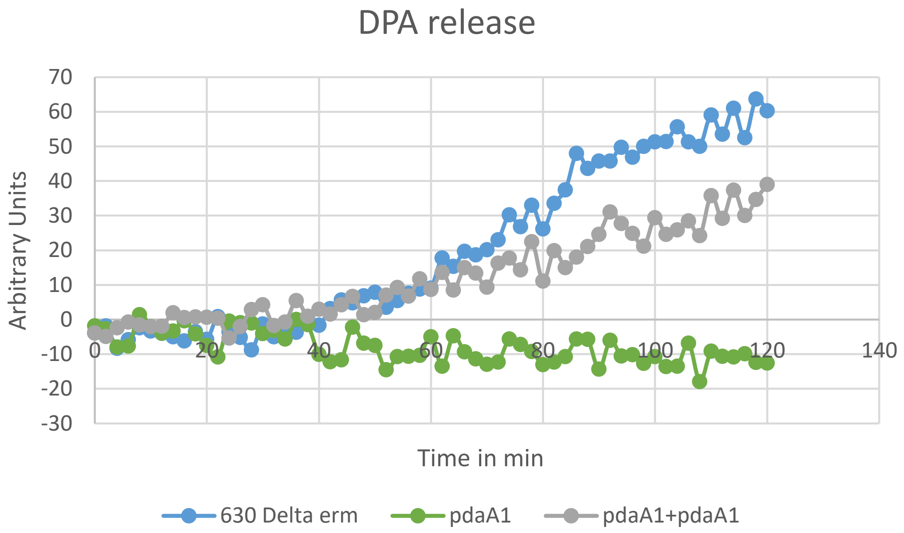
A



B

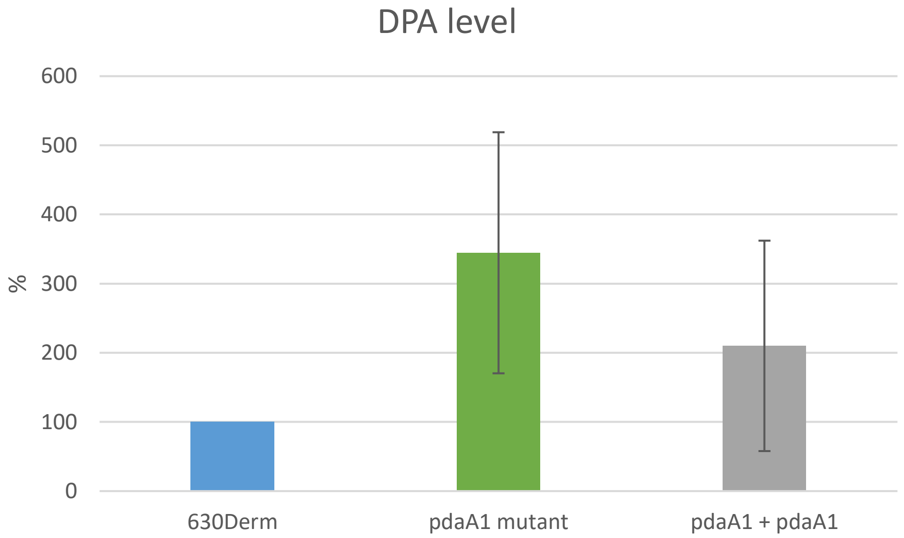


Figure S1. DPA release during germination and quantification of DPA contents of spores

Kinetics of DPA release after germination induction for spore suspensions of the strains 630Δ*erm*(pMTL84151) in blue, Δ*pdaA1(*pMTL84151) in green, and the complemented strain Δ*pdaA1*(pCH67) in gray in response to germinant (A). Total DPA amount of the parental (630Δ*erm*), the *pdaA1* mutant (pdaA1 mutant) and the complemented mutant spores (pdaA1+pdaA1) (B). The average of DPA for the parental strain was normalized at 100% and the DPA level of the mutant and complemented strains were presented as ratios relative to the parental.

DPA was measured by fluorescence, according to the protocol described in Donnelly et *al*. with the following difference, 2.5 X 10^7 spores were used in the terbium release assay. Fluorescence was measured using the spectrofluorometer SAFAS FLX-Xenius.

Reference:

Donnelly, M. L., Fimlaid, K. A., and Shen, A. (2016) Characterization of *Clostridium difficile* Spores Lacking Either SpoVAC or Dipicolinic Acid Synthetase. *Journal of bacteriology* **198**, 1694-1707

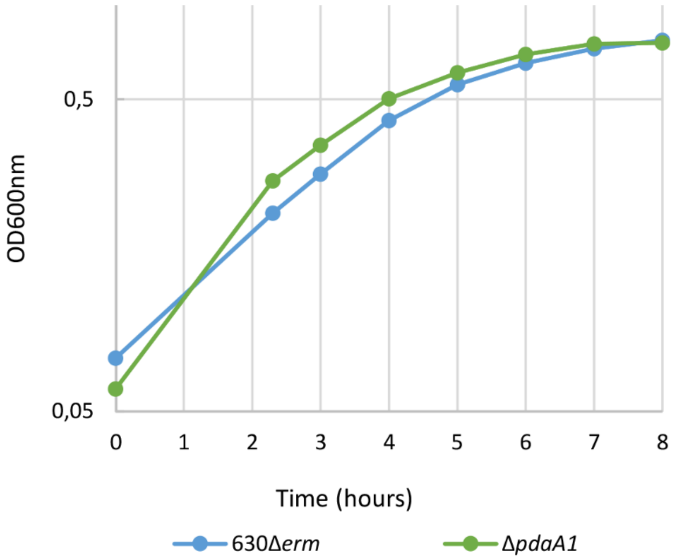


Figure S2. The *pdaA1* mutant and parental strains have a similar growth curve.
Exponential growth curve of the *pdaA1* mutant (green) and parental strain (blue) in BHI broth.

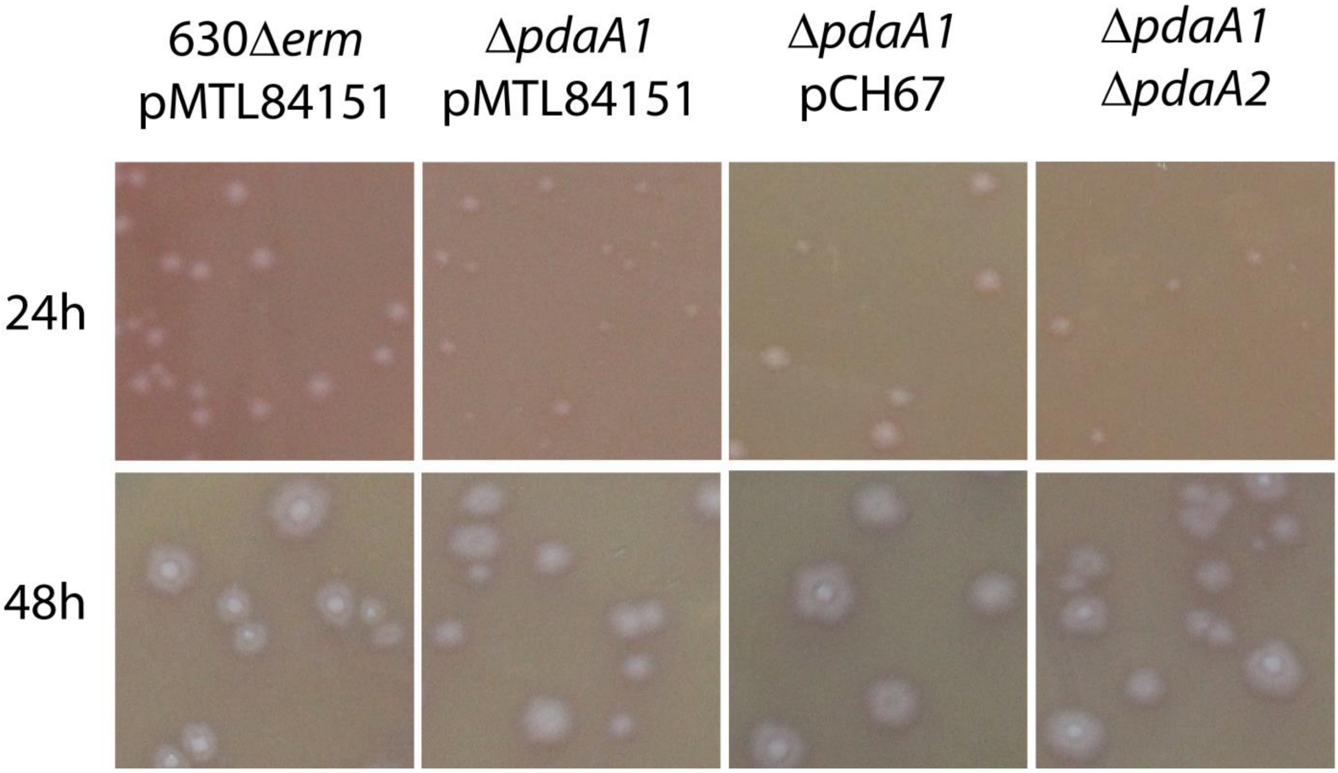


Figure S3. Additional germination assays in solid medium

Assessment of germination delay in solid BHI supplemented with horse blood and taurocholates was performed for the 630Δ*erm*(pMTL84151) parental strain, the Δ*pdaA1*(pMTL84151) mutant, the Δ*pdaA1*(pCH67) complemented strain, and the Δ*pdaA1* Δ*pdaA2* double mutant. Pictures are magnified at the same level, and they are representative of three independent experiments.

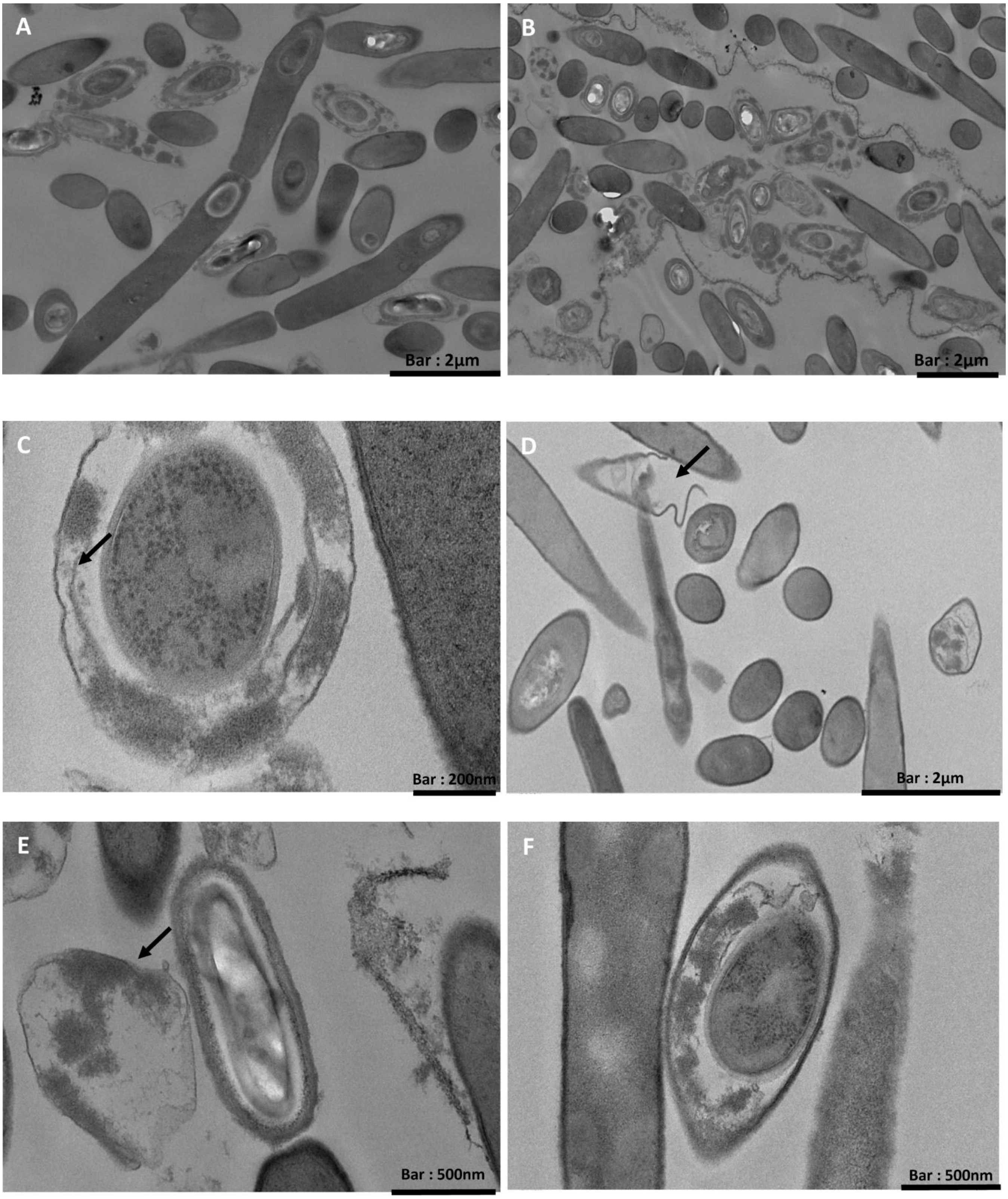
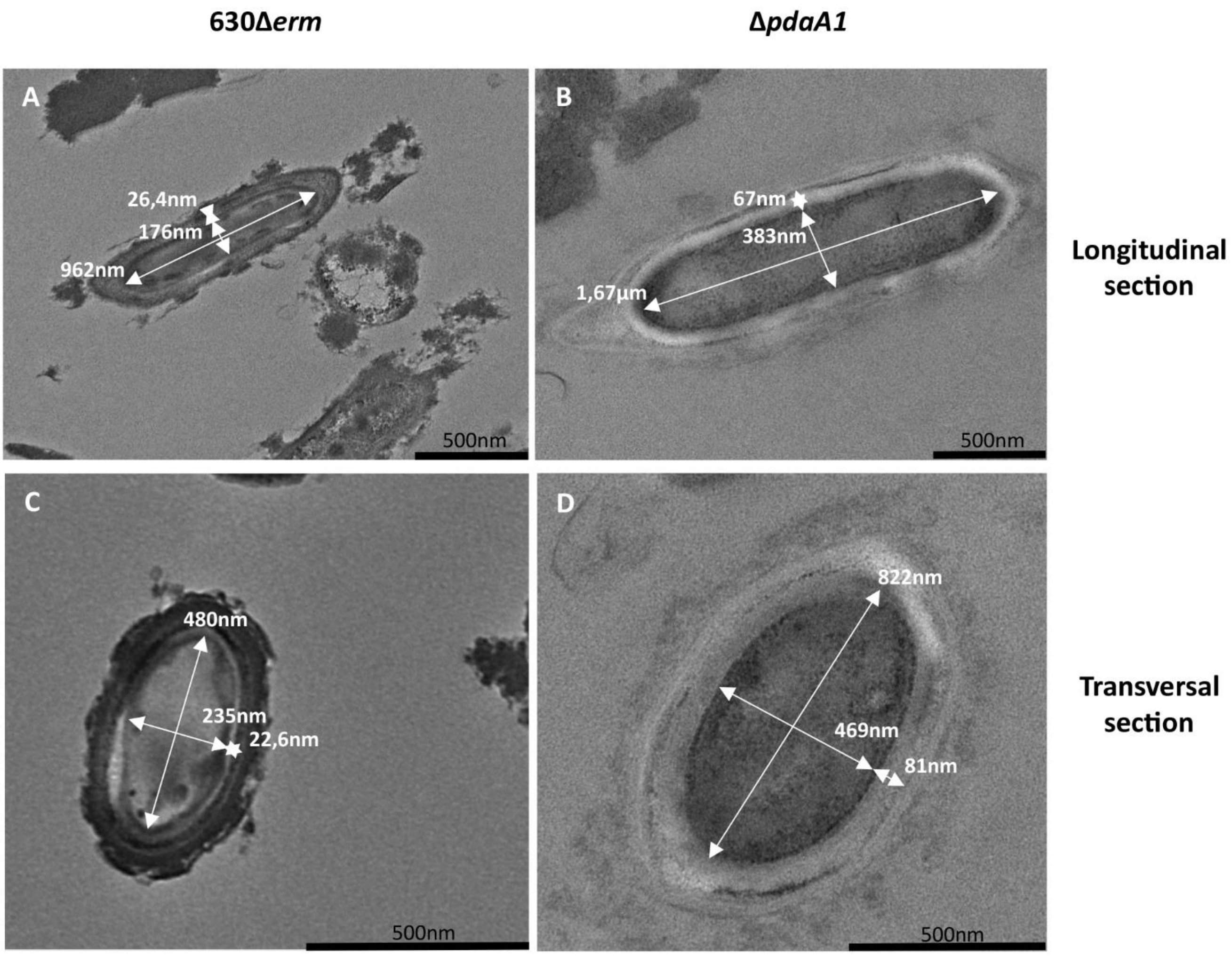


Figure S4. Additional TEM analysis
Global observation for the 630Δ*erm* (A) and Δ*pdaA1* strains (B). Additional abnormal morphology in Δ*pdaA1* endospore TEM analysis (C to F). Black arrows shows detached external structures.



Spore		Core length nm	Core width nm	Cortex thickness nm	Core volume nm^3	Sporoplast volume nm^3	P/S ratio
Longitudinal section	630Δ*erm*	912,90 ± 179,13	230,60 ± 39,60	34,17 ± 12,80	2,08E+08 ± 7,64E+07	2,83E+08 ± 9,67E+07	0,73 ± 0,08
	Δ*pdaA1*	**1122,00** **± 213,53**	**392,20** **± 49,22**	**74,54** **± 13,63**	**7,39E+08** **± 2,31E+08**	**1,11E+09** **± 3,02E+08**	**0,66** **± 0,06**
Transversal section	630Δ*erm*	455,80 ± 92,51	276,90 ± 73,30	36,85 ± 13,03	1,69E+08 ± 1,35E+08	2,31E+08 ± 1,68E+08	0,72 ± 0,07
	Δ*pdaA1*	**583,70** **± 179,06**	**418,70** **± 66,80**	**70,60** **± 12,22**	**4,63E+08** **± 2,11E+08**	**6,98E+08** **± 2,91E+08**	**0,65** **± 0,07**
Average	630Δ*erm*	684,35 ± 269,37	253,75 ± 63,29	35,51 ± 12,98	1,89E+08 ± 1,12E+08	2,57E+08 ± 1,39E+08	0,73 ± 0,08
	Δ*pdaA1*	**852,85** **± 333,57**	**405,45** **± 60,15**	**72,57** **± 13,09**	**6,01E+08** **± 2,61E+08**	**9,03E+08** **± 3,61E+08**	**0,65** **± 0,07**

Figure S5. TEM spore measurements

TEM images of *C. difficile* spore cross sections. Representative images of spores are shown in each panel, for the parental strain (A and C) and the *pdaA1* mutant (B and D). The scale is indicated in the bottom left corner of each image. Full measure results are detailed in the table: longitudinal and transversal values are averages of 10 measurement each ± standard deviation. The protoplast volume (core) and sporoplast volume (core and cortex) were calculated according to Beaman *et al.*: volume = $(4/3)\ \pi\ (\text{width}/2) \times 2 \times (\text{length}/2)$ (57). The protoplast-to-sporoplast (P/S) ratio was calculated by dividing the calculated core volume (protoplast) by the volume of the core plus cortex layer (sporoplast). Bold data indicate significant difference compared to the parental strain (Student test, $p < 0.05$).

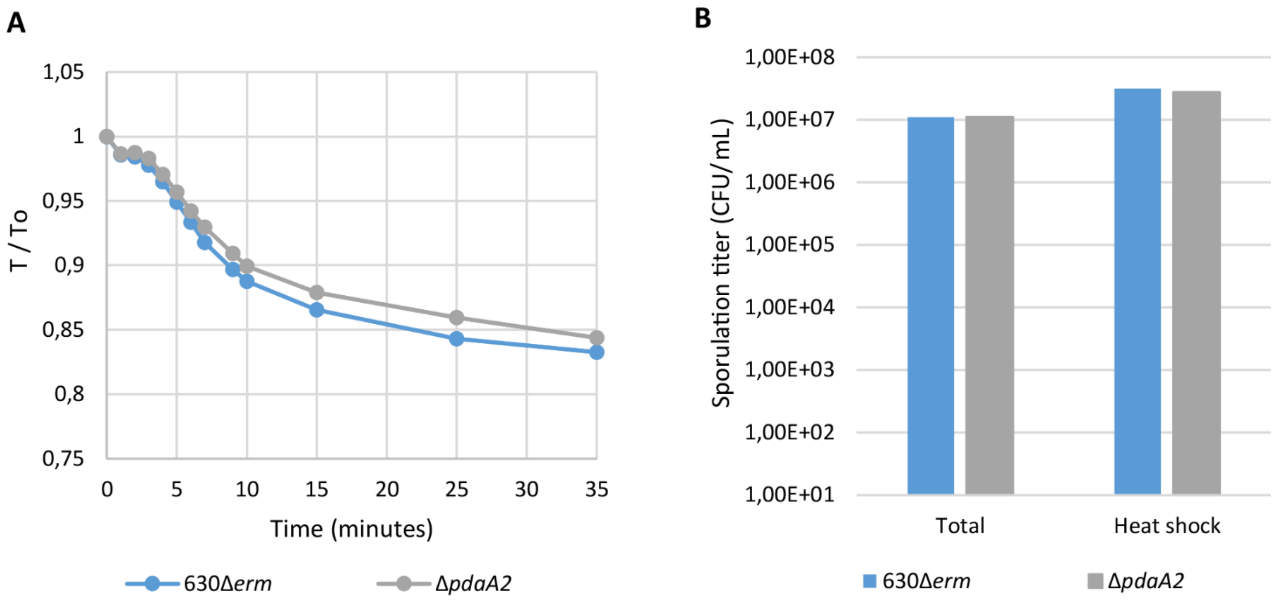


Figure S6. *pdaA2* mutant has similar germination and sporulation compared to the parental strain. Germination assay by optical density monitoring (A). OD_{600nm} observed at time point (T); initial OD_{600nm} at T=0 (To). Quantification of sporulation titers after 72h incubation in SM broth (B), for the 630Δ*erm* parental strain in blue and *pdaA2* mutant (CD630_27190) in grey.

Parameters		630Δ*erm*	Δ630_14300	ΔCD630_27190	ΔCD14300 ΔCD27190
Cross-linking	monomers	90,23	93,80	89,78	85,33
	dimers	9,77	6,20	10,22	14,67
	trimers	0,00	0,00	0,00	0,00
	cross-link	4,89	3,10	5,11	7,33
Side chains (% of muropeptides)	none	21,92	55,35	25,16	30,67
	dipeptide	5,78	8,17	7,01	6,59
	tripeptide	4,83	2,79	5,49	8,67
	tetrapeptide	43,52	33,30	40,61	54,08
	muramic-lactam	23,96	0,40	21,73	0,00
Saccharide chains	disaccharides	45,85	42,57	48,11	54,54
	tetrasaccharides	52,26	40,30	48,90	40,10
	hexasaccharides	1,80	11,57	2,85	3,93
	octasaccharides	0,10	5,56	0,14	1,43
N-deacetylation	N-deacetylated	54,73	51,85	49,15	57,65
	Acetylated	45,27	48,15	50,85	42,35

Table S2. Cortex parameters of the 630Δ*erm*, the *pdaA1* mutant (Δ630_14300) and the *pdaA2* mutant (ΔCD630_27190) strains. The cross-linking index was calculated with the formula (1/2Σdimers + 2/3Σtrimers)/ Σ all muropeptides (61).

Identity matrix	PdaA *B. subtilis*	PdaA *B. thuringiensis*	CD630_1430 *C. difficile*	CD630_27190 *C. difficile*
PdaA *B. subtilis*	100	52.33	36.72	34.66
PdaA *B. thuringiensis*	52.33	100	34.5	39.53
CD630_1430 *C. difficile*	36.72	34.5	100	40.67
CD630_2719 *C. difficile*	34.66	39.53	40.67	100

Table S3. Identity matrix of PdaA proteins

Identity matrix of protein sequences of PdaA from *B. subtilis*, PdaA from *B. thuringiensis*, CD630_14300 and CD630_27190 from *C. difficile*, obtained from ClustalW multiple sequence alignment (28).

Cortex purification and analysis yields

	Freeze-dried spores	Freeze-dried muropeptides	Total peaks area
630Δ*erm*	5,0mg	2,0mg	2,25E+09
Δ*pdaA*	5,3mg	2,0mg	1,17E+10

Table S5. Cortex analysis yields

Name	5' Sequence	3' Sequence	Use
M13F	/	tgtaaaacgacggccagt	Cloning screening
M13R	/	caggaaacagctatgacc	Cloning screening
TC157	/	gcagccagaagccatcgattacaaacgttg	Cloning screening
7315A	/	CCCGGGTACCGAGCTCGAATTCGCCCTTTA	pMTLSC7315 amplification
7315B	/	GATCCTCTAGAGTCGACGTCACGCGTCCATG	
HC174	taaagggcgaattcgagctcggtacccggg	GACCCAAAGAATCTAACACATTTGG	*CD630_14300* deletion – DNA amplification
HC175	CTATAAATATTCAAACTATATTATAAG	tatcataaatttaaaaaaactctatcatg	
HC176	catgatagagtttttttaaatttatgata	CTTATAATATAGTTTGAATATTTATAG	
HC177	catggacgcgtgacgtcgactctagaggatc	CATATTTAGTACATAAATAATTAGTAAG	
HC178	/	GCTTCCAGTCTATCTATACAAATATAG	*CD630_14300* deletion – mutant screening
HC179	/	CCTTTGATTATGTCATGTACAATTTC	
HC254	gaattcgagctc	CCTTGACAGCTC	*CD630_14300* complementation – DNA amplification
HC272	/	CTCTATCGAGAATTAAAGTATT	
HC185	taaagggcgaattcgagctcggtacccggg	TCCCCGTCAATTCCTTTGAGTTTCA	*CD630_27190* deletion - DNA aplification
HC186	CCAATTATAATAATACTATATGC	tatttataattacatctaaagtcta	
HC187	tagactttagatgtaattataaata	GCATATAGTATTATTATAATTGG	
HC188	catggacgcgtgacgtcgactctagaggatc	CAGCTGGTGCAGCAGGTGTTGCAGTATC	
HC189	/	GGTAAGGTTCTTCGCGTTGCTTCG	*CD630_27190* deletion - mutant screening
HC190	/	TCATGGTCTTGCCTTATCTACAGAC	

Table S6. Primers