Supplemental materials

Extracellular DNA filaments associated to surface polysaccharide II give *Clostridioides difficile* biofilm matrix a network-like structure

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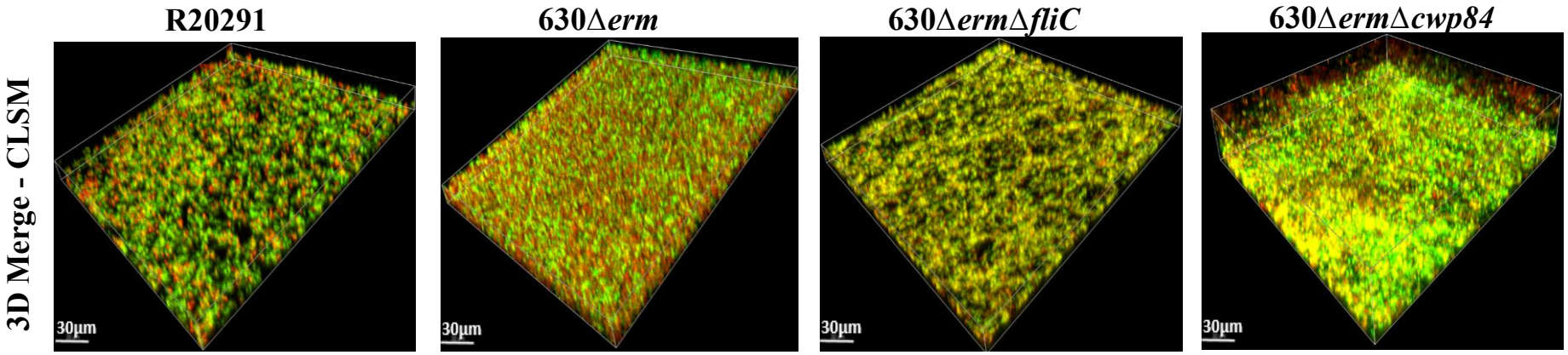

Figure S1. 3D biofilms structure observed using inverted CLSM and designated with Imaris software. 48h Biofilms were stained with Live/Dead, SYTOX Green, and SYTO 60 dyes, and the merged projection is represented in the figure. Images were acquired with a confocal microscope and the objective HC PL APO CS2 20x/0.75 DRY/air. The planes were used to construct the three-dimensional projection using Imaris software (Bitplane, Belfast, UK) 9.7.2 version.

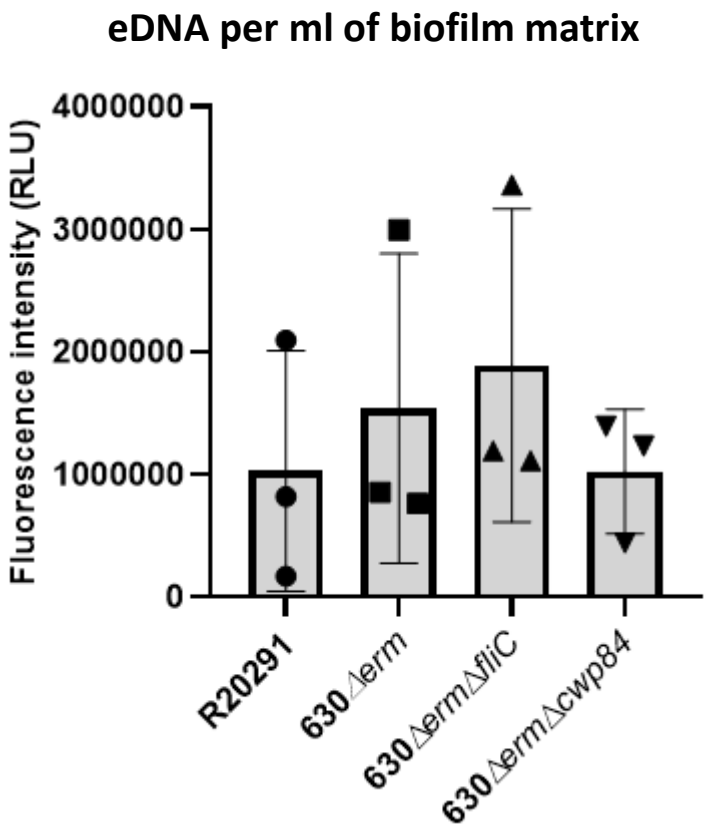

Figure S2. eDNA in biofilm matrix, quantified by fluorescence. The matrix was first extracted from biofilm and then marked with SYTOX Green for 15 minutes. The fluorescence was measured with the spectrophotometer (Synergy H1) after a dye excitation at 500 nm and emission gathered at 526 nm ; expressed in RFU (relative fluorescence unit). Each graph represents the means and standard deviations of 3 biological replicates; statistical normality was determined with the Shapiro-Wilk test and the Mann-Whitney test was used to perform statistical analysis (test (ns, non-significant).

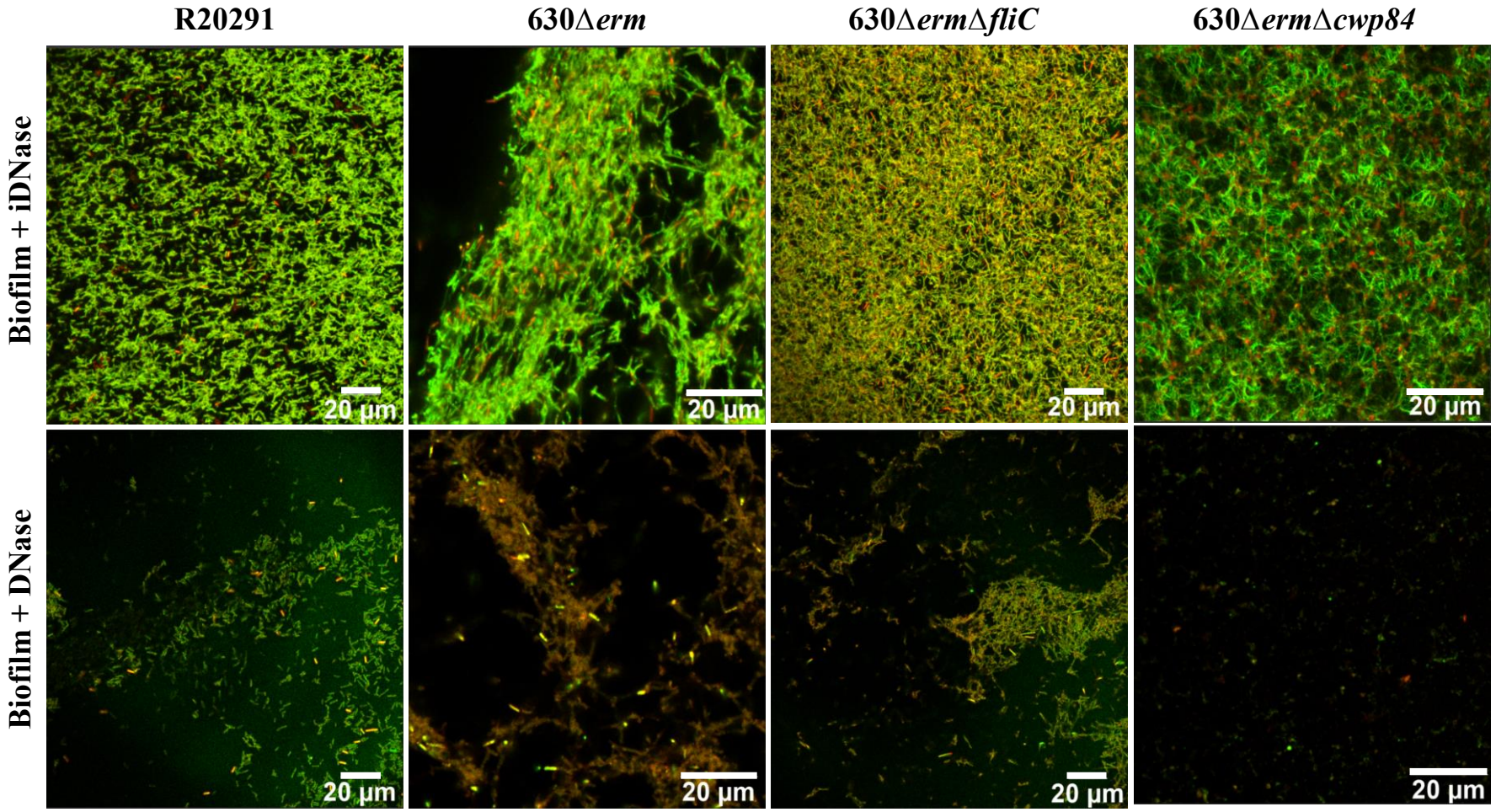

Figure S3. DNase dispersed *C. difficile* biofilm by disrupting eDNA filaments. The four strains biofilms were grown for 48h, treated with DNase, and inactivated DNase (iDNase) for control. Biofilms are stained with SYTOX Green and SYTO 60 and the merge of a Z-stack projections are represented on the figure. 4 biological replicates were realized, and the merge projections are represented in the figure, a representative image was chosen for each condition. Images were acquired with a confocal microscope and the objective HC PL APO CS2 20x/0.75 DRY/air.

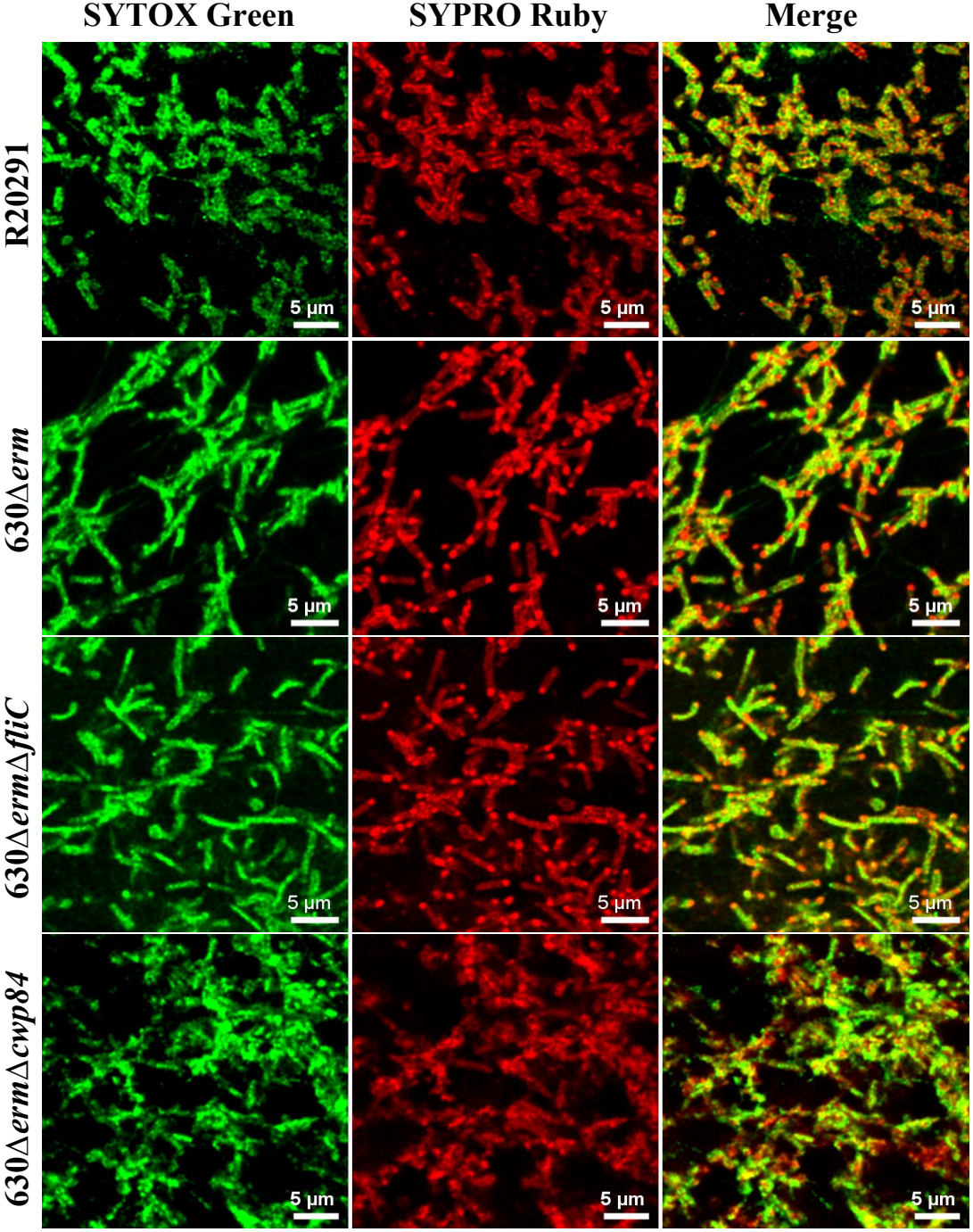

Figure S4. Proteins are one of the EPS components of *C. difficile* biofilm. Biofilms were marked with SYTOX Green (eDNA) and SYPRO Ruby (proteins). Proteins are present in the matrix but aren't on eDNA filaments. 4 biological replicates were realized, and, a representative image was chosen for each condition. Images were deconvolved with Huygens Professional v.20.04 (Scientific Volume Imaging).

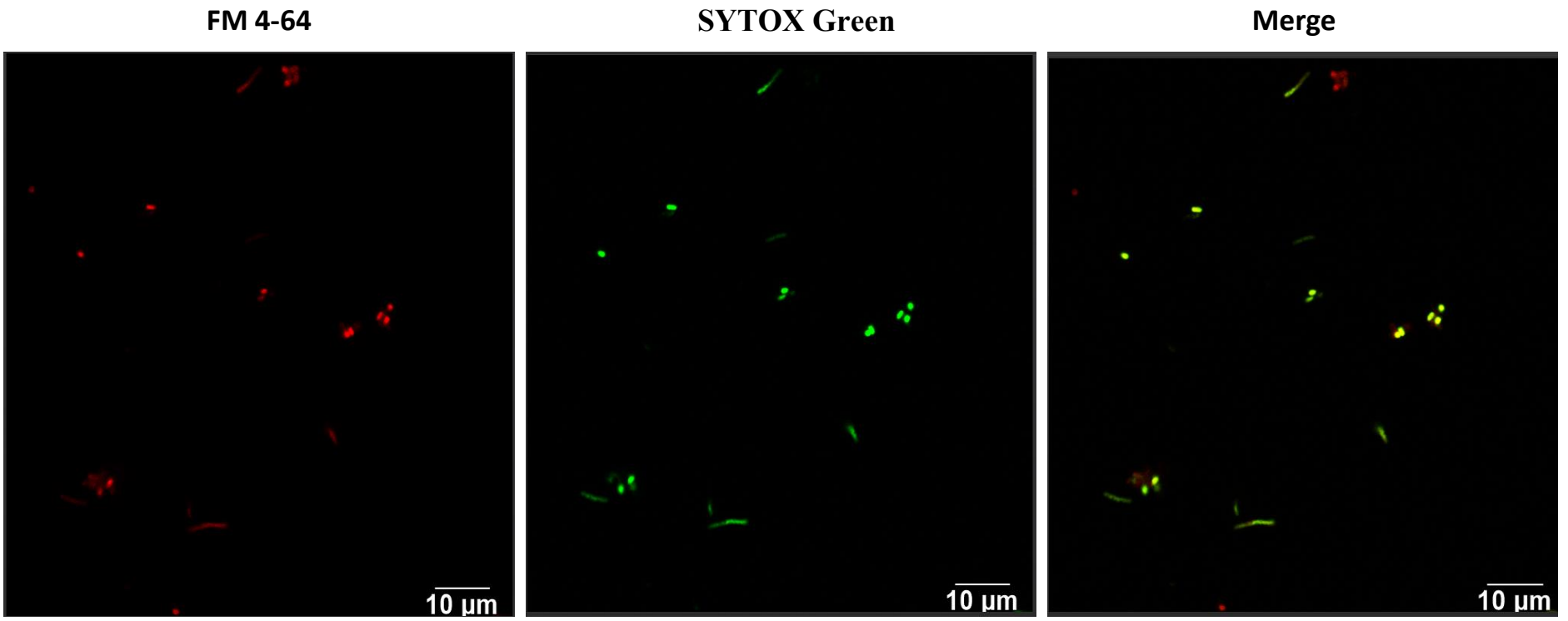

Figure S5. *C. difficile* spores stained with FM 4-64 (lipids) and SYTOX Green (eDNA), imaged with CLSM. SYTOX Green and FM 4-64 were able to penetrate inside the spores. The spores have a regular shape with a diameter of 1-2µm.

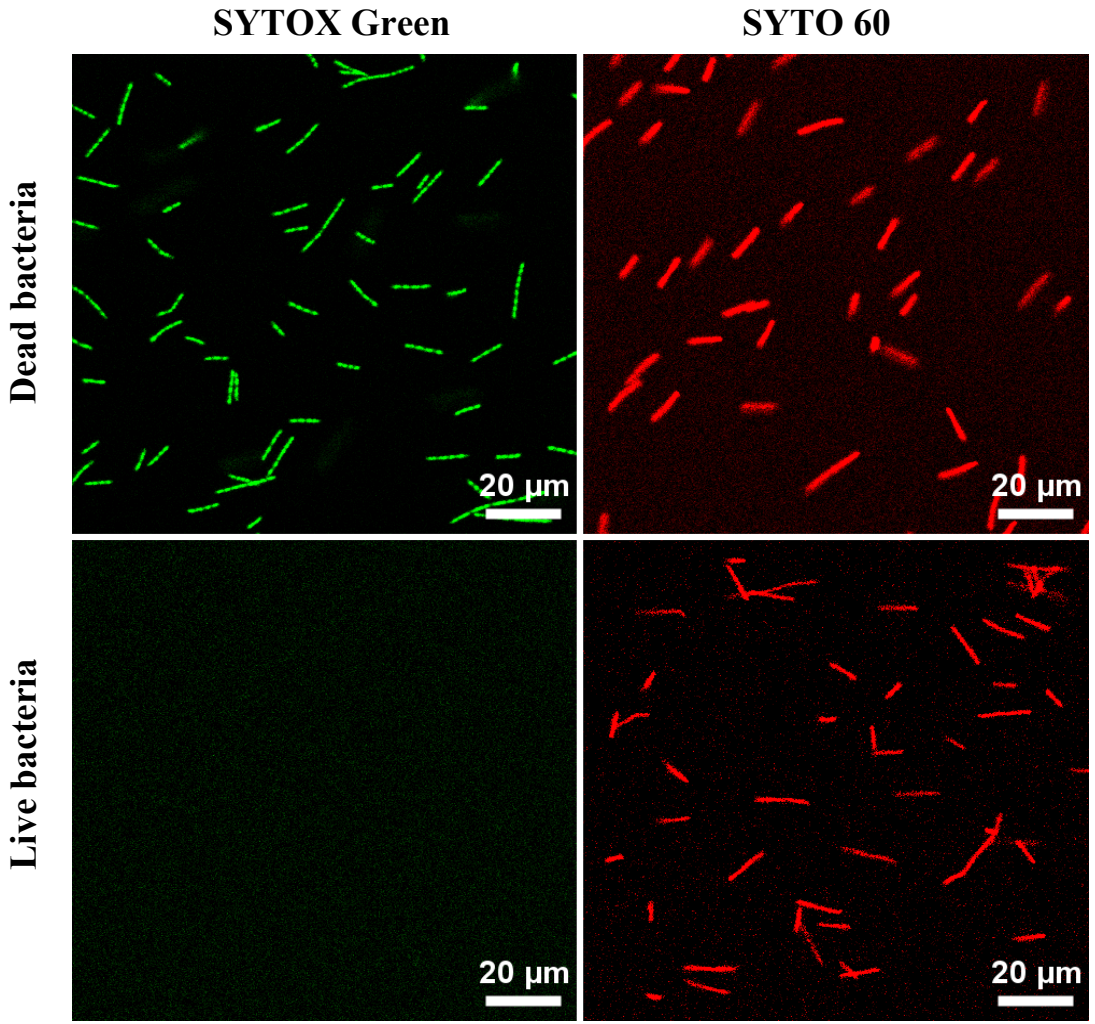

Figure S6. Specificity of Sytox green staining of eDNA and DNA from dead bacteria is maintained after 2 h 30 of aerobic staining. Live bacteria suspension of 630Δ*erm* is in the exponential growth phase (2h of culture, at absorbance$_{600}$ 0,6); part of the suspension is heated for 10 minutes at 100°C to obtain a suspension of dead bacteria. Live and dead bacteria were stained separately with SYTOX Green and SYTO 60 for 2h 30 in aerobic conditions. They were fixed with Carnoy solution and imaged with CLSM. The tests were carried out with all four strains of *C. difficile* and the same result was obtained for all strains.

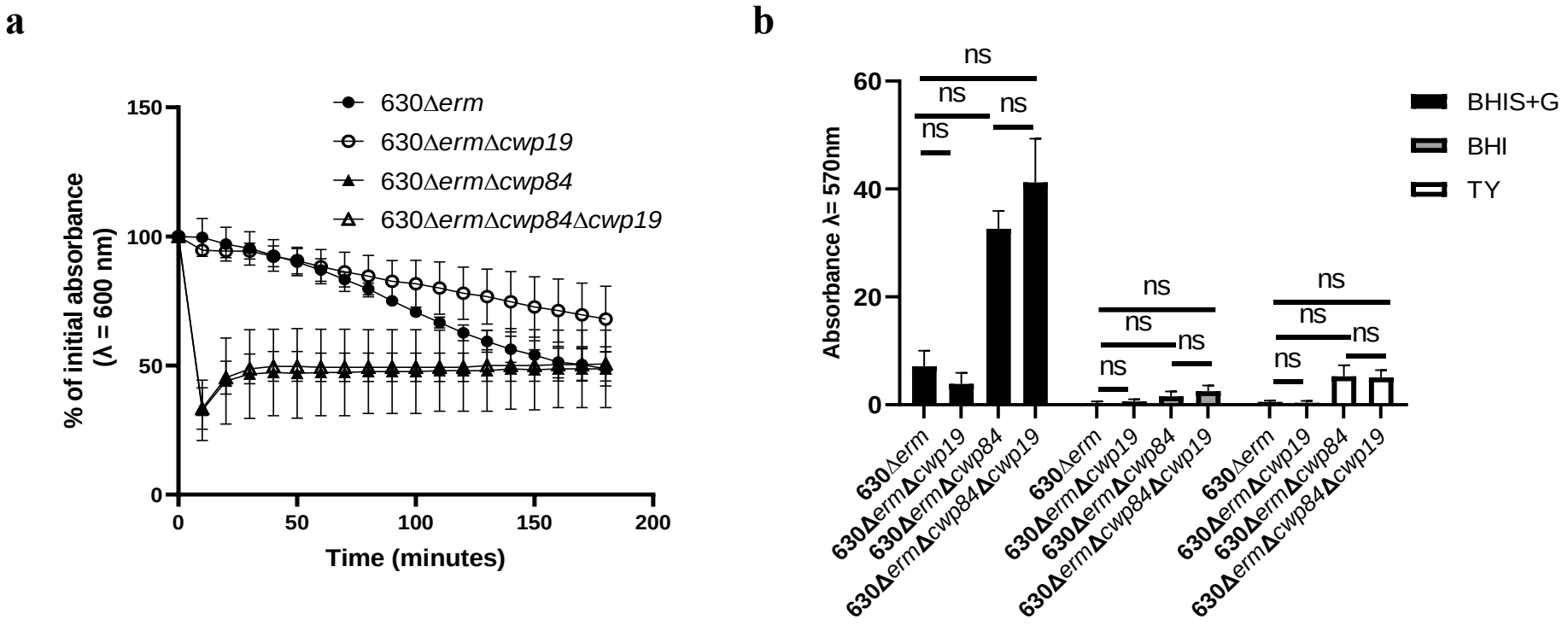

Figure S7. Impact of *cwp19* deletion on strains 630Δ*erm* and 630Δ*erm*Δ*cwp84.* (**a**) Autolysis capacity of *C. difficile* strains. Autolysis was induced with Triton X-100 and kinetics were measured for 3 h every 10 minutes by measuring absorbance (Absorbance λ= 600 nm). Each graph represents the means and standard deviations of 3 biological replicates. (**b**) Biofilm biomass of *cwp19* mutants and parental strains. Biofilms were grown for 48h in three different culture media, BHIS+G, BHI, and TY. Biomass was quantified using crystal violet staining and Absorbance λ = 570 nm measurement. Each graph represents the means and standard deviations of 3 biological replicates; statistical normality was determined with the Shapiro-Wilk test and the Mann-Whitney test was used to perform statistical analysis test (ns, non-significant).

Table S1. Raw quantification data for EPS matrix compounds.

Matrix components	R20291	630Δ*erm*	630Δ*ermΔfliC*	630Δ*ermΔcwp84*
	Quantity (in µg)			
Proteins	336.60	284.82	387.29	336.62
Polysaccharides	1769.17	1889.99	2691.75	5283.22
eDNA	73.84	105.36	184.61	149.91
Total of EPS amount	2179.60	2280.18	3263.65	5769.75
Standart deviation	1262.18	1322.18	1540.57	2405.95
Biofilm dry weight	14890	6670	15690	42610

Table S2. *E. coli* strains and plasmids used for mutants construction in *C. difficile* background.

		Genotypes
E. coli strains	TG1	*E. coli* k12 (F' tra D36 lacl^{q} lacZΔMIS, *proAB/SupE*, Δ(*hdsM-mcrB*)
	HB101 *tra* (pRK24)	*E. coli* (pRK24): F- Δ*(gpt-proA)62 leuB6 glnV44 ara14 galK2 lacY1* Δ*(mcrC-mrr) rpsL20* (Srt^{R}) *xyl5 mlt1 recA13* pRK24
Plasmids	pKNTS1	pJV7 derived, spec^{R-}, with CO1 and CO2 of *cwp19* gene
	pJV7	pMSRX*aadA*, spectinomycin resistance gene flanked by BsaI sites, Cm^{R}, and Sp^{R}
	pHC49	pMTL-SC7315 derived plasmid for fliC (CD630_02390) deletion
	pMTL-SC7315	Circular cloning vector, 6000 nucleotides, catP, codA

Table S3. Primers for *cwp19 and fliC* gene deletion.

	Name of primers	Primers sequences
cwp19 (CD630Δ*erm*_27670) deletion	F_primer_CO1 (TC370)	GGCTACGGTCTCGTTGCgctatggtgttaatagcag
	R_primer_CO1 (TC371)	GGCTACGGTCTCccttaatttttaaatttatttttctacaatatac
	F_primer_CO2 (TC372)	GGCTACGGTCTCCTAAGGGGGATGaataaaaaagttaatttaaaatacag agtattttataaatgtag
	R_primer_CO2 (TC373)	GGCTACGGTCTCTTGCCggaacagatgctagaccaaatg
fliC (CD630_02390) deletion	HC129	GGGCGAATTCGAGCTCGGTACCCGGGgctagtggaactaaatctgcag tagtatatgg
	HC159	CTTTTTTATTCAATATACTAAATTTATTttatacttttttattaaaaatattta c
	HC130	GTAAATATTTTTAATAAAAAAGTATAAaataaatttagtatattgaataa aaaag
	HC160	GACGCGTGACGTCGACTCTAGAGGATCctatctctctcaaccttcgaat ttggaatctc
pMTL-SC7315 amplification	7315A	cccgggtaccgagctcgaattcgcccttta
	7315B	gatcctctagagtcgacgtcacgcgtccatg

*CO for crossing over regions