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Supplemental Data

A Eukaryotic-like Ser/Thr Kinase

Signals Bacteria to Exit Dormancy

in Response to Peptidoglycan Fragments

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Supplemental Experimental Procedures

Reagents

Bryostatin and staurosporine were obtained from Calbiochem and Sigma, respectively. Muramyl-dipeptide was obtained from Sigma and tripeptide (Ala-Glu-Dpm) was obtained from Anaspec.

General Methods

B. anthracis Sterne spores were generated by growing cells for 4 days in modified G medium followed by repeated washing with dH_2O and storage at 4ºC.

Antibiotic Sensitivity

B. subtilis wild type spores were incubated with non-germinant buffer, muropeptide (GlcNAc-MurNAc tripeptide, 40 μM), *B. subtilis* cell free supernatant, or bryostatin (1 μM), for 60 min at 37°C prior to treatment with tetracycline (10 μg/ml for 60 min at 37°C). Percent loss in plating efficiency was calculated relative to that observed in the absence of germinant

Plasmid Construction

pIMS36 (His_6-YycI): Sequence corresponding to codons 31-280 (nt 93-840) of *yycI* was amplified from *B. subtilis* genomic DNA using primers that included six codons coding for histidine residues after the start codon. The resulting PCR product was digested with *Nco*I and *Xba*I and ligated to pBAD24 digested with *Nco*I and *Xba*I.

pIMS40 (His_6-$PASTA_{(Bs)}$): Sequence corresponding to codons 357-648 (nt 1071-1944) of *prkC* was amplified from *B. subtilis* genomic DNA using primers that included six codons coding for histidine residues after the start codon. The resulting PCR product was digested with *Nco*I and *Xba*I and ligated to pBAD24 digested with *Nco*I and *Xba*I.

pIMS41 (His_6-PrkC): Full length prkC was amplified from *B. subtilis* PY79 genomic DNA using primers that included the native *prkC* RBS followed by six codons coding for histidine residues after the start codon. The resulting PCR product was digested with *Spe*I and *Sph*I and the digested product was ligated to pDR111 digested with *Nhe*I and *Sph*I.

pIMS42 (His$_6$-AcmA): Sequence corresponding to codons 243-439 of *acmA* was amplified from *L. lactis* genomic DNA (kind gift from M. Belfort) using primers that included six codons coding for histidine residues after the start codon. The resulting PCR product was digested with *Nco*I and *Xba*I and ligated to pBAD24 digested with *Nco*I and *Xba*I.

pIMS44(His$_6$-PASTA$_{(Sa)}$): Sequence corresponding to codons 378-644 of S_TPK was amplified from *S. aureus NEWMAN* genomic DNA using primers that included six codons coding for histidine residues after the start codon. The resulting PCR product was digested with *Nco*I and *Xba*I and ligated to pBAD24 digested with *Nco*I and *Xba*I.

pIMS46 (His$_6$-PrkC$_{Sa}$): The gene encoding S_TKc was amplified from *S. aureus NEWMAN* genomic DNA using primers that included the *B. subtilis prkC* RBS followed by six codons coding for histidine residues after the start codon. The resulting PCR product was digested with *Nhe*I and *Sph*I and the digested product was ligated to pDR111 digested with *Nhe*I and *Sph*I.

pIMS47 (FLAG-PrkC$_{Bs}$): Full length prkC was amplified from *B. subtilis* genomic DNA from strain PY79 using primers that included the native *prkC* RBS followed by codons coding for FLAG tag after the start codon. The resulting PCR product was digested with *Spe*I and *Sph*I and the digested product was ligated to pDR111 digested with *Nhe*I and *Sph*I.

pIMS48 (FLAG-PrkC$_{Bs(K40A)}$): pIMS47 was subjected to site-directed mutagenesis with primers to substitute lysine at position 40 with an alanine. PCR products resulting from the 5' FLAG-*prkC* primer and (K40A) reverse primer as well as from K40A forward primer and 3' *prkC* primer were gel-purified and used as templates for PCR-SOEing using 5'FLAG-*prkC* and 3' *prkC* primers. The resulting PCR product was digested with *Spe*I and *Sph*I and the digested product was ligated to pDR111 digested with *Nhe*I and *Sph*I.

Strain Construction

JDB1980 (*ΔprkC amyE::P$_{spac}$-his6-prkC$_{Bs}$*): PB705 was transformed with pIMS41, selecting for Spec^R and screening for amy-.

JDB2226 (*ΔprkC amyE::P$_{spac}$*-FLAG-*prkC$_{Bs}$*): PB705 was transformed with pIMS47, selecting for Spec^R and screening for amy-.

JDB2227 (*ΔprkC amyE::P$_{spac}$*-FLAG-*prkC$_{Bs(K40A)}$*): PB705 was transformed with pIMS48, selecting for Spec^R and screening for amy-.

JDB2017 (*ΔprkC amyE*::P$_{spac}$-his$_6$-*prkC$_{Sa}$***):** PB705 was transformed with pIMS46, selecting for spec^R and screening for amy-.

JDB1930 (*B. anthracis* Δ*prkC*): We used the temperature sensitive plasmid pKS1 (Shatalin and Neyfakh, 2005) to construct a deletion mutation (Δ*prkC*::*aphA3).* We introduced a Kan^R cassette into the *bas3713* gene that had been amplified from *B. anthracis* Sterne 34F2 strain genomic DNA. This construct was then introduced into pKS1, and the resulting plasmid (pML280) was transformed into *B. subtilis* PY79. A midiprep of the plasmid amplified in *B. subtilis* was used to electroporate *B. anthracis* Sterne. We grew this strain at 37ºC without antibiotic and then selected for the integration of the pML280 plasmid into the *B. anthracis* chromosome using antibiotic selection (kanamycin, 10µg/ml) followed by PCR screening for the insertion in the correct locus. After a cycle at a permissive temperature (30ºC) with antibiotic, we selected for the excision of the plasmid (loss of the erythromycin resistance) and the insertion of the antibiotic cassette in the *prkC* gene using antibiotic selection and a PCR screen using flanking primers of the locus.

Figure Legends

Figure S1. Phase Contrast Images of Cells Exposed to Germinants. Wild type PY79 spores (wt), FB85 spores lacking all five nutrient germination receptors (Δ*ger5*) or PB705 spores lacking PrkC (Δ*prkC*) were incubated with germination buffer alone or with 10 mM L-alanine (Alanine); 1 μg/ml *B. subtilis* peptidogylcan (PG), or *B. subtilis* cell free supernatant (CFS, 10^{-3} dilution) for 60 min and 100X phase contrast images were subsequently acquired.

Figure S2. Kinetics of Germination. Wild type PY79 spores were incubated with germination buffer alone or with germination buffer containing 1 mM L-alanine (red) or cell free supernatant (blue) for times indicated and the percentage of heat sensitive (80ºC, 20 min) spores was determined.

Figure S3. Effect of Cell Free Supernatant Isolated from Non-growing Cells. *B. subtilis* cells were grown up to an A_{600} of 1.2, washed and transferred to non-growth promoting buffer (Tbase/10 mM $MgSO_4$) and incubated for 24 hours. Filtrate, referred to as CFS(NG) was subsequently isolated and used in a germination assay as described in Experimental Procedures along with L-alanine (1 mM) and cell-free supernatant (CFS) prepared as described in Experimental Procedures as controls. % Germination is shown.

Figure S4. Effect of Cortex PG on Spore Germinanation. PG from decoated spores was obtained as described in Experimental Procedures for vegetative PG by boiling in 4% SDS and washing extensively with dH_20. The resulting suspension was used at indicated concentrations in a germination assay. % germination is shown.

Figure S5. Effect of Δ*prkC* Mutation on Ca^{2+}-DPA Spore Germination. Wild type PY79 and PB705 Δ*prkC B. subtilis* spores were incubated with 1 mM L-alanine, 100 μg/ml PG, or 50 mM Ca^{2+}-DPA (Sigma) for 60 min and % germination was determined.

Figure S6. Effect of Δ*prkC* Mutation on *B. anthracis* Spore Germination. *B. anthracis* Sterne wild type or JDB1930 (Δ*prkC*) spores were incubated in the presence of 100 μg/ml of *B. anthracis* peptidoglycan (black) and % germination was determined. Error bars represent SD for triplicate samples.

Figure S7. Complementation of Δ$prkC_{K40A}$ Mutation. Spores generated from strains JDB3 (PY79, wild type), PB705 (Δ*prkC*) JDB2227 (Δ*prkC amyE*::P_{spac}-*FLAG-prkC*$_{Bs}$) and JDB2228 (Δ*prkC amyE*::P_{spac}-*FLAG-prkC*$_{Bs(K40A)}$) were exposed to 1 mM L-alanine (ala), 100 μg/ml PG (PG) or 20 μM disaccharide tripeptide (tri) for 60 min prior to measuring % germination.

Figure S8. Spore Fractionation. JDB2228 (Δ*prkC amyE*::P_{spac}-*FLAG-prkC*$_{Bs(K40A)}$), JDB1568 (*cotE-gfp*), and JDB1700 (P_{spank}-*gfp*) spores were fractionated according to the protocol described for the localization for FLAG-PrkC. Detection of Flag-$PrkC_{(K40A)}$ in

the P100 fraction (IM) using α-FLAG antibodies, CotE-GFP in the coat fraction (C) and GFP in the S100 fraction (S) by α-GFP antibodies (kind gift from H. Shuman) is shown.

Figure S9. Germination by *S. aureus* Cell-free Supernatant. JDB1980 (*ΔprkC amyE*::P_{spac}-his_6-$prkC_{Bs}$; red) or JDB2017 (*ΔprkC amyE*::P_{spac}-his_6-$prkC_{Sa}$; black) spores were incubated with *S. aureus* cell-free supernatant at a series of dilutions. Error bars represent SD for triplicate samples.

Table S1. Bacterial strains.

Strain	Genotype	Source
PY79	Wild type	Lab collection
EB1451	*hisA1 argC4 metC3 tagO*::*erm*	(D'Elia et al., 2006)
PB705	trpC2 *prkC*Δ1	(Gaidenko et al., 2002)
FB85	Δ*gerA::spc* Δ*gerB::cat* Δ*gerK::erm* Δ*yndDEF::tet* Δ*yfkQRT::neo*	(Paidhungat and Setlow, 2000)
JDB1930	*B. anthracis* Sterne *ΔprkC*	This study
JDB1980	*ΔprkCΔ1 amyE*::P_{spac}-his_6-$prkC_{Bs}$	This study
JDB2017	*ΔprkCΔ1 amyE*::P_{spac}-his_6-$prkC_{Sa}$	This study
JDB2226	*ΔprkCΔ1 amyE::*P_{spac}-FLAG-$prkC_{Bs}$	This study
JDB2227	*ΔprkCΔ1 amyE::*P_{spac}-FLAG-$prkC_{Bs(K40A)}$	This study
B. anthracis Sterne 34F2	Wild type	Lab collection
B. megaterium MS021	Δ*bgaR/bgaM*	Lab collection
C. acetobutylicum NCTC 619	Wild type	ATCC #4259
B. sphaericus 2362	Wild type	Lab collection
L. innocua	Wild type	D. Portnoy
E. coli DH5α	*hsdR17*($r_K^-m_K^+$) *supE44 thi*$^-$ *recA1 gyrA* (Nal^r) *relA1* D(*lacIZYA-argF*)U169 deo^R (F80Δ*lacD*(*lacZ*)M15)	Lab collection
S. aureus Newman	Wild type	F. Lowy
E. faecalis OG1RF	Wild type	D. Garsin
S. pyogenes	Wild type	A. Ratner
L. casei	Wild type	A. Ratner

Table S2. Stimulation of germination makes spores sensitive to an antibiotic

Germinant	% loss in viable cells
None	**0**
Muropeptide	**72 ± 3**
Cell free supernantant	**45 ± 2**
Bryostatin	**32 ± 7**

B. subtilis wild type spores were incubated with non-germinant buffer, muropeptide (GlcNAc-MurNAc tripeptide, 40 μM), *B. subtilis* cell free supernatant, or bryostatin (1 μM), for 60 min at 37°C prior to treatment with tetracycline (10 μg/ml for 60 min at 37°C). Percent loss in plating efficiency was calculated relative to that observed in the absence of germinant.

Supplemental References

D'Elia, M.A., Millar, K.E., Beveridge, T.J., and Brown, E.D. (2006). Wall teichoic acid polymers are dispensable for cell viability in Bacillus subtilis. J Bacteriol *188*, 8313-8316.

Gaidenko, T.A., Kim, T.J., and Price, C.W. (2002). The PrpC serine-threonine phosphatase and PrkC kinase have opposing physiological roles in stationary-phase Bacillus subtilis cells. J Bacteriol *184*, 6109-6114.

Paidhungat, M., and Setlow, P. (2000). Role of ger proteins in nutrient and nonnutrient triggering of spore germination in Bacillus subtilis. J Bacteriol *182*, 2513-2519.

Shatalin, K.Y., and Neyfakh, A.A. (2005). Efficient gene inactivation in Bacillus anthracis. FEMS Microbiol Lett *245*, 315-319.

Figure S1

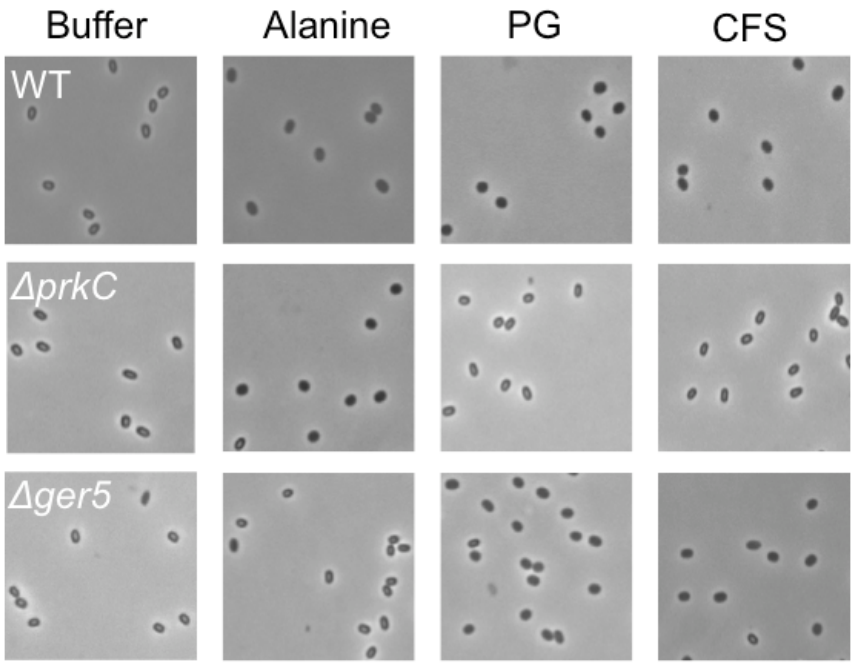
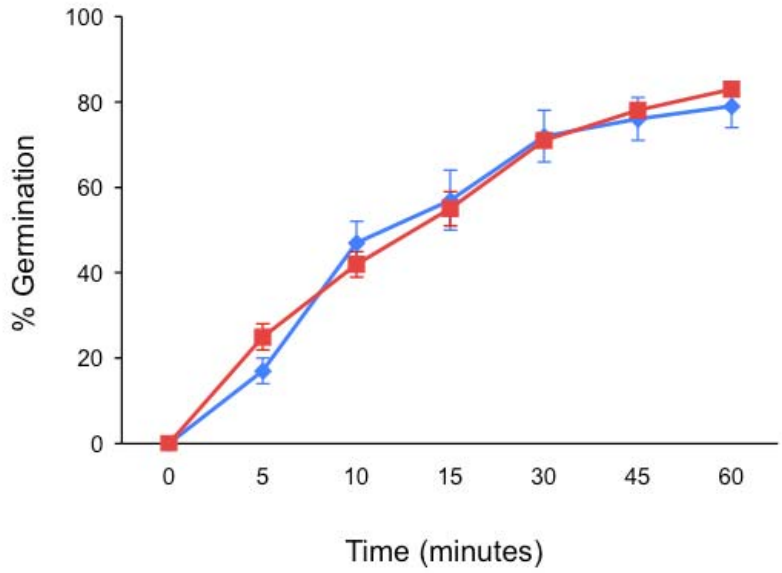


Figure S2



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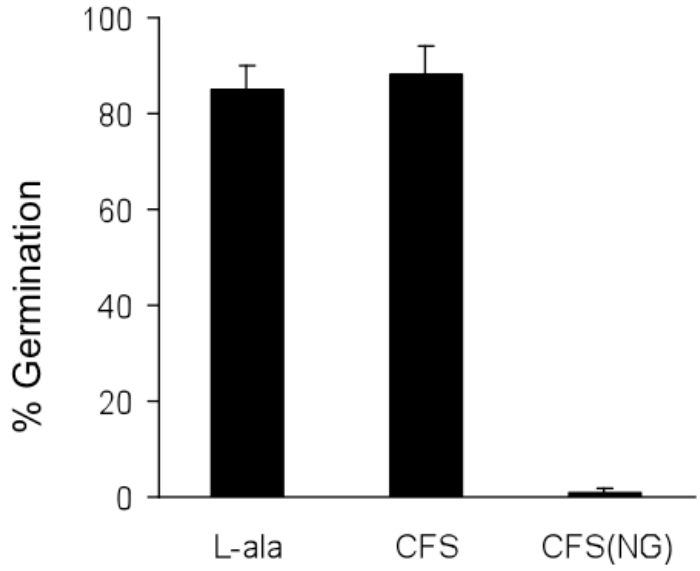

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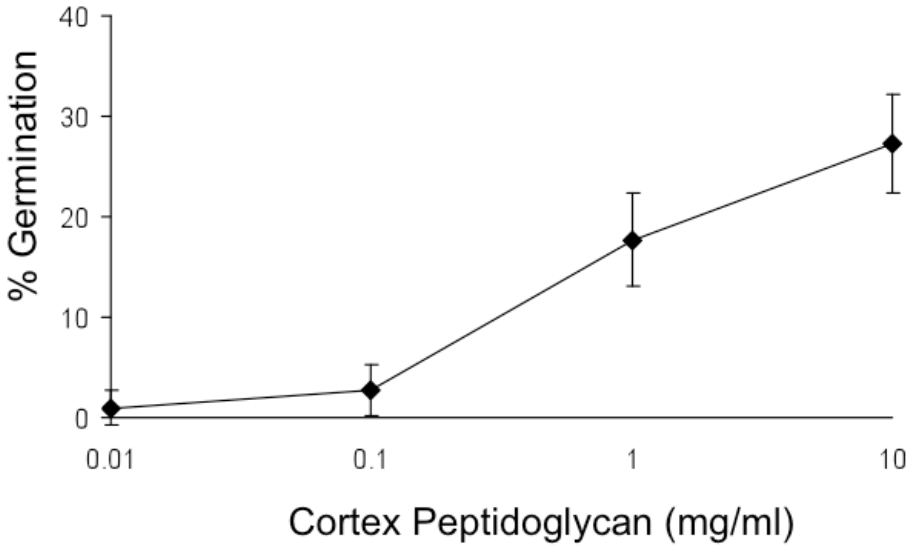

40
30
20
10
0
% Germination
0.01
0.1
1
10
Cortex Peptidoglycan (mg/ml)

Figure S5

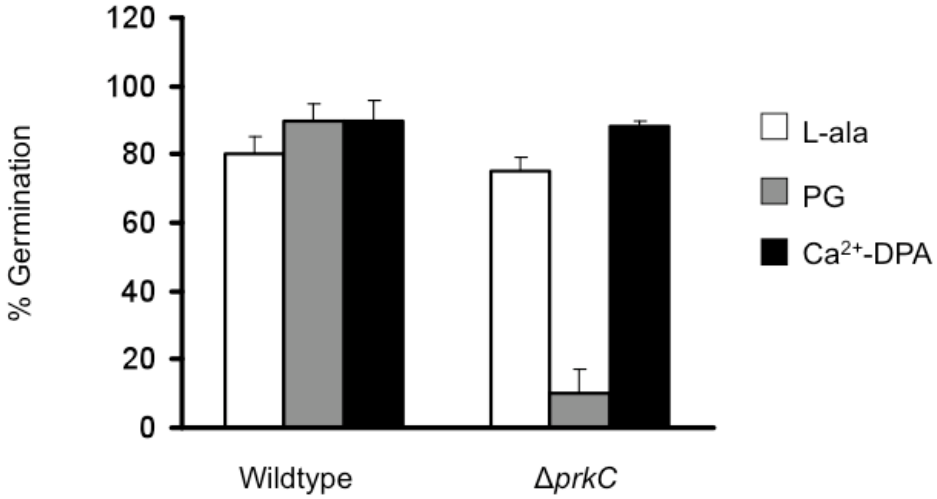

120
100
80
60
40
20
0
% Germination
Wildtype
ΔprkC
L-ala
PG
Ca2+-DPA

Figure S6

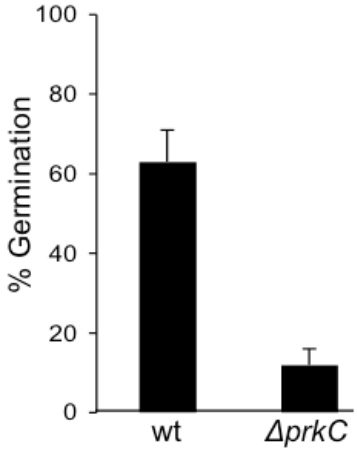


Figure S7

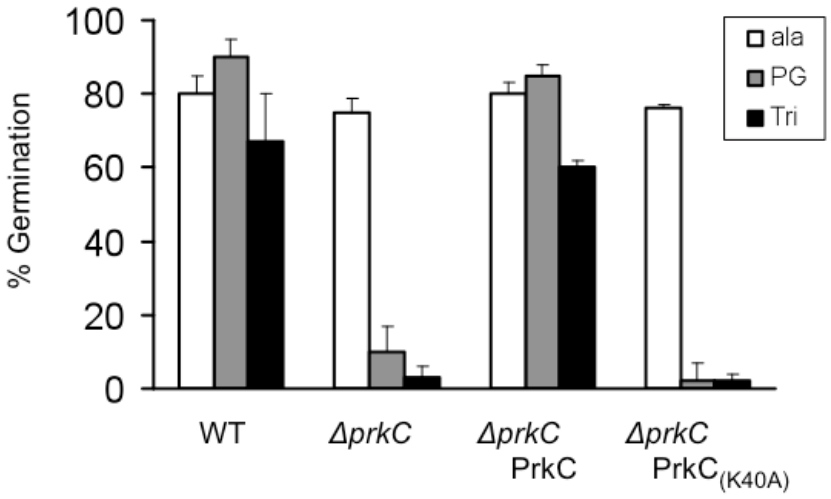

100
80
60
40
20
0
% Germination
ala
PG
Tri
WT
ΔprkC
ΔprkC PrkC
ΔprkC PrkC(K40A)

Figure S8

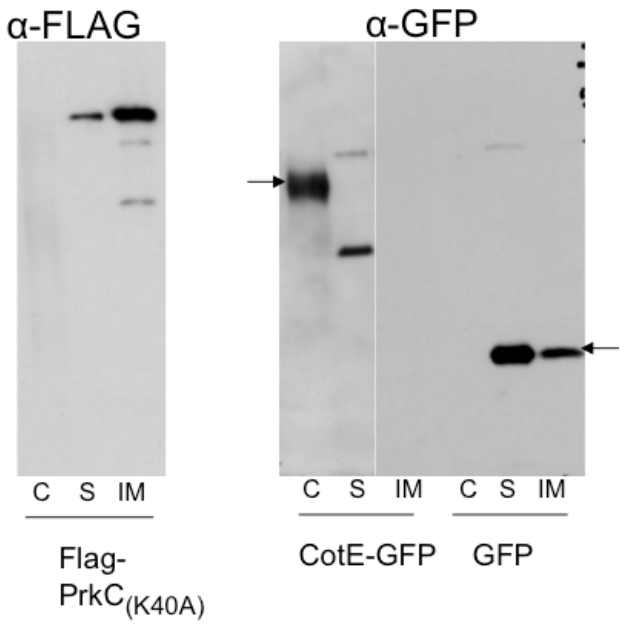
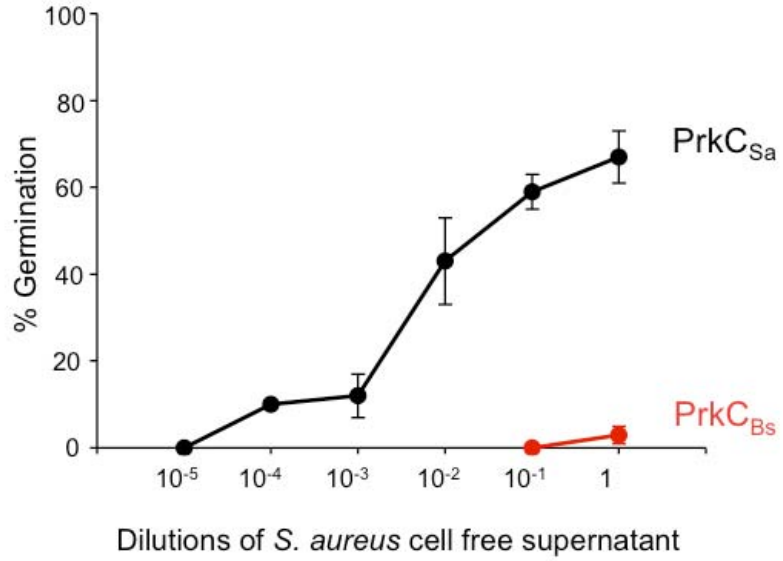


Figure S9


100
80
60
40
20
0
% Germination
PrkC$_{Sa}$
PrkC$_{Bs}$
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1
Dilutions of *S. aureus* cell free supernatant