Supplemental material

Improved Lethal Effect of Cpl-7, a Pneumococcal Phage Lysozyme of Broad Bactericidal Activity by Inverting Net Charge of its Cell Wall-Binding Module

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Running title: Improved Bactericidal Effect of a Phage Lysozyme

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[*] These authors contributed equally to this work.

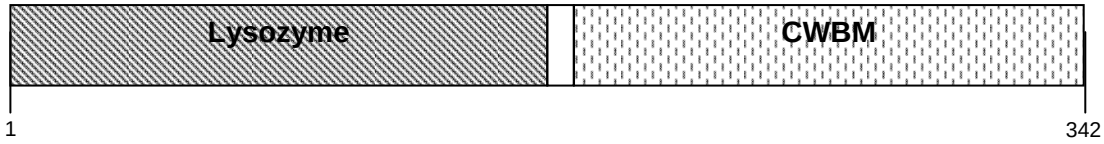
Contents:

• **Full-length Cpl-7S:** 1,029 bp, 342 amino acids, 38,581 Da, theoretical pI: 4.63.

• **N-terminal module:** Catalytic module from GH-25 family of glycosyl hydrolases. It cleaves *N*-acetylmuramoyl-(β 1,4)-N-acetylglucosamine bonds (amino acids 1–188).

• **Linker region:** Amino acids 189–204.

• **C-terminal module:** Cell wall-binding module (CWBM) formed by three identical CW_7 repeats (amino acids 205–342).



Nucleotide sequence:

```
        10         20         30         40         50         60
ATGGTTAAAA AGAACGACCT GTTCGTTGAC GTTGCGTCTC ACCAGGGTTA CGACATCTCT

        70         80         90        100        110        120
GGTATCCTGG AAGAAGCGGG TACCACCAAC ACCATCATCA AAGTTTCTGA ATCTACCAGC

       130        140        150        160        170        180
TACCTGAACC CGTGCCTGTC TGCGCAGGTT TCTCAGTCTA ACCCGATCGG TTTCTACCAC

       190        200        210        220        230        240
TTCGCGTGGT TCGGTGGTAA CGAAGAAGAA GCGGAAGCGG AAGCGCGTTA CTTCCTGGAC

       250        260        270        280        290        300
AACGTTCCGA CCCAGGTTAA ATACCTGGTT CTGGACTACG AAGACCACGC GTCTGCGTCT

       310        320        330        340        350        360
GTTCAGCGTA ACACCACCGC GTGCCTGCGT TTCATGCAGA TCATCGCGGA AGCGGGTTAC

       370        380        390        400        410        420
ACCCCGATCT ACTACTCTTA CAAACCGTTC ACCCTGGACA ACGTTGACTA CCAGCAGATC

       430        440        450        460        470        480
CTGGCGCAGT TCCCGAACTC TCTGTGGATC GCGGGTTACG GTCTGAACGA CGGTACCGCG

       490        500        510        520        530        540
AACTTCGAAT ACTTCCCGTC TATGGACGGT ATCCGTTGGT GGCAGTACTC TTCTAACCCG

       550        560        570        580        590        600
TTCGACAAAA ACATCGTTCT GCTGGACGAC GAAAAAGAAG ACAACATCAA CAACGAAAAC

       610        620        630        640        650        660
ACCCTGAAAT CTCTGACGAC CGTTGCGAAT GAAGTTATCC AAGGTAAATG GGGTAATGGC

       670        680        690        700        710        720
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CAGGAGCGCT ACAAATCTCT GGCCAACCGT GGTTACAATC CGCAGGCCGT GCAGAATAAA

       730        740        750        760        770        780
GTTAACGAGA TTCTGAATGC GCGTGAGATC GCGGATCTCA CTACGGTGGC AAACGAAGTG

       790        800        810        820        830        840
ATTCAAGGCA AGTGGGGGAA CGGGCAGGAA CGGTACAAGT CCTTGGCAAA CCGCGGCTAC

       850        860        870        880        890        900
AACCCACAGG CAGTACAGAA CAAGGTAAAC GAAATCTTGA ACGCTCGCGA AATTGCTGAC

       910        920        930        940        950        960
CTTACCACAG TAGCCAATGA GGTCATTCAG GGAAAATGGG GCAACGGTCA AGAACGTTAT

       970        980        990       1000       1010       1020
AAAAGTTTAG CGAATCGAGG GTATAATCCC CAAGCGGTTC AAAATAAAGT GAATGAATTA

CTTTCATAA
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Amino acid sequence:

```
         10         20         30         40         50         60
MVKKNDLFVD VASHQGYDIS GILEEAGTTN TIIKVSESTS YLNPCLSAQV SQSNPIGFYH

         70         80         90        100        110        120
FAWFGGNEEE AEAEARYFLD NVPTQVKYLV LDYEDHASAS VQRNTTACLR FMQIIAEAGY

        130        140        150        160        170        180
TPIYYSYKPF TLDNVDYQQI LAQFPNSLWI AGYGLNDGTA NFEYFPSMDG IRWWQYSSNP

        190        200        210        220        230        240
FDKNIVLLDD EKEDNINNEN TLKSLTTVAN EVIQGKWGNG QERYKSLANR GYNPQAVQNK
                                    *          *    *    *     *

        250        260        270        280        290        300
VNEILNAREI ADLTTVANEV IQGKWGNGQE RYKSLANRGY NPQAVQNKVN EILNAREIAD
                       *               *    *    *      *

        310        320        330        340
LTTVANEVIQ GKWGNGQERY KSLANRGYNP QAVQNKVNEL LS
           *          *    *  *       *
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* Mutated amino acids with respect to Cpl-7

Figure S1. Nucleotide and amino acid sequence of Cpl-7S. Characteristics of the two domains and the linker region are indicated, as well as the amino acid residues mutated compared to Cpl-7.

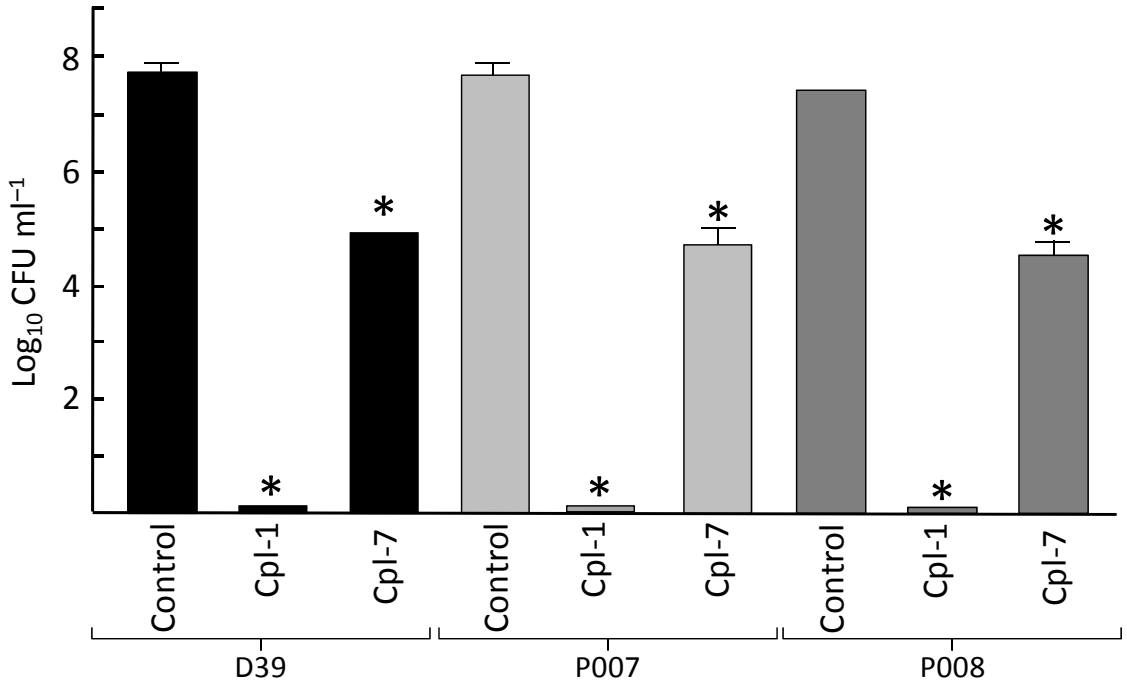

Figure S2. Bactericidal effects of Cpl-1 and Cpl-7 against different pneumococcal strains. Exponentially growing D39, P007 or P008 strains were washed, suspended in PBS at an $OD_{550} \approx 0.6$, and incubated in the absence or in the presence of the selected enzyme (5 μg · ml^{-1}) at 37°C. Viable cells, after 60 min incubation in the same conditions, were determined on blood-agar plates. Error bars represent standard deviations, and asterisks mark results that are statistically significant compared to controls in the absence of enzybiotics (one-way ANOVA with a post hoc Dunnet test; *, $P < 0.001$).

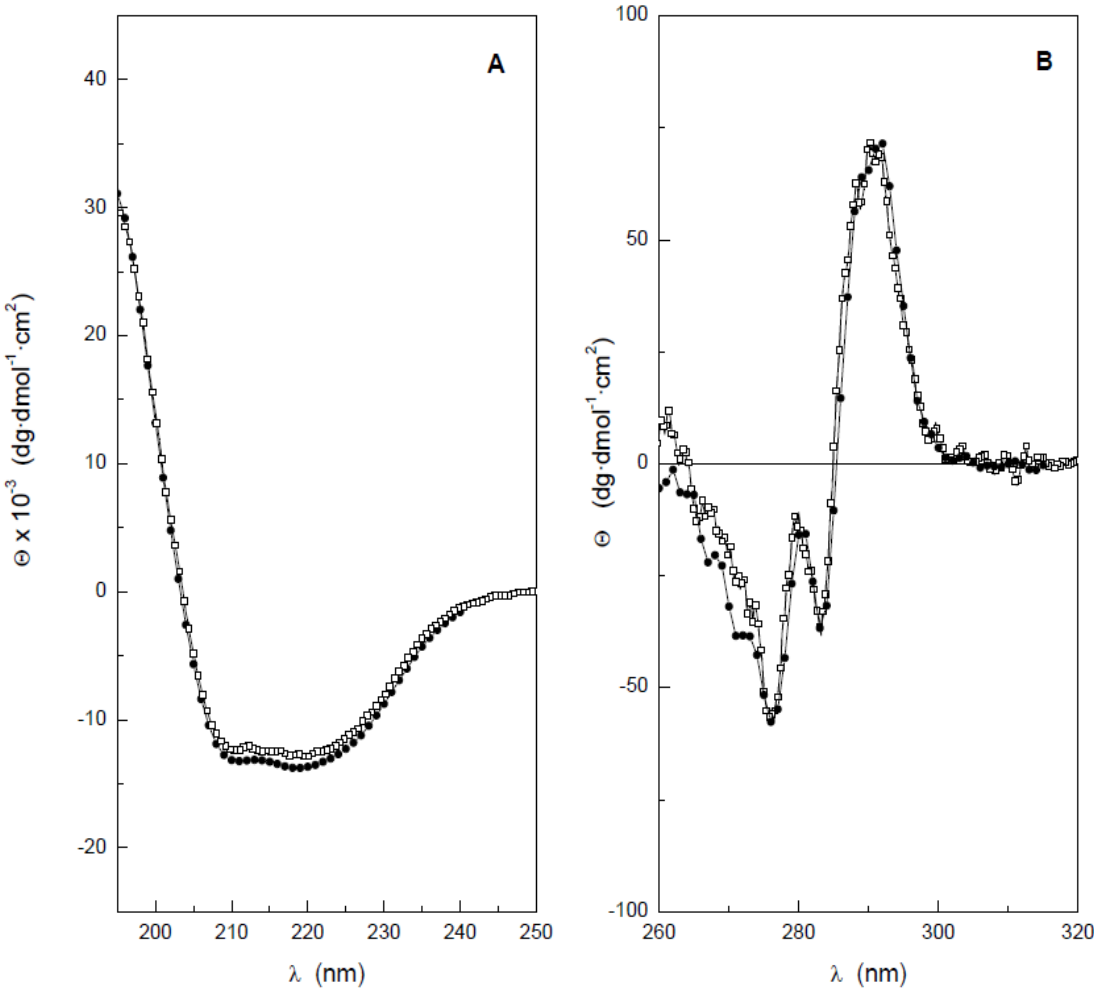

Figure S3. **Structure conservation in Cpl-7S lysozyme**. Comparison of far- (A) and near-UV (B) CD spectra of Cpl-7 (circles) and Cpl-7S (squares). Data were monitored at 0.2 mg · ml^{-1} (far UV-spectra) and 0.8 mg · ml^{-1} (near UV-spectra) in 20 mM phosphate buffer, pH = 7.0, at 20°C.

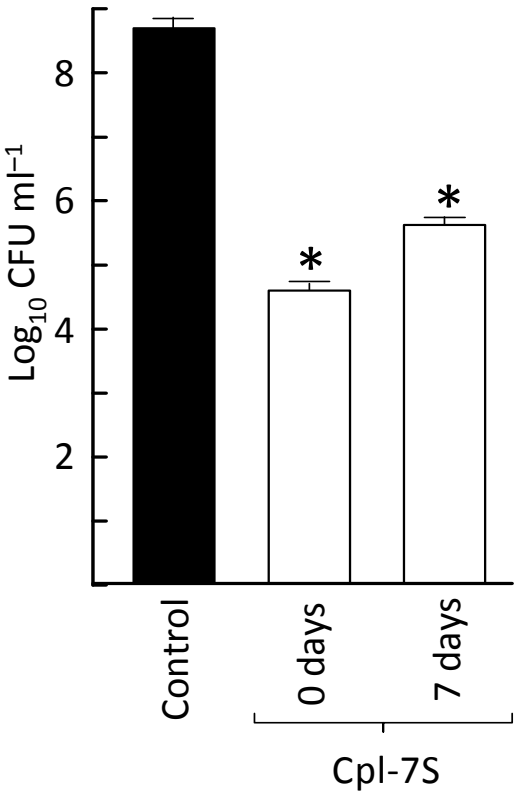

Figure S4. Variation of Cpl-7S bactericidal effect with incubation at 37ºC. Pneumococcal R6 cells were treated as in Fig. S2 and viable cells were determined on blood-agar plates after 60 min treatment with Cpl-7S recently prepared or kept at 37ºC for 7 days. Data are the mean of three independent experiments. Error bars represent standard deviations, and asterisks mark results that are statistically significant compared to controls in the absence of the enzyme (one-way ANOVA with a post hoc Dunnet test; *, P <0.001).

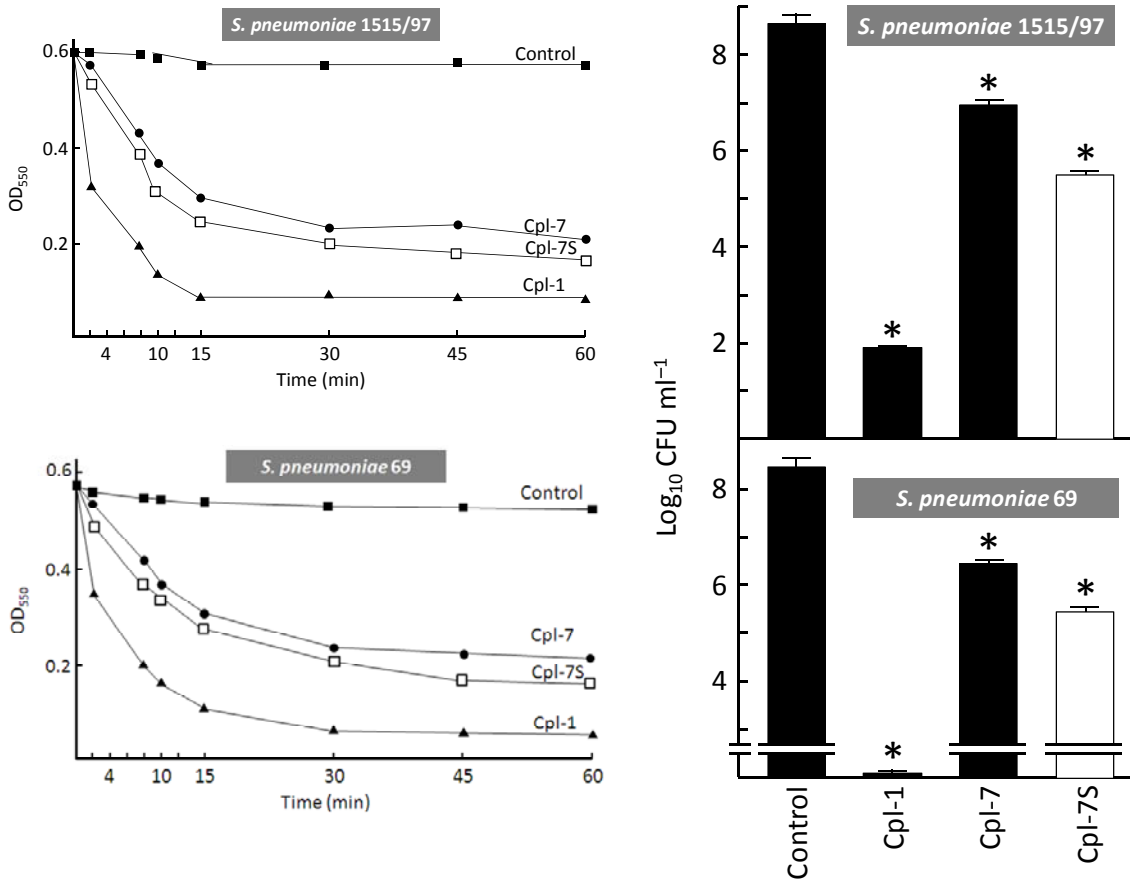

Figure S5. Bacteriolytic and bactericidal effects of different lysozymes against two pneumococcal multiresistant strains. Exponentially growing 1515/97 or 69 strains were washed, suspended in PBS at an $OD_{550} \approx 0.6$, and incubated in the absence or in the presence of the selected enzyme (5 μg · ml^{-1}) at 37°C. Viable cells, after 60 min incubation in the same conditions, were determined on blood-agar plates. Error bars represent standard deviations, and asterisks mark results that are statistically significant compared to controls in the absence of enzybiotics (one-way ANOVA with a post hoc Dunnet test; *, P <0.001).

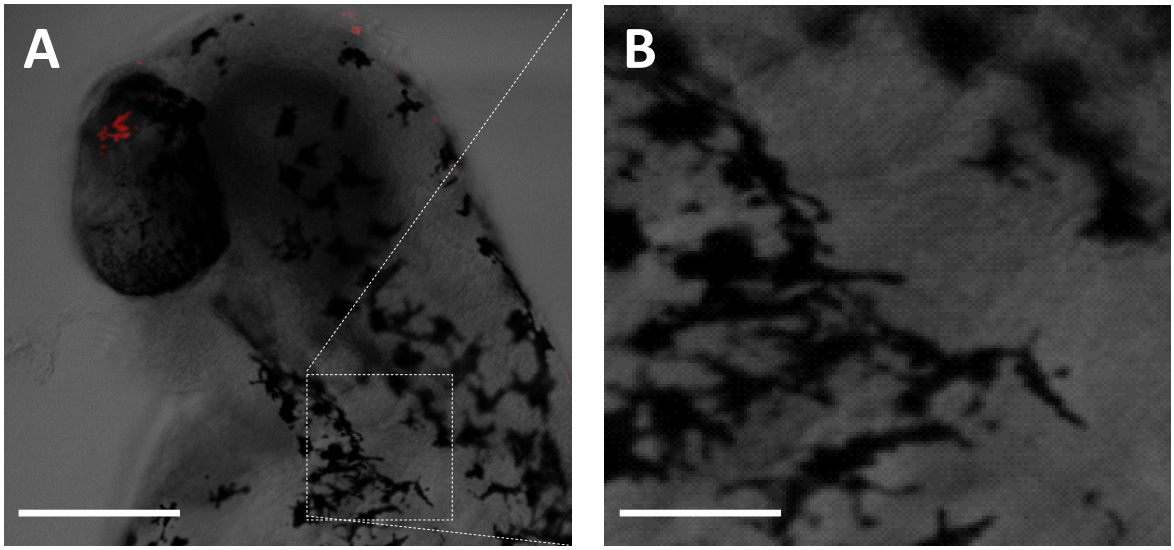

Figure S6. Representative whole-mount immunofluorescence of zebrafish embryos used as controls in infection assays. Maximal projections from 15 z-stacks were constructed from fluorescence and differential interference contrast confocal images. (A) Pneumococcal-free 5 d post fecundation embryo (20× objective) treated as in Fig. 7 showing transmitted light and red fluorescence overlay. (B) Details around the gills (40× objective). Bars (A), 250 μm; (B), 25 μm.

Table S1. Type of activity and net charge of relevant pneumococcal murein hydrolases.

Protein	**Activity**	Z_{CM}	Z_{link}	Z_{CWBM}	Z_{Total}
Cpl-7	Lysozyme (GH25)	–10.86	–3.98	–14.93	–29.77
Cpl-1	Lysozyme (GH25)	–9.86	–3.98	–0.98	–14.82
LytA	Amidase (Amidase_2)	–7.89	–1.99	–4.69	–14.57
Pal	Amidase (Amidase_5)	–4.74	1.00	–6.83	–10.57
Cpl-7S	Lysozyme (GH25)	–10.86	–3.98	3.0	–11.84

Charges refer to the catalytic module ($\mathbf{Z_{CM}}$), the linker ($\mathbf{Z_{link}}$), the cell wall-binding module ($\mathbf{Z_{CWBM}}$) and the overall sequence ($\mathbf{Z_{Total}}$).