**Table S1 Strains, plasmids, and antibodies used in this study**

|  |  |  |
| --- | --- | --- |
| **Strain and Plasmid** | **Feature** | **Source** |
| **Mycobacteria** |  |  |
| *M. smegmatis* mc2-155 | Wild type strain of *M. smegmatis* | From National Institutes for Food and Drug Control of China |
| Δ*cnpB* | *cnpB* deletion mutant strain | This study |
| Δ*cnpB*::C | *cnpB* complementary strain | This study |
| Δ*cnpB*::O | *cnpB* overexpressed strain | This study |
| *M. tuberculosis* H37Ra | Wild type of *M. tuberculosis* attenuated strain | National Institutes for Food and Drug Control of China |
| ***E. coli*** |  |  |
| DH5α  BL21 | Plasmid construction  Expression of protein | This study  This study |
| **Plasmids** |  |  |
| pJV53 | Expressing homologous recombination enzymes of gp60 and gp61, KanR | Addgene |
| pMSG360zeo | *loxp*-*zeo*-*loxp* | Addgene |
| PW75 | Recombinant pMSG360zeo carrying *cnpB* upstream and downstream fragments, ZeoR | This study |
| PW86 | Mycobacterium-*E. coli* *cnpB* single copy shuttle expression vector, HygR | This study |
| PW87 | Mycobacterium-*E. coli* *cnpB* multiple copy shuttle expression vector, HygR | This study |
| PW92 | CnpB prokaryotic expression vector of pET28a(+)-*cnpB*, KanR | This study |
| **Antibodies** |  |  |
| anti-CnpB pAb | Mouse polyclonal antibody to CnpB (MSMEG\_2630) | Our lab |
| anti-Ag85 pAb | Mouse polyclonal antibody to Ag85 (MSMEG\_6398), | Our lab |
| anti-PdxH pAb | Mouse polyclonal antibody to PdxH (MSMEG\_5675) | Our lab |

**Table S2 Primers used in this study**

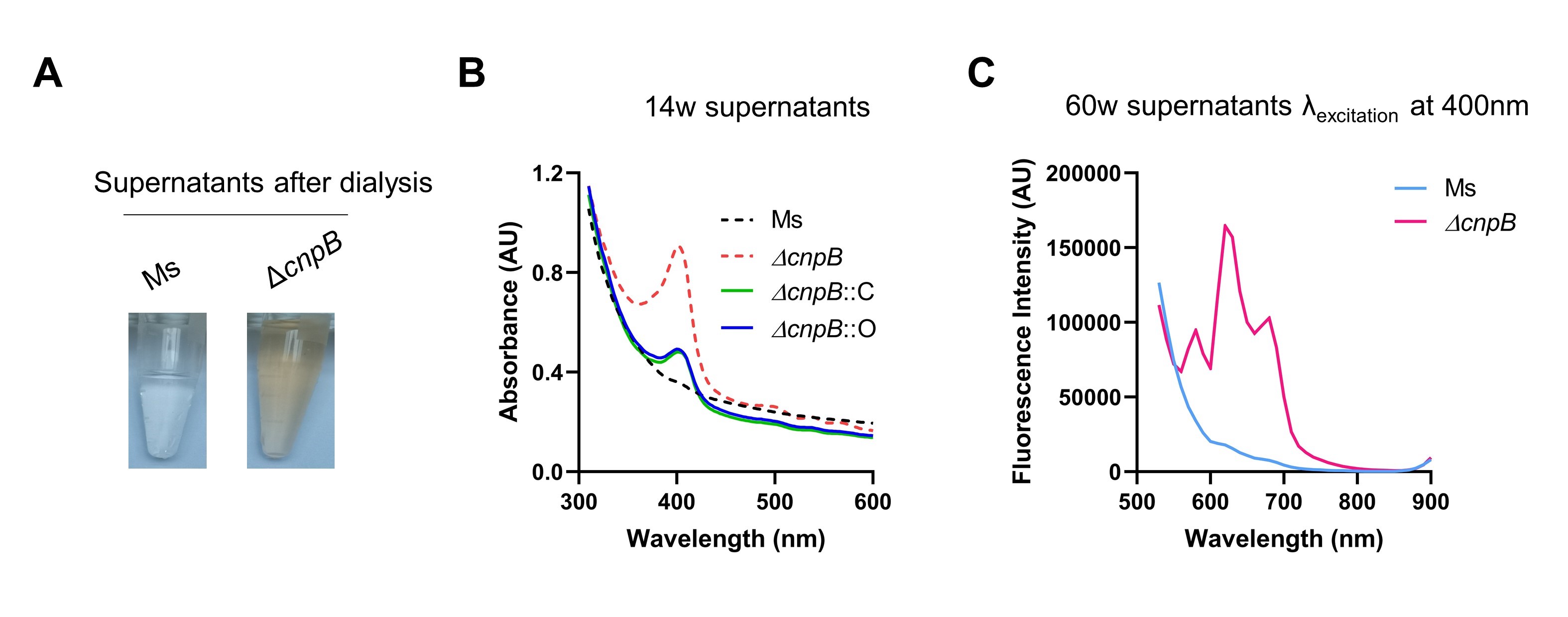
|  |  |
| --- | --- |
| **Oligo sequence (5’ to 3’)** *a* | **Description** |
| **Strains construction and verification** |  |
| tttgatatcggccatcgagtacgagatc | *cnpB*upstream for knockout, forward |
| gcgaagcttggtcttgggatcggttgtcgtc | *cnpB*upstream for knockout, reverse |
| ttttctagagcgggatactccgcgacc | *cnpB*downstream for knockout, forward |
| gcgggtaccgaaagcatcggcaccgcaac | *cnpB*downstream for knockout, reverse |
| aactacatcgacccttccgc | *cnpB* internal, forward |
| gtgcatcggttcgatctcct | *cnpB* internal, reverse |
| gtgatctcggtgagccactc | *MSMEG\_6080* internal, forward |
| aagtccagcagttcgctgtc | *MSMEG\_6080* internal, reverse |
| tttggtaccatgccggtgacgacaacc | *cnpB*complementation, forward |
| tttggatcctcagccaagggcccgtgc | *cnpB*complementation, reverse |
| tttaagcttatgccggtgacgacaacc | *cnpB*overexpression, forward |
| tttaagctttcagccaagggcccgtgc | *cnpB*overexpression, reverse |
| **qRT-PCR** |  |
| cgacgacctcgactccgacgac | *MSMEG\_2758* (*sigA*), forward |
| gccttcctggatgaggtcgagga | *MSMEG\_2758* (*sigA*), reverse |
| agctgtacctgatcttcccg | *MSMEG\_1875 (mtrB)*, forward |
| ctcttcgagctgctggatct | *MSMEG\_1875 (mtrB)*, reverse |
| ggagccgacgactatgtgat | *MSMEG\_1874 (mtra)*, forward |
| gtatccccacacctgttcga | *MSMEG\_1874 (mtra)*, reverse |
| gatgcggcacaactcgatc | *MSMEG\_0246 (prrB)*, forward |
| cgctcaacaagatggagtcg | *MSMEG\_0246 (prrB)*, reverse |
| tacctgaccaaaccgttcga | *MSMEG\_0244 (prrA)*, forward |
| aagtcgtagccccacacc | *MSMEG\_0244 (prrA)*, reverse |
| cggctacgaagaggtgctc | *MSMEG\_2618*, forward |
| tgcatcccaattcactaacg | *MSMEG\_2618*,reverse |
| cctacgacgaggttttcgac | *MSMEG\_0954*, forward |
| ccaccggtcttgatcatctc | *MSMEG\_0954*, reverse |
| ccatcctgttctccgacatc | *MSMEG\_2780*, forward |
| caccaggtaggaggccaac | *MSMEG\_2780*, reverse |
| aggtcaccacagaagcgaac | *MSMEG\_4525*, forward |
| agatgaggtcgaggttgacg | *MSMEG\_4525*, reverse |
| gattccctggactggctgg | *MSMEG\_1638*, forward |
| tagccgttctccatctcgac | *MSMEG\_1638*, reverse |
| ctactacaaccccggcatga | *MSMEG\_0234 (zmp1)*, forward |
| tacttggcaccctgatcgtc | *MSMEG\_0234 (zmp1)*, reverse |
| acagaacaggtatgg | *MSMEG\_0066 (esat-6)*, forward |
| tcaggcaaacattcc | *MSMEG\_0066 (esat-6)*, reverse |
| catctgggtgtactgcggta | *MSMEG\_6398(ag85)*, forward |
| ctctgcgggaagttgaacac | *MSMEG\_6398(ag85)*, reverse |
| aagaatctgcccctcacaca | *MSMEG\_5350 (PPE 63)*, forward |
| acgtccttggtttccgtact | *MSMEG\_5350 (PPE 63)*, reverse |
| accaccatgagcacctatca | *MSMEG\_0619 (PPE4)*, forward |
| gtcatgaagtccgtcagcag | *MSMEG\_0619 (PPE4)*, reverse |
| tacagaacttcgtctcggcc | *MSMEG\_5392 (kdpA)*, forward |
| ggtgcccagttctttgatgg | *MSMEG\_5392 (kdpA)*, reverse |
| agtacttcgcgatcatcccg | *MSMEG\_5393 (kdpB)*, forward |
| cgatgagtttgatgccgagg | *MSMEG\_5393 (kdpB)*, reverse |
| aaggtgtacgggtcgagtg | *MSMEG\_5394 (kdpC)*, forward |
| caccgagaaatcccagcatg | *MSMEG\_5394 (kdpC)*, reverse |
| gccatcagatgtttcacgca | *MSMEG\_5395 (kdpD)*, forward |
| tacagaacttcgtctcggcc | *MSMEG\_5395 (kdpD)*, reverse |
| cctttggcatggacgagttc | *MSMEG\_5396 (kdpE)*, forward |
| cgcgtaaatagtgggtctcg | *MSMEG\_5396 (kdpE)*, reverse |
| gagaccgagaccaccaagtg | *MSMEG\_2771 (trkA)*, forward |
| tcattcggaatccaggtcgt | *MSMEG\_2771 (trkA)*, reverse |
| gcgcaacgaatggcttttc | *MSMEG\_2769 (trkB)*, forward |
| cagttcgtcaccaccttcga | *MSMEG\_2769 (trkB)*, reverse |
| *a* Underlined oligo DNA is restriction site. | |



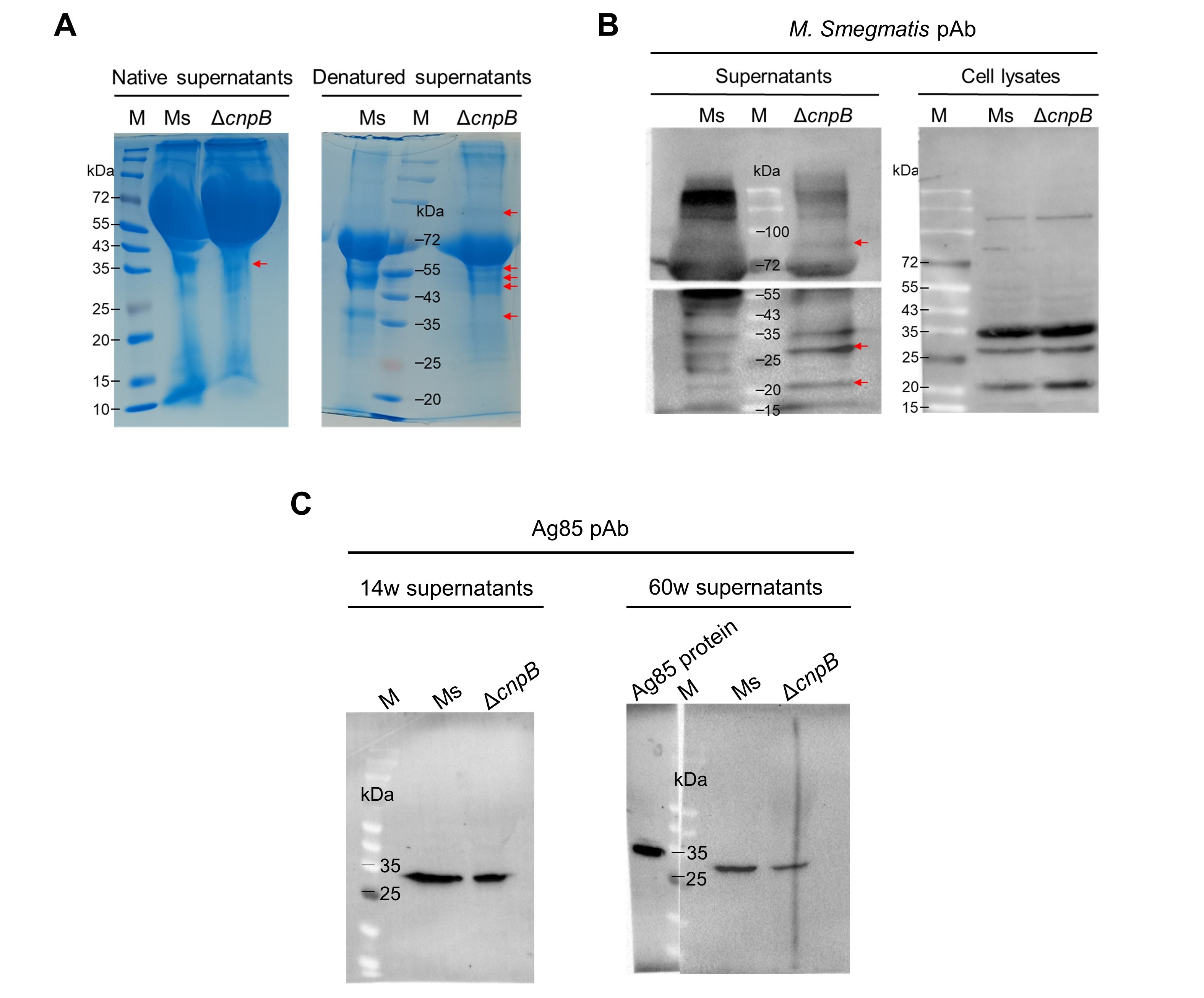
**Figure S1 CnpB amino acid sequences alignment between *M. tuberculosis* and *M. smegmatis*.** (A) Alignment of *M. tuberculosis* CnpB (Query) and *M. smegmatis* CnpB (Sbjct) sequences. The amino acids shown in the red rectangle is DHH motif. (B) Amino acid sites of DHH and DHHA1 domains of CnpB in *M. tuberculosis* and *M. smegmatis*, respectively.



**Figure S2** **Detection of bacteria growth in broth liquid media (80rpm).** Each strain was inoculated at 2.5×106 CFU/mL in media of 7H9 (A), 7H9+OADC (C), Sauton’s (E), Sauton’s +OADC (G). Bacteria were monitored at OD600 (A, C, E, G) and corresponding CFUs (B, D, F, H) numeration at indicated time points, respectively. The growth curves were generated according to three repeats.



**Figure S3** ***ΔcnpB* produceds more brown pigment during the long-term static culture.** (A) Observation of 14w supernatants after dialysis. (B) Absorption spectra of 14-week supernatants was recorded by full-wavelength (100 - 1 000 nm) scanning. (C) Fluorescence measurements of 60w supernatants were analyzed at the excitation wavelength (λexcitation) 400 nm.

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**Figure S4**  ***ΔcnpB* produce** (A) After 14-week of stationary culture, native and boil-denatured supernatants were analyzed by SDS-PAGE. The red arrows indicated possible new protein bands of *ΔcnpB*. (B) After 14-week of stationary culture, supernatants were condensed by saturated ammonium sulfate. Condensed-supernatants and bacteria lysates were analyzed by Western blot using *M. smegmatis* polyclonal antibody. The red arrows indicated the *M. smegmatis*-specific differential protein bands in *ΔcnpB* supernatant after 14-week culture. (C) Western blot analysis of Ag85 secretion levels in concentrated-supernatants at 14 and 60 weeks.