Journal Name

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Supporting information

Materials and methods

Bacterial strains and growth conditions

Bacterial strains used in this study: *B. thuringiensis* 407 Cry⁻ *plcA'Z* (Bt A'Z) and the PapR null-mutant 407 Cry⁻ Δ*papR plcA'Z* (Bt ΔpapR A'Z) strains, containing a transcriptional fusion between the promoter of *plcA* and the *lacZ* reporter gene (as described previously[6, 17]); *B. cereus* strain ATCC 14579.

Unless otherwise noted, cells were grown in modified LB medium (16 g/L tryptone, 8 g/L yeast extract, 5 g/L NaCl) at 37°C and stored at −80°C in LB containing 25% glycerol. Kanamycin (200 µg/mL) was used for the selection of *B. thuringiensis.*

Solid phase peptide synthesis methodology (SPPS)

All the peptides were synthesized using standard Fmoc-based solid-phase peptide synthesis (SPPS), microwave irradiation, procedures on Rink Amide resin (substitution 0.5 mmol/g, 25 µmol) in SPE polypropylene Single-Fritted tubes. The Fmoc-protecting group was then removed by treating the resin with 20 % (v/v) piperidine diluted in dimethylformamide (DMF) followed by heating to 80°C in the microwave (MARS, CEM, USA; 2-minute ramp to 80°C, 2-minute hold at 80°C) with stirring. To couple each amino-acid, Fmoc-protected amino acids (4 equiv. relative to the overall loading of the resin), were dissolved in DMF and mixed with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; 4 equiv.) and diisopropylethylamine (DIEA; 4 equiv.). The solution was allowed to pre-activate for 5 min before being added to the resin, and heated to 70°C in a multimode microwave (2-minute ramp to 70°C, 4-minute hold at 70°C) with stirring. After each coupling/deprotection cycle the resin was drained and washed with DMF (3 × 5 mL). Once peptide synthesis was completed, the peptide was cleaved from the resin, by mixing the resin with 3 mL cleavage cocktail of 95% trifluoroacetic acid (TFA), 2.5% triisopropylsilane (TIPS), and 2.5% deionized water for 3 h with agitation. The peptide mixture was precipitated from the TFA solution by the addition of cold ether and collected by centrifugation (Eppendorf R5810 8000 rpm for 10 min). The ether was then removed, and the peptide was dried under a stream of nitrogen, and lyophilized, before HPLC purification.

Peptide Purification

Crude peptides were purified with RP-HPLC. The crude peptides were diluted to a final concentration of 10 mg/ml in a solution of 20% acetonitrile (ACN) in water (v/v) or dimethyl sulfoxide (DMSO). A semi-preparative Phenomenex Kinetex C18 (5 µm, 10 X 250 mm) was used (Shimadzu Scientific Instruments, MD, USA). Standard RP-HPLC conditions were as follows: flow rates = 5 mL min^{-1}; mobile phase A = 18 MΩ water + 0.1% TFA; mobile phase B = ACN. Purities were determined by integration of peaks with UV detection at 220 nm using a linear gradient (first prep 5% B → 65% B over 60 min and second prep 26% B → 36% B over 20 min). MALDI-TOF spectrometry (Bruker Daltonik, Germany) was used to validate the presence of synthesized peptides. The purified peptides were lyophilized and stored at -20°C until use.

β-galactosidase assay

β-galactosidase activity was measured as described previously, except that samples were incubated at 30°C instead of room temperature[15]. Specific activity is expressed in units of β-galactosidase per mg of protein. Each assay was repeated at least three times.

Analysis of PapR–PlcR interactions using β-galactosidase assay

A. PlcR activation studies by β-galactosidase assay

Bt ΔpapR A'Z cells were grown over night in LB medium with selective antibiotic. The cells were diluted 10^{-3} in modified LB to a final volume of 1 liter and incubated at 37°C with shaking (200 rpm) until onset of the stationary phase of bacterial growth (OD_{600} 3 ± 0.5). Various concentrations of synthetic peptides were added to 2 ml aliquots of culture, which were incubated for 1 h before centrifugation (Eppendorf centrifuge R5810, 4000 rpm for 5 min) and quantification of β-galactosidase.

B. Competition studied of $PapR_7$-derived peptides by β-galactosidase assay

Bt A'Z cells were grown over night in LB medium. The cells were diluted 10^{-3} in modified LB to a final volume of 1 liter and incubated at 37°C with shaking (200 rpm) until the end of the lag or late-exponential/early-stationary phase of bacterial growth (OD_{600} 0.1 ± 0.03; 1.8 or 2.5 ± 0.1 respectively). Different concentrations of synthetic peptides were added to 2 ml aliquots of culture and incubated for various times (1 - 6.5 h) before centrifugation (Eppendorf centrifuge R5810, 4000 rpm for 5 min) and quantification of β-galactosidase activity.

Analysis of PapR–PlcR interactions using hemolytic assay

A. Hemolytic activity on blood sheep agar plates

Bt A'Z or *B. cereus* ATCC 14579 cells were grown over night in LB medium. The cells were diluted 10^{-3} in modified LB to a final volume of 1 liter and incubated at 37°C with shaking (200 rpm) until the end of the lag phase of bacterial growth (OD_{600} 0.1 ± 0.03). Different concentrations of synthetic peptides were added to 2 ml aliquots of bacterial culture and incubated for 2.5 h before centrifugation

(Eppendorf centrifuge R5810, 4000 rpm for 5 min), separation and filtration (0.2µm filter) of the supernatants of the treated cultures. 20 µl of filtered supernatants were placed into holes in sheep blood agar plate and incubated for 24 hours at 37°C.

B. Hemolytic activity of human red blood cells

Bt A'Z or *B. cereus* ATCC 14579 cells were grown over night in LB medium. The cells were diluted 10^{-3} in modified LB to a final volume of 1 liter and incubated at 37°C with shaking (200 rpm) until the end of the lag phase of bacterial growth (OD_{600} 0.1 ± 0.03). Different concentrations of synthetic peptides were added to 2 ml aliquots of culture and incubated for 2.5 h before centrifugation (Eppendorf centrifuge R5810, 4000 rpm for 5 min), separation and filtration (0.2µm filter) of the supernatants of the treated cultures. Analysis of hemolytic activity were conducted as previously described using human red blood cells[38,39]. Bacterial supernatants serially diluted in Tris-buffered saline (pH 7.2, 10 mM Tris-HCl, 155 mM NaCl) with 1% human red blood cells (hRBC) suspension and were incubated for 30 min at 37°C. Hemolytic activities were measured by monitoring the absorbance at 420 nm.

Statistical analysis

Unless otherwise noted, the results are presented as the mean ± SEM. One-way analysis ANOVA of variance, followed by Tukey post-hoc analysis was used for statistical analysis. The results were considered to be statistically significant if $p < 0.01$.

Table S1: Sequences and molecular weight (MW) of $PapR_7$-derived peptides

Peptide name	Sequence	Calculated MW	Observed MW	Retention time (min)
$PapR_7$	**A-D-L-P-F-E-F**	**837.9**	**837.76**	**9.118**
$PapR_7$ - D2A	A-**A**-L-P-F-E-F	793.9	793.78	15.05
$PapR_7$ - L3A	A-D-**A**-P-F-E-F	795.83	795.73	11.39
$PapR_7$ - P4A	A-D-L-**A**-F-E-F	811.87	811.57	13.39
$PapR_7$ - F5A	A-D-L-P-**A**-E-F	761.81	761.65	9.612
$PapR_7$ - E6A	A-D-L-P-F-**A**-F	779.87	779.61	15
$PapR_7$ - F7A	A-D-L-P-F-E-**A**	761.81	761.58	9.972
$PapR_7$ - dA	**dA**-D-L-P-F-E-F		837.95	8.794
$PapR_7$ - dD	A-**dD**-L-P-F-E-F		837.96	9.373
$PapR_7$ - dL	A-D-**dL**-P-F-E-F		838.55	7.021
$PapR_7$ - dP	A-D-L-**dP**-F-E-F	837.9	837.78	10.03
$PapR_7$ - dF_5	A-D-L-P-**dF**-E-F		837.87	9.917
$PapR_7$ - dE	A-D-L-P-F-**dE**-F		837.9	7.439
$PapR_7$ - dF_7	A-D-L-P-F-E-**dF**		837.93	8.408

Sequences and molecular weight (MW) of the synthetic $PapR_7$ derived peptides. Six of the seven residues of $PapR_7$ were modified to alanine amino-acid (shown in the top of the table), while all seven residues of $PapR_7$ were modified to their D-enantiomers, (presented in the lower half of the table).

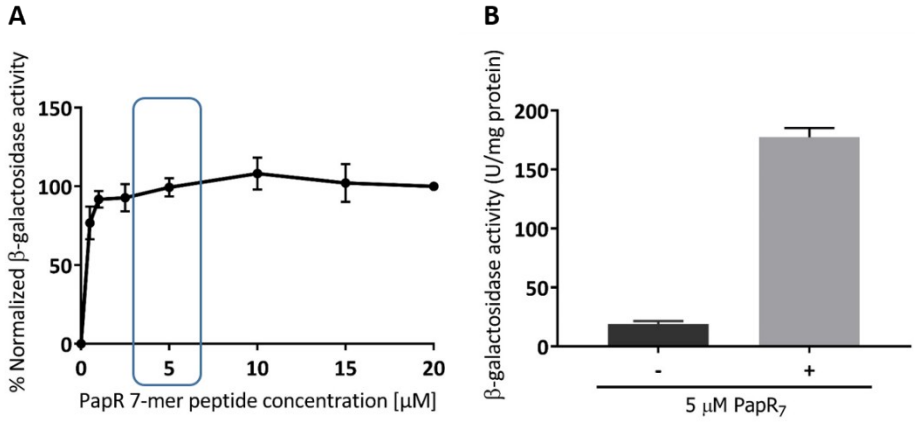

Figure S1: Studying PlcR regulon activation. (A) β-galactosidase of Bt ΔpapR A'Z activity induced by the addition of various concentrations of $PapR_7$ synthetic peptide (0.5-20 µM) at onset of the stationary

phase of bacterial growth (OD_{600} of 3 ± 0.5; mean ± SEM, n = 6). (B) Effect of 5 µM synthetic $PapR_7$ peptide was examined by β-galactosidase activity of Bt ΔpapR A'Z at onset of the stationary phase of bacterial growth (OD_{600} of 3 ± 0.5; mean ± SEM, n = 3).

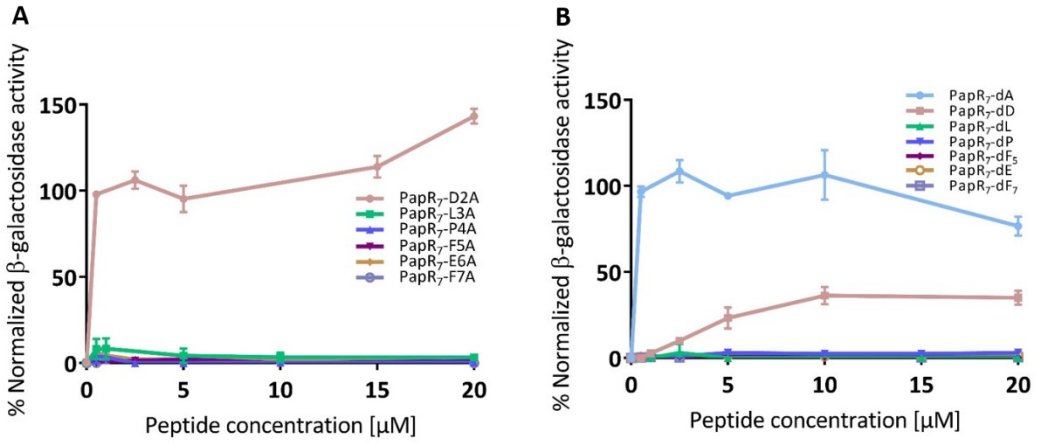

Figure S2: PlcR regulon activation by $PapR_7$-derived peptides. β-galactosidase activity of Bt ΔpapR A'Z induced by the addition of 0.5-20 µM (A) Alanine and (B) D-amino $PapR_7$-derived peptides normalized to synthetic $PapR_7$ peptide at onset of the stationary phase of bacterial growth (OD_{600} of 3 ± 0.5; mean ± SEM, n = 3).

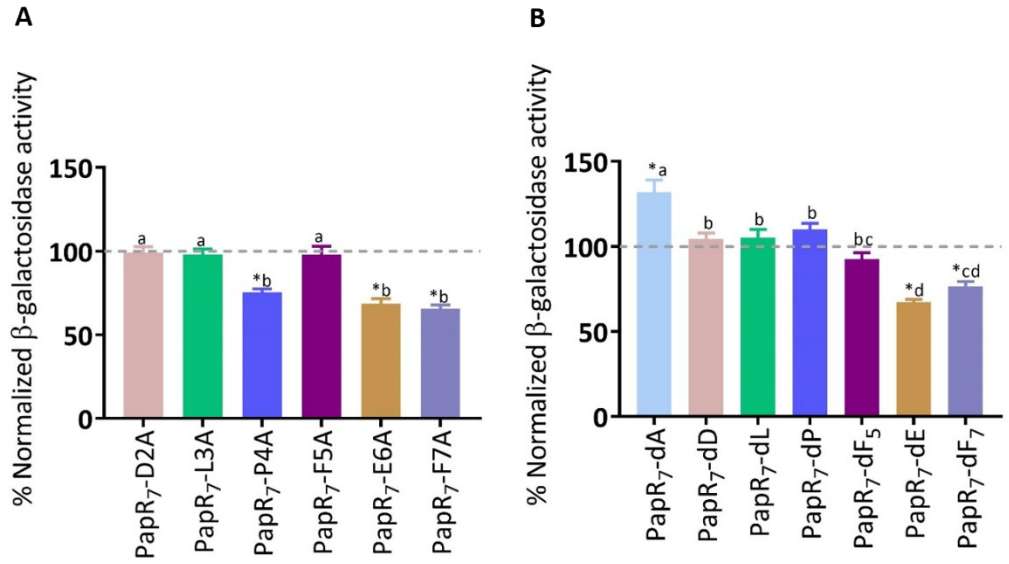

Figure S3: Competition studies with $PapR_7$-derived peptides. β-galactosidase activity of Bt A'Z induced by the addition of 2.5 µM (A) Alanine and (B) D-amino $PapR_7$-derived peptides normalized to untreated bacterial cells at early-stationary phase of bacteria growth (OD_{600} of 2.5 ± 0.1; mean ± SEM, n = 9). $^{*}p < 0.01$ indicates a statistically significant difference between untreated Bt A'Z and addition of $PapR_7$-derived peptides. ^{a,b,c,d} $p < 0.01$ indicates a statistically significant difference between $PapR_7$-derived peptide treatments.

Table S2: IC_{50} values of five $PapR_7$-derived QS peptidic inhibitors as determined by the *lacZ*-based reporter assay

$PapR_7$-derived peptides	IC_{50} [µM]
$PapR_7$-P4A	2.901 ± 0.194
$PapR_7$-E6A	1.698 ± 0.111
$PapR_7$-F7A	1.637 ± 0.102
$PapR_7$-dE	0.977 ± 0.04
$PapR_7$-dF_7	1.223 ± 0.07

IC_{50} values were calculated by GraphPad Prism 7, using the nonlinear inhibitor vs. normalized response method.

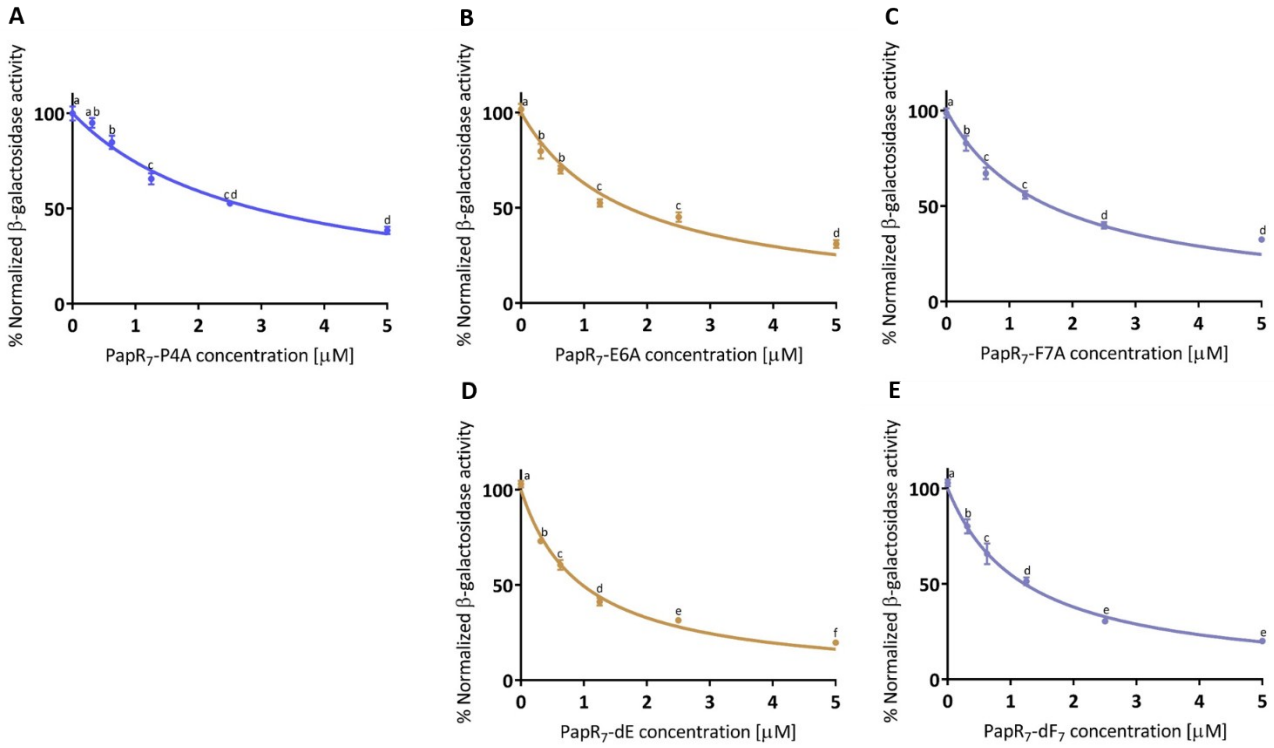

Figure S4: Quantification of $PapR_7$-derived peptides competition studies. β-galactosidase activity of Bt A'Z induced by the addition of (A) $PapR_7$-P4A, (B) $PapR_7$-E6A, (C) $PapR_7$-F7A, (D) $PapR_7$-dE and (E) $PapR_7$-dF7 derivatives in several concentrations normalized to untreated bacterial cells at late-exponential of bacterial growth (OD_{600} of 1.8 ± 0.1; mean ± SEM, n=9). $^{a,b,c,d,e,f}p<0.01$ indicates statistically significant difference between untreated Bt A'Z and addition of $PapR_7$-derived peptides.

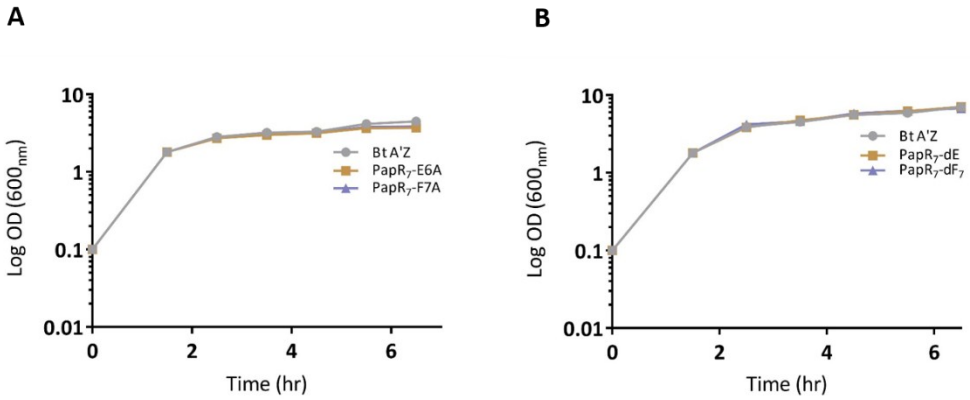

Figure S5: PapR$_7$-derived peptides effect on bacterial growth. OD_{600} measurements (logarithmic scale) of Bt A'Z cultures treated with (A) Alanine and (B) D-amino acid PapR$_7$-derived peptides over time compared to untreated bacterial cells (Mean ± SE, n = 3).
Bt A'Z cells were grown at 37°C in modified LB medium. PapR$_7$-derived peptides were added at final concentration of 10 µM at the end of the lag phase of bacterial growth (OD_{600} of 0.1 ± 0.03) and incubated for up to 6.5 h. OD_{600} values were measured and then bacterial cells were harvested at each time point (starting from 2.5 h) and the β-galactosidase activity was assayed.

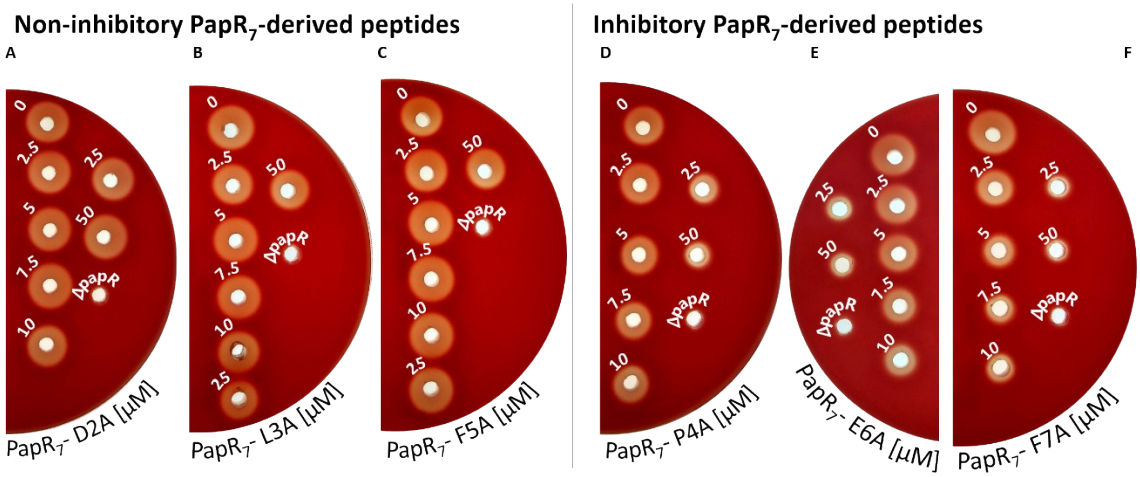

Figure S6: Alanine PapR$_7$-derived peptides effect on hemolytic activity of sheep red-blood cells. Hemolytic activity on sheep blood agar of supernatants Bt A'Z treated cultures with a range of concentration of (A) PapR$_7$ – D2A, (B) PapR$_7$ – L3A, (C) PapR$_7$ – F5A, (D) PapR$_7$ – P4A, (E) PapR$_7$ – E6A, and (F) PapR$_7$ – F7A peptides.

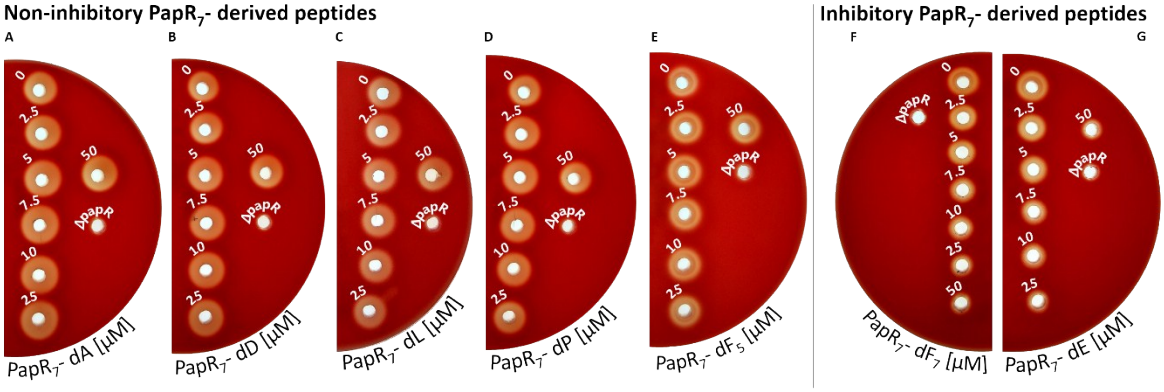

Figure S7: D-amino PapR$_7$-derived peptides effect on hemolytic activity of sheep red-blood cells. Hemolytic activity on sheep blood agar of supernatants Bt A'Z treated cultures with a range of concentration of (A) PapR$_7$ – dA, (B) PapR$_7$ – dD, (C) PapR$_7$ – dL, (D) PapR$_7$ – dP, (E) PapR$_7$ – dF$_5$, (F) PapR$_7$ – dF$_7$ and (G) PapR$_7$ – dE peptides.

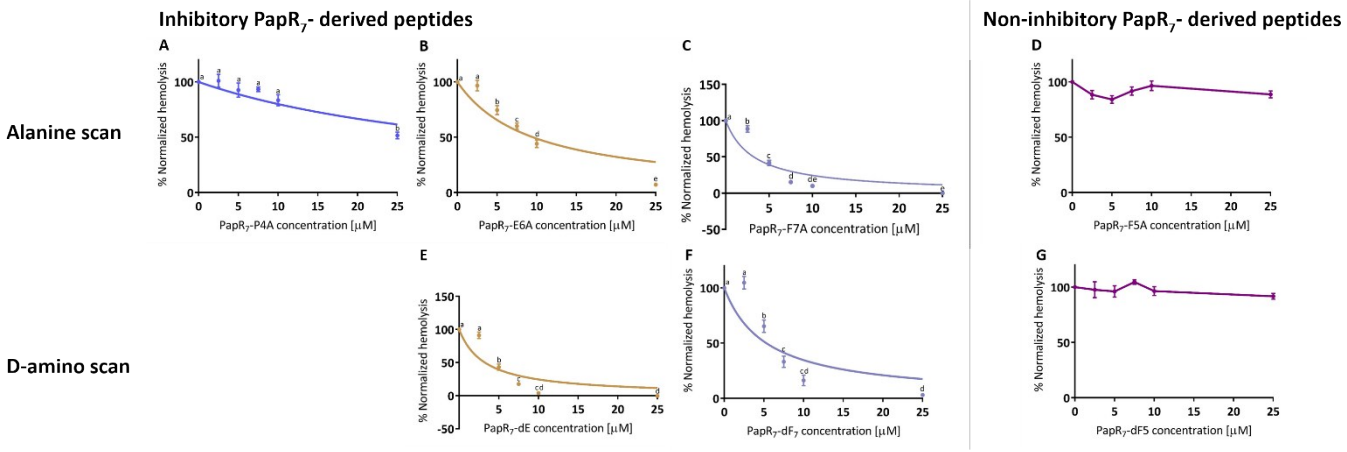

Figure S8: Quantifying PapR$_7$-derived peptides hemolytic activity effect on human red-blood cells. Hemolysis inhibition dose response curves of Bt A'Z treated supernatants cultures with range of concentration of (A) PapR$_7$ – P4A, (B) PapR$_7$ – E6A, (C) PapR$_7$ – F7A, (D) PapR$_7$ – F5A, (E) PapR$_7$ – dE, (F) PapR$_7$ – dF$_7$ and (G) PapR$_7$ – dF$_5$ normalized to untreated Bt A'Z supernatants cultures (mean ± SEM, n = 9). $^{a,b,c,d,e}p < 0.01$ indicates a statistically significant difference between untreated Bt A'Z and addition of inhibitory PapR$_7$-derived peptides. The results show no statistically significant difference between untreated Bt A'Z supernatants cultures and addition of non-inhibitory PapR$_7$-F5A and PapR$_7$-dF$_5$ peptides.

Table S3: IC$_{50}$ values of five PapR$_7$-derived QS peptidic inhibitors as determined by the hemolytic assay

PapR$_7$-derived peptides	IC$_{50}$ [µM]
PapR$_7$-P4A	40.03 ± 5.293
PapR$_7$-E6A	9.532 ± 0.947
PapR$_7$-F7A	3.229 ± 0.426
PapR$_7$-dE	3.242 ± 0.474
PapR$_7$-dF$_7$	5.304 ± 0.817

IC$_{50}$ values were calculated by GraphPad Prism 7, using the nonlinear inhibitor vs. normalized response method.

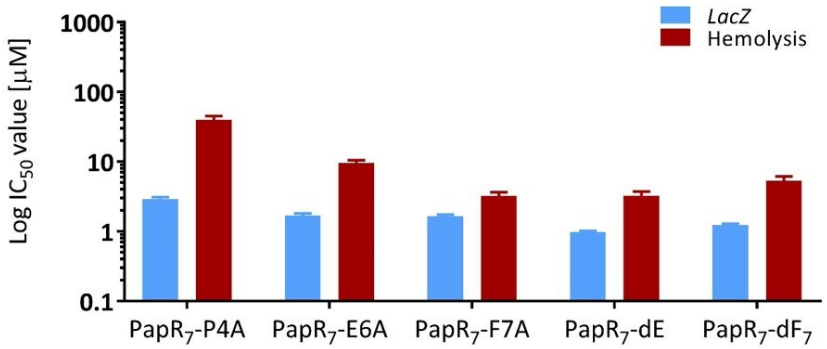

Figure S9: IC$_{50}$ values differences of *B. cereus* QS system peptidic inhibitors. IC$_{50}$ values of five PapR$_7$-derived QS peptidic as determined by the *lacZ*-reporter assay (blue) and hemolytic assay with hRBC suspension (red).