

Supplementary material

Hydrogen peroxide is crucial for NLRP3 inflammasome mediated IL-1 β production and cell death in pneumococcal infections of bronchial epithelial cells

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Short Title: H₂O₂ activates NLRP3 inflammasome

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Supplementary Figures

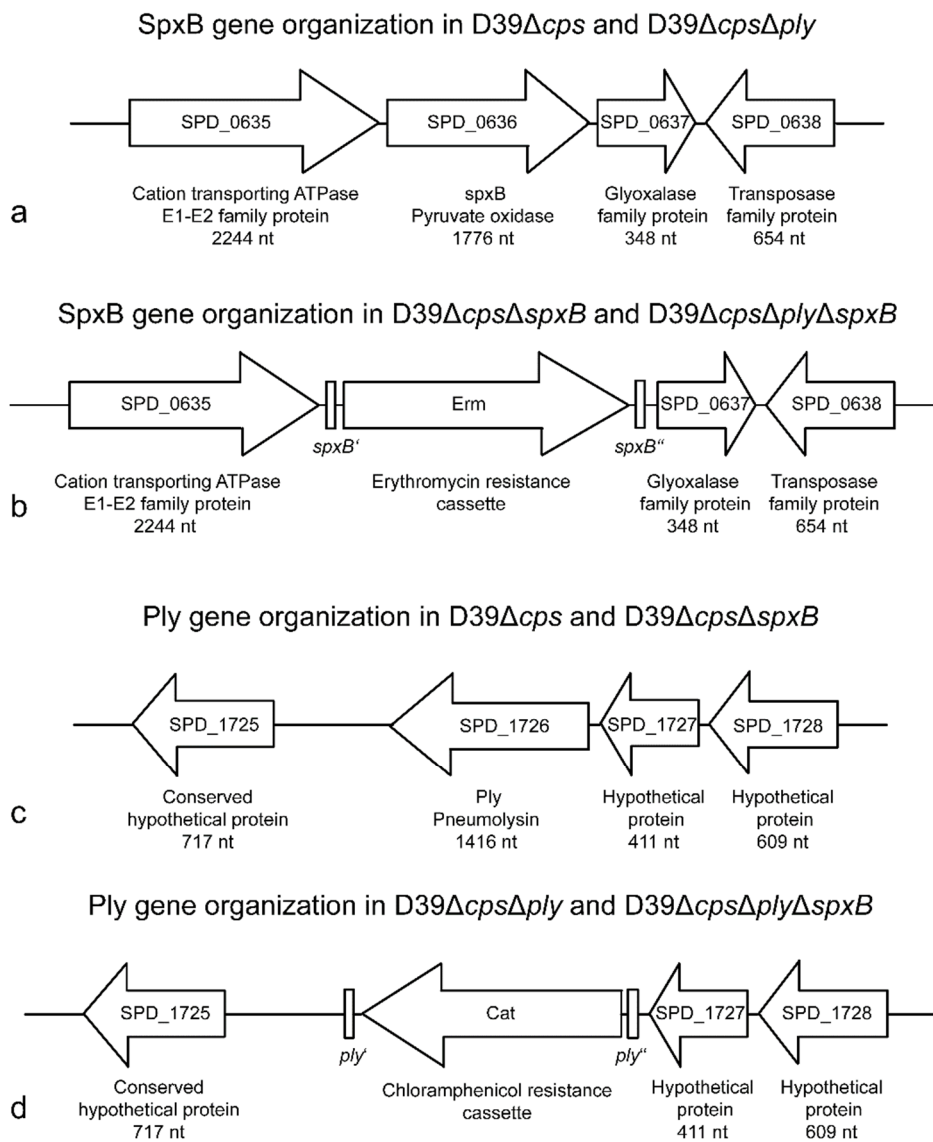


Fig. S1. Gene organization in *S. pneumoniae* D39Δ*cps* mutants. Gene organization of *spxB* (SPD_0636) in (a) D39Δ*cps*, D39Δ*cps*Δ*ply*, (b) D39Δ*cps*Δ*spxB* and D39Δ*cps*Δ*ply*Δ*spxB*. Gene organization of *ply* (SPD_1726) in (c) D39Δ*cps*, D39Δ*cps*Δ*spxB*, (d) D39Δ*cps*Δ*ply* and D39Δ*cps*Δ*ply*Δ*spxB*. The gene organization maps were created using KEGG database.

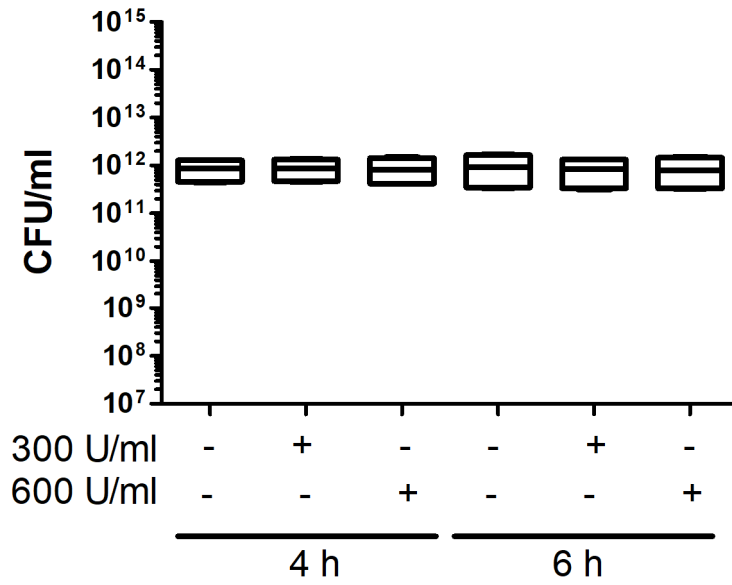


Fig. S2. Catalase has no bactericidal activity. *S. pneumoniae* D39 Δ *cps* was incubated with 300 and 600 U/ml catalase at 37°C. After 4 and 6 h (equivalent to the infection time in Fig. 1) of incubation, bacterial counts were determined by serial dilution. The data are displayed as box plots.

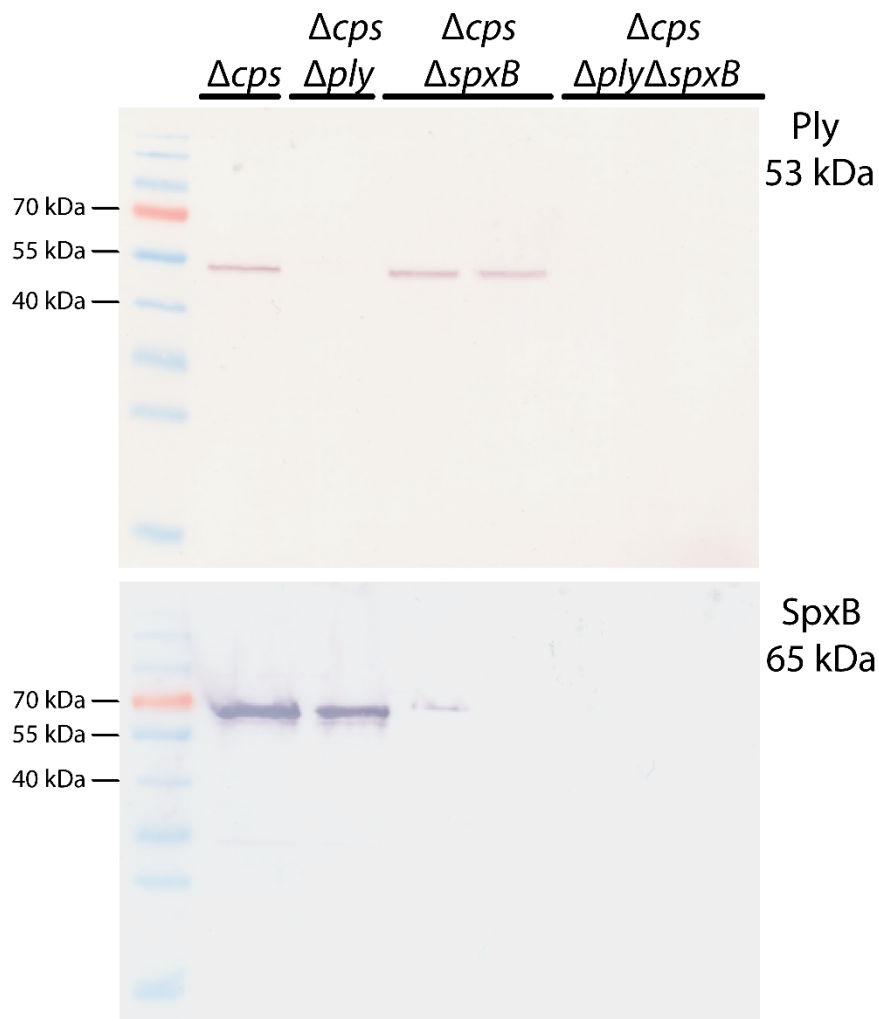


Fig. S3. Ply and SpxB Immunoblots. Bacterial lysates of D39 Δcps and the isogenic mutants were analysed for the production of Ply and SpxB protein by performing immunoblot. Bacterial lysates were separated using a 12% SDS gel and blotted onto a PVDF membrane. The membrane was blocked with 5% (v/v) skim milk prior to primary antibody incubations. 1:1000 dilution of mice anti-sera against Ply and SpxB were used. Anti-mice IgG Alkaline phosphatase-linked Antibody was used as a secondary antibody.

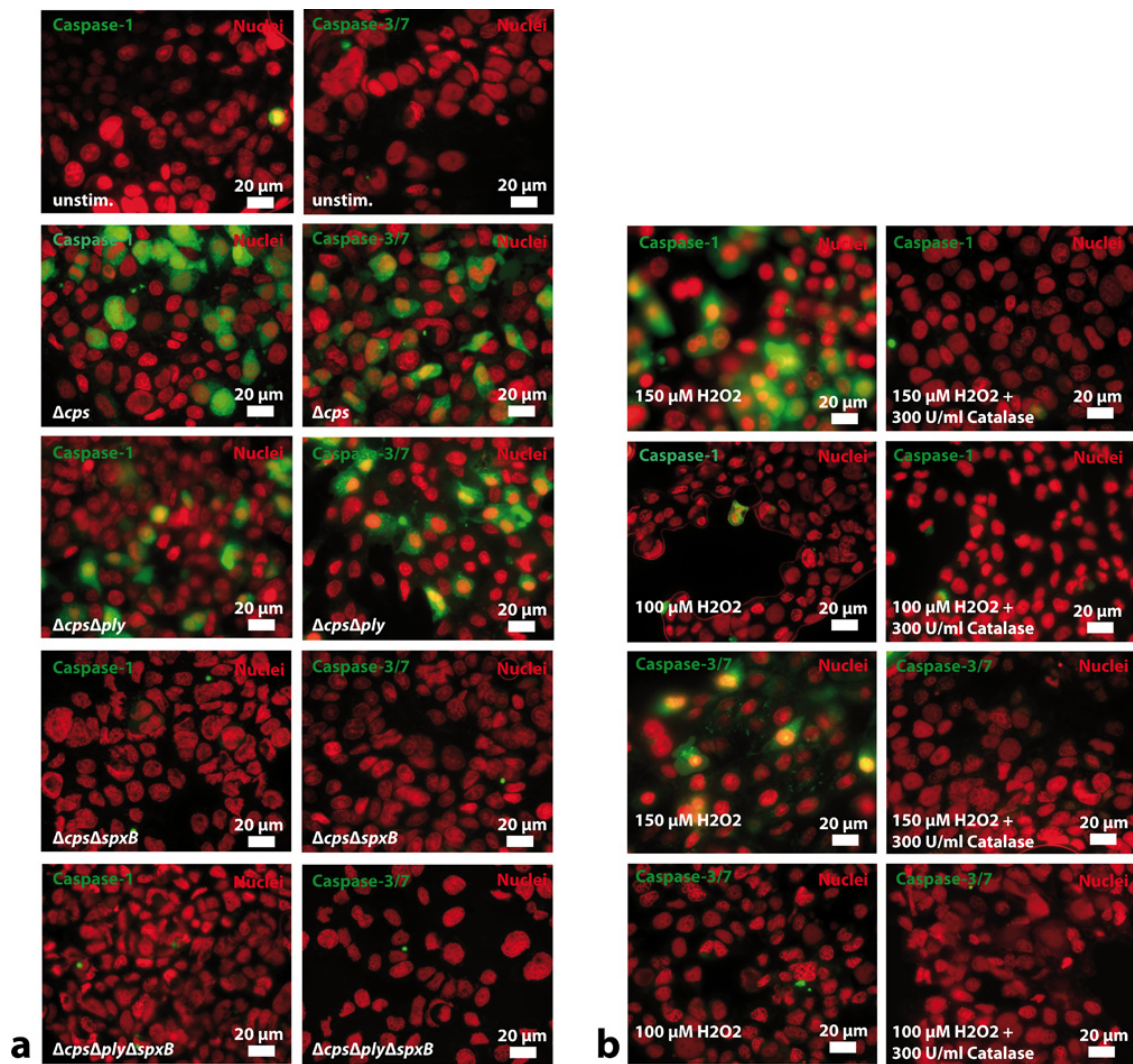


Fig. S4. H_2O_2 and *S. pneumoniae* strains with functional SpxB activate caspase-1 and caspase-3/7 in 16HBE cells. LPS-primed 16HBE cells were infected with D39 Δcps and the isogenic mutants at MOI 50 (a), or stimulated with 150 μM and 100 μM H_2O_2 in the presence or absence catalase (b) for 4 h and caspase-1 and caspase 3/7 activation was assessed. The cells were stained using fluorescent inhibitor probe FAM-YVAD-FMK and FAM-DEVD-FMK to microscopically visualize active caspase-1 and caspase-3/7, respectively. Nuclear-ID stain was used to visualize cell nuclei. Representative images of four independent experiments are shown.

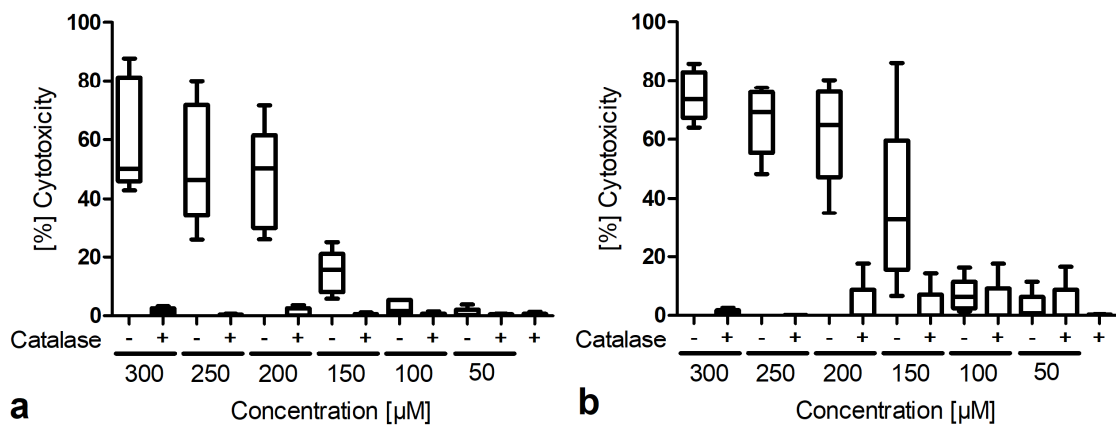


Fig. S5. Hydrogen peroxide kills 16HBE cells. Unprimed human bronchial epithelial cells were stimulated with various concentrations of H_2O_2 in the presence or absence of catalase for 4 h (**a**) and 6 h (**b**). Cytotoxicity was evaluated at the indicated time points. The data are displayed as box plots (n=5).

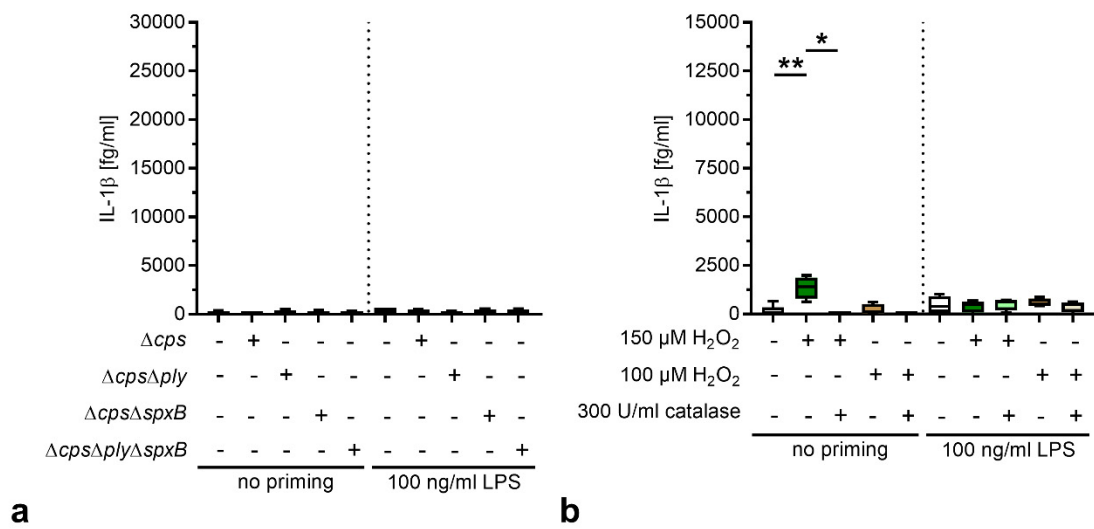


Fig. S6. IL-1 β release after 4 h of bacterial infection or H₂O₂ stimulation. Unprimed or LPS-primed human bronchial epithelial cells were infected with D39 Δcps and the isogenic mutants at MOI 50 (a), or stimulated with 150 μM and 100 μM H₂O₂ in the presence or absence of catalase (b). IL-1 β release was evaluated at 4 h post infection or stimulation. The data are displayed as box plots. The level of significance was determined using Kruskal Wallis test with Dunn's post-test ($n \geq 4$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

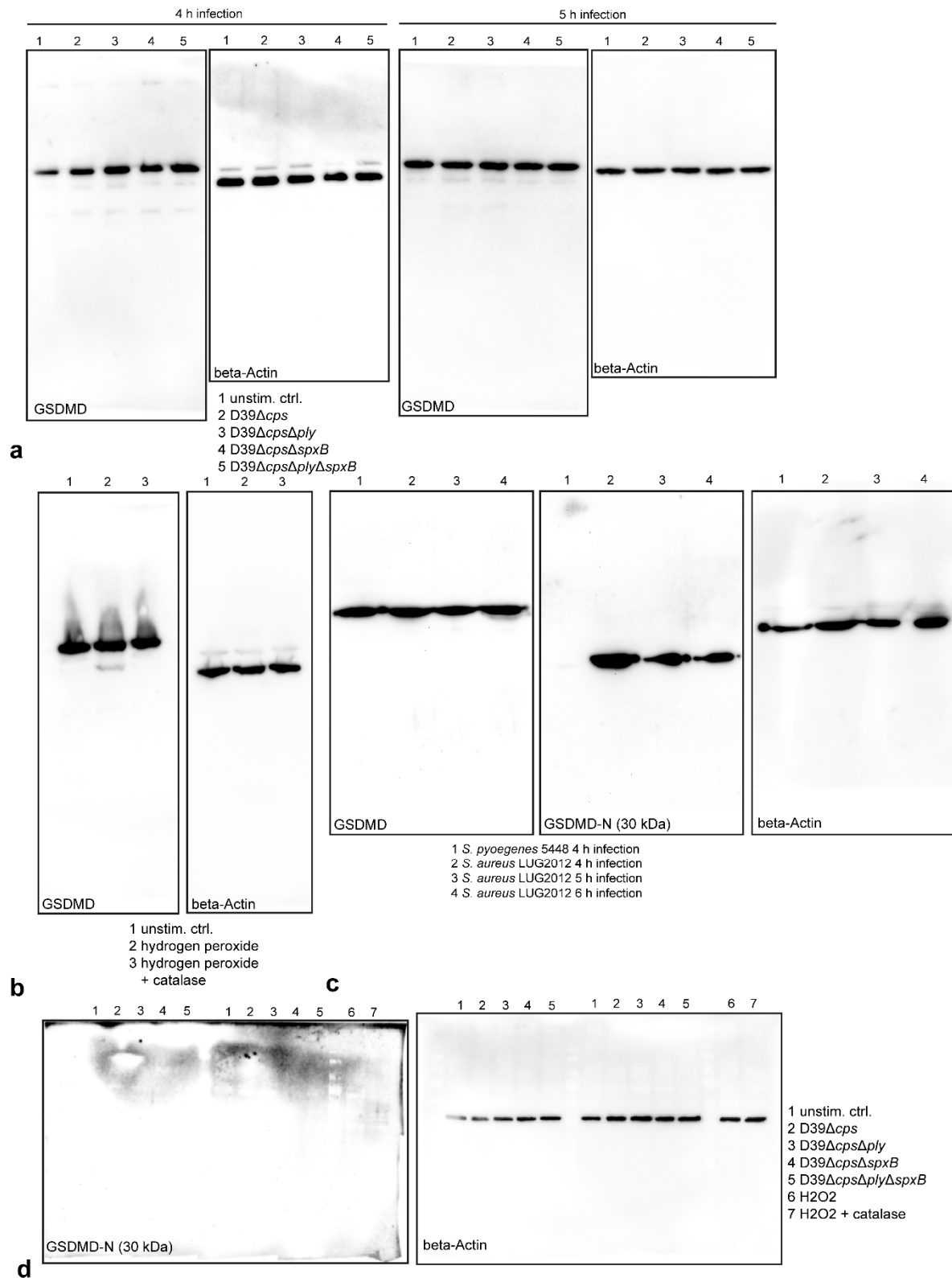


Fig. S7. Original Western blots of blots displayed in Figure 6. (a) Analyses of bacterial (D39Δcps and mutants) infections. (b) Analysis of hydrogen peroxide stimulations. (c) Analyses of bacterial (*S. pyogenes* and *Staphylococcus aureus*) infections. (d) Specific GSDMD-N analysis in pneumococcal infections (lanes 1-5 left: 4 h infections; lanes 1-5 right: 5 h infections; lanes 6-7: 4 hH₂O₂ stimulations).

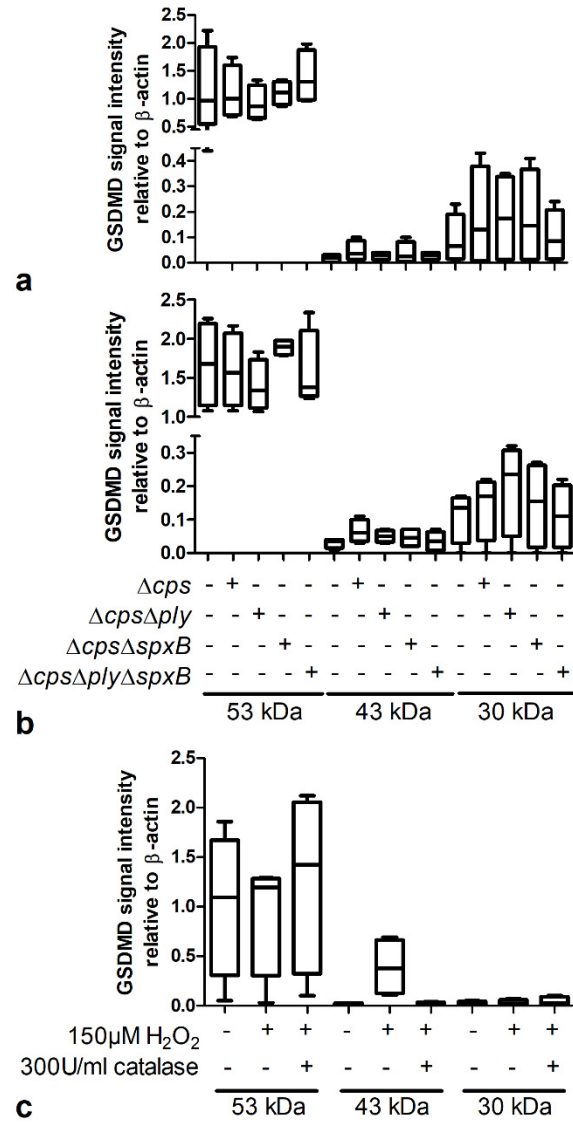


Fig. S8. Quantification of Gasdermin D Western blots as displayed in Figure 6e-f and Figure S7. Quantification of Gasdermin D blots after (a) 4 h and (b) 5 h of bacterial (D39 Δcps and mutants) infections. (c) Quantification of Gasdermin D blots post hydrogen peroxide stimulations.