

Figure S1. Kinetics and domain structure of PlyPy. MGAS315 resuspended in PBS was incubated with PlyPy at various concentrations (50 U/ml, 25 U/ml, 12.5 U/ml, 6 U/ml, 3 U/ml, 1.5 U/ml); reduction in OD₅₉₅ was monitored for 30 minutes (1A). PlyPy was added at different concentrations to both exponentially growing and stationary phase MGAS315, and live bacteria was measured after 60 minutes incubation (1B). PlyPy has an N-terminal CHAP domain, and a C-terminal SH3 domain, similar to the domain structure of the *Streptococcus suis* phage lysin PlySs2 (C).

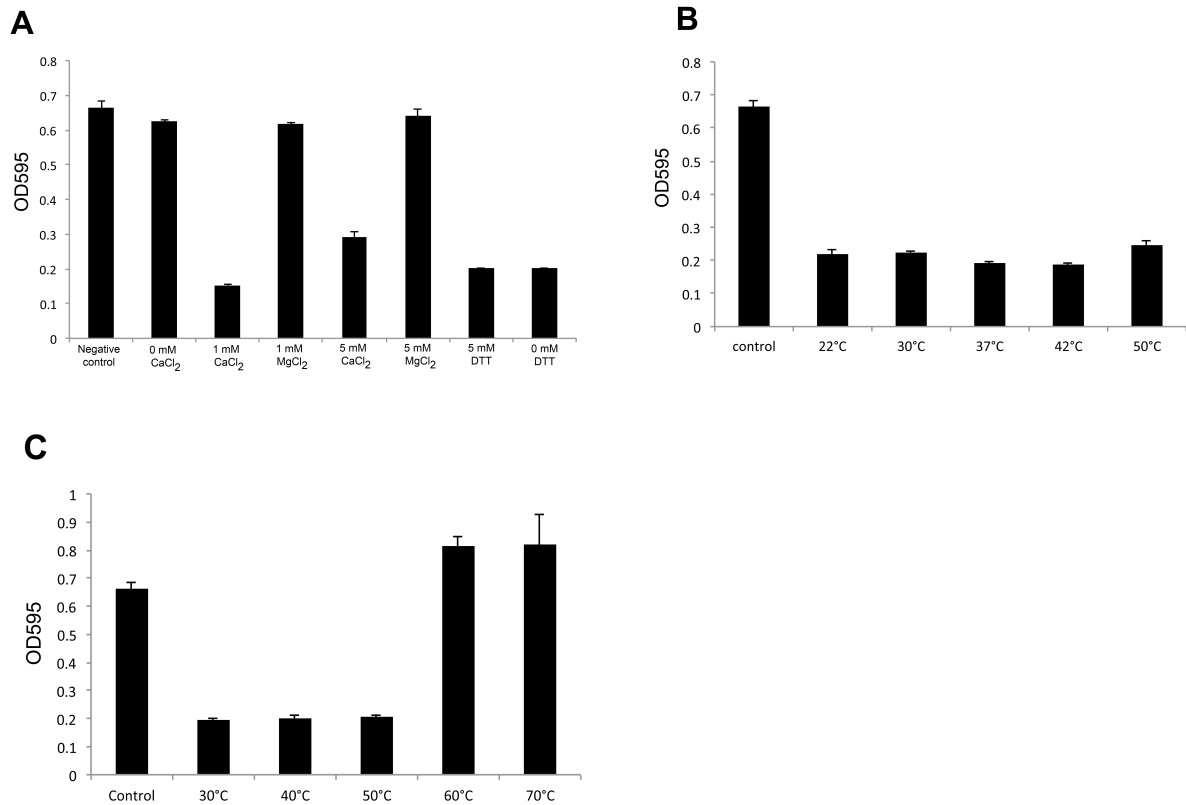


Figure S2. Enzymatic properties of PlyPy. Exponentially growing MGAS315 resuspended in 20 mM Tris-HCl pH 6.8, supplemented with 100 mM NaCl, was incubated with 5 $\mu\text{g}/\text{ml}$ PlyPy and varying concentrations of CaCl₂ and MgCl₂ at 37°C. Reduction in OD₅₉₅ was measured for 10 minutes (A). To investigate the influence of any reducing agents, PlyPy was pre-incubated with 5 mM DTT for 1 hour, before analyzing the effect of PlyPy on MGAS315 (A). The temperature optimum was determined by incubating PlyPy (5 $\mu\text{g}/\text{ml}$) with MGAS315 at different temperatures for 10 minutes; reporting the change in optical density (B). The effect of pre-incubation of the enzyme only at different temperatures for 30 minutes before analyzing its lytic activity was also investigated (C).

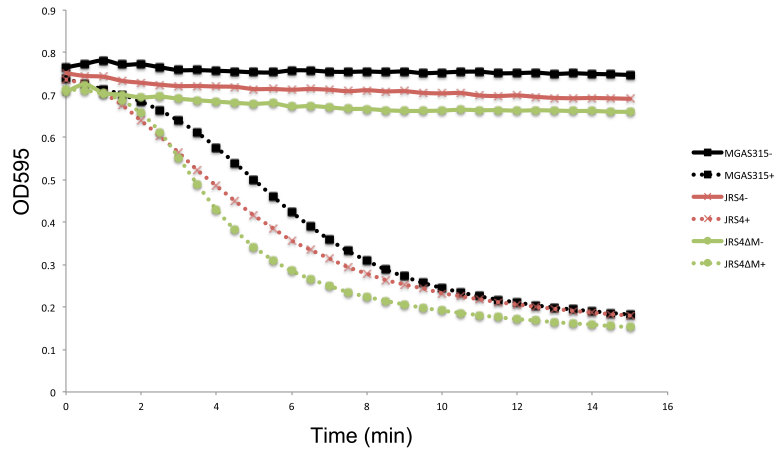


Figure S3. Effect of the M-protein on the activity of PlyPy. To investigate if the presence of M-protein on the surface of *S. pyogenes* may influence the activity of PlyPy, we compared the activity of PlyPy against MGAS315 (control), JRS4 (parental strain) and JRS4ΔM (M-protein knockout). Dashed lines indicate the presence of 5 µg/ml PlyPy.

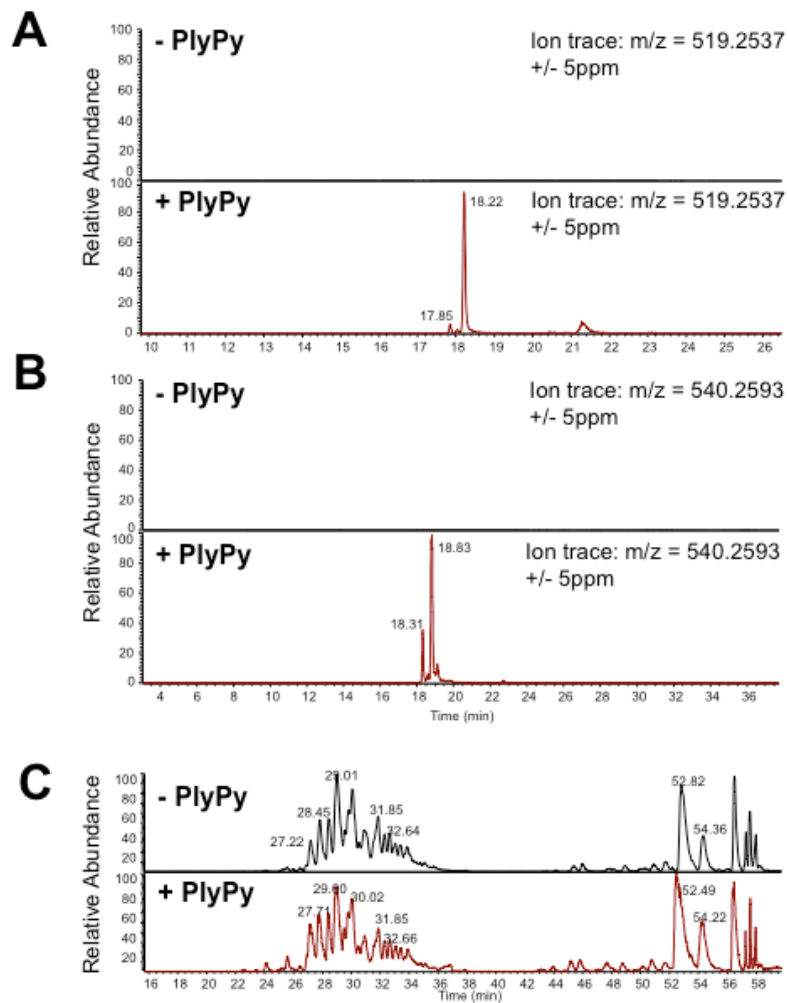


Figure S4. Ion traces for ions identified as PlyPy cleavage peptidoglycan products. In panel A and B are shown the ion traces generated for the doubly charged peptidoglycan PlyPy products measured at m/z 519.2537 (Fig. S4A) and m/z 540.2593 (Fig. S4B). Upper panels show ion traces measured in the samples without treatment (-PlyPy), while lower panels are the traces measured after PlyPy treatment (+PlyPy). The ion traces are extracted with a mass tolerance of 5 ppm. The y-scale is percent abundance normalized to the signal measured in the PlyPy treated sample. C) For comparison, the base peak chromatograms for the two samples, without (upper panel) and after treatment (lower panel) with PlyPy.

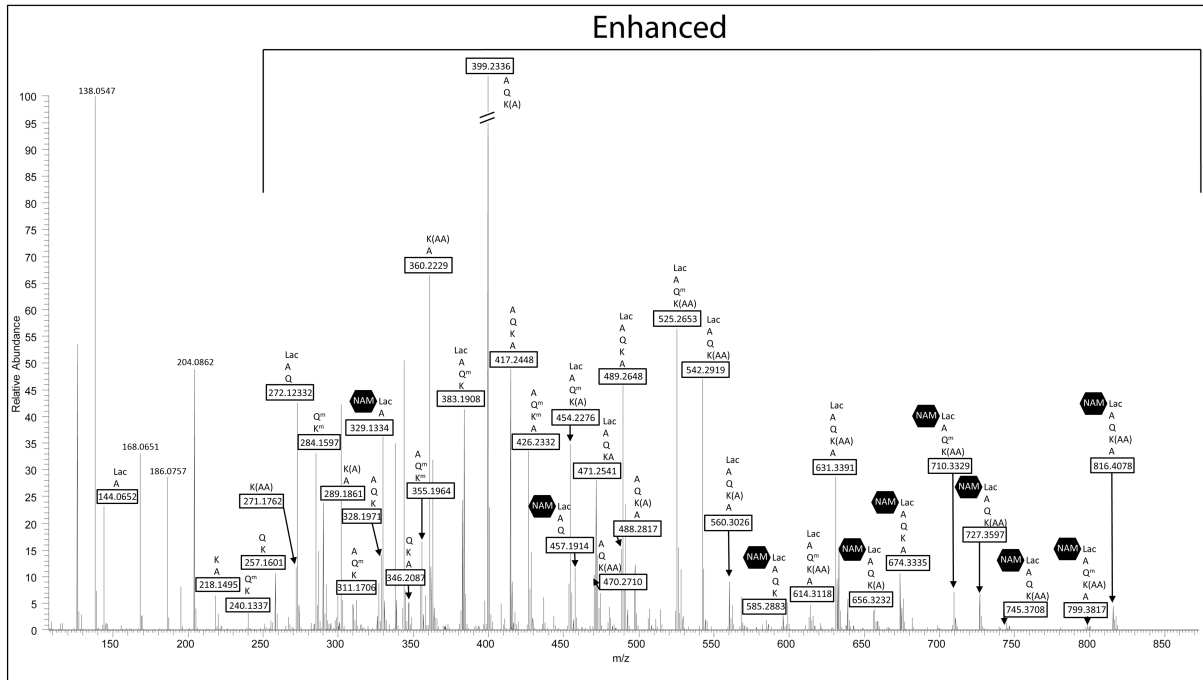


Figure S5. Tandem MS spectrum (m/z 100 to 1080) of the doubly charged PlyPy peptidoglycan cleavage product measured at m/z 519.2537 (1.35 ppm) with important fragment ions annotated. The intense fragment ions at m/z 204.086, 186.078, 168.065 and 138.058 are diagnostic for carbohydrates. To better appreciate the back-bone fragmentation, the intensity scale has been enhanced for the m/z region >250. The MS/MS spectrum (collision energy:29) was recorded with a Q-Exactive mass spectrometer using an isolation window of 3.5 Th and a resolution of 35,000 at m/z 200.

Table S1. Strains used to determine PlyPy specificity

Bacillus cereus RS46
Enterococcus faecalis V583
Enterococcus faecium EFSK2
Staphylococcus aureus RN4220
Staphylococcus epidermidis HER1292
Streptococcus agalactiae 090R
Streptococcus agalactiae type II
Streptococcus dysgalactiae CTF
Streptococcus dysgalactiae equisimilis 26RP66
Streptococcus equi ATCC9528
Streptococcus gordonii 10558
Streptococcus mutans U159
Streptococcus oralis 35037
Streptococcus pneumoniae serotype 6 DCC1850
Streptococcus pneumoniae serotype 11
DCC1811
Streptococcus porcinus K131
Streptococcus pyogenes M6 D471
Streptococcus pyogenes NZ131
Streptococcus pyogenes SF370
Streptococcus pyogenes MGAS8332
Streptococcus pyogenes MGAS10390
Streptococcus pyogenes MGAS5005
Streptococcus pyogenes MGAS315
Streptococcus rattus BHT
Streptococcus sanguinis 10556
Streptococcus sobrinus 6715
Streptococcus suis serotype 1 6112
Streptococcus suis serotype 2 S735
Streptococcus suis serotype 7 7197
Streptococcus suis serotype 9 7997
Streptococcus uberis #24
Streptococcus uberis #27
Streptococcus uberis #69
Streptococcus uberis ATCC 27985
Streptococcus uberis ATCC 700407
Streptococcus uberis ATCC BAA-854

Table S2. Annotation of tandem MS spectrum of the doubly charged PlyPy peptidoglycan cleavage product measured at m/z 519.2537

| m/z | interpretation | alternative | Observed | Theoretical | mass accuracy | O18 |
|-----------|-----------------------|-------------|-----------|-------------|---------------|-----|
| 1037.4988 | NAG-NAM-Lac-AQK(AA)A* | | 1037.4988 | 1037.5002 | 1.35 ppm | nd |
| 816.40747 | NAM-Lac-AQK(AA)A* | | 816.40747 | 816.4103 | 3.47 ppm | yes |
| 799.38171 | NAM-Lac-AQK(AA)A* | | 799.38171 | 799.38377 | 2.58 ppm | yes |
| 745.37079 | NAM-Lac-AQK(A)A* | | 745.37079 | 745.37319 | 3.22 ppm | yes |
| 727.35968 | NAM-Lac-AQK(AA) | | 727.35968 | 727.36263 | 4.06 ppm | no |
| 710.33289 | NAM-Lac-AQK(AA) | | 710.33289 | 710.3361 | 4.52 ppm | no |
| 674.3335 | NAM-Lac-AQKA* | | 674.3335 | 674.33608 | 3.83 ppm | yes |
| 656.3232 | NAM-Lac-AQK(A) | | 656.3232 | 656.32552 | 3.53 ppm | no |
| 631.33911 | Lac-AQK(AA)A* | | 631.33911 | 631.34149 | 3.77 ppm | yes |
| 614.31177 | Lac-AQK(AA)A* | | 614.31177 | 614.31496 | 5.19 ppm | yes |
| 585.2883 | NAM-Lac-AQK | | 585.2883 | 585.28841 | 0.19 ppm | no |
| 560.30255 | Lac-AQK(A)A* | | 560.30255 | 560.30438 | 3.27 ppm | yes |
| 542.29193 | Lac-AQK(AA) | | 542.29193 | 542.29382 | 3.49 ppm | no |
| 525.26526 | Lac-AQK(AA) | | 525.26526 | 525.26729 | 3.87 ppm | no |
| 489.2648 | Lac-AQKA* | | 489.2648 | 489.26727 | 5.05 ppm | nd |
| 488.28171 | AQK(A)A* | QK(AA)A* | 488.28171 | 488.28325 | 3.15 ppm | nd |
| 471.25406 | Lac-AQK(A) | | 471.25406 | 471.25671 | 5.62 ppm | nd |
| 470.27097 | AQK(AA) | | 470.27097 | 470.27269 | 3.66 ppm | nd |
| 457.19138 | NAM-Lac-AQ | | 457.19138 | 457.19345 | 4.53 ppm | no |
| 454.22763 | Lac-AQK(A) | | 454.22763 | 454.23018 | 5.61 ppm | no |
| 426.23322 | AQK(A) | | 426.23322 | 426.23524 | 4.74 ppm | yes |
| 417.24475 | AQKA* | QK(A)A* | 417.24475 | 417.24614 | 3.33 ppm | yes |
| 399.23361 | AQK(A) | QK(AA) | 399.23361 | 399.23558 | 4.93 ppm | no |
| 383.1908 | Lac-AQK | | 383.1908 | 383.19307 | 5.92 ppm | no |
| 360.22287 | K(AA)A* | | 360.22287 | 360.22467 | 5.00 ppm | yes |
| 355.19644 | AQK | | 355.19644 | 355.19813 | 4.76 ppm | yes |
| 346.2087 | QKA* | | 346.2087 | 346.20903 | 0.95 ppm | yes |
| 329.13342 | NAM-Lac-A | | 329.13342 | 329.13487 | 4.41 ppm | no |
| 328.1971 | AQK | QK(A) | 328.1971 | 328.19847 | 4.17 ppm | no |
| 311.17059 | AQK | | 311.17059 | 311.17194 | 4.34 ppm | no |
| 289.1861 | K(A)A* | | 289.1861 | 289.18756 | 5.05 ppm | yes |
| 284.15976 | QK | | 284.15976 | 284.16102 | 4.43 ppm | yes |
| 272.12332 | Lac-AQ | | 272.12332 | 272.12464 | 4.85 ppm | nd |
| 271.1762 | K(AA) | | 271.1762 | 271.177 | 2.95 ppm | nd |
| 257.1601 | QK | | 257.1601 | 257.16136 | 4.90 ppm | no |
| 240.13368 | QK | | 240.13368 | 240.13483 | 4.79 ppm | no |
| 218.14946 | KA* | | 218.14946 | 218.15045 | 4.54 ppm | yes |
| 144.0652 | Lac-A | | 144.0652 | 144.06606 | 5.97 ppm | no |

Q = deamidated glutamine (NH₃)

K = acetylated lysine (C₂H₄O)

A* = Alanine with a free carboxy group (eg. D-Ala)