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Clostridium difficile Extracytoplasmic Function σ Factor σ^V Regulates Lysozyme Resistance and Is Necessary for Pathogenesis in the Hamster Model of Infection

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***Clostridium difficile* is a clinically important pathogen and the most common cause of hospital-acquired infectious diarrhea. Expression of the *C. difficile* gene *csfV*, which encodes σ^V, an extracytoplasmic function σ factor, is induced by lysozyme, which damages the peptidoglycan of bacteria. Here we show that σ^V is required for lysozyme resistance in *C. difficile*. Using microarray analysis, we identified the *C. difficile* genes whose expression is dependent upon σ^V and is induced by lysozyme. Although the peptidoglycan of wild-type *C. difficile* is intrinsically highly deacetylated, we have found that exposure to lysozyme leads to additional peptidoglycan deacetylation. This lysozyme-induced deacetylation is dependent upon σ^V. Expression of *pdaV*, which encodes a putative peptidoglycan deacetylase, was able to increase lysozyme resistance of a *csfV* mutant. The *csfV* mutant strain is severely attenuated compared to wild-type *C. difficile* in a hamster model of *C. difficile*-associated disease. We conclude that the σ^V signal transduction system, which senses the host innate immune defense enzyme lysozyme, is required for lysozyme resistance and is necessary during *C. difficile* infection.**

Clostridium *difficile* is an anaerobic, Gram-positive, spore-forming opportunistic pathogen and is the most common cause of hospital-acquired infectious diarrhea in the United States (1–3). *C. difficile* infections can range from mild diarrhea to a life-threatening pseudomembranous colitis. In recent years, both the incidence and severity of *C. difficile* infections have increased in susceptible populations as well as in groups not traditionally considered at risk (4–6).

The major known virulence determinants of *C. difficile* are TcdA and TcdB, members of the large clostridial glucosylating toxin family (7, 8). TcdB and, to a lesser degree, TcdA are required for *C. difficile* pathogenesis, but not colonization, in the hamster model of *C. difficile* infections (7, 9, 10). Expression of the toxin genes is tightly regulated by a number of factors. The alternative σ factor TcdR is required for *tcdA* and *tcdB* transcription (11). In addition to TcdR, expression of the toxin genes is negatively regulated by two global regulators, CodY and CcpA (12, 13). Although the toxins are required for the more severe sequelae of a *C. difficile* infection, virulence factors that enable evasion of host immune defenses and intestinal colonization likely play important roles during the course of infection.

Peptidoglycan, the major component of the cell wall, is essential for bacterial survival. Peptidoglycan confers shape and rigidity to the bacterial cell and prevents osmotic lysis. The peptidoglycan of most bacteria is composed of repeating subunits of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), which form a polysaccharide backbone that is cross-linked by peptide side chains. Lysozyme is a hydrolytic enzyme which cleaves the β(1-4) linkage between MurNAc and GlcNAc in the backbone of peptidoglycan. Lysozyme is a critical component of the innate immune system which can be found in abundance in macrophages and neutrophils (14).

Increased lysozyme resistance is an important virulence factor for numerous Gram-positive bacteria, including *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Listeria monocytogenes* (15–20). To resist killing by lysozyme, bacteria have evolved several mechanisms to modify the acetylation patterns of peptidoglycan, resulting in increased lysozyme resistance (14). There are four known mechanisms of peptidoglycan modification which provide lysozyme resistance. *Bacillus subtilis*, *S. aureus*, and *E. faecalis* utilize *O*-acetyltransferases to acetylate the OH group at C-6 of MurNAc (15, 16, 21, 22), while *Lactobacillus plantarum* adds an acetyl group to GlcNAc subunits (23). Additional acetylation of these sugars results in increased lysozyme resistance. Other bacteria increase their resistance to lysozyme by deacetylating the peptidoglycan polysaccharide backbone. Lysozyme resistance in *Bacillus cereus*, *Bacillus anthracis*, *Streptococcus suis*, and *S. pneumoniae* is increased by polysaccharide deacetylases which remove the acetyl group from the GlcNAc subunit of their peptidoglycan (20, 24–26). Similarly, a deacetylase, PdaC, from *B. subtilis* was recently found to be capable of removing an acetyl group from MurNAc (27). In these organisms, deacetylation of the peptidoglycan backbone results in increased lysozyme resistance.

Extracytoplasmic function (ECF) σ factors are often involved in response to extracytoplasmic stress and represent the "third pillar" of bacterial signal transduction (28–30). *C. difficile* encodes at least three ECF σ factors, σ^T, σ^U, and σ^V. The expression of the

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Clostridium difficile strains		
JIR8094	Spontaneous erythromycin-sensitive derivative of 630	36
TCD20	JIR8094 $csfV_{63}$::*ltrB*::*ermB*	
TCD28	JIR8094 $csfV_{63}$::*ltrB*::*ermB*/pMC123	
TCD25	JIR8094 $csfV_{63}$::*ltrB*::*ermB*/pTHE1073 (P_{xyl}-*csfV*)	
CDE2023	JIR8094 $csfV_{63}$::*ltrB*::*ermB*/pRPF185	
CDE2025	JIR8094 $csfV_{63}$::*ltrB*::*ermB*/pTHE1070 (P_{tet}-*pdaV*)	
TCD1250	JIR8094 $csfV_{63}$::*ltrB*::*ermB*/pTHE1070 (P_{tet}-*prsA2*)	
CDE2026	JIR8094 $csfV_{63}$::*ltrB*::*ermB*/pTHE1070 (P_{tet}-*pdaV-prsA2*)	
CDE1938	JIR8094 $cd0739_{48}$::*ltrB*::*ermB*	
CDE1930	JIR8094 $cd1607_{117}$::*ltrB*::*ermB*	
Escherichia coli strains		
Omnimax-2 T1R	F′ [*pro*AB+ *lacI*^{q} *lacZ*ΔM15 Tn*10*(TetR) Δ(*ccdAB*)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD*	Invitrogen
HB101/pRK24	F$^-$ *mcrB mrr hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*(*SmR*) *glnV44* λ$^-$ pRK24	51
Plasmids		
pCE240	P_{fac} *ltrB*::*ermBRAM ltrA cat repA oriT*	31
pBL100	P_{fac} *ltrB*::*ermBRAM colE1 bla cat CD6ori repA orfB oriT*	39
pCE294	pDR160-RfA *ccdB cat*	
pMC123	*colE1 bla cat CD6ori repA orfB oriT*	43
pTHE163	pENTR-D-*csfV*	31
pDR160	*colE1 bla aad9 xylR* P_{xyl}	D. Rudner
pRPF185	P_{tet} *colE1 bla cat repA orfB oriT aad9*	44
pCE380	pRPF185-P_{tet}-RfC.1 *ccdB cat*	
pTHE519	P_{fac} $ltrB_{csfV}$::*ermBRAM ltrA cat colE1 repA oriT*	
pCE356	P_{fac} $ltrB_{cd1607}$::*ermBRAM ltrA cat colE1 bla cat CD6ori repA orfB oriT*	
pCE358	P_{fac} $ltrB_{cd0739}$::*ermBRAM ltrA cat colE1 bla cat CD6ori repA orfB oriT*	
pCE327	pENTR-D-*pdaV*	
pTHE1057	pENTR-D-*prsA2*	
pTHE1058	pENTR-D-*pdaV prsA2*	
pTHE1069	*colE1 bla cat repA orfB oriT aad9 xylR* P_{xyl} RfA *ccdB cat*	
pTHE1072	*colE1 bla cat repA orfB oriT aad9 xylR* P_{xyl}-*csfV*	
pCE382	P_{tet}-*pdaV colE1 bla cat repA orfB oriT aad9*	
pCE400	P_{tet}-*prsA2 colE1 bla cat repA orfB oriT aad9*	
pCE383	P_{tet}-*pdaVprsA2 colE1 bla cat repA orfB oriT aad9*	

genes encoding these ECF σ factors is induced in response to extracellular stresses, including antimicrobial peptides or lysozyme (31). The *C. difficile* σ^V system is homologous to the σ^V systems found in *E. faecalis* and *B. subtilis*. In these organisms, σ^V is activated by lysozyme and transcribes the genes required to provide resistance to lysozyme (21, 32, 33). Activation of σ^V is achieved via the proteolytic destruction of an anti-σ factor RsiV (34, 35).

Previously, we demonstrated that lysozyme induced expression of *csfV*, which encodes σ^V (31). Here we report that σ^V is required for *C. difficile* lysozyme resistance. In response to lysozyme, σ^V induces deacetylation of GlcNAc of the peptidoglycan backbone. We demonstrate that σ^V-dependent lysozyme resistance and peptidoglycan deacetylation are partially due to the activity of a putative polysaccharide deacetylase, PdaV. Our studies indicate that *C. difficile* pathogenesis requires σ^V.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids used in this study are described in Table 1. The *C. difficile* strains are isogenic derivatives of JIR8094 (36), an erythromycin-sensitive derivative of sequenced clinical isolate 630 (37). *C. difficile* was grown in or on brain heart infusion medium (Gibco) supplemented with 0.5% yeast extract, 0.4% glucose, and 0.1% L-cysteine (BHIS) or in TY medium (0.4% tryptone, 0.5% yeast extract, 0.1% L-cysteine). *C. difficile* spores were germinated on cycloserine-cefoxitin-fructose agar (CCFA) and 0.1% sodium taurocholate (Sigma-Aldrich) plates (38). All *C. difficile* growth was conducted at 37°C in an anaerobic chamber (Coy Lab Products) with an atmosphere of 10% H_2, 5% CO_2, and 85% N_2. The following antibiotics (RPI Corp.) for *C. difficile* were used as needed: thiamphenicol (Thi; 10 μg/ml), erythromycin (Ery; 2.5 μg/ml), kanamycin (Kan; 50 μg/ml), and cefoxitin (Fox; 16 μg/ml) as needed. *Escherichia coli* strains were grown in or on LB medium at 37°C with ampicillin (Amp; 50 μg/ml), spectinomycin (Spt; 100 μg/ml), or chloramphenicol (Chl; 10 μg/ml) as needed.

Plasmid and bacterial strain construction. The oligonucleotide primers used in this work are listed in Table S1 in the supplemental material and were synthesized by IDT DNA Inc. (Coralville, IA). Plasmid cloning was performed in *E. coli* strain Omnimax-2 (Invitrogen). To construct the *csfV*, *cd0739*, and *cd1607* mutants, we retargeted plasmid pCE240 (31) or pBL100 (39) using oligonucleotides designed by a targetron algorithm generously provided by Rob Britton (Michigan State University) or ClosTron (40). The intron was PCR amplified using *Taq*

polymerase (NEB) from a targetron template using the EBS universal primer CDE914 in combination with the following primer sets: for $csfV_{63}$, CDEP958, CDEP956, and CDEP957; for $cd0739_{48}$, CDEP1497, CDEP1498, and CDEP1499; and for $cd1607_{117}$, CDEP1494, CDEP1495, and CDEP1496. The resulting PCR products were digested with HindIII and BsrGI and cloned into pCE240 or pBL100 digested with the same restriction enzymes. The resulting plasmids, pTHE519 ($csfV_{63}$), pCE356 ($cd1607_{117}$), and pCE358 ($cd0739_{48}$), were moved into *C. difficile* JIR8094 via the *E. coli* conjugation donor HB101/pRK24 (12). The $csfV_{63}$, $cd0739_{48}$, and $cd1607_{117}$ intron insertion mutants were generated as previously described (31, 41). The insertion of introns into *csfV*, *cd0739*, and *cd1607* was confirmed by PCR using primers complementary to the chromosome and/or intron.

The entire genome of the *csfV* mutant (TCD20) was sequenced using the Illumina platform (Iowa State University DNA Facility). This resulted in 10,522,538 reads, with an average coverage of 96 reads. Analysis of these sequences found that 86 reads contained overlap of at least 10 bp with the intron and the *C. difficile* chromosome. In all cases, the sequence indicates that the intron is inserted into *csfV*. This demonstrated that there was a single intron insertion in this strain located within *csfV* in an otherwise wild-type (WT) strain background. To exogenously express *csfV* in *C. difficile*, we constructed an *E. coli-C. difficile* shuttle vector with a P_{xyl} promoter from *B. subtilis* (42). We generated pCE294, a destination vector for the Gateway system (Invitrogen), which placed the RfA Gateway conversion cassette behind the xylose promoter of pDR160 (David Rudner, Harvard University) in a blunt-ended HindIII site. The spectinomycin resistance gene *aad9*, *xylR*, xylose promoter, and RfA Gateway cassette (BsrBI digested) were subcloned from pCE294 onto the pMC123 plasmid (43) (AfeI digested) to generate the pTHE1069 plasmid. The *csfV* gene was moved onto pTHE1069 from pJK3 (31) using LR clonase (Invitrogen) according to the manufacturer's instructions.

A tetracycline-inducible Gateway destination vector was constructed by digesting pRPF185 (44) with BamHI and SacI, generating blunt ends using Klenow and then cloning the RfA cassette with T4 ligase, resulting in pCE380. The *pdaV*, *prsA2*, or *pdaV-prsA2* genes were cloned onto the pENTR-D-TOPO plasmid using directional TOPO cloning (Invitrogen). The *pdaV*, *prsA2*, or *pdaV-prsA2* genes were moved from the pENTR vectors (pCE327, pTHE1057, or pTHE1058, respectively) onto the pCE380 plasmid using LR clonase (Invitrogen). The resulting plasmids, pCE382 (*pdaV*), pCE400 (*prsA2*), and pCE383 (*pdaV-prsA2*), were transformed into the conjugation donor HB101/pRK24 (12) to transfer these plasmids into *C. difficile* as previously described (31, 41). The strains containing the plasmids were confirmed by PCR using primers homologous to the plasmid-borne *catP* gene.

***C. difficile* virulence assays.** *C. difficile* spores used for infections were prepared as previously described (7, 45). The *C. difficile* wild-type and *csfV* mutant strains were spread on TY plates and incubated for 3 days at 37°C in an anaerobic chamber. The cells were resuspended in phosphate-buffered saline (PBS), and the vegetative cells were killed by heat inactivation at 65°C aerobically for 30 min. Spore preparations were washed extensively in PBS and stored at 4°C prior to use. Spore counts were determined by plating serial dilutions on BHI plates containing 0.1% taurocholate.

Adult Syrian gold hamsters (~90 to 120 g; Harlan Sprague-Dawley, Inc., Indianapolis, IN) were inoculated orally with clindamycin (Sigma; 30 mg/kg of body weight) 5 days prior to infection (7, 9, 45). Hamsters were inoculated with 1,000 spores of either wild-type or *csfV* mutant *C. difficile*. Hamsters were monitored twice daily for signs of severe morbidity prior to death and were euthanized. Fecal samples were obtained from each hamster daily beginning 1 day prior to clindamycin treatment until animals were moribund. Fecal samples were homogenized and serial dilutions plated on cycloserine-cefoxitin-fructose agar (CCFA) plus 0.1% taurocholate; data were used to determine the colonization state of the hamsters. The limit of detection for colonization in this assay is $\geq$1,000 spores/ml of resuspended stool. The data were graphed by Kaplan-Meier survival analysis (46) and compared for determination of statistical significance using the log rank test and the software GraphPad Prism (GraphPad Software, San Diego, CA). The animal experiments performed in this study were approved by the University of Iowa Institutional Animal Care and Use Committee.

Disk diffusion assays. Disk diffusion assays were performed by spreading 0.3 ml of *C. difficile* strains grown overnight in BHIS onto a BHIS plate and allowing the strains to dry for 15 min. Filter disks of Whatman no. 2 paper (~8-mm diameter) were saturated with 10 μl of hen egg white lysozyme (100 mg/ml), human lysozyme (100 mg/ml), bacitracin (20 mg/ml), vancomycin (4.5 mg/ml), ampicillin (1 mg/ml), nisin (15 mg/ml), and colistin (50 mg/ml) and allowed to dry for 15 min. After drying, filter disks were placed onto a lawn of *C. difficile* and incubated anaerobically at 37°C for 24 h before the zones of inhibition were measured.

MIC assays. To determine the MICs for antibiotic killing, we grew *C. difficile* strains overnight in TY broth. Overnight *C. difficile* cultures were diluted (1:40) in TY media containing different concentrations of hen egg white lysozyme, human lysozyme, bacitracin, vancomycin, ampicillin, nisin, and colistin in a 96-well microtiter plate. For all plasmid-containing strains, media were supplemented with thiamphenicol (10-μg/ml final concentration). For P_{tet} promoter plasmids, tetracycline was added to a final concentration of 100 ng/ml. After 20 h of anaerobic incubation, growth (optical density at 600 nm [OD_{600}]) was measured using a Tecan F50 microplate reader. Positive growth was considered an OD_{600} reading of >0.05.

Microarray analysis of σ^V regulon. Overnight cultures of *C. difficile* were grown in TY medium at 37°C. These were diluted 1:50 into 50 ml of fresh TY medium, grown to an OD_{600} of 0.8, and then exposed for 1 h to hen egg white lysozyme (50 μg/ml for the WT and 5 μg/ml for the *csfV* mutant). A 10-fold-lower concentration of lysozyme was used for the *csfV* mutant, as it is more sensitive to lysozyme than the wild type, in order to maintain similar effective lysozyme concentrations. The cells were harvested and fixed with an equal volume of a 50:50 mixture of acetone-ethanol and placed at −80°C until purification of RNA.

RNA samples and microarrays were processed by the University of Iowa Carver Center for Genomics (Iowa City, IA). RNA quality was assessed using the Experion automated electrophoresis system (Bio-Rad, Hercules, CA). Double-stranded cDNA was synthesized from 10 μg of total RNA in the presence of random primers using the SuperScript double-stranded cDNA synthesis kit (Invitrogen). Labeled cDNAs were prepared using a hybridization kit (Roche NimbleGen), and two technical replicates per sample were loaded on a custom gene expression microarray designed by Roche NimbleGen, Inc. (Prokaryote Expression 12x135K custom array delivery; design name, 101230_VR_Cdiff_strains_expr_HX12; design no. 546991). Each of the 12 subarrays included 18 probes (60 oligomers each) homologous to 3,786 of the 3,787 open reading frames of the *C. difficile* 630 genome as well as negative hybridization controls. The microarray slide was hybridized and scanned using a NimbleGen MS 200 microarray scanner (Roche NimbleGen, Inc.).

The 12 TIFF images were subjected to a robust multichip average (RMA) algorithm included in the NimbleScan software using the default settings. During the RMA analyses, the probe raw intensities were corrected for background noise and quantile normalized between arrays, and normalized probe intensity values were averaged to give a single intensity value per transcript and per sample. For each strain and condition assayed, RNA from three independent biological replicates grown on different days was used. Each of the biological replicates was tested in technical duplicate on the microarray slides. Data analysis work flow was performed with the Partek Genomics Suite (Partek Inc.). Lists of regulated genes were created using a 3-fold change cutoff and *P* value limit of ≤0.05.

Quantitative reverse transcriptase PCR (qRT-PCR). *C. difficile* chromosomal DNA was purified as previously described (31). *C. difficile* RNA was isolated from bacteria grown in TY medium. For each sample, a single colony of *C. difficile* was inoculated in TY medium and grown for 24 h. The *C. difficile* strains were diluted 1:25 into TY medium and grown to an

OD_{600} of 0.8. At this time, lysozyme was added to wild-type (600 μg/ml total) and *csfV* mutant (30 μg/ml total) cells, and the cells were allowed to grow for 1 h. Cells were fixed and RNA was purified as described above.

To generate cDNA from RNA samples, Superscript II was used according to the manufacturer's protocol (Invitrogen). The resulting cDNA samples were diluted 1:5 in diethyl pyrocarbonate (DEPC)-treated water. For each quantitative RT-PCR, 5 μl of sample was added to 10 μl of power SYBR green master mix (Applied Biosystems) and 5 μl of gene-specific primers (2.5 μM each primer). The following oligomer pairs were used to measure cDNA levels: for *rpoB*, TEQ009 and TEQ010; for *pdaV*, TEQ041 and TEQ42; for *prsA2*, TEQ043 and TEQ044; for *csfV*, TEQ007 and TEQ008; for *cd0738*, CDEP1445 and CDEP1446; for *cd0739*, CDEP1447 and CDEP1448; and for *cd1607*, CDEP1449 and CDEP1450. Experiments were performed in technical triplicates on three biologically independent replicates. Data were normalized to RNA levels of *C. difficile* housekeeping gene *rpoB*.

Peptidoglycan isolation and muropeptide analysis. Peptidoglycan was purified from 100-ml cultures of *C. difficile* grown to an OD_{600} of 0.6 to 0.8 in TY broth. For lysozyme experiments, bacteria were grown to an OD_{600} of 0.3 to 0.4 and exposed to hen egg white lysozyme (wild-type *C. difficile*, 100 μg/ml; *csfV* mutant, 20 μg/ml). The cells were then incubated until the OD_{600} reached 0.6 to 0.8. All plasmid-containing bacteria were grown in the presence of 10 μg/ml of thiamphenicol. To induce expression of genes under the control of P_{tet}, strains bearing P_{tet} vectors were grown in 2 μg/ml of tetracycline. Peptidoglycan was purified using a modified *B. subtilis* peptidoglycan purification protocol (24, 47, 48). Briefly, cells were harvested by centrifugation, and the supernatant was discarded. The cells were boiled in 4% sodium dodecyl sulfate (SDS) for 60 min. Samples were pelleted by centrifugation, and the supernatant was discarded. The samples were resuspended in sterile water; this process was repeated until SDS could not be detected (49). Nucleotides were removed by treating the samples with DNase I (NEB) and RNase A (Sigma-Aldrich) at 37°C for 2 h and then digested with pronase (Sigma-Aldrich) at 37°C for 16 h. The samples were washed extensively with sterile water. Teichoic acids were removed by resuspending the pellet in 1 ml of 49% hydrofluoric acid (Thermo Scientific) and incubated with rocking for 48 h at 4°C. The isolated peptidoglycan was digested with mutanolysin for 16 h at 37°C, and the solubilized muropeptides were reduced and separated by reverse-phase high-pressure liquid chromatography (HPLC) as previously described (47, 48). Muropeptides separated using a phosphate buffer and methanol gradient were collected and further purified using HPLC and a trifluoroacetic acid-acetonitrile buffer system. The muropeptides were collected, lyophilized, and analyzed using two mass spectrometry (MS) methods.

For electrospray MS analyses, the muropeptides were dissolved in 0.1% aqueous acetic acid-acetonitrile (9:1 [vol/vol]) and submitted to HPLC-MS analyses using a triple quadrupole mass spectrometer (ABSciex API3200) interfaced with an HPLC (Agilent 1100). Separations (10-μl injections) were performed on a Kinetex C_{18} reversed-phase column (2.6-μm particle size; 100 by 3 mm; Phenomenex) at 250 μl/min. The elution conditions were 99% solvent A (0.1% aqueous acetic acid) and 1% solvent B (acetonitrile) for 1 min, followed by a linear gradient to 95% solvent B over a period of 34 min. After a 5-min hold at 5% and 95% (A and B), the system was returned to 99% and 1% (A and B) for reequilibration. Under these conditions, the muropeptides were eluted between 1.5 and 11 min. Mass spectrometer conditions were (positive-ion, single-quad mode) as follows: temperature, 420°C; voltage, 4.2 kV; gases (curtain, gas supply 1 [GS1], GS2), 10; mass range, 120 to 1,800 amu; and declustering/entrance potentials (DP/EP), 50/9.

For muropeptide fragmentation and structural analyses, a 4800 matrix-assisted laser desorption ionization–time of flight/time of flight (MALDI-TOF/TOF) mass spectrometer (Applied Biosystems) was used. Muropeptides were dissolved in HPLC-grade water, and 1 μl of each muropeptide sample was air dried on a MALDI plate. The muropeptide spots were overlaid with 1 μl of 4-mg/ml α-cyano-4-hydroxycinnamic acid in 50% (vol/vol) acetonitrile, 0.2% (vol/vol) trifluoroacetic acid, and 20 mM ammonium chloride and allowed to air dry. A mass spectrum was acquired for each muropeptide sample in positive-ion mode for the mass-to-charge range of 680 to 3,500, averaging data from 1,000 individual laser shots. The primary parent ions, matching those observed using electrospray MS, were subsequently chosen for fragmentation. Tandem mass spectra were obtained utilizing the MS/MS 1-kV positive operating mode. Each tandem mass spectrum was typically the sum of 2,500 individual laser shots. A peak list for each muropeptide sample was generated using 4000 Series Explorer software.

RESULTS

The ECF σ factor σ^V is required for *C. difficile* lysozyme resistance. We previously demonstrated that *csfV* expression was induced by lysozyme but not by other cell envelope stresses (31). To begin characterizing the role of σ^V in *C. difficile*, we constructed a *csfV* mutant. We retargeted the *ltrB*::*ermB* intron from pCE240 to insert 66 bp downstream of the start of the *csfV* open reading frame (Fig. 1A). The resulting *csfV*::*ltrB*::*ermB* insertion was confirmed by PCR and sequencing as described in Materials and Methods.

We compared the sensitivity of the wild type and the *csfV* mutant to a variety of antimicrobial compounds using disk diffusion assays. Filter disks containing antimicrobial compounds were placed onto confluent lawns of *C. difficile* cells, and the zones of inhibition were determined after 24 h of incubation. We found that hen egg white lysozyme produced a small zone of inhibition, <6 mm in diameter, when placed on a confluent lawn of wild-type *C. difficile* (Table 2). In contrast, hen egg white lysozyme created a substantially larger zone of inhibition (14 mm) when placed on a lawn of the *csfV* mutant (Table 2). Since *C. difficile* is a human pathogen, we also tested *C. difficile* sensitivity to human lysozyme, which is 78% similar and 63% identical to hen egg white lysozyme. We found that similar to hen egg white lysozyme, human lysozyme showed very little inhibition of wild-type *C. difficile* growth, but human lysozyme produced a substantially larger zone of inhibition when placed on a lawn of the *csfV* mutant (Table 2). We tested a number of other antimicrobial compounds that damage the cell envelope, including ampicillin, bacitracin, colistin, nisin, and vancomycin. A mutation in *csfV* did not alter the size of the zones of inhibition created by these antimicrobial compounds compared to those with the wild type (Table 2).

To further quantify the observation that the *csfV* mutant strain was more sensitive to lysozyme, we determined the MICs of hen egg white lysozyme and human lysozyme for both wild-type and *csfV* mutant strains. We established that the MICs of hen egg white and human lysozymes for wild-type *C. difficile* were greater than 1,000 μg/ml. Because lysozyme concentrations higher than 1,000 μg/ml resulted in lysozyme precipitation in the growth medium, we were unable to determine the exact lysozyme MIC of wild-type *C. difficile*. The lysozyme MIC of the *csfV* mutant was 50 μg/ml (Table 2). Thus, the *csfV* mutant was at least 20-fold more sensitive to lysozymes than the wild type (Table 2). The MICs for ampicillin, bacitracin, colistin, nisin, and vancomycin were nearly identical for the wild type and the *csfV* mutant strains (Table 2 and Fig. 1B). These data suggest that σ^V is required for *C. difficile* resistance to lysozymes but not for other antimicrobial compounds. Since hen egg white and human lysozymes behaved similarly, we used hen egg white lysozyme in all subsequent lysozyme experiments.

To demonstrate that the *csfV* mutation was responsible for the defect in lysozyme resistance, we generated a plasmid which expresses *csfV* (42). A *csfV* mutant containing the *csfV*-expressing

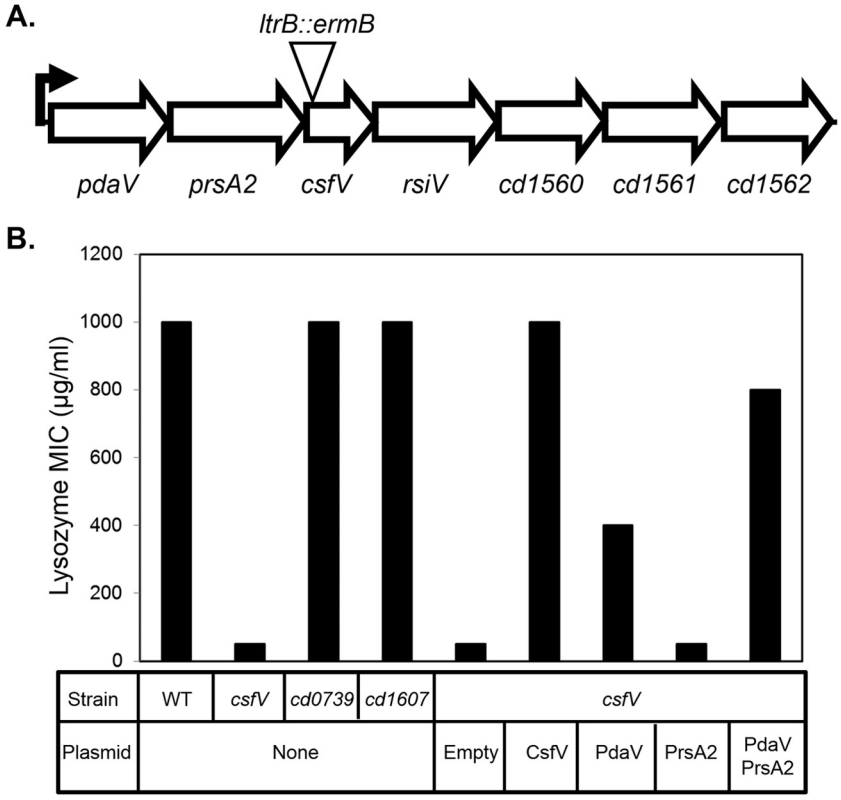

FIG 1 Role of σ^V regulon in lysozyme resistance. (A) Structure of *csfV* operon to scale. *csfV*::*ltrB*::*ermB* is indicated by a triangle. (B) The MIC of HEW lysozyme was determined for the WT (JIR8094) and *csfV* (TCD20; $csfV_{63}$::*ltrB*::*ermB*), *cd0739* (CDE1938; $cd0739_{48}$::*ltrB*::*ermB*), and *cd1607* (CDE1930; $cd1607_{117}$::*ltrB*::*ermB*) mutants. We also compared the abilities of different genes to complement the *csfV* mutant; plasmids are labeled as empty (TCD28; $csfV_{63}$::*ltrB*::*ermB* pMC123), CsfV (TCD25; $csfV_{63}$::*ltrB*::*ermB* pTHE1072), PdaV (CDE2025; $csfV_{63}$::*ltrB*::*ermB* pCE382), pPrsA2 (TCD1250; $csfV_{63}$::*ltrB*::*ermB* pCE400), and PdaV-PrsA2 (CDE2026; $csfV_{63}$::*ltrB*::*ermB* pCE383). The MIC is defined as the concentration showing no growth (OD_{600} less than 0.05) after 16 h at 37°C in a 96-well plate. The MIC experiments were performed in technical triplicates and repeated on different days. Data are from one representative experiment. The MIC varies slightly between days or experimenters; however, the fold changes always remain the same.

plasmid had a lysozyme MIC that was similar to that of the wild type and was at least 20-fold higher than that of the *csfV* mutant containing an empty vector control (Fig. 1B). This suggests that σ^V is required for lysozyme resistance.

Identification of genes induced by lysozyme and dependent upon σ^V. Because a *csfV* mutant was more sensitive to lysozyme, we reasoned that σ^V controls expression of genes required for lysozyme resistance. To identify *C. difficile* genes whose expression was dependent upon σ^V, we used microarrays to compare global RNA transcriptome levels of the wild type and the *csfV* mutant strain grown in the presence of lysozyme. These experiments were performed in biological triplicates and technical duplicates. Using a fold change cutoff of >3-fold and a *P* value limit of 0.05, we identified 30 genes whose expression was dependent upon σ^V (Table 3). In addition, we identified 9 genes whose expression was reduced by lysozyme in a σ^V-dependent manner (see Table S2 in the supplemental material).

Since σ factors are most commonly associated with increased expression of target genes, we focused on the genes whose expression was σ^V dependent. In a previous study, we demonstrated that the *csfV* gene is the third gene in a 7-gene operon (31). We found that several of the genes in the *csfV* operon (*cd1556*, *cd1557*, *csfV*, *rsiV*, and *cd1560*) had between 14- and 183-fold increases in σ^V-dependent expression in the presence of lysozyme (Table 3). In addition, we identified a number of genes outside the *csfV* operon which were also induced by lysozyme in a σ^V-dependent manner, including *cd0738*, *cd0739*, and *cd1606* to -11, whose expression appeared lysozyme induced 117-, 39-, and ~9-fold, respectively (Table 3). The *cd0738* and *cd0739* genes are divergently transcribed on the chromosome and appear to be unique to *C. difficile*. The *cd1606* to -11 genes appear to be an operon encoding a putative GntR-like regulator (CD1606) and a putative ABC transporter (CD1607 to -11).

TABLE 2 Zones of inhibition and MICs for the wild type and *csfV* mutant

Antibiotic	Diam of inhibition area (mm)		MIC (μg/ml)	
	Wild type	*csfV* mutant	Wild type	*csfV* mutant
Ampicillin	19	22	3.1	3.1
Bacitracin	9	9	125	125
Colistin	<6	<6	625	625
Lysozyme, hen	<6	14	>1,000	50
Lysozyme, human	<6	14	>1,000	50
Nisin	8	8	50	50
Vancomycin	22	22	2.8	1.4

TABLE 3 Genes induced by lysozyme in a σ^V-dependent manner

Gene name[a]	Function of corresponding protein[b]	Fold change[c]	*P* value[d]
cd0738	Putative exported protein	117.17	1.49E−07
cd0739	Putative exported protein	39.46	1.64E−09
cd0740	Putative aminotransferase	8.504	1.66E−10
cd1101	Putative mobilization protein	4.12	1.01E−03
cd1114	Hypothetical protein	4.33	2.10E−03
cd1117	Conserved hypothetical protein	5.18	5.94E−04
cd1555	Putative amino acid permease	4.70	2.06E−03
cd1556 (*pdaV*)	Putative polysaccharide deacetylase	183.81	1.62E−09
cd1557 (*prsA2*)	Putative peptidyl-prolyl isomerase	14.27	1.85E−09
cd1558 (*csfV*)	RNA polymerase σ factor	125.4	1.00E−12
cd1559 (*rsiV*)	Conserved hypothetical protein	119.55	9.64E−12
cd1560	Putative exported protein	50.52	6.28E−10
cd1561	Putative dehydrogenase accessory protein	3.86	7.85E−09
cd1562	Conserved hypothetical protein	6.45	5.86E−09
cd1565	Ketol-acid reductoisomerase	3.10	3.14E−04
cd1605	Conserved hypothetical protein	3.27	9.60E−04
cd1606	GntR family transcriptional regulator	9.10	5.12E−07
cd1607	ABC transporter, ATP-binding protein	7.57	2.26E−07
cd1608	Putative ABC transporter, permease protein	10.5	1.71E−06
cd1609	Putative exported protein	9.01	1.29E−06
cd1610	Putative membrane protein	9.57	2.24E−06
cd1611	Putative exported protein	7.68	3.61E−06
cd3329	Conjugative transposon conserved hypothetical protein	3.16	3.97E−06
cd3339	Conjugative transposon membrane protein	7.28	1.05E−03
cd3342a	Conjugative transposon conserved hypothetical protein	5.21	6.07E−04
cd3343	Putative conjugative transposon replication initiation factor	7.13	5.85E−04
cd3344	Conjugative transposon-related FtsK/SpoIII-related protein	3.88	1.10E−03
cd3345	Conjugative transposon conserved hypothetical protein	6.54	6.52E−04
cd3346	Conjugative transposon conserved hypothetical protein	8.54	4.56E−05
cd3384	Conjugative transposon membrane protein	3.01	9.60E−04

[a] Locus tags as listed in GenBank.
[b] Putative functions as determined by current annotation of the *C. difficile* genome.
[c] Fold changes (type *C. difficile* compared to the *csfV* mutant) are averages from three biological replicates, with each performed in technical duplicate. Fold changes signify expression that is increased in wild-type *C. difficile* compared to the *csfV* mutant.
[d] *P* values listed are averages from three biological replicates, with each done in technical replicates.

To validate the results of the microarray analysis, we determined the relative expression levels of putative σ^V-regulated genes using quantitative PCR analysis on reverse-transcribed RNA (qRT-PCR) from wild-type and *csfV* strains in the absence and presence of lysozyme. Consistent with our microarray data, qRT-PCR validated that exposure to lysozyme resulted in >17-fold σ^V-dependent induction of *cd1556* (hereafter referred to as *pdaV* [polysaccharide deacetylase V]), *cd1557* (hereafter referred to as *prsA2* [putative peptidyl-prolyl *cis-trans* isomerase]), *csfV*, *cd0739*, and *cd1607* (Fig. 2). This lysozyme induction of gene expression was not observed in the *csfV* mutant (Fig. 2). We found that although *cd0738* RNA levels increased upon exposure to lysozyme, there was no significant difference between the wild type and the *csfV* mutant strains (Fig. 2). This suggests that while *cd0738* expression may be lysozyme induced, it is likely not dependent upon σ^V. Overall, these data suggest that σ^V strongly regulates at least 3 genetic units, including the *csfV* operon, *cd0739*, and *cd1607* to -11 operon.

Role of σ^V-regulated genes in lysozyme resistance. To determine which of the σ^V-regulated genes were required for lysozyme resistance, we constructed mutations in *cd0739* and *cd1608* using the retargeted *ltrB*::*ermB* intron from pBL100. The mutants were confirmed by PCR and were tested for increased lysozyme sensitivity. We determined that neither the *cd0739*::*ltrB*::*ermB* nor the *cd1608*::*ltrB*::*ermB* insertions led to an increase in lysozyme susceptibility (Fig. 1B). This suggests that these are not essential for lysozyme resistance in *C. difficile*.

Construction of mutations in individual genes within the *csfV* operon is complicated by the fact that insertion of *ltrB*::*ermB* into a gene is likely polar on the expression of downstream genes. Thus, any insertion upstream of *csfV* would likely be polar on *csfV*. To determine if any of the genes in the *csfV* operon affected lysozyme resistance, we placed the expression of either *pdaV*, *pdaV-prsA2*, or *prsA2* under the control of an exogenous promoter in the *csfV* mutant strain (44). A *csfV* mutant with an empty vector control remained as sensitive to lysozyme as the *csfV* mutant alone (Fig. 1B). We found that exogenous expression of either *pdaV* or *pdaV-prsA2* in the *csfV* mutant resulted in increased lysozyme resistance (Fig. 1B). Exogenous expression of only *prsA2* in the *csfV* mutant did not affect lysozyme resistance (Fig. 1B). Our data suggest that exogenous expression of *pdaV*, which encodes a putative polysaccharide deacetylase, confers increased lysozyme resistance on the *C. difficile csfV* mutant.

Lysozyme-induced deacetylation of *C. difficile* peptidoglycan is dependent on σ^V. Peptidoglycan deacetylation is often associated with increased lysozyme resistance (14). Thus, we sought to determine if σ^V regulates modifications to the *C. difficile* peptidoglycan. We isolated peptidoglycan from the wild type and the *csfV* mutant grown in the presence or absence of subinhibitory lysozyme. The peptidoglycan was digested with mutanolysin to produce peptidoglycan fragments (muropeptides), which were then separated by charge and size using reversed-phase HPLC. The structures for these muropeptides were determined using mass spectrometry and numbered as previously described (25) (Table 4; see also Fig. S1 in the supplemental material).

The majority of the muropeptide fragments from different samples were unaffected by either the *csfV* mutation or lysozyme exposure (Table 4; see also Fig. S1 in the supplemental material). However, the ratio of muropeptides containing *N*-acetylgluco-

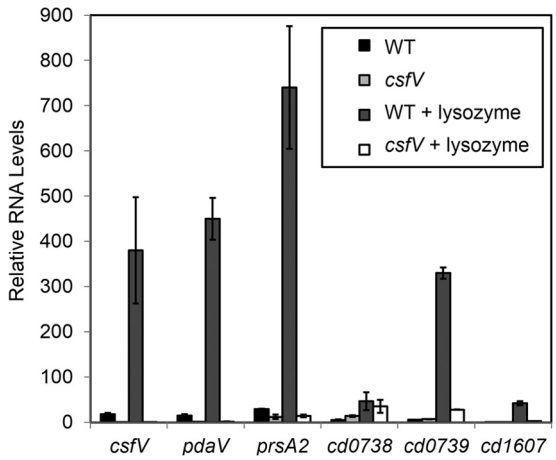

FIG 2 Expression of the *csfV* operon is dependent upon σ^V. Shown are relative RNA levels of *cd1556* (the first gene in the *csfV* operon) (TEQ041 and TEQ42), *prsA2* (TEQ043 and TEQ044), *csfV* (TEQ007 and TEQ008), and *tcdB* (TEQ037 and TEQ008) in wild-type *C. difficile* (JIR8094) or the *csfV* mutant (TCD20) in the absence or the presence of lysozyme. RNA levels were normalized to the housekeeping gene *rpoB* (TEQ009 and TEQ010). Experiments were performed in technical and biological triplicates, and standard deviations are noted.

TABLE 4 σ^V is required for lysozyme-induced peptidoglycan deacetylation

Peak[a]	Muropeptide structure[f]	*m/z*		Area (%) of each muropeptide peak (mean ± SD)							
		Predicted[b]	Observed[b]	WT ($n = 3$)	WT + Lys ($n = 4$)	*csfV* ($n = 3$)	*csfV* + Lys ($n = 2$)	*csfV* pEmpty ($n = 2$)	*csfV* pXyl-*csfV* ($n = 2$)	*csfV* pTet ($n = 2$)	*csfV*/pTet-*pdaV* ($n = 2$)
1	Tri, deAc	851.4	851.4	2.7 ± 0.2	6.8 ± 5.4	1.5 ± 0.2	1.7 ± 0.2	1.7 ± 0.3	1.8 ± 0.1	2.2 ± 0.1	2.4 ± 0.1
4	Tri-Gly, deAc	908.4	908.4	2.7 ± 0.3	3.5 ± 1.3	4.5 ± 0.4	3.9 ± 0.5	3.3 ± 1.3	4.0 ± 0.2	2.4 ± 0.1	2.3 ± 0.2
6	Tetra	964.4	964.5	3.1 ± 0.1	0.8 ± 0.3	7.7 ± 0.3	7.4 ± 0.8	8.6 ± 1.4	5.6 ± 0.2	11.4 ± 0.7	10.0 ± 1.4
7	Tetra, deAc	922.4	922.4	30.0 ± 1.3	28.7 ± 2.6	28.2 ± 1.3	25.7 ± 3.0	26.8 ± 0.5	30.8 ± 1.5	21.9 ± 0.1	24.3 ± 0.3
11a	Tri-tri-Gly, deAcX2	1,718.7	1,718.8	2.5 ± 0.6	3.3 ± 1.0	3.2 ± 0.3	3.7 ± 0.3	2.5 ± 0.3	2.9 ± 0.4	2.1 ± 0.1	2.0 ± 0.2
11b	Tri-tri, deAcX2	1,661.7	1,661.8	1.9 ± 0.2	6.6 ± 6.2	0.7 ± 0.1	1.1 ± 0.1	1.0 ± 0.4	0.9 ± 0.1	1.1 ± 0.1	1.3 ± 0.1
12	Tri-tetra	1,816.8	1,816.8	3.4 ± 0.4	0.6 ± 0.4	8.1 ± 0.5	9.2 ± 0.6	10.1 ± 2.1	5.2 ± 1.3	12.2 ± 0.8	10.5 ± 0.8
13/14	Tri-tetra, deAcX1	1,774.8	1,774.8	5.8 ± 2.2	3.6 ± 4.2	7.6 ± 0.6	8.2 ± 0.1	8.3 ± 0.4	6.3 ± 0.0	9.4 ± 0.5	8.9 ± 0.3
15a	Tri-tetra, deAcX2	1,732.7	1,732.7	25.0 ± 1.4	25.1 ± 2.0	18.6 ± 0.8	20.6 ± 1.5	16.8 ± 2.5	22.1 ± 0.0	14.7 ± 0.6	16.4 ± 1.1
16	Tri-tetra, deAcX1	1,752.5[c]	1,753.3[c]	0.2 ± 0.3	1.6 ± 1.6	0.1 ± 0.2	0.1 ± 0.2	0.0 ± 0.0	0.1 ± 0.2	0.2 ± 0.3	0.2 ± 0.3
17	Tri-tetra, deAcX2	1,732.7	1,732.7	3.6 ± 1.4	4.7 ± 0.2	2.4 ± 0.3	2.7 ± 0.0	2.2 ± 0.2	2.1 ± 0.0	1.1 ± 0.1	1.4 ± 0.1
17a[d]	Tetra-tetra	1,887.8	1,887.7	0.8 ± 0.1	0.1 ± 0.1	2.2 ± 0.2	1.9 ± 0.2	2.7 ± 0.0	1.5 ± 0.2	5.1 ± 0.4	4.5 ± 0.9
19	Tetra-tetra, deAcX2	1,803.8	1,803.6	7.7 ± 0.2	6.6 ± 1.0	6.1 ± 0.5	5.3 ± 0.1	6.6 ± 0.4	7.9 ± 0.1	5.0 ± 0.3	5.6 ± 0.7
20	Tetra-tetra, deAcX1	1,845.8	1,845.7	2.2 ± 0.5	1.4 ± 1.0	3.9 ± 0.6	3.1 ± 0.5	4.9 ± 0.9	3.1 ± 0.0	5.6 ± 0.3	4.8 ± 0.1
21	Tetra-tetra, deAcX2	1,803.8	1,803.6	3.7 ± 0.5	2.6 ± 0.3	2.7 ± 0.2	2.1 ± 0.4	1.7 ± 1.0	2.7 ± 0.4	2.5 ± 0.1	2.2 ± 0.6
23	Tri-tri-tetra, deAcX3	2,543.1	2,542.8	4.6 ± 1.2	3.9 ± 0.5	2.5 ± 0.9	3.3 ± 1.7	2.7 ± 0.7	2.8 ± 0.2	3.1 ± 0.3	3.0 ± 0.4
% NAG residues[e]				11.4	4.8	23.8	24.1	28.0	17.1	36.3	32.0

[a] Peak name as identified by Peltier et al. (48).
[b] *m/z* for $[M+Na]^+$ ion.
[c] *m/z* for $[M+H]^+$ ion.
[d] This muropeptide was not observed previously (48).
[e] Sum of peak area percentage times the fraction of acetylated NAG residues in each muropeptide.
[f] deAcX1 refers to the presence of a single deacylated disaccharide. deAcX2 refers to the presence of two deacylated idsaccharides.

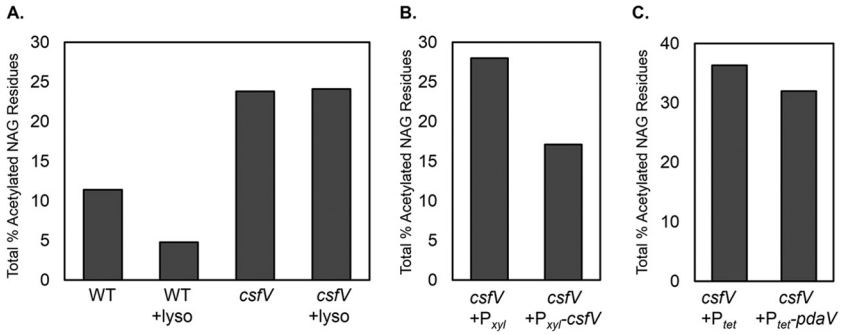

FIG 3 Lysozyme-induced decrease in *C. difficile* peptidoglycan acetylation is dependent upon the presence of ECF σ^V. Total percentages of acetylated NAG residues are shown for the wild-type strain grown in the absence or presence of lysozyme (WT and WT+lyso) and the *csfV* mutant grown in the absence or presence of lysozyme (*csfV* and *csfV*+lyso) (A), the *csfV* mutant containing the empty vector control (*csfV* + P_{xyl}) and the *csfV* mutant containing the *csfV*-expressing plasmid (*csfV*+P_{xyl}-*csfV*) (B), and the *csfV* mutant containing the empty vector control (*csfV*+P_{tet}) and the *csfV* mutant containing the *pdaV*-expressing plasmid (*csfV*+P_{tet}-*pdaV*) (C).

samine (GlcNAc) versus deacetylated GlcNAc (glucosamine) was altered by growth in the presence of lysozyme, in a σ^V-dependent manner (Fig. 3A and Table 4). These changes can be seen on individual muropeptide peaks. For example, we observed that incubation of wild-type *C. difficile* with subinhibitory lysozyme led to a decrease in the level of acetylated tri-tetra cross-linked muropeptides (peak 12) and a concomitant increase in the percentage of deacetylated tri-tetra cross-linked muropeptides (peaks 15a, 16, and 17) (Table 4). In the *csfV* mutant, the percentage of the acetylayed glucosamine (GlcNAc) tri-tetra cross-linked muropeptides (peak 12) was higher than that in the wild type (Table 4). We also observed a concomitant decrease in the deacetylated glucosamine tri-tetra cross-linked muropeptides (peaks 15a, 16, and 17) in the *csfV* mutant compared to the wild type (Table 4). For example, peak 15a decreased from 25% in the wild type to ~19% in the *csfV* mutant, and peak 17 decreased from ~4% to ~2.5% (Table 4).

To confirm that the deacetylation we detected was occurring on GlcNAc, we performed MS fragmentation on the most abundant muropeptides (peaks 1, 7, 12, 13, 15a, 19, and 21). In each case where the parent ion mass indicated the presence of deacetylation (peaks 1, 7, 19, and 21) a fragment matching the loss of a deacetylated glucosamine (160.9) but not GlcNAc (loss of 202.9) was always observed. This suggests that GlcNAc had been deacetylated (see Table S3 in the supplemental material). In contrast, where the parent ion (peak 12) mass indicated no deacetylation, we detected only the loss of GlcNAc (202.9) and never a loss of deacetylated glucosamine (see Table S3). For peak 13, which consists of tri-tetra cross-linked muropeptides with only one site of deacetylation, we detected fragment ions indicating a loss of either glucosamine, GlcNAc, or even both (see Table S3). In no case were there fragment ions that could be matched to a deacetylated MurNAc moiety. Thus, we conclude that nearly all of the deacetylation we observed was due to deacetylation of GlcNAc.

We calculated the total percentage of GlcNAc in muropeptides (Fig. 3A and Table 4). In wild-type cells, in the absence of lysozyme, 11% of the glucosamine residues were acetylated (Fig. 3A and Table 4). When cells were grown in the presence of subinhibitory lysozyme, the percentage of GlcNAc decreases 2-fold, to 4.8%, suggesting further peptidoglycan deacetylation (Fig. 3A and Table 4). In contrast, the quantity of GlcNAc in the *csfV* mutant was ~24% in both the presence and absence of lysozyme (Fig. 3A and Table 4). Thus, there is a 5-fold increase in the percentage of GlcNAc in *csfV* mutant cells compared to wild-type cells when grown in the presence of lysozyme. We also observed that expression of *csfV* from an exogenous promoter decreased the quantity of GlcNAc from 28% to 17% (Fig. 3B and Table 4). Taken together, these data demonstrate that σ^V plays a role in maintaining the high basal level of peptidoglycan deacetylation in *C. difficile*, as well as being required for the additional lysozyme-inducible deacetylation observed in wild-type *C. difficile*.

PdaV increases lysozyme resistance and can deacetylate *C. difficile* peptidoglycan. Exposure to lysozyme increased deacetylation of *C. difficile* peptidoglycan in a σ^V-dependent manner. Based upon our microarray analysis, *pdaV* was the only putative polysaccharide deacetylase whose expression was increased in response to lysozyme in a σ^V-dependent manner (see Table S4 in the supplemental material). Since expression of the putative polysaccharide deacetylase, encoded by *pdaV*, increases lysozyme resistance of a *csfV* mutant, we tested if expression of *pdaV* could increase peptidoglycan deacetylation. Consistent with the partial complementation of the *csfV* mutant, expression of *pdaV* resulted in an overall decrease in the percentage of GlcNAc in the peptidoglycan (Fig. 3C and Table 4). This decrease could also be seen in the individual muropeptide peaks. The percentage of the GlcNAc tri-tetra cross-linked muropeptide (peak 12) was lower when *pdaV* was expressed in the *csfV* mutant (Table 4). As expected, there was an increase in the percentage of deacetylated glucosamine tri-tetra cross-linked muropeptides (peaks 15a and 17) (Table 4). This suggests that PdaV has peptidoglycan deacetylase activity and that this activity is at least partially responsible for the increase in lysozyme resistance conferred by σ^V.

The ECF σ factor σ^V mutant is severely attenuated in a hamster model of *C. difficile* infection. Since lysozyme is a major component of the innate immune system's defense against bacterial infection, we reasoned that σ^V may be important for *C. difficile* pathogenesis. To determine the role of σ^V in *C. difficile* pathogenesis, we tested the ability of a *csfV* mutant to colonize and cause illness in a hamster model of infection. Approximately 1,000 spores of either the wild type (JIR8094) or a *csfV* mutant (TCE20) were used to infect clindamycin-treated hamsters as previously described (7, 45). Fecal samples from all hamsters inoculated with either the wild type or the *csfV* mutant tested positive for the presence of *C. difficile* by day 2 or 3 postinfection and showed similar levels of colonization (Fig. 4A). This indicated that both

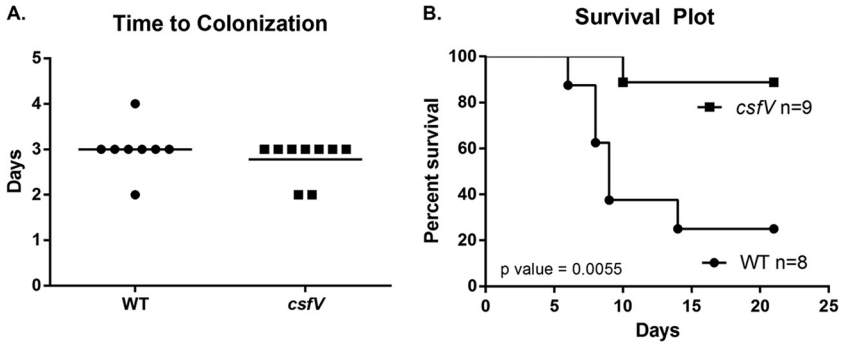

FIG 4 σ^V is required for *C. difficile* pathogenesis in hamsters. Hamsters were treated with clindamycin (30 mg/kg) 3 days prior to inoculation with 1,000 wild-type (JIR8094) or *csfV* mutant (TCD20) spores. (A) Time to first detectable colonization. Fecal samples were taken daily from each animal. One stool pellet was resuspended in 1 ml of PBS, and dilutions were plated on BHIS media containing 0.1% taurocholate, to germinate spores. The limit of detection for colonization in this assay is ≥1,000 spores/ml of resuspended stool. (B) The length of time between inoculation with *C. difficile* and morbidity was plotted using the Kaplan-Meier method (Mantel-Cox [log rank] test *P* value = 0.0055).

the wild type and the *csfV* mutant were able to colonize the hamsters. Five days postinfection, 6 out of 8 hamsters infected with wild-type *C. difficile* began to display symptoms suggestive of a *C. difficile* infection (wet tail, diarrhea, and lethargy). All six of these hamsters were moribund between 6 and 12 days postinoculation and required euthanasia (Fig. 4B). In contrast, we found that only 1 of the 9 hamsters infected with the *csfV* mutant showed signs of illness and succumbed to the infection (Fig. 4B). In addition, the 8 hamsters that survived infection with the *csfV* mutant cleared the infection by day 6. While the times to colonization are similar between wild-type- and *csfV* mutant-infected animals, hamsters infected with the *csfV* mutant clear bacteria and survive infection.

To ensure that the defects we observed were not due to a simple growth defect, we compared the growth of a *csfV* mutant to that of wild-type *C. difficile* in TY media; we observed no significant growth defect (see Fig. S1 in the supplemental material). We also investigated the effect of the *csfV* mutant on sporulation and observed no significant difference compared to the effect of the wild type (see Fig. S2 in the supplemental material). Together, these data indicate that σ^V is required for *C. difficile* pathogenesis.

DISCUSSION

Lysozyme is an important and abundant factor of the innate immune system. *C. difficile* is naturally highly resistant to lysozyme. We had previously found that *csfV* expression was induced by lysozyme but not other cell envelope stresses (31). Here we show that the *C. difficile* ECF σ factor σ^V is required for resistance to lysozyme but not antibiotics which inhibit peptidoglycan synthesis. Homologs of σ^V are found almost exclusively in Firmicutes, low-GC Gram-positive bacteria, and represent a subgroup of the ECF σ factor clade ECF30 (28). Homologs of σ^V that have been identified in the pathogenic bacterium *Enterococcus faecalis* and the model organism *B. subtilis* are also lysozyme inducible and required for lysozyme resistance (21, 32, 33). Thus, the σ^V signal transduction pathway is used in other bacteria to sense and provide resistance to lysozyme.

Using microarray analysis, we identified the σ^V regulon in *C. difficile*. We found that disruption of two of the most highly induced operons outside the *csfV* operon had no detectable effect on lysozyme resistance. It is possible that a decrease in lysozyme resistance may be observed only when multiple mutations are combined. Alternatively, these genes may not be directly involved in lysozyme resistance but are involved in some other process which uses lysozyme as a signal to induce their expression under optimal conditions.

We observed that the *csfV* operon itself was highly induced by lysozyme in a σ^V-dependent manner. Several other lysozyme-induced, σ^V-dependent genes identified in our studies had higher expression in the wild type than in the *csfV* strain but were not induced to the same degree as the *csfV* operon. Interestingly, microarray analysis of *B. subtilis sigV* mutants revealed a similar result, in which the *sigV-rsiV-oatA-yrhK* operon was induced >63-fold by lysozyme and a large number of other genes were induced only 2- to 3-fold (33).

Peltier et al. previously determined that peptidoglycan from vegetatively growing *C. difficile* is highly deacetylated (48). Here we report that in addition to the high degree of innate deacetylation of *C. difficile* peptidoglycan, there is also lysozyme-inducible deacetylation of peptidoglycan. Our data demonstrate that σ^V is required for the lysozyme-inducible deacetylation of peptidoglycan. Peltier et al. determined that GlcNAc was the primary sugar deacetylated in *C. difficile* peptidoglycan (48). Our data confirm this finding and demonstrate that lysozyme, through σ^V, induces further deacetylation of GlcNAc in *C. difficile* peptidoglycan. This further regulation results in a striking level (95%) of total PG deacetylation, which contributes directly to *C. difficile*'s high level of lysozyme resistance.

C. difficile contains 10 putative polysaccharide deacetylases, of which only 1, encoded by *pdaV*, is significantly induced by σ^V in a lysozyme-dependent manner. PdaV belongs to the PgdA family of polysaccharide deacetylases, several of which have been implicated in increased lysozyme resistance (19, 20, 24). We found that expression of *pdaV* in the *csfV* mutant increased lysozyme resistance and peptidoglycan deacetylation. This suggests that PdaV can function as a peptidoglycan deacetylase.

In our experiments, the PdaV-expressing plasmid was unable to fully complement the *csfV* mutant. This may be due to the lower expression levels from the plasmid promoter than from its native σ^V-dependent promoter. Another possibility is that additional genes are required for σ^V-induced lysozyme resistance. Indeed, lysozyme resistance in *E. faecalis* and *S. aureus* is dependent upon peptidoglycan modification in conjunction with other factors, including D-alanylation of the cells' lipoteichoic acids, resulting in

increased lysozyme resistance (15, 21). In these organisms, disruption of both peptidoglycan modification and D-alanylation of the lipoteichoic acids results in a significantly larger increase in lysozyme sensitivity than the single mutant alone (15, 21, 32). It is also interesting that we observed a slight increase in lysozyme resistance when we expressed *pdaV-prsA2* in a *csfV* mutant compared to expression of *pdaV* alone in the *csfV* mutant (Fig. 1B). Since PrsA2 is homologous to peptidyl-prolyl *cis-trans* isomerases, it is tempting to hypothesize that PrsA2 may be required for secretion or proper folding of PdaV.

σ^V homologs from *B. subtilis* and *E. faecalis* are also required for lysozyme resistance by inducing expression of peptidoglycan modification genes. In *B. subtilis*, σ^V is required for expression of *oatA*, which encodes a peptidoglycan *O*-acetyltransferase that increases lysozyme resistance (22, 32, 33). In *E. faecalis*, σ^V is required for expression of a peptidoglycan deacetylase, PgdA (19, 21). We found that in response to lysozyme, *C. difficile* σ^V induces expression of a peptidoglycan deacetylase, which results in deacetylation of the *C. difficile* peptidoglycan. While the signal transduction systems from these three organisms are similar, the mechanism of peptidoglycan modification differs. These three different Gram-positive bacteria use the same mechanism to detect lysozyme but in response use different strategies to modify the peptidoglycan and protect against lysozyme.

The activity of *C. difficile* σ^V is controlled by lysozyme. Similarly, the activity of σ^V homologs in both *B. subtilis* and *E. faecalis* is induced by lysozyme. Recent evidence from *B. subtilis* and *E. faecalis* suggests that activity of σ^V is controlled by regulated intramembrane proteolysis of the anti-σ factor RsiV. We hypothesize that a similar mechanism controls activation of σ^V in *C. difficile*. However, it is not known how degradation of RsiV is initiated, nor have the factors required for degradation of RsiV been identified.

Our observation that *csfV* mutants were severely attenuated in the hamster model of infection suggests that σ^V is an important virulence factor in *C. difficile*. Our data suggest that the *csfV* mutant can initially colonize the animal to levels similar to those of the wild type but is more easily cleared. *C. difficile* likely encounters and must resist lysozyme during infection. The *csfV* mutant is less able to resist lysozyme stress and therefore might not survive as well as the more lysozyme-resistant wild type. This is supported by the high level of lysozyme resistance we observed in *C. difficile* strains (MIC $>$ 1 mg/ml). As a comparison, *B. subtilis* and *L. monocytogenes* are 10- to 100-fold more sensitive to lysozyme than *C. difficile* (17, 18). Similarly, the lysozyme MIC for *C. perfringens*, a close relative of *C. difficile*, is almost 10-fold lower than that of *C. difficile* (50). Interestingly, a homolog of σ^V in *E. faecalis* has been shown to be required for virulence in systemic and urinary tract models of mouse infection (21), suggesting that σ^V homologs may be required for virulence in multiple pathogens. In fact, the role of σ^V in *E. faecalis* pathogenesis appears to be resistance to lysozyme (19). In *S. pneumoniae*, a homolog of PgdA was found to deacetylate peptidoglycan and is essential for virulence and lysozyme resistance (24). Although σ^V is necessary during a *C. difficile* infection, we do not yet know which components of the σ^V regulon are required during an infection. It is tempting to speculate that σ^V is simply required for lysozyme resistance during an infection; however, at this time we cannot rule out the possibility that σ^V is required for pathogenesis due to regulation of other virulence genes we have not yet identified.

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REFERENCES

1. **Kyne L, Hamel MB, Polavaram R, Kelly CP.** 2002. Health care costs and mortality associated with nosocomial diarrhea due to Clostridium difficile. Clin. Infect. Dis. **34:**346–353. http://dx.doi.org/10.1086/338260.
2. **Bartlett JG.** 1992. Antibiotic-associated diarrhea. Clin. Infect. Dis. **15:** 573–581. http://dx.doi.org/10.1093/clind/15.4.573.
3. **Kelly CP, LaMont JT.** 1998. Clostridium difficile infection. Annu. Rev. Med. **49:**375–390. http://dx.doi.org/10.1146/annurev.med.49.1.375.
4. **Pépin J, Valiquette L, Alary M, Villemure P, Pelletier A, Forget K, Pépin K, Chouinard D.** 2004. Clostridium difficile-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. CMAJ **171:**466–472. http://dx.doi.org/10.1503/cmaj.1041104.
5. **McFarland LV, Clarridge JE, Beneda HW, Raugi GJ.** 2007. Fluoroquinolone use and risk factors for Clostridium difficile-associated disease within a Veterans Administration health care system. Clin. Infect. Dis. **45:**1141–1151. http://dx.doi.org/10.1086/522187.
6. **McFarland LV, Mulligan ME, Kwok RYY, Stamm WE.** 1989. Nosocomial acquisition of Clostridium difficile infection. N. Engl. J. Med. **320:** 204–210. http://dx.doi.org/10.1056/NEJM198901263200402.
7. **Lyras D, O'Connor JR, Howarth PM, Sambol SP, Carter GP, Phumoonna T, Poon R, Adams V, Vedantam G, Johnson S, Gerding DN, Rood JI.** 2009. Toxin B is essential for virulence of Clostridium difficile. Nature **458:**1176–1179. http://dx.doi.org/10.1038/nature07822.
8. **Voth DE, Ballard JD.** 2005. Clostridium difficile toxins: mechanism of action and role in disease. Clin. Microbiol. Rev. **18:**247–263. http://dx.doi.org/10.1128/CMR.18.2.247-263.2005.
9. **Kuehne SA, Cartman ST, Heap JT, Kelly ML, Cockayne A, Minton NP.** 2010. The role of toxin A and toxin B in Clostridium difficile infection. Nature **467:**711–713. http://dx.doi.org/10.1038/nature09397.
10. **Kuehne SA, Cartman ST, Minton NP.** 2011. Both, toxin A and toxin B, are important in Clostridium difficile infection. Gut Microbes **2:**252–255. http://dx.doi.org/10.4161/gmic.2.4.16109.
11. **Mani N, Dupuy B.** 2001. Regulation of toxin synthesis in Clostridium difficile by an alternative RNA polymerase sigma factor. Proc. Natl. Acad. Sci. U. S. A. **98:**5844–5849. http://dx.doi.org/10.1073/pnas.101126598.
12. **Dineen SS, Villapakkam AC, Nordman JT, Sonenshein AL.** 2007. Repression of Clostridium difficile toxin gene expression by CodY. Mol. Microbiol. **66:**206–219. http://dx.doi.org/10.1111/j.1365-2958.2007.05906.x.
13. **Antunes A, Martin-Verstraete I, Dupuy B.** 2011. CcpA-mediated repression of Clostridium difficile toxin gene expression. Mol. Microbiol. **79:** 882–899. http://dx.doi.org/10.1111/j.1365-2958.2010.07495.x.
14. **Callewaert L, Michiels CW.** 2010. Lysozymes in the animal kingdom. J. Biosci. **35:**127–160. http://dx.doi.org/10.1007/s12038-010-0015-5.
15. **Herbert S, Bera A, Nerz C, Kraus D, Peschel A, Goerke C, Meehl M, Cheung A, Götz F.** 2007. Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. PLoS Pathog. **3:**e102. http://dx.doi.org/10.1371/journal.ppat.0030102.
16. **Bera A, Herbert S, Jakob A, Vollmer W, Götz F.** 2005. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of Staphylococcus aureus. Mol. Microbiol. **55:**778–787. http://dx.doi.org/10.1111/j.1365-2958.2004.04446.x.
17. **Aubry C, Goulard C, Nahori M-A, Cayet N, Decalf J, Sachse M, Boneca IG, Cossart P, Dussurget O.** 2011. OatA, a peptidoglycan O-acetyltransferase involved in Listeria monocytogenes immune escape, is critical for virulence. J. Infect. Dis. **204:**731–740. http://dx.doi.org/10.1093/infdis/jir396.
18. **Boneca IG, Dussurget O, Cabanes D, Nahori M-A, Sousa S, Lecuit M, Psylinakis E, Bouriotis V, Hugot J-P, Giovannini M, Coyle A, Bertin J, Namane A, Rousselle J-C, Cayet N, Prévost M-C, Balloy V, Chignard M, Philpott DJ, Cossart P, Girardin SE.** 2007. A critical role for peptidoglycan N-deacetylation in Listeria evasion from the host innate immune

system. Proc. Natl. Acad. Sci. U. S. A. **104:**997–1002. http://dx.doi.org/10.1073/pnas.0609672104.
19. **Benachour A, Ladjouzi R, Le Jeune A, Hébert L, Thorpe S, Courtin P, Chapot-Chartier M-P, Prajsnar TK, Foster SJ, Mesnage S.** 2012. The lysozyme-induced peptidoglycan N-acetylglucosamine deacetylase PgdA (EF1843) is required for Enterococcus faecalis virulence. J. Bacteriol. **194:**6066–6073. http://dx.doi.org/10.1128/JB.00981-12.
20. **Fittipaldi N, Sekizaki T, Takamatsu D, de la Cruz Domínguez-Punaro M, Harel J, Bui NK, Vollmer W, Gottschalk M.** 2008. Significant contribution of the pgdA gene to the virulence of Streptococcus suis. Mol. Microbiol. **70:**1120–1135. http://dx.doi.org/10.1111/j.1365-2958.2008.06463.x.
21. **Le Jeune A, Torelli R, Sanguinetti M, Giard J-C, Hartke A, Auffray Y, Benachour A.** 2010. The extracytoplasmic function sigma factor SigV plays a key role in the original model of lysozyme resistance and virulence of Enterococcus faecalis. PLoS One **5:**e9658. http://dx.doi.org/10.1371/journal.pone.0009658.
22. **Laaberki M-H, Pfeffer J, Clarke AJ, Dworkin J.** 2011. *O*-Acetylation of peptidoglycan is required for proper cell separation and S-layer anchoring in Bacillus anthracis. J. Biol. Chem. **286:**5278–5288. http://dx.doi.org/10.1074/jbc.M110.183236.
23. **Bernard E, Rolain T, Courtin P, Guillot A, Langella P, Hols P, Chapot-Chartier M-P.** 2011. Characterization of O-acetylation of N-acetylglucosamine: a novel structural variation of bacterial peptidoglycan. J. Biol. Chem. **286:**23950–23958. http://dx.doi.org/10.1074/jbc.M111.241414.
24. **Vollmer W, Tomasz A.** 2000. The pgdA gene encodes for a peptidoglycan N-acetylglucosamine deacetylase in Streptococcus pneumoniae. J. Biol. Chem. **275:**20496–20501. http://dx.doi.org/10.1074/jbc.M910189199.
25. **Psylinakis E, Boneca IG, Mavromatis K, Deli A, Hayhurst E, Foster SJ, Vårum KM, Bouriotis V.** 2005. Peptidoglycan N-acetylglucosamine deacetylases from Bacillus cereus, highly conserved proteins in Bacillus anthracis. J. Biol. Chem. **280:**30856–30863. http://dx.doi.org/10.1074/jbc.M407426200.
26. **Milani CJE, Aziz RK, Locke JB, Dahesh S, Nizet V, Buchanan JT.** 2010. The novel polysaccharide deacetylase homologue Pdi contributes to virulence of the aquatic pathogen Streptococcus iniae. Microbiology **156:**543–554. http://dx.doi.org/10.1099/mic.0.028365-0.
27. **Kobayashi K, Sudiarta IP, Kodama T, Fukushima T, Ara K, Ozaki K, Sekiguchi J.** 2012. Identification and characterization of a novel polysaccharide deacetylase C (PdaC) from Bacillus subtilis. J. Biol. Chem. **287:**9765–9776. http://dx.doi.org/10.1074/jbc.M111.329490.
28. **Staroń A, Sofia HJ, Dietrich S, Ulrich LE, Liesegang H, Mascher T.** 2009. The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) sigma factor protein family. Mol. Microbiol. **74:**557–581. http://dx.doi.org/10.1111/j.1365-2958.2009.06870.x.
29. **Helmann JD.** 2002. The extracytoplasmic function (ECF) sigma factors. Adv. Microb. Physiol. **46:**47–110. http://dx.doi.org/10.1016/S0065-2911(02)46002-X.
30. **Ho TD, Ellermeier CD.** 2012. Extracytoplasmic function σ factor activation. Curr. Opin. Microbiol. **15:**182–188. http://dx.doi.org/10.1016/j.mib.2012.01.001.
31. **Ho TD, Ellermeier CD.** 2011. PrsW is required for colonization, resistance to antimicrobial peptides, and expression of extracytoplasmic function σ factors in Clostridium difficile. Infect. Immun. **79:**3229–3238. http://dx.doi.org/10.1128/IAI.00019-11.
32. **Ho TD, Hastie JL, Intile PJ, Ellermeier CD.** 2011. The Bacillus subtilis extracytoplasmic function σ factor σ(V) is induced by lysozyme and provides resistance to lysozyme. J. Bacteriol. **193:**6215–6222. http://dx.doi.org/10.1128/JB.05467-11.
33. **Guariglia-Oropeza V, Helmann JD.** 2011. Bacillus subtilis σ(V) confers lysozyme resistance by activation of two cell wall modification pathways, peptidoglycan O-acetylation and D-alanylation of teichoic acids. J. Bacteriol. **193:**6223–6232. http://dx.doi.org/10.1128/JB.06023-11.
34. **Hastie JL, Williams KB, Ellermeier CD.** 2013. The activity of σV, an extracytoplasmic function σ factor of Bacillus subtilis, is controlled by a regulated proteolysis of the anti-σ factor RsiV. J. Bacteriol. **195:**3135–3144. http://dx.doi.org/10.1128/JB.00292-13.
35. **Varahan S, Iyer VS, Moore WT, Hancock LE.** 2013. Eep confers lysozyme resistance to Enterococcus faecalis via the activation of the extracytoplasmic function sigma factor SigV. J. Bacteriol. **195:**3125–3134. http://dx.doi.org/10.1128/JB.00291-13.
36. **O'Connor JR, Lyras D, Farrow KA, Adams V, Powell DR, Hinds J, Cheung JK, Rood JI.** 2006. Construction and analysis of chromosomal Clostridium difficile mutants. Mol. Microbiol. **61:**1335–1351. http://dx.doi.org/10.1111/j.1365-2958.2006.05315.x.
37. **Sebaihia M, Wren BW, Mullany P, Fairweather NF, Minton N, Stabler R, Thomson NR, Roberts AP, Cerdeño-Tárraga AM, Wang H, Holden MTG, Wright A, Churcher C, Quail MA, Baker S, Bason N, Brooks K, Chillingworth T, Cronin A, Davis P, Dowd L, Fraser A, Feltwell T, Hance Z, Holroyd S, Jagels K, Moule S, Mungall K, Price C, Rabbinowitsch E, Sharp S, Simmonds M, Stevens K, Unwin L, Whithead S, Dupuy B, Dougan G, Barrell B, Parkhill J.** 2006. The multidrug-resistant human pathogen Clostridium difficile has a highly mobile, mosaic genome. Nat. Genet. **38:**779–786. http://dx.doi.org/10.1038/ng1830.
38. **Wilson KH, Kennedy MJ, Fekety FR.** 1982. Use of sodium taurocholate to enhance spore recovery on a medium selective for Clostridium difficile. J. Clin. Microbiol. **15:**443–446.
39. **Bouillaut L, Self WT, Sonenshein AL.** 2013. Proline-dependent regulation of Clostridium difficile Stickland metabolism. J. Bacteriol. **195:**844–854. http://dx.doi.org/10.1128/JB.01492-12.
40. **Heap JT, Kuehne SA, Ehsaan M, Cartman ST, Cooksley CM, Scott JC, Minton NP.** 2010. The ClosTron: mutagenesis in Clostridium refined and streamlined. J. Microbiol. Methods **80:**49–55. doi.org/10.1016/j.mimet.2009.10.018.
41. **Heap JT, Pennington OJ, Cartman ST, Carter GP, Minton NP.** 2007. The ClosTron: a universal gene knock-out system for the genus Clostridium. J. Microbiol. Methods **70:**452–464. http://dx.doi.org/10.1016/j.mimet.2007.05.021.
42. **Kim L, Mogk A, Schumann W.** 1996. A xylose-inducible Bacillus subtilis integration vector and its application. Gene **181:**71–76. http://dx.doi.org/10.1016/S0378-1119(96)00466-0.
43. **McBride SM, Sonenshein AL.** 2011. Identification of a genetic locus responsible for antimicrobial peptide resistance in Clostridium difficile. Infect. Immun. **79:**167–176. http://dx.doi.org/10.1128/IAI.00731-10.
44. **Fagan RP, Fairweather NF.** 2011. Clostridium difficile has two parallel and essential Sec secretion systems. J. Biol. Chem. **286:**27483–27493. http://dx.doi.org/10.1074/jbc.M111.263889.
45. **Sambol SP, Tang JK, Merrigan MM, Johnson S, Gerding DN.** 2001. Infection of hamsters with epidemiologically important strains of Clostridium difficile. J. Infect. Dis. **183:**1760–1766. http://dx.doi.org/10.1086/320736.
46. **Kaplan EL, Meier P.** 1958. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc. **53:**457–481. http://dx.doi.org/10.1080/01621459.1958.10501452.
47. **McPherson DC, Popham DL.** 2003. Peptidoglycan synthesis in the absence of class A penicillin-binding proteins in Bacillus subtilis. J. Bacteriol. **185:**1423–1431. http://dx.doi.org/10.1128/JB.185.4.1423-1431.2003.
48. **Peltier J, Courtin P, El Meouche I, Lemée L, Chapot-Chartier M-P, Pons J-L.** 2011. Clostridium difficile has an original peptidoglycan structure with a high level of N-acetylglucosamine deacetylation and mainly 3–3 cross-links. J. Biol. Chem. **286:**29053–29062. http://dx.doi.org/10.1074/jbc.M111.259150.
49. **Hayashi K, October R, Spectronic L, Division B.** 1975. A rapid determination of sodium dodecyl sulfate with methylene blue. Anal. Biochem. **506:**503–506.
50. **Zhang G, Darius S, Smith SR, Ritchie SJ.** 2006. In vitro inhibitory effect of hen egg white lysozyme on Clostridium perfringens type A associated with broiler necrotic enteritis and its alpha-toxin production. Lett. Appl. Microbiol. **42:**138–143. http://dx.doi.org/10.1111/j.1472-765X.2005.01812.x.
51. **Trieu-Cuot P, Carlier C, Poyart-Salmeron C, Courvalin P.** 1991. Shuttle vectors containing a multiple cloning site and a lacZα gene for conjugal transfer of DNA from Escherichia coli to Gram-positive bacteria. Gene **102:**99–104. http://dx.doi.org/10.1016/0378-1119(91)90546-N.