Supplemental Material

A LysM domain intervenes in sequential protein-protein and protein-peptidoglycan interactions important for spore coat assembly in *Bacillus subtilis*

Fatima C. Pereira[1, #,¶], Filipa Nunes[1,¶], Fernando Cruz[1], Catarina Fernandes[1,&], Anabela L. Isidro[1,§], Diana Lousa[1], Cláudio M. Soares[1], Charles P. Moran, Jr.[2], Adriano O. Henriques[1] and Mónica Serrano[1,*]

[1]Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Avenida da República, Apartado 127, 2781-901 Oeiras Codex, Portugal; [22]Emory University School of Medicine, Department of Microbiology and Immunology, Atlanta GA 30322, USA

[¶]Equal contributions.

[#]**Present address**: Division of Microbial Ecology, Department of Microbiology and Ecosystem Science, Research Network Chemistry Meets Microbiology, University of Vienna, Althanstrasse 14, Vienna, Austria

[&]**Present address**: Hovione, 2674 – 506 Loures, Portugal.

[§]**Present address**: Fundação para a Ciência e Tecnologia, avenida D. Carlos I, 126, 1249-074 Lisboa, Portugal.

***Corresponding authors**: Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da República, Apartado 127, 2781-901 Oeiras Codex, Portugal; Telephone: +351-21-4469521; Fax: +351-21-4411277; e-mail: aoh@itqb.unl.pt or serrano@itqb.unl.pt

Running title: *Role of a LysM domain in spore morphogenesis*

Keywords: sporulation, LysM domain, peptidoglycan-binding protein, SpoVID, spore coat, spore cortex.

Supplemental Materials and Methods

***B. subtilis* strains expressing *safA* alleles.** We also constructed strains with the in-frame deletion of *safA* and expressing *safA* alleles from the non-essential locus *amyE*. By SOE-PCR, we obtained a DNA fragment containing the *safA* coding sequence with an in-frame deletion that eliminates residues 46-249, as well as *safA* flanking regions (1). The fragment was digested with SalI and EcoRI and cloned into the same sites of pUC18 (New England Biolabs), yielding pCF72. Then, *B. subtilis* cells were co-transformed with pDG364 (2) and pCF72 to create a *safA* null mutant strain bearing the chloramphenicol resistance *cat* gene at *amyE* (AH10297). The presence of the in-frame deletion in *safA* was confirmed by PCR, and supported by the lysozyme sensitivity of the cells (3). Plasmid pCF75 is a derivative of pMLK83 (4) with the *safA* coding sequence and its flanking regions bordered by 5' and 3' regions of *amyE*, was used as template for insertion of alanine substitutions in LysM residues by site-directed mutagenesis. The resulting vectors, (pCF181-185) as well as pCF75, were transferred to AH10297, yielding strains AH10302, AH10555-10558 and AH10561.

***B. subtilis* strains expressing YFP or GFP fusions fusions.** To obtain strains expressing *safA-yfp* variants, we digested the vector pCF149, containing *safA-fl3-yfp* fusion (1), with HindIII and BamHI. A DNA fragment with the terminus of *safA* followed by the linker *fl3* and *yfp* was released and inserted in the same cloning sites of pCF181-185, yielding vectors pCF186-190. These vectors, as well as pCF149, were then used to substitute of the *cat* gene for *safA-fl3-yfp* fusion at the *amyE* locus of AH10297, originating strains AH10487 and AH10562-10566. Also, strains MB24 and AH10297 were transformed with the chromosomal DNA of JDB1752 (5), in order to knock-out *spoVE*.

These cells were then transformed with pCF149 or with chromosomal DNA of PE1478 (6) generating strains expressing *safA-yfp* or *spoVID-gfp* in the absence of *spoVE*. For cells expressing *yaaH-gfp* in the presence of the various *safA* alleles, we transformed AH10297, AH10302 and AH10555-10561 with chromosomal DNA prepared from PE659 (6).

LysM-(GFP) Strep II *tag* fusions. The coding sequence of the LysM domain of *safA* was PCR amplified, digested with XbaI and NcoI, and cloned into the same sites of pASK-IBA3 (IBA GmBH) to create pTC178. Then, the *gfp* gene, amplified from pEA18, was inserted into the AatII site of pTC178, yielding pTC182 (for expression of $LysM_{SafA}$-GFP-StrepTagII). This vector was used as template to introduce the alanine substitutions D10A, S11A, L12A, N30A or I39A at the LysM by site-directed mutagenesis, yielding plasmids pAI4, pAI1, pAI2, pAI5 and pAI3, respectively. For GFP-StrepTagII overproduction, the *gfp* gene was, digested with XbaI and Eco47III and cloned into the same sites of pASK-IBA3, generating pAI12. These vectors are then cloned in *E. coli* DH5α for protein overproduction (strains AH4504-4509, AH4520 and AH4522). For overproduction of $LysM_{SpoVID}$-StrepTagII, the coding region of the LysM domain of SpoVID was amplified, digested with XbaI and NcoI and inserted into pASK-IBA3. The resulting vector was transferred to *E. coli* BL21(DE3), resulting in strain AH5104.

Strains for the overproduction of various SafA variants. The vector pFN76 (1), a derivative of pACYCDuet-1 (Merk, Milipore) with the *safA* coding sequence, was used as template for insertion of alanine substitutions in LysM residues by site-directed mutagenesis. The resulting vectors, (see table S3) were used to transform *E. coli* BL21

(DE3) (see table S1).

Supplemental Figure Legends

Figure S1 - Accumulation of the various forms of SafA. The WT, the complementation strain (WT^C), and strains of a Δ*safA* in-frame deletion mutant expressing alleles of *safA* coding for proteins with the indicated substitutions in the LysM domain at *amyE*, were induced to sporulate by the ressuspension method. Samples were withdrawn at the indicated times, in hours, after ressuspension (defined as the onset of sporulation), and whole cell lysates prepared. Proteins in the extracts were resolved by SDS-PAGE and the gel subject to immunoblotting with anti-$SafA_{FL}$ antibodies. The position of $SafA_{FL}$ and $SafA_{C30}$ is indicated by arrows. The substitutions are colour coded as described in the legend for figure 1: in red, the substitutions that cause early localization defects of SafA-YFP; in blue, the substitutions that cause localization defects late in morphogenesis. The position of molecular weight markers (in kDa) is shown in the left side of the panels.

Figure S2 – Amino acid substitutions in $SafA_{LysM}$ affect the localization of the SafA-dependent YaaH-GFP. A: The localization of YaaH-GFP was examined in the WT, the Δ*safA* in-frame deletion mutant and in strains producing forms of SafA with the D10A and I39A single amino acid substitutions. Samples were taken from sporulating cultures 6 hours after the onset of sporulation induced by ressuspension. The cells were stained with membrane dye FM4-64 and examined by phase contrast and fluorescence microscopy. Scale bar, 1 μm. **B**: scoring of the percentage of sporangia showing the YaaH-GFP localization pattern depicted schematically. The scoring refers to the microscopy experiment represented in A.

Figure S3 – Time-course of the localization of SafA-YFP in WT and *spoVE* sporangia. The localization of SafA-YFP was examined in the WT background and in cells bearing a *spoVE*::*tet* insettional allele. Samples were taken from sporulating cultures 2, 4 and 6 hours after the onset of sporulation induced by ressuspension. The cells were stained with membrane dye FM4-64 and examined by phase contrast and fluorescence microscopy. Scale bar, 1 μm. The scoring of the percentage of sporangia showing the SafA-YFP localization pattern depicted schematically is shown below the microscopy images. Arrows: white, single cap; blue, double cap. See also Fig. 6A for cells examined at hour 8 of sporulation.

References

1. Nunes F, Fernandes C, Freitas C, Marini E, Serrano M, Moran CP, Jr., Eichenberger P, Henriques AO. 2018. SpoVID functions as a non-competitive hub that connects the modules for assembly of the inner and outer spore coat layers in Bacillus subtilis. Mol Microbiol doi:10.1111/mmi.14116.
2. Cutting SaHPB. 1990. Genetic Analysis. John Wiley and sons, Lta.
3. Nicholson WLaS, P. 1990. Sporulation, Germination and Outgrowth, p 391-450. *In* S.M. HCRaC (ed), Molecular Biology Methods for Bacillus. John Wiley & Sons Ltd, Chichester.
4. Karow ML, Piggot PJ. 1995. Construction of gusA transcriptional fusion vectors for Bacillus subtilis and their utilization for studies of spore formation. Gene 163:69-74.
5. Real G, Fay A, Eldar A, Pinto SM, Henriques AO, Dworkin J. 2008. Determinants for the subcellular localization and function of a nonessential SEDS protein. J Bacteriol 190:363-76.
6. Wang KH, Isidro AL, Domingues L, Eskandarian HA, McKenney PT, Drew K, Grabowski P, Chua MH, Barry SN, Guan M, Bonneau R, Henriques AO, Eichenberger P. 2009. The coat morphogenetic protein SpoVID is necessary for spore encasement in Bacillus subtilis. Mol Microbiol 74:634-49.
7. Ozin AJ, Samford CS, Henriques AO, Moran CP, Jr. 2001. SpoVID guides SafA to the spore coat in Bacillus subtilis. J Bacteriol 183:3041-9.

Supplemental Tables

Table S1 - Bacterial strains.

Strain	Relevant Genotype/Phenotype	Origin/ Reference
E. coli		
DH5α	F⁻ Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK⁻, mK⁺) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1	Invitrogen
BL21	F⁻ dcm ompT hsdS(rB⁻ mB⁻) gal λ(DE3)	Promega
AH2687	BL21(DE3)/pTC55 Cm^r Amp^r	(7)
AH2979	BL21(DE3)/pTC169 Cm^r Amp^r	(7)
AH4504	DH5α /pTC182 Amp^r	This work
AH4505	DH5α /pAI1 Amp^r	"
AH4506	DH5α /pAI2 Amp^r	"
AH4507	DH5α /pAI3 Amp^r	"
AH4508	DH5α /pAI4 Amp^r	"
AH4509	DH5α /pAI5 Amp^r	"
AH4522	DH5α /pAI12 Amp^r	"
AH5104	BL21(DE3)/ pFN45 Amp^r	"
AH5236	BL21(DE3)/ pFN76 Cm^r	(1)
AH5459	BL21(DE3)/ pFN132 Cm^r	This work
AH5460	BL21(DE3)/ pFN133Cm^r	"
AH5461	BL21(DE3)/ pFN134 Cm^r	"
AH5462	BL21(DE3)/ pMS550 Cm^r	"
AH5463	BL21(DE3)/ pMS551 Cm^r	"
B. subtilis		
MB24	*trp2 metC3*, Spo^+	Lab. stock
AH4634	*trp2 metC3 spoVID::spoVID-gfp* Sp^r	"
AH4808	*trp2 metC3 spoVID::spoVID-gfp , ΔspoVE::tet* Sp^r Neo^r	This work
AH5429	*trp2 metC3 ΔsafA*, *amyE'::safA-yfp::'amyE, ΔspoVE::tet* Neo^r	"
AH10297	*trp2 metC3 ΔsafA ΔamyE::cm*	"
AH10302	*trp2 metC3 ΔsafA*, *amyE'::safA::'amyE* Neo^r	"
AH10555	*trp2 metC3 ΔsafA*, *amyE'::safA*$_{D10A}$*::'amyE* Neo^r	"
AH10556	*trp2 metC3 ΔsafA*, *amyE'::safA*$_{S11A}$*::'amyE* Neo^r	"
AH10557	*trp2 metC3 ΔsafA*, *amyE'::safA*$_{L12A}$*::'amyE* Neo^r	"
AH10558	*trp2 metC3 ΔsafA*, *amyE'::safA*$_{N30A}$*::'amyE* Neo^r	"
AH10561	*trp2 metC3 ΔsafA*, *amyE'::safA*$_{I39A}$*::'amyE* Neo^r	"
AH10487	*trp2 metC3 ΔsafA*, *amyE'::safA-yfp::'amyE* Neo^r	"
AH10562	*trp2 metC3 ΔsafA*, *amyE'::safA*$_{D10A}$*-yfp::'amyE* Neo^r	"
AH10563	*trp2 metC3 ΔsafA*, *amyE'::safA*$_{S11A}$*-yfp::'amyE* Neo^r	"
AH10564	*trp2 metC3 ΔsafA*, *amyE'::safA*$_{L12A}$*-yfp::'amyE* Neo^r	"
AH10565	*trp2 metC3 ΔsafA*, *amyE'::safA*$_{I39A}$*-yfp::'amyE* Neo^r	"
AH10566	*trp2 metC3 ΔsafA*, *amyE'::safA*$_{N30A}$*-yfp::'amyE* Neo^r	"
AH10612	*trp2 metC3 ΔsafA yaaH::yaaH-gfp* Sp^r	"
AH10613	*trp2 metC3 ΔsafA*, *amyE'::safA::'amyE yaaH::yaaH-gfp* Sp^r Neo^r	"
AH10614	*trp2 metC3 ΔsafA*, *amyE'::safA*$_{D10A}$*::'amyE yaaH::yaaH-gfp* Sp^r Neo^r	"
AH10616	*trp2 metC3 ΔsafA*, *amyE'::safA*$_{L12A}$*::'amyE yaaH::yaaH-gfp* Sp^r Neo^r	"

[a] Amp^r, ampiclin resistant; Sp^r, spectinomycin resistant; Nm^r, neomycin resistant; Cm^r, chloramphenicol resistant; Erm^r, eritromycin resistant, Tet^r tetracycline resistant.

136 **Table S2 – Oligonucleotides used in this study.**

Primer	Sequence (5´to 3´)[a]
safA-364D	GCAAGTCGACAATCGGGACAGAAATGAATCTTG
safA+135R	CCCGGGTCATACTGAGGCGATACTATTTTCATTCCAGGCATGATTAGTC
safA+748D	CATGCCTGGAATGAAAATAGTATCGCCTCAGTATGACCCGGGTTATG
safA+1250R	GGGAATTCTAAGCGTGTCAGTTCTCTCCATTTG
safA-IBA3d	CAAAAATCTAGATAACGAGGGCAAAAAATGAAAATCCATATCGTTCAAAAAGGCG
safA-IBA3r	CTGAGACCATGGACGTCTCCTTCTGACGGCACTTTTATTTTCATTCC
gfpmut2+4D	CCGACGTCAGTAAAGGAGAAGAAC
gfpmut2+714R	CCGACGTCTTTGTATAGTTCATCC
gfpinpASKDir	CAAAAATCTAGATAACGAGGGCAAAAAATGAGTAAAGGAGAAGAACTTTTCACTG GAG
gfpinpASKRev	TGCCAGCGCTTTTGTATAGTTCATCCATGCCATGTG
safAD10A-d	GAAAATCCATATCGTTCAAAAAGGCGCTTCGCTCTGGAAAATAGCTG
safAD10A-r	CAGCTATTTTCCAGAGCGAAGCGCCTTTTTGAACGATATGGATTTTC
safAS11A-d	GAAAATCCATATCGTTCAAAAAGGCGATGCGCTCTGGAAAATAGCTGAAAAGTAC
safAS11A-r	GTACTTTTCAGCTATTTTCCAGAGCGCATCGCCTTTTTGAACGATATGGATTTTC
safAL12A-d	CCATATCGTTCAAAAAGGCGATTCGGCCTGGAAAATAGCTGAAAAGTACGG
safAL12A-r	CCGTACTTTTCAGCTATTTTCCAGGCCGAATCGCCTTTTTGAACGATATGG
safAN30A-d	GATGTTGAGGAAGTGAAAAAACTCGCTACACAGCTTAGCAATCCAGAC
safAI39A-d	GTCTGGATTGCTAAGCTGTGTAGCGAGTTTTTTCACTTCCTCAACATC
safAI39A-r	CAGCTTAGCAATCCAGACTTAGCCATGCCTGGAATGAAAATAAAAGTGCCG
VID-LysM1761D	GCTCTAGATAACGAGGGCAAAAAATGAAAATTTGTATTGTGCAGCAGG
VID-LysM1928R	GCCCATGGACGTCCGCATGGCTATTTTTATATTGAGG

137 [a] Restriction sites are underlined
138

Table S3 – Plasmids

Plasmid	Relevant features
pAI1	pASK-IBA3 derivative with *safA*$_{LysM\ S11A}$-*gfp*
pAI2	pASK-IBA3 derivative with *safA*$_{LysM\ L12A}$-*gfp*
pAI3	pASK-IBA3 derivative with *safA*$_{LysM\ I39A}$-*gfp*
pAI4	pASK-IBA3 derivative with *safA*$_{LysM\ D10A}$-*gfp*
pAI5	pASK-IBA3 derivative with *safA*$_{LysM\ N30A}$-*gfp*
pAI12	pASK-IBA3 derivative with *gfp*
pCF72	pUC18 derivative to perform an *in frame* deletion in *safA*
pCF75	pMLK83 derivative for insertion of *safA* at *amyE* locus
pCF149	pMLK83 derivative for insertion of *safA-yfp* at *amyE* locus
pCF181	pMLK83 derivative for insertion of *safA*$_{D10A}$ at *amyE* locus
pCF182	pMLK83 derivative for insertion of *safA*$_{S11A}$ at *amyE* locus
pCF183	pMLK83 derivative for insertion of *safA*$_{L12A}$ at *amyE* locus
pCF184	pMLK83 derivative for insertion of *safA*$_{I39A}$ at *amyE* locus
pCF185	pMLK83 derivative for insertion of *safA*$_{N30A}$ at *amyE* locus
pCF186	pMLK83 derivative for insertion of *safA*$_{D10A}$-*yfp* at *amyE* locus
pCF187	pMLK83 derivative for insertion of *safA*$_{S11A}$-*yfp* at *amyE* locus
pCF188	pMLK83 derivative for insertion of *safA*$_{L12A}$-*yfp* at *amyE* locus
pCF189	pMLK83 derivative for insertion of *safA*$_{I39A}$-*yfp* at *amyE* locus
pCF190	pMLK83 derivative for insertion of *safA*$_{N30A}$-*yfp* at *amyE* locus
pFN45	pASK-IBA3 derivative with *spoVID*$_{LysM}$
pFN76	pACYDuet-1 derivative with safA
pFN132	pACYDuet-1 derivative with *safA*$_{D10A}$
pFN133	pACYDuet-1 derivative with *safA*$_{N30A}$
pFN134	pACYDuet-1 derivative with *safA*$_{I390A}$
pMS550	pACYDuet-1 derivative with *safA*$_{S11A}$
pMS551	pACYDuet-1 derivative with *safA*$_{L12A}$
pTC55	pGEX-4T3 derivative for GST overexpression
pTC182	pASK-IBA3 derivative with *safA*$_{LysM}$-gfp
pOZ169	pGEX-4T3 derivative for GST-SpoVID overexpression

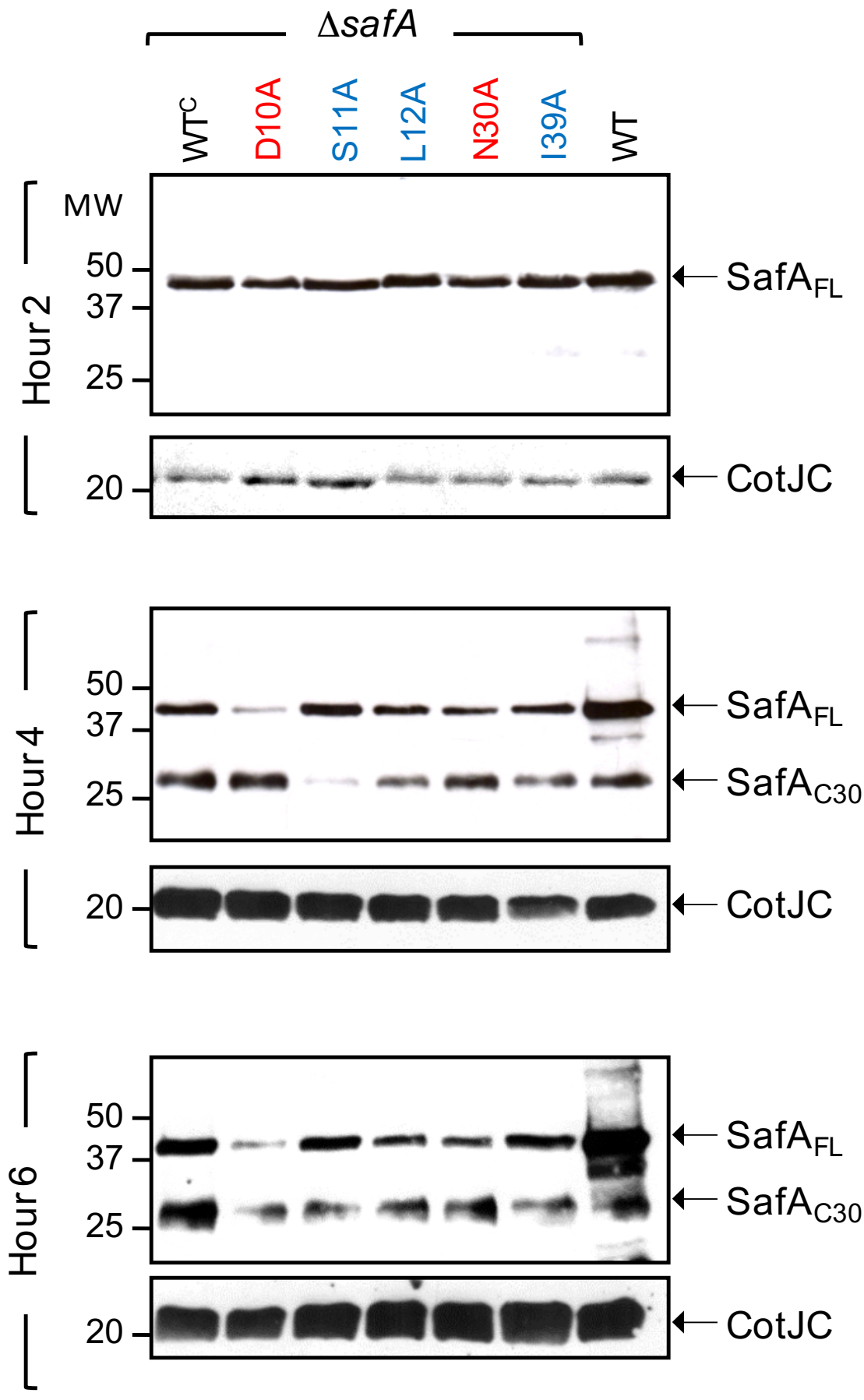
142 **Table S4 – Spore heat and lysozyme resistance of various strains.**

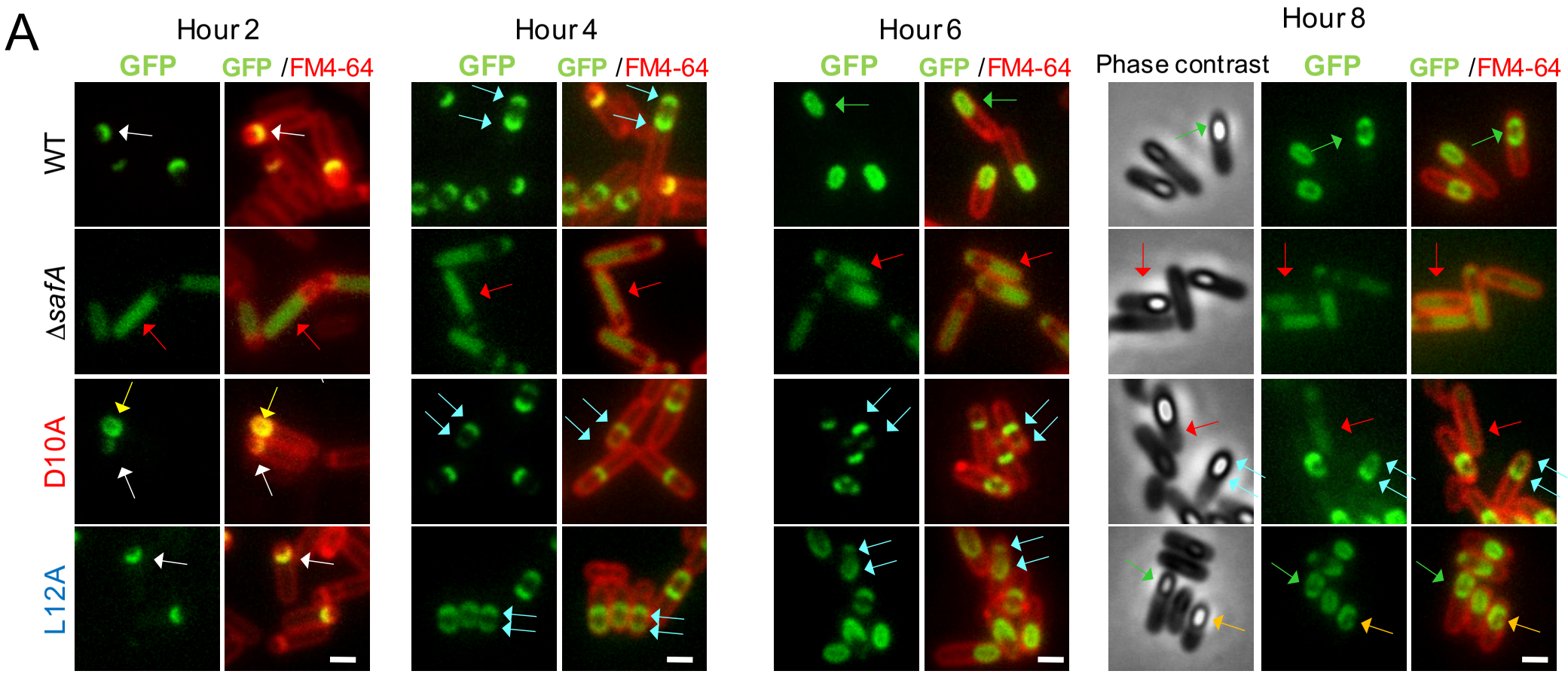
143
144

	Titer of spores (CFU/ml*)							
	WT	**WT^{c}**	**Δ*safA***	**D10A**	**S11A**	**L12A**	**N30A**	**I39A**
Viable**	$3.6x10^{7}$	$6.2x10^{7}$	$2.2x10^{7}$	$4.4x10^{7}$	$7.8x10^{7}$	$3.8x10^{7}$	$4.8x10^{7}$	$6.9x10^{7}$
Heat**	$1.7x10^{7}$	$1.7x10^{7}$	$9.2x10^{6}$	$2.5x10^{7}$	$6.3x10^{7}$	$1.1x10^{7}$	$1.1x10^{7}$	$3.8x10^{7}$
Lysozyme**	$1.6x10^{7}$	$1.5x10^{7}$	$7.5x10^{6}$	$1.6x10^{7}$	$4.4x10^{7}$	$1.5x10^{7}$	$1.3x10^{7}$	$2.8x10^{7}$

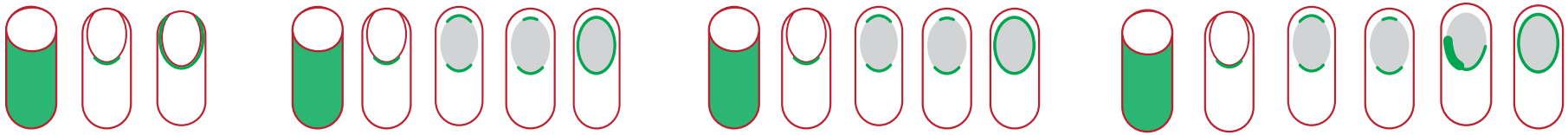
145
146 *Colony-forming units; **Viable, heat and lysozyme resistant CFU´s/ml; WT^{c}, completation strain (Δ*safA* mutant
147 with the WT *safA* allele at *amyE*).

148
149
150
151
152

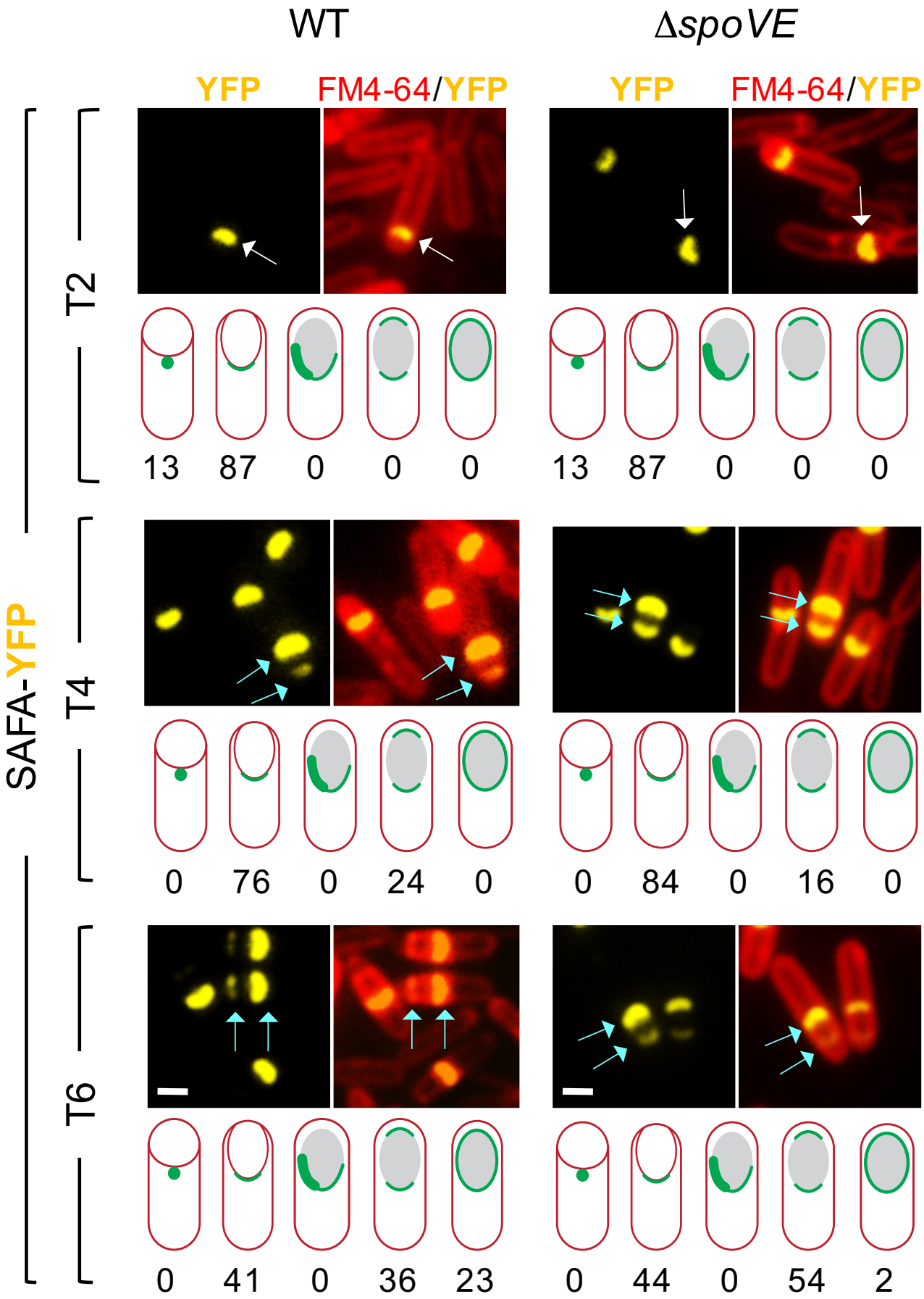

ΔsafA
WTc
D10A
S11A
L12A
N30A
I39A
WT
MW
Hour 2
50
37
25
SafAFL
20
CotJC
Hour 4
50
37
25
SafAFL
SafAC30
20
CotJC
Hour 6
50
37
25
SafAFL
SafAC30
20
CotJC



B



WT	0	100	0	0	30	54	12	4	0	4	22	5	69	0	3	14	4	0	79
ΔsafA	100	0	0	100	0	0	0	0	100	0	0	0	0	100	0	0	0	0	0
D10A	0	95	5	0	26	15	57	2	0	7	10	65	18	14	1	6	54	0	25
L12A	0	96	4	0	21	57	10	12	0	4	17	18	61	1	3	12	0	14	70


WT
ΔspoVE
YFP
FM4-64/YFP
YFP
FM4-64/YFP
SAFA-YFP
T2
T4
T6
13 87 0 0 0
13 87 0 0 0
0 76 0 24 0
0 84 0 16 0
0 41 0 36 23
0 44 0 54 2