution at −80°C. All animal studies were performed with prior approval from the Emory University Institutional Animal Care and Use Committee (IACUC). Differences in spores recovered from fecal samples were analyzed by analysis of variance (single factor; Excel; Microsoft). Differences in hamster survival between those infected with *C. difficile* 630Δ*erm* and those infected with either MC296 (*opp*), MC301 (*app*), or MC307 (*opp app*) were analyzed using the log rank test (GraphPad Prism).

Nucleotide sequence accession numbers. The nucleotide sequences of *C. difficile* strains 630 and R20291 have been deposited in GenBank under accession numbers NC_009089.1 and NC_013316.1, respectively.

RESULTS

Identification of Opp and App transporters in *C. difficile*. The *opp* and *app* operons were identified on the chromosome of sequenced strain 630 as CD0853 to CD0857 and CD2670 to CD2674, respectively (53, 54). The *app* genes are arranged in two apparently divergently transcribed operons (*appABC* and *appDF*), while the *opp* genes appear to be organized as a single transcription unit (*oppBCADF*) (Fig. 1). Similar to the oligopeptide transporters found in other Gram-positive bacteria, both the *app* and *opp* regions encode an apparent extracellular substrate binding protein (*appA* and *oppA*), two apparent integral membrane permeases (*appBC* and *oppBC*), and two apparent cytoplasmic ATP-binding proteins (*appDF* and *oppDF*). Orthologs of the *opp* and *app* oligopeptide transporters are common to many bacterial species, and often, two or more analogous transport systems are encoded by the genomes of Gram-positive bacteria (17, 55–58). In

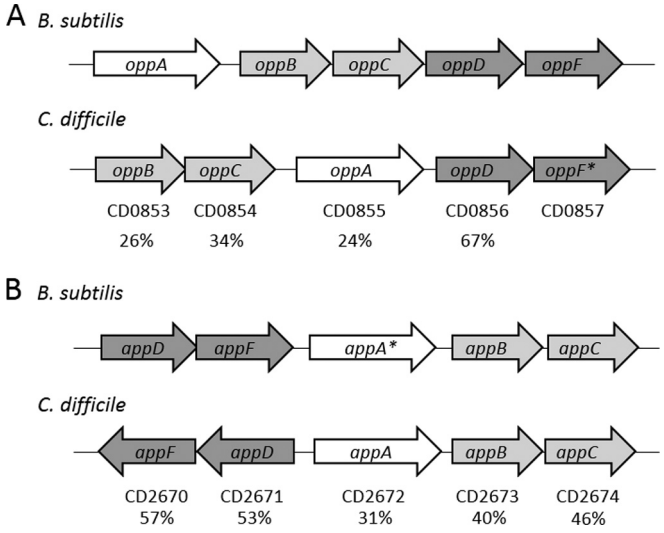


FIG 1 Identification of oligopeptide transporter systems in *C. difficile*. Comparison of the *opp* (A) and *app* (B) operons in *B. subtilis* 168 (GenBank accession no. AL009126) and *C. difficile* 630 (GenBank accession no. AM180355). OppA/AppA are solute-binding lipoproteins, OppBC/AppBC are transmembrane proteins, and OppDF/AppDF are ATP-binding proteins. *, a pseudogene; OppF is annotated as a pseudogene in *C. difficile* 630 as well as AppA in *B. subtilis* 168 (57). Numbers below the arrows represent percent identity to proteins in the corresponding *B. subtilis* sequences.

the latter cases, the systems may have overlapping or redundant functions (57). Although the predicted App and Opp protein sequences are orthologous to the oligopeptide transport systems found in other bacteria (Fig. 1), the individual App and Opp proteins of *C. difficile* have only modest amino acid identity to one another (e.g., 29% identity between OppA and AppA); therefore, the specific functions of these transporters in *C. difficile* could not be assumed from their sequence similarities. In previous *C. difficile* studies, the *opp* operon was found to be repressed by the global transcriptional regulators CodY and CcpA and by glucose, implying that the Opp transporter is involved in adaptation to nutrient-limiting conditions (42, 59). In addition, SigH, the alternative sigma factor involved in the transition to stationary phase, was shown to differentially affect transcription of the *app* and *opp* genes (60). In a *sigH* mutant, transcription of the *opp* genes was decreased, while *app* transcripts were increased, suggesting that the transporters are regulated differently during the adaptation to stationary phase in TY medium (60). However, our transcriptional analysis of *app* and *opp* expression in cells grown in sporulation medium (70:30 broth) (36) revealed that the expression of *appA* and *oppB* genes increases similarly throughout the transition to stationary phase (see Fig. S2A in the supplemental material).

Disruption of the *opp* and *app* operons results in increased spore formation. In the model spore-forming firmicute *Bacillus subtilis*, the Opp (Spo0K) and App transporters are responsible for the uptake of small, quorum-signaling peptides that stimulate competence and sporulation (12, 57). In *B. subtilis*, the absence of functional App and Opp transporters results in a significant sporulation defect (61, 62). *C. difficile* does not carry orthologs of either the PhrA peptide or the competence-stimulating factor (CSF) PhrC (18), but unrelated peptides with similar activities could potentially be transported by the oligopeptide transporters. To test whether the Opp and App transporter systems have a role in regulating the entry into sporulation in *C. difficile*, we introduced insertional mutations into the first genes of the *app* and *opp* operons (*appA* and *oppB*, respectively). To do so, we used a TargeTron-based group II intron, which was retargeted for integration into *appA* at nucleotide 439 and into *oppB* at nucleotide 178 of the coding sequences (see Materials and Methods). The locations of the insertions were confirmed by Southern blotting (see Fig. S3 in the supplemental material). Both the *appA* and *oppB* insertional mutations greatly reduced the expression of all the downstream genes in each operon, as evidenced by qRT-PCR analysis (see Fig. S2B and C in the supplemental material)

The *app*- and *opp*-null mutants (MC296 and MC301, respectively) were tested for the frequency of spore formation after 24 h of growth in 70:30 sporulation medium by measuring the number of ethanol-resistant spores recovered and normalizing this to the number of viable cells present at T_2, as described in Materials and Methods (36). We observed a sporulation frequency of ~0.1% in the parent strain, 630Δ*erm* (Fig. 2A). Low sporulation frequencies in *C. difficile* have been observed in other studies (1, 63, 64); however, the percentage of spore formation reported is widely variable, depending on the strain, medium, and methods used to enumerate spores (63). As shown in Fig. 2A, the *opp* mutant had the same low frequency of spore formation as the parent strain. In contrast, the *app*-null mutant produced an average of 10-fold more ethanol-resistant spores than did the parent strain (Fig. 2A). To determine if the Opp and App transporter systems have overlapping or redundant roles in mediating sporulation frequency, a double mutant strain containing both the *oppB* and *appA* insertional disruptions was created (MC307). The *opp app* double mutant produced ~20-fold more spores than did the parent strain (Fig. 2A). These data suggest that the App transporter influences the sporulation frequency in *C. difficile*, while the Opp transporter does not appear to affect sporulation *in vitro*.

Using phase-contrast microscopy, we followed the rate of sporulation in the parent and the transporter mutant strains (Fig. 3A). Phase-dark prespores and phase-bright spores were visible by the second hour of stationary phase (T_2) in all strains; however, the *app* and *opp app* mutants produced more spores than the wild type by T_2 (3.7-fold and 4.3-fold higher, respectively) and T_{24} (14.1-fold and 12.5-fold higher, respectively) (Fig. 2B). To further define sporulation initiation in the parent strain and the *opp app* mutant, we performed fluorescence microscopy using the membrane-specific dye FM4-64 (65, 66). This technique enables the identification of cells that have asymmetric sporulation septa (stage II) as well as partial or complete engulfment of the prespore (stage III). At T_4, more cells of the transporter mutant (43.2% ± 7.8%) than of the wild-type strain (8.3% ± 3.0%) were in stage II or beyond (Fig. 2C and 3B). There was no significant difference in the growth rate of the parent, *app*, *opp*, or *opp app* strain in 70:30 or BHIS medium (Fig. 2C; data not shown). These results indicate that these oligopeptide transporters are not essential for growth or for detection of population density under the conditions tested. Surprisingly, the increased sporulation rate of *C. difficile* App transporter mutants contrasts dramatically with the roles of analogous transporters in other spore-forming species, suggesting that peptide transport plays a distinctly different role in *C. difficile* spore formation (21, 57).

The sporulation phenotypes of the *opp* and *opp app* transporter mutants were fully rescued when plasmid copies of the *opp* operon were used to complement the disrupted transporters. Expression

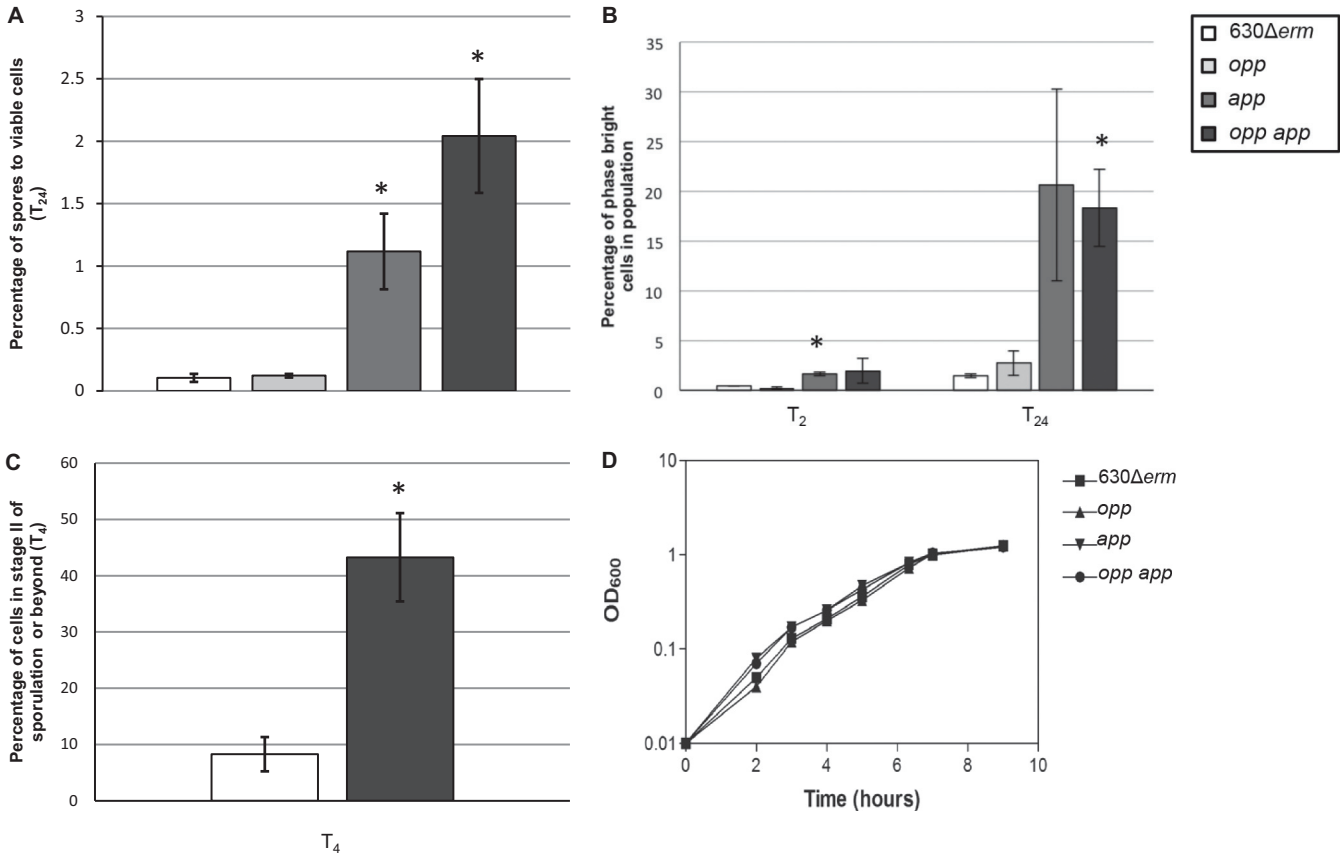


FIG 2 Disruption of the *opp* and *app* operons results in an increase in sporulation frequencies. (A) Sporulation frequency (CFU ml^{-1} ethanol-resistant spores) of 630Δ*erm*, MC296 (*opp*), MC301 (*app*), and MC307 (*opp app*) grown in 70:30 sporulation medium at T_{24}. (B) Percentage of phase-bright spores assessed by phase-contrast microscopy. (C) Percentage of cells that have a polar septum or partially/completely engulfed forespores, assessed by fluorescence microscopy using the membrane-specific dye FM4-64. (D) Representative growth curve of 630Δ*erm*, MC296 (*opp*), MC301 (*app*), and MC307 (*opp app*) grown in 70:30 sporulation medium. The means and standard errors of the means for spore counts of at least two biological replicates are shown. *, $P \leq 0.05$ by two-tailed Student's *t* test.

of the *app* operons from their native promoters on a plasmid did not effectively complement either the *app* or the *opp app* double mutant (see Fig. S4 in the supplemental material). We performed transcriptional analysis to determine the levels of *opp* and *app* expression from plasmids in the complemented strains. Transcript levels of *oppB* were increased ~25-fold in the respective complemented strains (MC322 and MC323), while *appA* and *appD* transcript levels were upregulated ~60- to 120-fold in the respective complemented strains (MC331 and MC334; see Fig. S5 in the supplemental material). These data reveal that expression of the *app* operons in *trans* results in significantly increased levels of the *app* transcript compared to the levels of the native *app* transcript during log-phase growth. Increased *app* transcription may result in a dysfunctional App system that may cause additional stress on the strains harboring plasmid copies of the *app* operons. We were unable to complement the transporter mutants in a single copy on the chromosome with current technologies due to size constraints (>5 kb) and toxicity issues. Notably, the sporulation frequency was increased in all strains containing a plasmid, including those with only the vector control (data not shown). This increase in sporulation may be due to the energy burden of the plasmid or the addition of thiamphenicol to the medium for plasmid maintenance, although these possibilities are not mutually exclusive.

Loss of Opp and App affects the expression of genes required for initiation of spore formation. The increased sporulation frequencies of the *app* and *opp app* transporter mutants may result from earlier and/or higher transcription of key sporulation-specific genes or other post-exponential-phase control genes during growth. To determine if sporulation-dependent gene expression correlates with the increased sporulation phenotype of the *app* and *opp app* mutants, we analyzed early sporulation gene expression during growth in 70:30 sporulation medium, starting with the master regulator of sporulation, Spo0A. Spo0A is a DNA-binding protein that directly and indirectly regulates many stationary-phase processes, including sporulation. *spo0A* transcription is regulated by multiple global regulators, including positive autoregulation of its own transcription, and Spo0A activity in other sporeformers is tightly controlled at multiple levels by transcriptional, posttranscriptional, and posttranslational mechanisms (3, 59, 60, 67, 68). Expression of *spo0A* was slightly elevated (~1.5-fold) in the *opp app* double mutant during exponential growth phase compared to that in the parent strain (Fig. 4A). We also analyzed the expression of the *murG* gene, which is a direct target of active Spo0A (69). *murG* transcript levels increased in the *app* and *opp app* mutants at the T_4 and T_8 time points (~3-fold compared to those for the parent strain; Fig. 4B), indicating that

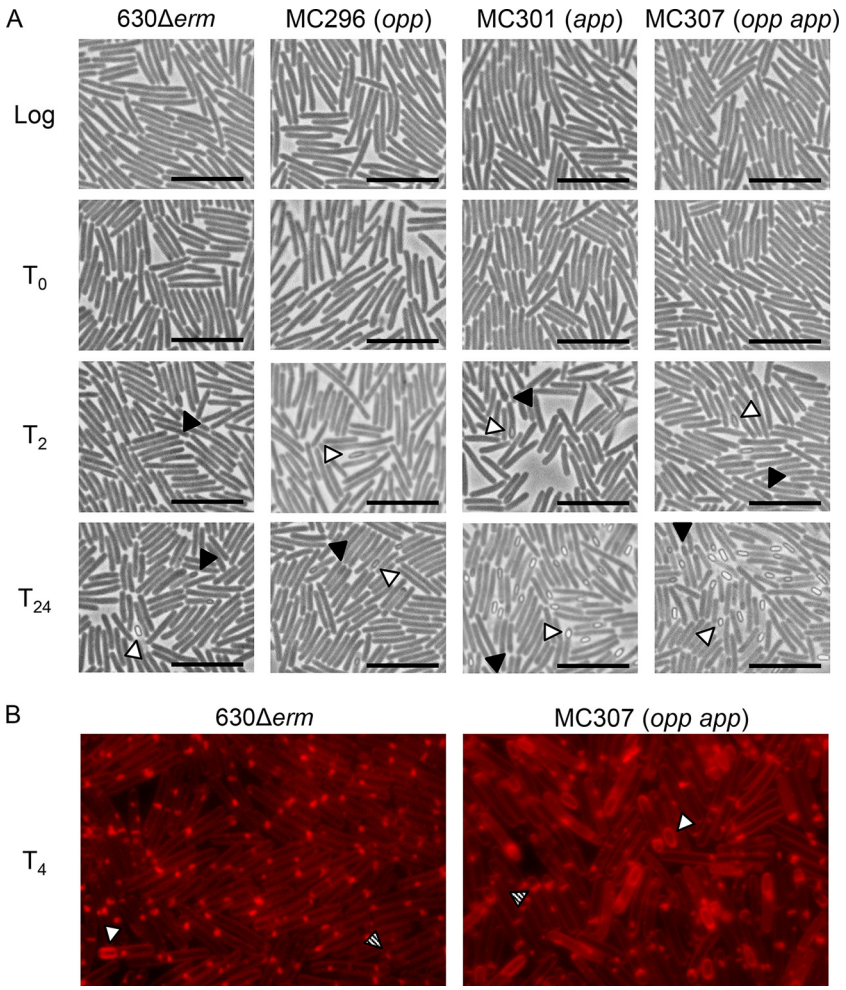
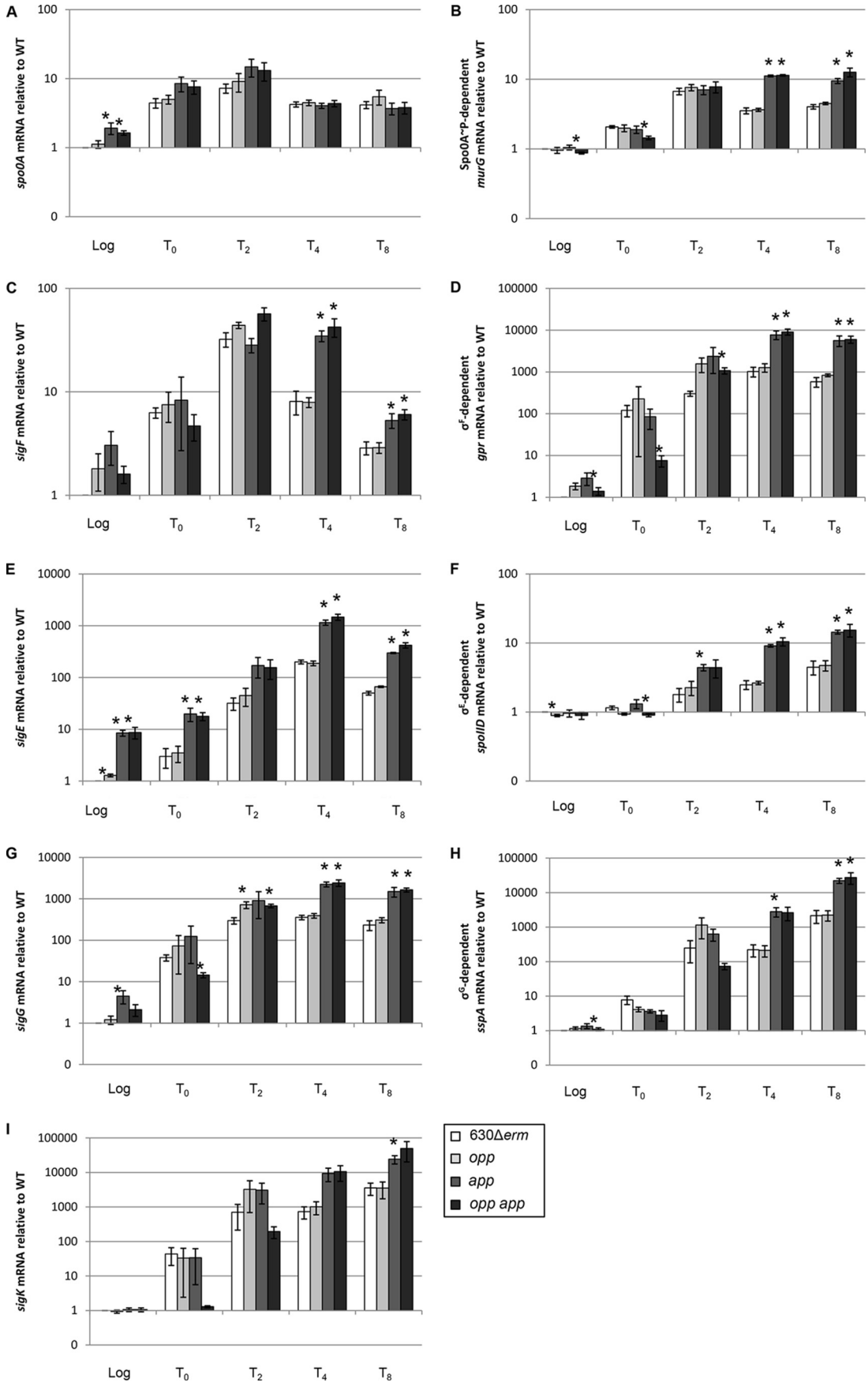


FIG 3 Oligopeptide transporter mutants hypersporulate and form spores earlier than the wild-type strain. (A) Phase-contrast microscopy of 630Δ*erm*, MC296 (*opp*), MC301 (*app*), and MC307 (*opp app*) grown in 70:30 sporulation medium. Filled arrowheads, phase-dark prespores; open arrowheads, phase-bright mature spores. Bars, 10 μm. (B) Fluorescence microscopy of 630Δ*erm* and MC307 (*opp app*) grown in 70:30 sporulation medium and stained with FM4-64. Open arrowheads, partially or completely engulfed forespores; patterned arrowheads, cells with polar septa. For both phase-contrast and fluorescence microscopy, samples were removed at the indicated times and prepared for microscopy as described in Materials and Methods.

more Spo0A is active and Spo0A-dependent gene expression is upregulated in the absence of the App transporter.

To determine if subsequent sporulation-specific gene transcription differs in the transporter mutants, we used qRT-PCR to analyze the expression of sporulation sigma factors and genes dependent upon activation of each sigma factor. qRT-PCR analysis was performed to assess the expression and activities of the sporulation sigma factors. SigF (σ^F) controls early forespore-specific sporulation gene expression (47, 69, 70). Transcript analysis revealed that the level of *sigF* mRNA is increased in the *app* and the *opp app* transporter mutants at T_4 and T_8 (~4-fold and ~2-fold, respectively; Fig. 4C), and expression of a σ^F-dependent gene, *gpr*, is also up at T_4 (~8-fold) and T_8 (~10-fold) compared to that in the parent strain (Fig. 4D). *gpr* transcript levels were ~4- to 8-fold higher in the *app* single mutant and the *opp app* double mutant at T_2. These data indicate that σ^F gene expression and activity are increased in the App transporter mutants in stationary phase. SigE (σ^E) is responsible for transcription of early sporulation-specific genes in the mother cell compartment (71), and *sigE* transcription is directly upregulated only by active Spo0A (47, 69, 70, 72). The *app* and *opp app* mutants expressed more *sigE* transcript than the parent cells during logarithmic and stationary phases of growth (~5- to 10-fold; Fig. 4E). In addition, analysis of the σ^E-dependent transcript, *spoIID*, showed that σ^E-dependent gene expression is increased ~3-fold at stationary phase in the *app* and *opp app* mutants compared to that in the parent or *opp* mutant strain (Fig. 4F) (73). These data indicate that early sporulation-specific gene expression proceeds earlier and is increased at stationary phase in the *app* and *opp app* mutants.

To further understand sporulation progression in the transporter mutants, we analyzed expression of additional mid- to late-stage sporulation genes. In contrast to *B. subtilis*, the order and synchronization of sporulation sigma factor expression and activation are not tightly regulated in *C. difficile* (47, 69, 70), but increased expression of these sigma factors and genes dependent on their activation is positively correlated with the later stages of spore development. Transcription of *sigG*, the forespore-specific late sigma factor, was higher (~2- to 3-fold) in the App transporter mutants by T_2 and was increased even further (~6- to 7-fold) by later time points (Fig. 4G). Transcription of the σ^G-


A
spo0A mRNA relative to WT
B
Spo0A~P-dependent murG mRNA relative to WT
C
sigF mRNA relative to WT
D
σF-dependent gpr mRNA relative to WT
E
sigE mRNA relative to WT
F
σE-dependent spoIID mRNA relative to WT
G
sigG mRNA relative to WT
H
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I
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T8
630Δerm
opp
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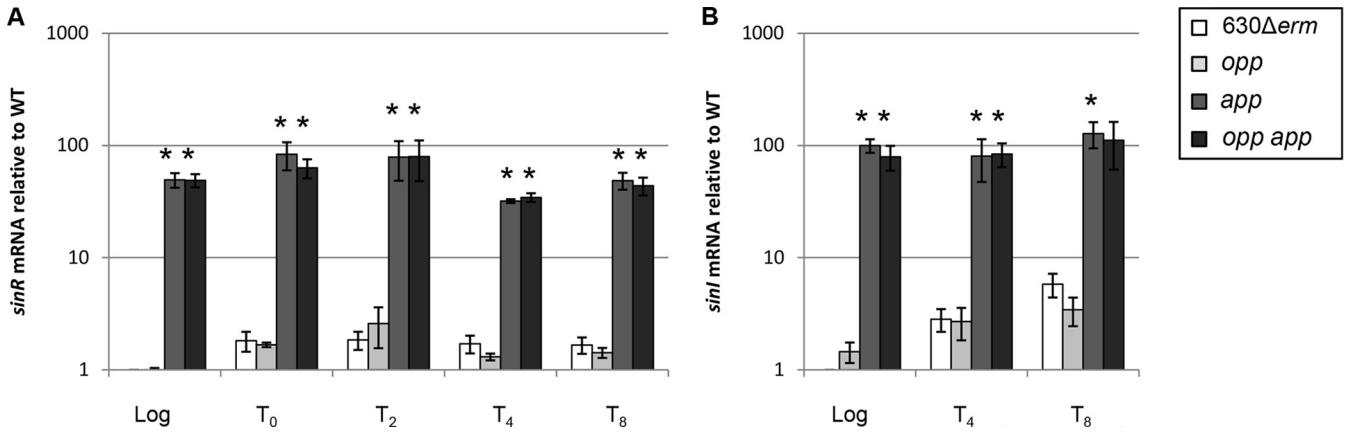

FIG 5 Effects of oligopeptide transporters on regulation of *sinR* and *sinI* gene expression. qRT-PCR analysis of expression of *sinR* (A) and *sinI* (B) in 630Δ*erm*, MC296 (*opp*), MC301 (*app*), and MC307 (*opp app*) grown in 70:30 sporulation medium to OD_{600}s of 0.5 (log phase) and 1.0 (start of stationary phase; T_0) and 2, 4, or 8 h after T_0 (T_2, T_4, and T_8), as described in Materials and Methods. The means and standard errors of the means of at least three biological replicates are shown. *, $P \leq 0.05$ by a two-tailed Student's *t* test.

dependent gene, *sspA*, was also increased in stationary phase in the *app* and *opp app* mutants (~10- to 13-fold; Fig. 4H). σ^K, the late-stage mother cell sigma factor, contains a 14.6-kb *skin*^{cd} element that must be excised prior to translation (74). σ^K activity is necessary for efficient sporulation but is not strictly required for the formation of heat-resistant spores (70). Expression of *sigK* in the App transporter mutants was upregulated ~7- to 15-fold at T_4 and T_8 (Fig. 4I). Together, these results demonstrate that sporulation genes are expressed earlier and at higher levels in the *app* and *opp app* mutants, consistent with their increased sporulation phenotype observed *in vitro*.

In *B. subtilis*, transcription of *opp* and *app* is repressed by the DNA-binding negative regulator of sporulation, ScoC (75). *C. difficile* contains a putative *scoC* ortholog (CD0852) encoded upstream of the *opp* operon. Preliminary data suggest that overexpression of CD0852 in *C. difficile* negatively impacts sporulation (A. N. Edwards and S. M. McBride, unpublished data). qRT-PCR analysis revealed that *oppB*, *appA*, and *appD* transcript levels at stationary phase of growth (T_2) were unchanged between the wild-type strain and a CD0852-overexpressing strain (see Fig. S2D in the supplemental material), suggesting that the *opp* and *app* operons are not under the same regulatory control in *C. difficile* as they are in *B. subtilis*. Altogether, these results indicate that the Opp and App transporters play a different role in controlling sporulation in *C. difficile* than in other spore-forming bacteria, such as *B. subtilis*.

The absence of Opp and App transporters influences expression of the transcriptional regulator SinR and its putative inhibitor, SinI. As the Opp and App transporters are not predicted to have direct DNA-binding domains or regulatory capabilities, the changes in gene expression observed in the transporter mutants likely result from the lack of peptides feeding into nutritional and/or regulatory pathways. The two global regulatory proteins known to control cellular responses to nutrient availability in *C. difficile* are CodY and CcpA (39, 42, 76–78). CodY is a DNA-binding transcriptional regulator that controls the expression of hundreds of genes in response to the intracellular availability of GTP and branched-chain amino acids (76, 79). CcpA mediates carbon catabolite repression in many Gram-positive bacteria, including *C. difficile*, and controls the expression of a wide range of genes involved in sugar uptake, fermentation, and amino acid metabolism (59, 77). Both CodY and CcpA directly control the transcription of gene products required for sporulation initiation and toxin synthesis in *C. difficile* and other sporulating pathogens (59, 76, 77, 80, 81). To determine if either of these global regulatory proteins is involved in mediating the increase in spore formation observed in the *app* and *opp app* mutants, we investigated the effects of the transporter mutations on CodY-mediated and CcpA-mediated gene regulation. qRT-PCR analysis of samples taken during logarithmic- and stationary-phase growth in 70:30 sporulation medium revealed that the expression of multiple CodY- and CcpA-dependent genes was not greatly influenced in the transporter mutants (see Fig. S6 in the supplemental material), suggesting that inactivation of the transporters is likely not signaling through CodY or CcpA to control sporulation. However, we found that expression of the CcpA-dependent *sinR* transcript was greatly elevated (~30- to 80-fold) in logarithmic and stationary phases in the *app* and *opp app* mutants (Fig. 5A) (59). In other species, SinR acts as a repressor of sporulation by directly inhibiting Spo0A transcription (82). Although transcription of *sinR* is elevated, the SinR protein can be held inactive in a complex with its repressor, SinI (83). *C. difficile* encodes a *sinI*-like regulator adjacent to *sinR*. Transcription of the putative *sinI* was also amplified in the *app* and *opp app* mutants (~80- to 125-fold; Fig. 5B), suggesting that the inhibitory effects of SinR on sporulation are negated by SinI in *C. difficile*. These results suggest that the func-

FIG 4 Effects of oligopeptide transporters on the regulation of sporulation-specific gene transcription. qRT-PCR analysis of *spo0A* (A), the Spo0A-dependent gene *murG* (B), *sigF* (C), the σ^F-dependent gene *gpr* (D), *sigE* (E), the σ^E-dependent gene *spoIID* (F), *sigG* (G), the σ^G-dependent gene *sspA* (H), and *sigK* (I) expression in 630Δ*erm*, MC296 (*opp*), MC301 (*app*), and MC307 (*opp app*) grown in 70:30 sporulation medium to OD_{600}s of 0.5 (log phase) and 1.0 (start of stationary phase; T_0) and 2, 4, and 8 h after T_0 (T_2, T_4, and T_8, respectively), as described in Materials and Methods. The means and standard errors of the means of at least three biological replicates are shown. WT, wild type. *, $P \leq 0.05$ by a two-tailed Student's *t* test.

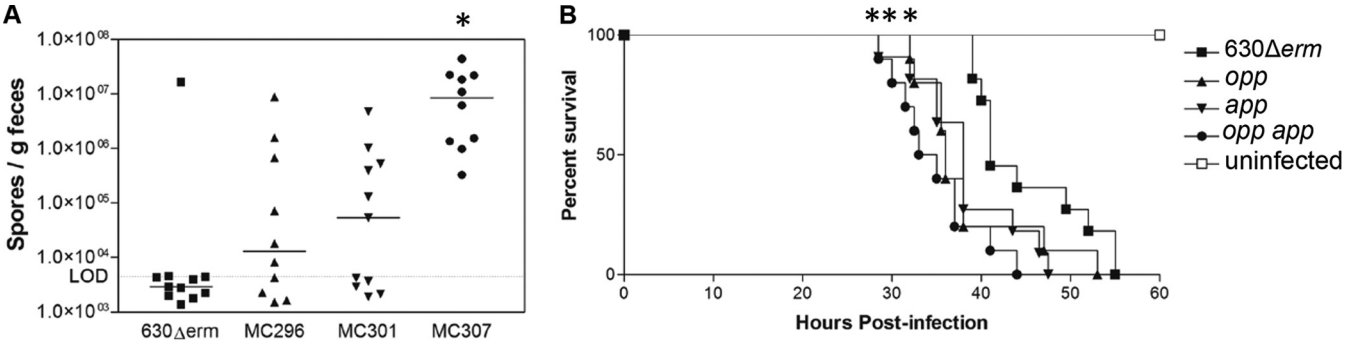

FIG 6 Effects of oligopeptide transporters on fecal spore output and virulence in the hamster model of CDI. (A) Total number of *C. difficile* spores per gram of feces recovered at 24 h postinfection from two independent experiments of clindamycin-treated Syrian golden hamsters inoculated with 500 spores of *C. difficile* 630Δ*erm*, MC296 (*opp*), MC301 (*app*), or MC307 (*opp app*). Fecal samples were weighed, resuspended in 1× PBS, and plated onto TCCFA plates (95, 96), and *C. difficile* colonies were enumerated after 48 h. Solid lines, the median for each strain; dotted line, limit of detection (4.51×10^3 CFU/g). (B) Kaplan-Meier survival curve representing the cumulative results from the same two independent experiments described in the legend to panel A. Hamsters infected with the mutant strains succumbed more rapidly than those infected with the parent strain. Mean times to morbidity were as follows: 630Δerm, 45.1 ± 6.4 h; MC296 (*opp*), 38.4 ± 6.6 h; MC301 (*app*), 38.2 ± 5.8 h; and MC307 (*opp app*), 35.0 ± 4.9 h ($P < 0.02$ for all mutants, log rank test).

tion of the App transporter influences *sinR* and *sinI* gene expression and, potentially, the initiation of spore formation under the conditions tested.

Loss of Opp and App transporters results in increased virulence and spore formation in an animal model of CDI. The conditions that lead to the sporulation of *C. difficile* within the intestinal environment are not known. What is known is that *C. difficile* initiates sporulation *in vivo* within hours after germination and that *de novo* spores can be detected within 12 h postinoculation (84, 85). To determine if the Opp and App transporters influence sporulation frequency in the intestinal environment, we examined the effects of the *opp*, *app*, and *opp app* mutations on spore production and virulence in the hamster model of *C. difficile* infection (CDI), a model that has been used for more than 35 years to study acute disease caused by *C. difficile* (52, 86, 87). Female Syrian golden hamsters were infected by gavage with approximately 500 spores of either the parental strain or the transporter mutants. Animals were closely monitored for symptoms of disease, and fecal samples were retrieved to quantify the number of spores shed during infection, as described in Materials and Methods. As shown in Fig. 6A, hamsters infected with the *opp*-, *app*-, and both *opp*- and *app*-null mutants shed greater numbers of spores in feces than those infected with the parent strain at 24 h postinfection. On average, *opp app* mutant-infected animals shed approximately 8.5 times the number spores per gram of feces than wild-type-infected animals. The number of vegetative cells present in feces was not calculated, as exposure to oxygen could not be eliminated from our test conditions. Likewise, the number of spores located throughout the gastrointestinal tract was not assessed.

All of the hamsters infected with transporter mutant strains succumbed to disease more rapidly than those infected with the parent strain ($P < 0.02$, log rank test; mean times to morbidity, 45.1 ± 6.4 h for 630Δ*erm*, 38.4 ± 6.6 h for MC296 [*opp*], 38.2 ± 5.8 h for MC301 [*app*], and 35.0 ± 4.9 h for MC307 [*opp app*]; Fig. 6B). Hamsters infected with the transporter mutant strains lost weight and displayed symptoms earlier than wild-type-infected animals, but no statistically significant difference in weight loss at the time of death between hamsters infected with different strains was observed (data not shown). All animals lost a minimum of 6% of their body weight during infection. As described in other studies, a few animals did not exhibit wet tail prior to becoming moribund (88). The hypervirulent phenotypes of the transporter mutants demonstrate that these transporters are not required for growth *in vivo* but instead may indirectly function to influence toxin production or other virulence phenotypes in the host. Interestingly, the *opp* mutant exhibited a phenotype similar to that of the *app* mutant in the hamster model of CDI, despite the fact that no significant phenotype was observed *in vitro*. These data demonstrate that the strain 630 *opp* operon, containing *oppF* as a pseudogene, has an important function *in vivo*.

Production of both toxin A and toxin B by *C. difficile* 630 is required for full virulence in the hamster model of CDI; however, toxin A or toxin B alone is sufficient to cause disease (89). It was previously established that toxin production in *C. difficile* is upregulated in response to nutrient limitation, including amino acid starvation (39, 76–78, 90). We asked whether expression of the toxin genes *tcdA* and *tcdB* was upregulated in the transporter mutants *in vitro*, potentially explaining the observed hypervirulent phenotype. qRT-PCR analysis of the *tcdA* and *tcdB* transcripts revealed that there was no difference in toxin expression between the parent strain and the *opp*, *app*, and *opp app* mutants, except for a modest decrease in *tcdA* transcript levels in the *app* and the *opp app* mutants in late stationary phase, when grown in 70:30 medium (Fig. 7A and B). To determine if toxin production is upregulated *in vivo*, RNA was isolated from cecal contents obtained at the time of death from hamsters infected with the parental or *opp app* mutant strain. As a control, we analyzed RNA isolated from the cecal contents of an uninfected animal. We recovered insignificant levels of the *rpoC* transcript and were unable to detect specific *C. difficile* gene transcripts in uninfected controls (data not shown). qRT-PCR analysis revealed that *tcdA* and *tcdB* gene expression was slightly decreased in hamsters infected with the *opp app* double mutant compared to those infected with the parent strain (Fig. 7C). Additionally, the transcript levels of the sporulation-specific gene *sigE* and the negative transcriptional regulator *sinR* in RNA isolated from cecal contents were slightly increased in the *opp app* double mutant (Fig. 7C). These results suggest that the hypervirulent phenotype observed in hamsters infected with the oligopeptide transporter mutants is not directly correlated with toxin pro-

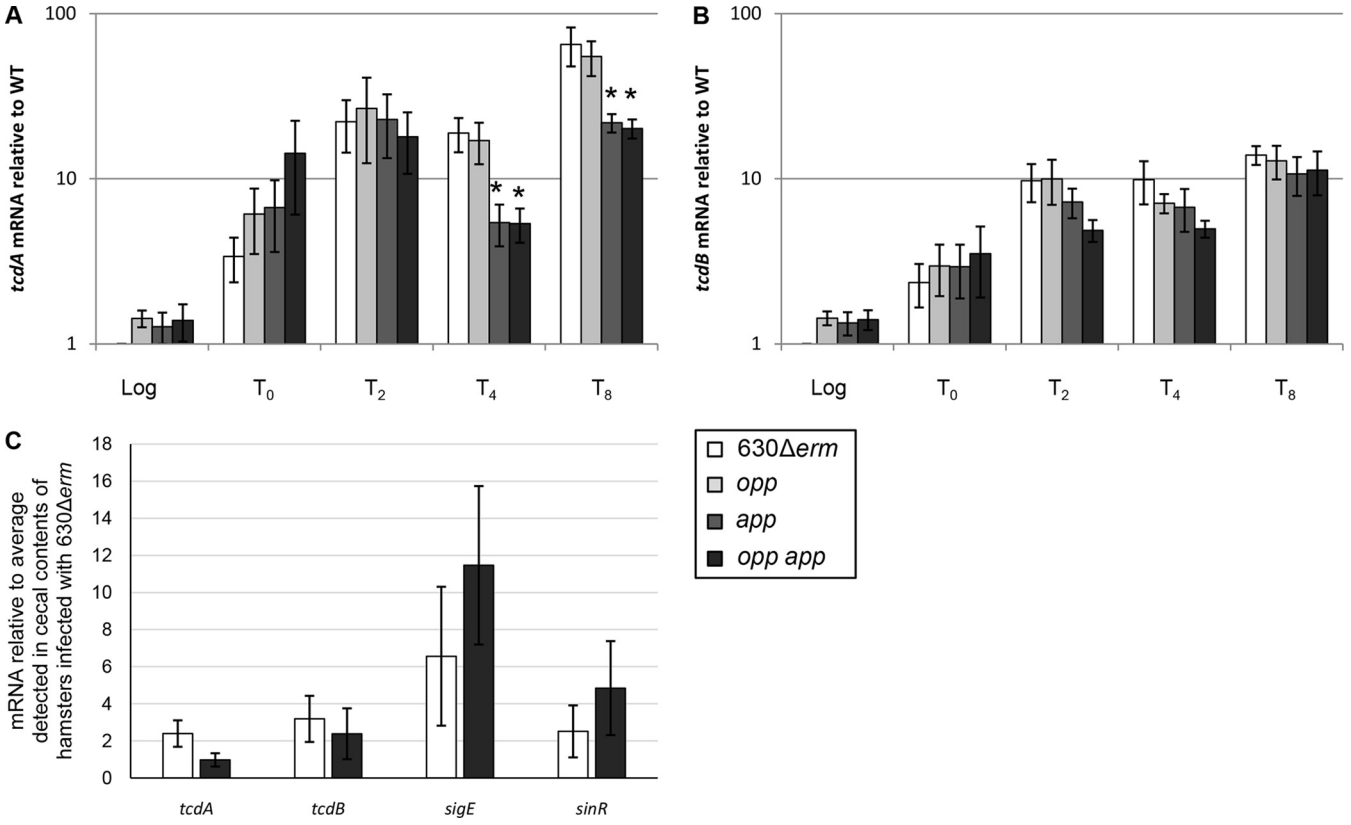

FIG 7 Effects of oligopeptide transporters on toxin gene expression. (A, B) qRT-PCR analysis of *tcdA* (A) and *tcdB* (B) expression in 630Δ*erm*, MC296 (*opp*), MC301 (*app*), and MC307 (*opp app*) grown in 70:30 sporulation medium to OD_{600}s of 0.5 (log phase) and 1.0 (start of stationary phase; T_0) and 2, 4, or 8 h after T_0 (T_2, T_4, and T_8), as described in Materials and Methods. The means and standard errors of the means of four biological replicates are shown. *, $P \leq 0.05$ by a two-tailed Student's *t* test. The key at the bottom right applies to panels A and B. (C) qRT-PCR analysis of *tcdA*, *tcdB*, *sigE*, and *sinR* gene expression in cecal contents from hamsters infected with either 630Δ*erm* or MC307 (*opp app*). The means and standard errors of the means of samples from at least 10 animals per strain are shown. *, $P \leq 0.05$ by a two-tailed Student's *t* test.

duction but may be related to the increased sporulation phenotype characterized *in vitro* and *in vivo*.

DISCUSSION

As an obligate anaerobe, the ability of *C. difficile* to form dormant spores is required for the effective spread of this pathogen from host to host. Although spore morphology and the stages of spore formation are similar among the spore-forming *Firmicutes*, the regulatory pathways and environmental triggers that control the initiation of sporulation are not conserved in *C. difficile* (2). While spore formation begins in response to nutrient deprivation and cell density in the well-studied model organism *B. subtilis* (91), these signals were not previously proven to influence the sporulation of *C. difficile* (59, 77). Oligopeptide transporters have been shown to activate sporulation in other bacteria through the import of specific quorum-sensing peptides that indirectly upregulate early sporulation-specific gene transcription (11–13). In the current work, we demonstrate that the Opp and App transporters play a significant role in *C. difficile* sporulation and pathogenesis *in vitro* and *in vivo*, but the role of these transporters in *C. difficile* sporulation differs from that in *B. subtilis*.

Our initial hypothesis was that the *C. difficile* oligopeptide transporters could be stimulating sporulation in a cell density-dependent manner through the uptake of small secreted quorum-signaling peptides, similar to the Phr peptides found in spore-forming *Bacillus* species (11–13). Surprisingly, the Opp and App transporters negatively impact both the initiation of spore formation and the total number of spores formed both *in vitro* and *in vivo* (Fig. 2A to C, 3, and 6A). The data indicate that in *C. difficile* these transporters are not required for cell density-dependent signaling. This conclusion is supported by the absence of *phr* orthologs in the *C. difficile* genome (2, 53, 54). Furthermore, addition of spent medium to exponentially growing wild-type *C. difficile* did not affect early sporulation gene expression (data not shown). One hypothesis is that *C. difficile* could import a signaling peptide that inhibits sporulation at low cell density; however, this is unlikely, given the ecological niche that *C. difficile* inhabits. Alternatively, other quorum-sensing mechanisms, such as *agr*, may potentially fill the role of the Phr system. *C. difficile* may not maintain a high population density within the gastrointestinal tract due to frequent diarrhea, which results in the transient availability of nutrients and other metabolites. We favor the hypothesis that the Opp and App transporters serve to import general oligopeptides that influence the intracellular nutrient availability (Fig. 8). In an effort to demonstrate directly the function of the transporters, we examined the efficacy of peptide uptake (random 5- and 10-amino-acid peptides) in the wild-type and mutant strains in the absence of other amino acid sources; however, both the wild-type and transporter mutants were unable to utilize 5- or 10-amino-

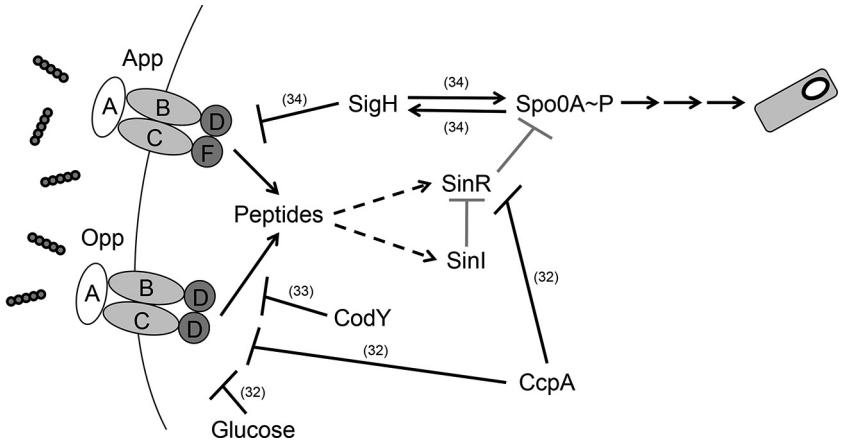

FIG 8 Proposed model of Opp and App influence on sporulation initiation in *C. difficile*. Opp and App mediate the uptake of peptides as a nutrient source. The acquisition of peptides indirectly influences both *sinR* and *sinI* gene expression through an unknown mechanism which represses sporulation-specific gene expression. Gray arrows, putative regulatory effects, hatched arrows, indirect effects. References for experimentally proven interactions are denoted in parentheses.

acid peptides as their sole source of amino acids in minimal defined medium (data not shown) (39). Our data suggest that without the Opp and App transporters, sporulation-specific gene programming is initiated, likely due to intracellular signals of poor nutritional and environmental conditions. Finally, the regulation of *opp* and *app* expression is different in *C. difficile* and *B. subtilis* (see Fig. S2D in the supplemental material), suggesting that *opp* and *app* are not influencing sporulation similarly in both species.

In contrast to *B. subtilis*, mutants defective in the Opp and App transporters express the genes for certain sporulation regulatory proteins (Spo0A, σ^F, and σ^E) prematurely, suggesting that their normal activities cause the uptake of peptide nutrients that block the initiation of sporulation (Fig. 4). Although the complete genetic pathway through which transporter activity signals sporulation initiation remains unclear, our data suggest that these signals may involve both SinR and SinI (Fig. 5). In *B. subtilis*, SinR functions as a homotetramer and directly represses a number of genes involved in sporulation initiation, including *spo0A* (82). However, SinR repression is relieved by SinI, which inhibits SinR-mediated repression by disrupting the SinR homotetramer and forming an inactive heterodimer with SinR (83). In *C. difficile*, *sinR* and *sinI* (CD2214 and CD2215, respectively) appear to form an operon. Our preliminary data indicate that the increase in *sinI* transcript levels was greater than the increase in *sinR* levels in the *app* and *opp app* mutants as cells entered sporulation (Fig. 5). These data suggest that *sinR* and *sinI* are differentially expressed, as they are in *B. subtilis* (92). A greater increase in SinI levels than SinR levels would allow the efficient deactivation of SinR-mediated *spo0A* repression if these proteins function like the *B. subtilis* orthologs. Even a minimal increase in *spo0A* transcription and subsequent activation may lead to the increases in transcription observed for the phosphorylated Spo0A (Spo0A~P)-dependent gene, *sigE* (Fig. 4E), and influence overall sporulation frequencies in the transporter mutants.

The regulatory pathway through which the intracellular amino acid available is signaled to initiate sporulation is unknown. Our data suggest that CodY and CcpA are not involved in this regulatory pathway, as expression of many direct targets, including the *tcdA* and *tcdB* genes (76, 77), are unchanged or nonuniformly changed in the transporter mutants (Fig. 7; see also Fig. S6 in the supplemental material). Determining the intermediates of the genetic pathway linking Opp and App transporter activity and sporulation initiation would provide further evidence of the environmental signals that regulate the initiation of spore formation and would fill a gap in the regulatory mechanisms governing the early stages of sporulation in *C. difficile* as well as nutrient acquisition.

The Opp and App transporters are not expected to be the only mechanisms by which *C. difficile* could obtain extracellular amino acids. *C. difficile* has orthologs of several individual amino acid importer proteins found in other species, as well as putative di- and tripeptide transporters. Additional oligopeptide transporters present in other spore-forming species include the Dpp and DtpT transporter systems (93, 94). However, the *C. difficile* genome does not contain a full *dpp* transporter complement. The *C. difficile* genome possesses two DtpT orthologs (CD2260 and CD3036), but their role in peptide uptake is unknown.

In our assays, the Opp transporter did not have a significant effect on sporulation *in vitro*. However, overexpression of the *opp* operon from its native promoter in the *opp app* mutant fully restored wild-type levels of sporulation, indicating that this operon encodes a functional oligopeptide transporter (see Fig. S4 in the supplemental material). In addition, the effects of the loss of *opp* function *in vivo* were similar to those of the loss of *app* function, and the *opp app* double mutant resulted in more fecal spores and a shorter time to morbidity compared to the number of fecal spores and time to morbidity obtained with the single mutants in the hamster model of infection (Fig. 6). Interestingly, a functional *oppF* gene is required in *B. subtilis* for competence but not for sporulation (61, 62). However, no natural competence system in *C. difficile* has been described, nor does the genome contain many of the orthologs required for competence in *Bacillus* species (53).

It is unclear why the transporter mutants are hypervirulent, despite no apparent increase in toxin expression within the population, *in vitro* or *in vivo* (Fig. 7). Our data do not account for differences in secreted toxin between the transporter mutants and the parent strain. When these experiments were performed, we did not anticipate the increased virulence of the mutants or the need to acquire additional samples for toxin assays. However, no

difference in the rate of lysis, which would result in toxin release, was observed between the parent strain and the transporter mutants *in vitro* (see Fig. S7 in the supplemental material). It is possible that the inability to import peptides as a nutrient source affects growth rates or results in the upregulation of other virulence factors, such as motility or adherence, *in vivo*. We did not observe a difference in growth rates (Fig. 2D) or in motility (data not shown) between the parent strain and the transporter mutants *in vitro*. Although inactivation of Opp and App results in increased sporulation and increased virulence *in vivo*, it does not necessarily suggest that these phenotypes are controlled through the same regulatory pathways. Further studies are needed to determine how nutrient limitation and increased sporulation contribute to more severe disease *in vivo*.

Finally, differences in ecological niches could explain much of the divergence in the genetic mechanisms of spore initiation and patterns of gene expression between bacterial sporeformers. We hypothesize that *C. difficile* does not require a quorum-sensing system to signal sporulation because *C. difficile* never inhabits an environment where cell population density directly correlates with nutrient availability. Rather, *C. difficile* relies on directly linking the availability of environmental nutrients, such as amino acids, to the regulatory mechanisms controlling sporulation initiation. This direct link between nutrient availability and sporulation allows *C. difficile* to quickly respond to changing conditions as the organism transits through the gastrointestinal tract. The findings presented here provide the first evidence of a nutritional link to spore formation in *C. difficile* and represent a mechanism that could potentially be exploited to prevent spore formation and, thereby, the spread of the pathogen.

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