Supplementary File S1: Recipe for Modified Minimal Medium (MMM)

Modified Minimal Medium (adapted from Cartman *et al.,* 2012)

Component	**Final Concentration (mg ml^{-1})**
Amino Acids	
Casamino acids	1.25
L-Tryptophan	0.0625
L-Cysteine	0.0625
Salts	
Na_2HPO_4	5
$NaHCO_3$	5
KH_2PO_4	0.9
NaCl	0.9
Trace salts	
$(NH_4)_2SO_4$	0.04
$CaCl_2 \cdot 2H_2O$	0.026
$MgCl_2 \cdot 6H_2O$	0.02
$MnCl_2 \cdot 4H_2O$	0.01
$CoCl_2 \cdot 6H_2O$	0.001
Iron	
$FeSO_4 \cdot 7H_2O$	0.004
Vitamins	
D-Biotin	0.001
Calcium-D-pantothenate	0.001
Pyridoxine	0.001
D-Glucose*	0.9 (5 mM)
Ethanolamine-HCl*	1.46 (15 mM)

*included as indicated in text

Supplementary File S2: Vector Construction

pMC253: The group II intron of pCE240 was retargeted to *eutA* using primers oMC665, oMC666, oMC667 and EBSu as outlined in the Targetron users manual (Sigma-Aldrich). The primers used for intron retargeting were generated using the Jpintronator algorithm provided by J.P. van Pijkeren and Rob Britton of Michigan State University. The retargeted group II intron was TA cloned into pCR2.1 to generate pMC253.

pMC256: The group II intron of pCE240 was retargeted to *eutG* using primers oMC674, oMC675, oMC676 and EBSu. The primers used were generated by the Intron Site Finder. The retargeted group II intron was TA cloned into pCR2.1 to generate pMC256.

pMC257: The *eutA*-targeted group II intron in pMC253 was cloned into pCE240 using *Bsr*GI and *Hind*III to generate pMC257.

pMC260: The *eutG*-targeted group II intron in pMC256 was cloned into pCE240 using *Bsr*GI and *Hind*III to generate pMC260.

pMC263: pMC257 was digested with *Sfo*I and *Sph*I. The resulting 5.8 kb fragment was cloned into pMC123 digested with *Sph*I and *Sna*BI, generating pMC263.

pMC266: pMC260 was digested with *Sfo*I and *Sph*I. The resulting 5.8 kb fragment was cloned into pMC123 digested with *Sph*I and *Sna*BI, generating pMC266.

Figure S3

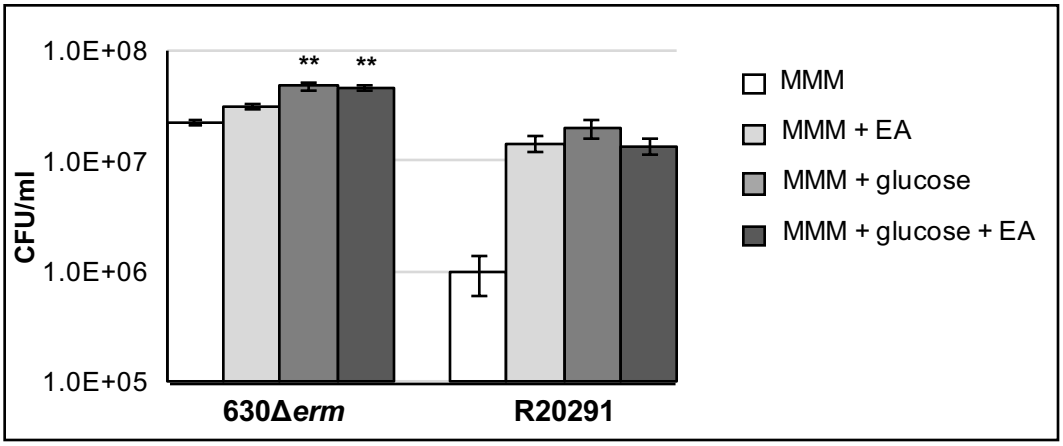

Figure S3. Enumeration of *C. difficile* grown in MMM. Colony forming units (CFU/ml) of *C. difficile* strains 630Δ*erm* and R20291 after 12 h of growth in modified minimal medium (MMM) or MMM supplemented with 15 mM ethanolamine (EA), and/or 5 mM D-glucose. CFU/ml for each condition was compared to the same strain grown in MMM without supplementation. Averages and standard error of the mean for a minimum of three biological replicates is shown. ** $P \leq 0.001$ by one-way ANOVA and Dunnett's test for multiple comparisons.

Figure S4

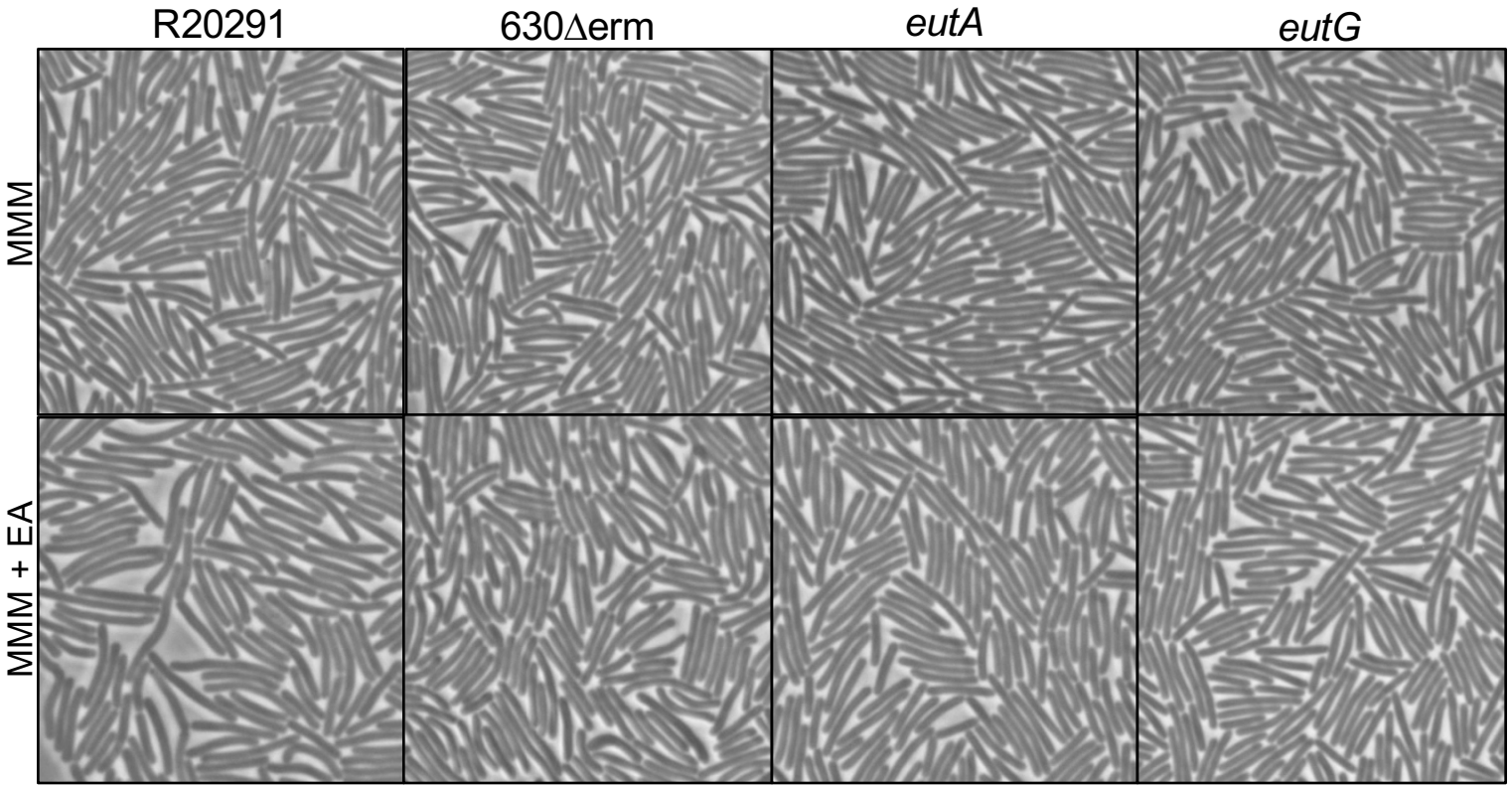

Figure S4. Cellular morphology of *C. difficile* grown in MMM. Phase contrast micrographs of *C. difficile* strains R20291, 630Δ*erm*, MC394 (*eutA*) and MC346 (*eutG*) after 12 h of growth in modified minimal medium (MMM) or MMM supplemented with 15 mM ethanolamine (EA).

Figure S5

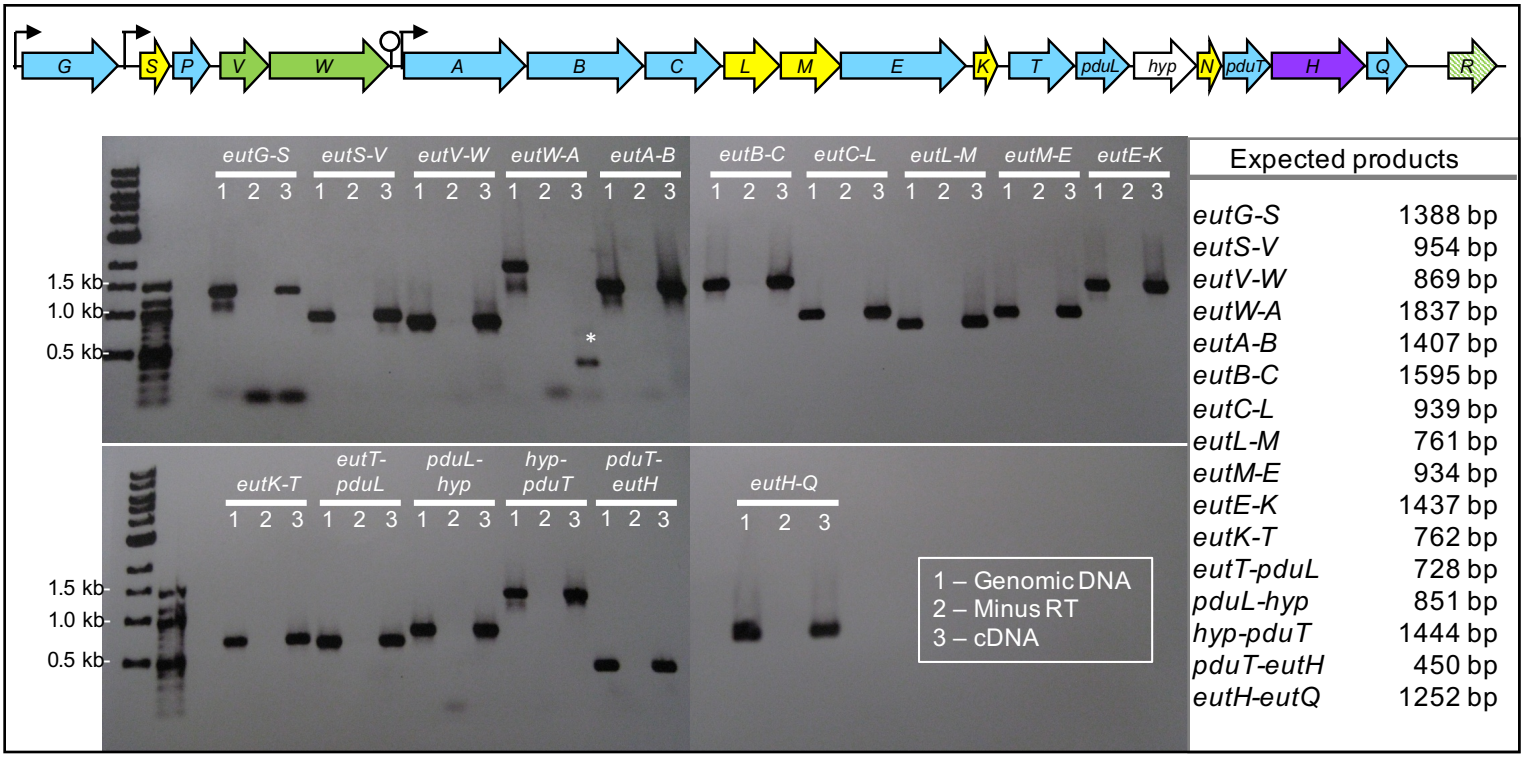

Figure S5. Analysis of transcriptional units within the *eut* gene cluster. Transcriptional units were examined by PCR using primers designed to generate a product if adjacent *eut* open reading frames are present on the same transcript. PCRs were performed using the primer pairs listed below to generate the predicted products listed in the column on the right. Templates for each primer pair were as follows: 1) 630Δ*erm* genomic DNA (positive control), 2) mock cDNA reaction without reverse transcriptase (negative control), and 3) cDNA generated from *C. difficile* grown with ethanolamine. RNA was isolated from *C. difficile* strain 630Δ*erm* grown in 70:30 broth containing 15 mM ethanolamine, and used to generate cDNA from random primers as described in the Methods. Primer pairs: *eutG-S,* oMC655/856; *eutS-V*, oMC855/662; *eutV-W*, oMC661/664; *eutW-A*, oMC663/658; *eutA-B*, oMC657/660; *eutB-C*, oMC659-860; *eutC-L*, oMC859/862; *eutL-M*, oMC861/864; *eutM-E*, oMC863/866; *eutE-K*, oMC865/868; *eutK-T*, oMC867/870; *eutT-pduL*, oMC869/872; *pduL-CD1921* (*hyp*), oMC871/874; *CD1921*(*hyp*)*-pduT*, oMC873/878; *pduT-eutH*, oMC877-880; *eutH-Q,* oMC879/882. *This product was sequenced and was a non-specific artifact. Suspected promoters and terminators are shown as arrows and lollipops, respectively. Liquid ethanolamine was used in this experiment, though the same results were obtained using ethanolamine hydrochoride (not shown). The New England Biolabs (NEB) 1 kb and 100 bp ladders are shown.

Figure S6

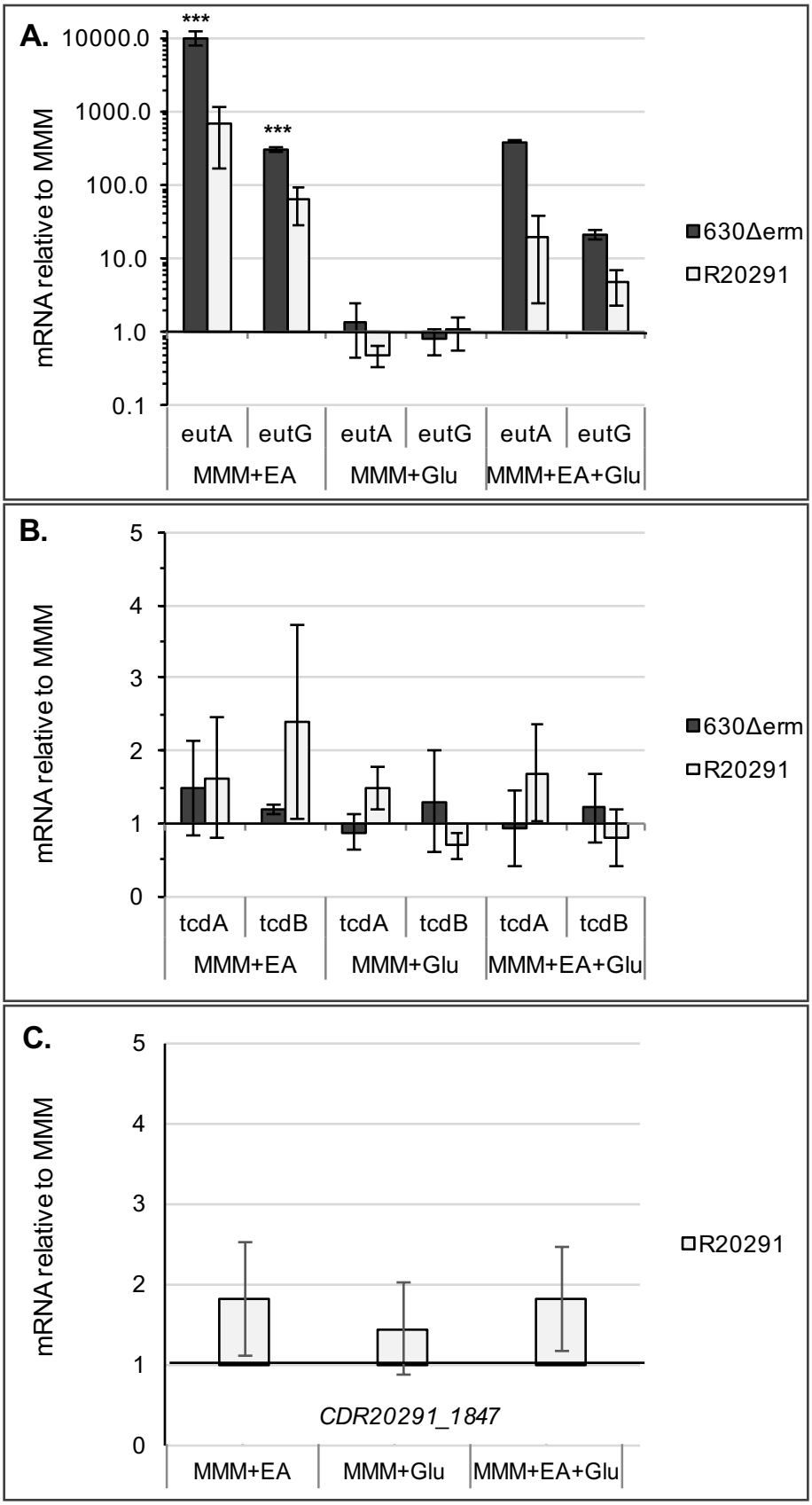

Figure S6. Gene expression in *C. difficile* strains grown in MMM with and without supplementation. Strains 630Δ*erm* and R20291 were grown in Modified Minimal Medium (MMM), MMM containing 15 mM ethanolamine (EA), MMM containing 5 mM D-glucose, or MMM with 5 mM D-glucose and 15 mM EA. Samples for RNA isolation were collected during logarithmic growth (condition-dependent, OD_{600} of 0.06-0.15). qRT-PCR analysis was performed to assess the relative expression of **A)** *eutA* and *eutG*, **B)** *tcdA* and *tcdB* or **C)** the putative regulator, *CDR20291_1847.* Expression is shown relative to the same strain grown in MMM without supplementation. The means and standard error of the means for a minimum of three biological replicates are shown. Statistical significance was assessed by one-way ANOVA and Dunnett's test for multiple comparisons. ** $P \leq 0.001$

Supplementary File S7: Predicted functions of Eut proteins

EutG: Alcohol dehydrogenase

EutS: Putative carboxysome structural protein

EutP: Acetate kinase

EutV: Two component system sensor histidine kinase

EutW: Two component system response regulator

EutA: Lyase reactivating factor

EutB: Ethanolamine ammonia-lyase large subunit

EutC: Ethanolamine ammonia-lyase small subunit

EutL: Carboxysome structural protein

EutM: Carboxysome structural protein

EutE: Aldehyde oxidoreductase

EutK: Carboxysome structural protein

EutT: Corrinoid adenosyltransferase

PduL: Phosphotransacylase

Hyp: hypothetical factor of unknown function

EutN: Carboxysome structural protein

PduT: Carboxysome structural protein

EutH: Ethanolamine transporter

EutQ: Acetate kinase

EutR* (putative): MarR-family transcriptional regulator
(*not encoded in strain 630; CDR20291_1847 in R20291)

Figure S8

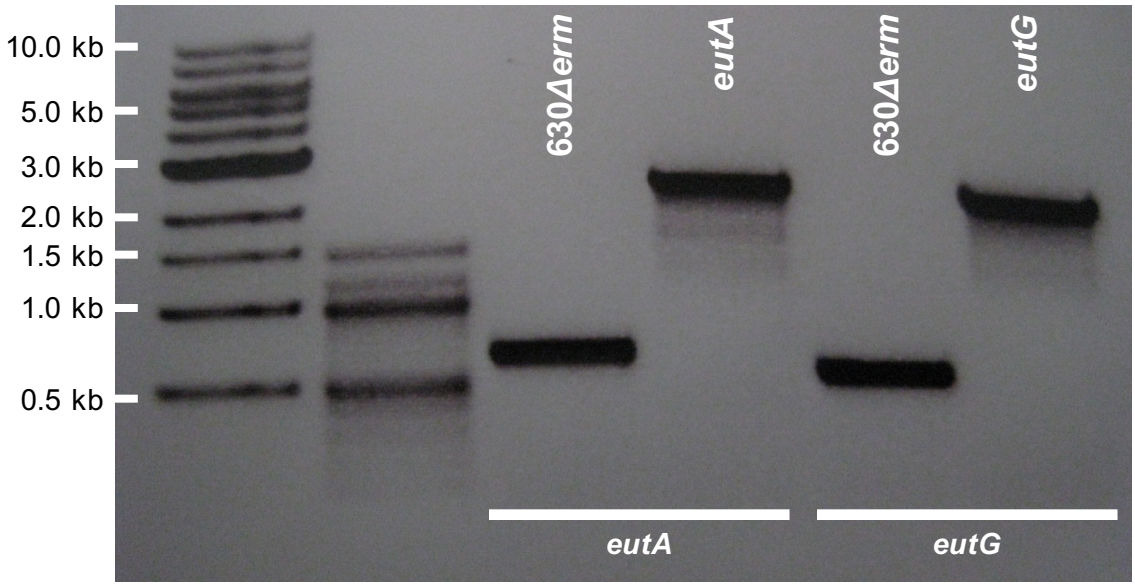

Figure S8. PCR confirmation of insertional disruption of *eutA* and *eutG*. PCR amplification across the regions of intron insertion for *eutA::erm* (MC394; primers oMC715/oMC716) and *eutG::erm* (MC346; oMC717/oMC718).

Figure S9

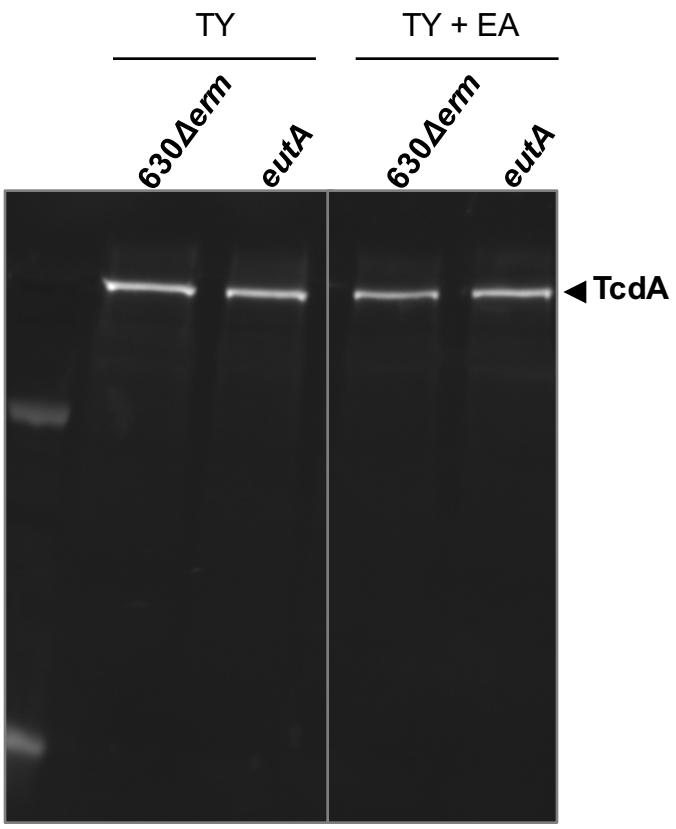

Figure S9. Western blot analysis of TcdA in strain 630Δ*erm* and *eutA* (MC394). Strains were grown 24 h in TY medium +/- 15 mM ethanolamine (EA). Representative images of strains from the same gel and blot are shown. No statistically significant differences were observed between strains or conditions.

Figure S10

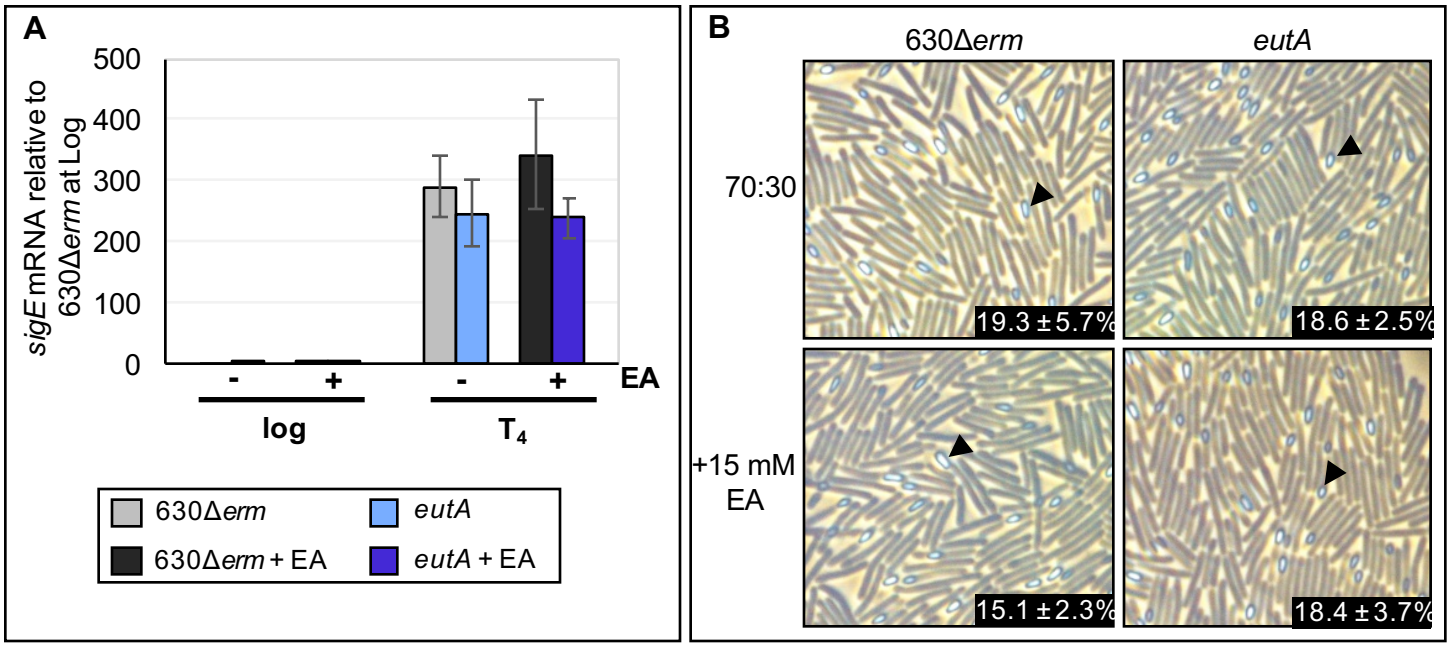

Figure S10. Ethanolamine utilization does not impact sporulation frequency. **A)** qRT-PCR analysis of the sporulation-specific sigma factor, *sigE,* in 630Δ*erm* and the *eutA* mutant (MC394) grown in 70:30 liquid sporulation medium with and without 15 mM ethanolamine (EA). Samples for RNA isolation were collected during logarithmic growth (Log, OD_{600} of 0.5) and four hours after the transition into stationary phase (T_4, late stationary). The means and standard error of the means of four biological replicates are shown. Gene expression of 630Δ*erm* and the *eutA* mutant were compared at each timepoint and condition using the Student's two-tailed *t*-test. (no statistically significant differences were observed). **B)** Phase contrast microscopy was performed on 630Δ*erm* and the *eutA* strain (MC394), grown for 24 hours on 70:30 sporulation agar with or without 15 mM EA. A representative image is shown for each strain and condition. Arrowheads indicate phase-bright spores. Sporulation frequency was determined by direct count (see methods). The means and standard deviations of four biological replicates are shown. Sporulation frequencies of 630Δ*erm* and the *eutA* mutant grown with and without EA were compared using a two-way ANOVA (no statistically significant differences were observed).

Figure S11

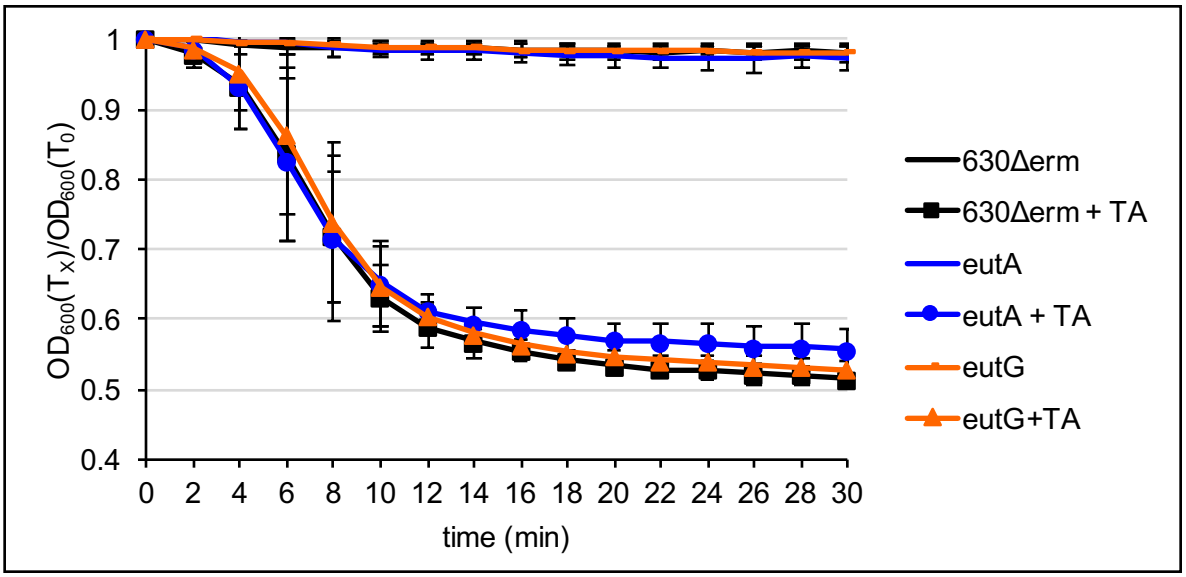

Figure S11. Germination rate is not impacted in ethanolamine pathway mutants. Spores of strains 630Δ*erm*, MC394 (*eutA*) and MC346 (*eutG*) were purified and heat activated prior to germination assessments, as described in the methods. Spores were suspended in BHIS broth to a starting OD_{600} of approximately 0.3. The *C. difficile* germinant, taurocholate was added to a final concentration of 5 mM, as indicated and the optical density of spore samples were assessed every two minutes for 30 minutes. The ratio of the OD_{600} measurement at each timepoint (T_x) were plotted against the density observed at T_0. Results are represented as the mean and standard deviations for three independent biological replicates. No statistically significant differences in germination were detected by one-way ANOVA analysis.