## Supporting Information

# Acyldepsipeptide antibiotics kill mycobacteria by preventing the physiological functions of the ClpP1P2 protease

Kirsten Famulla[1], Peter Sass[1], Imran Malik[1], Tatos Akopian[2], Olga Kandror[2], Marina Alber[3], Berthold Hinzen[4], Helga Ruebsamen-Schaeff[5], Rainer Kalscheuer[3], Alfred Goldberg[2], and Heike Brötz-Oesterhelt[1]

[1]Department of Microbial Bioactive Compounds, Interfaculty Institute of Microbiology and Infection Medicine, University of Tuebingen, Germany; [2]Department of Cell Biology, Harvard Medical School, Boston, MA; [3]Institute for Medical Microbiology and Hospital Hygiene, University of Duesseldorf; [4]Bayer Pharma AG, Wuppertal, Germany; [5]AiCuris GmbH & Co. KG, Wuppertal, Germany.

**Table S1.** Primer used in this study.

| primer | sequence | restriction site |
|---|---|---|
| Construction of *M. bovis* BCG Pasteur *clpP1-tet off:* | | |
| *clpP1*-F1-fwd | 5`-TTTTTTTTCCATAAATTGGAGCCCGGTTCAATGAGTTGC-3` | *Van*91I |
| *clpP1*-F1-rev | 5`-TTTTTTTTCCATTTCTTGGTCAGGGGCACCTGCTTTCC-3` | *Van*91I |
| *clpP1*-F2-fwd | 5`-TTTTTTTGCATCTTTTGCCAAACCGTATTCCAGGG-3` | *Bst*API |
| *clpP1*-F2-rev | 5`-TTTTTTTTGCATAGATTGCATGAGCCAAGTGACTGACATGC-3` | *Bst*API |
| c-*clpP1*-fwd | 5'-ACGAAGCGACAACGTGAC-3' | |
| c-*clpP1*-rev | 5'-GGGAATTCACTGTGCTTCTC-3' | |
| seq-*clpP1*-fwd | 5'-CACGACTTCGAGGTGTTC-3' | |
| seq-*clpP2*-rev | 5'-GGGAATTCACTGTGCTTCTC-3' | |
| *rev-tetR*-fwd | 5´-TTTTTGAATTCATGAGCACGATCCGCGGTACCATC-3´ | *Eco*R1 |
| *rev-tetR*-rev | 5´-TTTTTAAGCTTAGGAGCCGCTCTCGCACTTCAG-3´ | *Hin*dIII |
| Cloning of MTB *ftsZ:* | | |
| MTB *ftsZ*-fwd | 5'-TTTCCATGGCCCCCCCGCACAACTACC-3' | *Nco*I |
| MTB *ftsZ*-rev | 5'-AAAAAGCTTGCGGCGCATGAAGGGCGG-3' | *Hin*dIII |
| qRT-PCR of MTB *clpP* expression: | | |
| q*clpP1*-fwd | 5'- TGAGCCAAGTGACTGACAT-3' | |
| q*clpP1*-rev | 5'- GATGTAGAGGCTGATGTCCT-3' | |

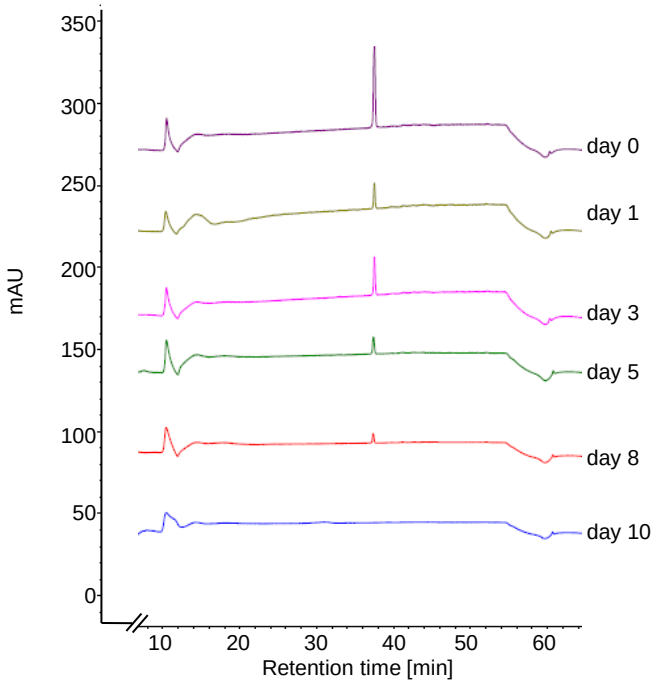



**Figure S1.** Chemical stability of ADEP2 in culture broth is limited. HPLC chromatograms at different times of incubation of ADEP2 in minimal medium at 37 °C.

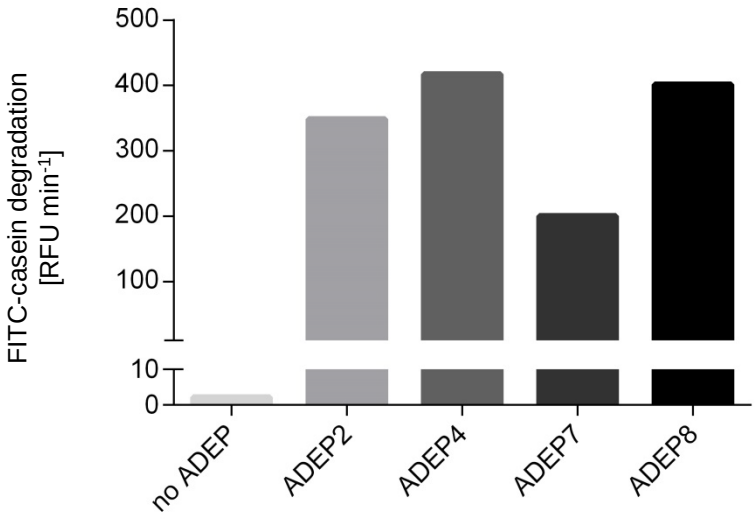
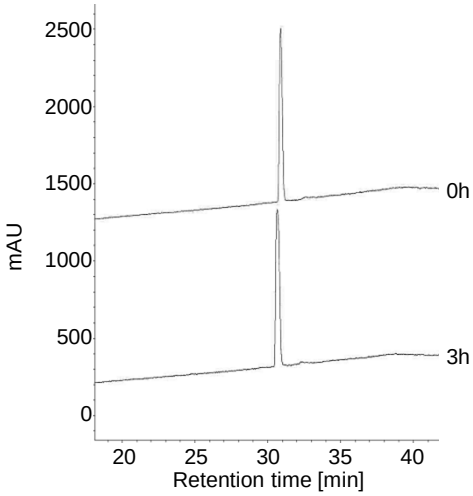



**Figure S2.** Degradation of FITC-casein by BS ClpP. Reaction rate [increase in RFU $min^{-1}$] calculated from the initial linear period of enzyme activity (10 min). ADEPs activate BS ClpP much more strongly than MTB ClpP1P2 under the same assay conditions.




| | retention time [min] | area [mAu*s] |
|---|---|---|
| 0 h | 30.66 | 1.79E+04 |
| 3 h | 30.67 | 1.52E+04 |

**Figure S3.** The HPLC chromatogram shows that Z-LL is not degraded by BS ClpP after 3 hours incubation at 37 °C.

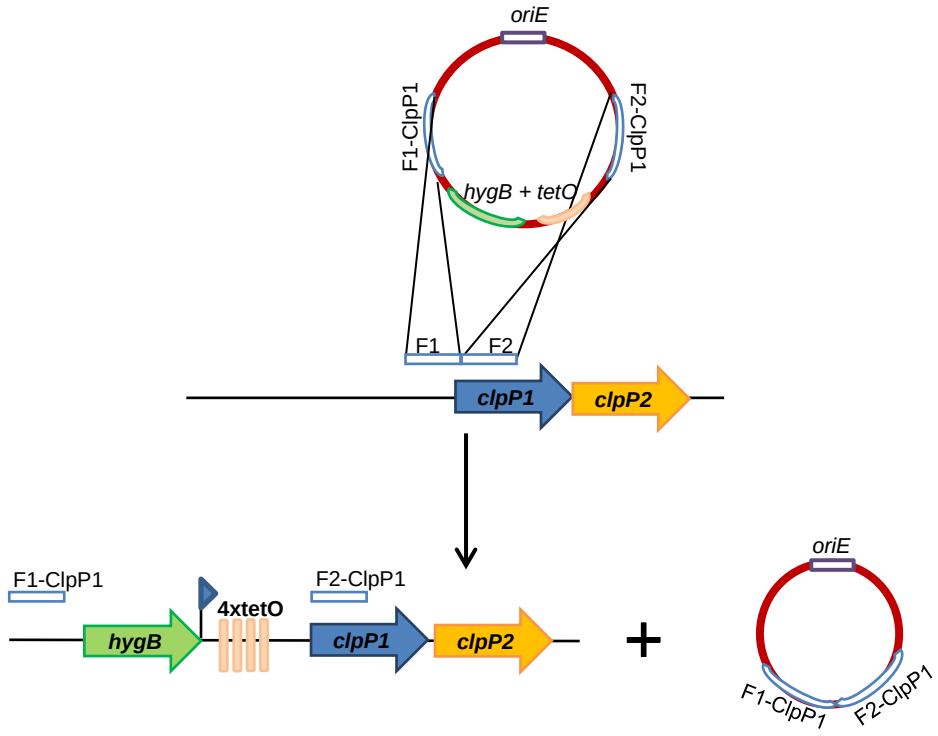



**Figure S4.** Construction of *M. bovis* BCG *clpP1-tetoff.* The *hyg*-P*myc1*-4x*tetO* cassette was inserted upstream of the *clpP1P2* operon via homologous recombination. Then, the plasmid which expresses the TetR repressor was introduced to obtain *M. bovis* BCG *clpP1-tetoff*.

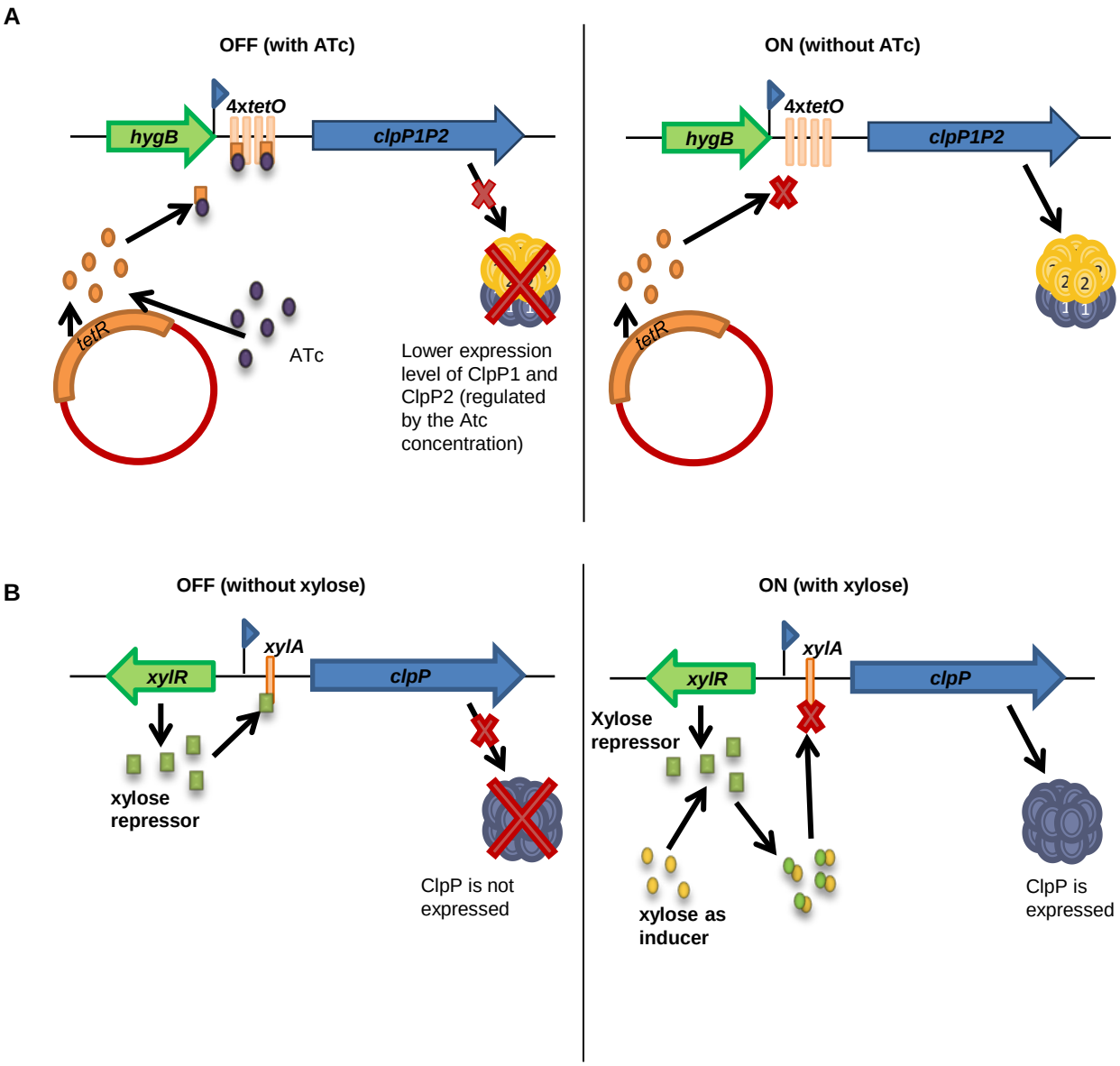



**Figure S5.** Schematic representation of the inducible mutants used in this study.
A. Operating mode of the Tet-Off system in strain *M. bovis* BCG *clpP1-tetoff* in the absence and presence of ATc. In the absence of ATc, the TetR repressor cannot bind to the *tetO* sites upstream the *clpP1P2* operon, *clpP1* and *clpP2* are expressed. In the presence of ATc, TetR undergoes a conformational change that now allows binding to the *tetO* sites. Thus, the expression level of *clpP1P2* can be regulated via the ATc concentration.
B. Operating mode of the Xyl-On system in *B. subtilis* 168-pX2-*clpP* in the absence and presence of xylose. In the absence of xylose, the repressor XylR can binds to the *xylA* operator palindrome upstream of *clpP,* thereby silencing *clpP* expression. Xylose functions as an inducer in this system. The presence of xylose leads to a conformational change in XylR, which is now incapable to bind to *xylA* operator, thereby allowing *clpP* expression