**SUPPLEMENTAL MATERIALS**

**Induction of the *cydAB* operon encoding the *bd* quinol oxidase under respiration-inhibitory conditions by the major cAMP receptor protein MSMEG\_6189 in *Mycobacterium smegmatis***

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**MATERIALS AND METHODS**

**Construction of mutant strains of *M. smegmatis***

A *crp2* (*MSMEG\_0539*) mutantof *M. smegmatis* was constructed by allelic exchange mutagenesis using the temperature-sensitive suicide plasmid pKOTscrp2 containing a temperature-sensitive replication origin as described previously (Jeong et al., 2013). pKOTscrp2 was introduced into *M. smegmatis* by electroporation. Transformants were selected at 30°C (replication-permissive temperature) on 7H9-glucose agar plates containing hygromycin, and the selected transformants were grown in 7H9-glucose liquid medium supplemented with hygromycin for 3 - 5 days at 30°C. Heterogenotes of *M. smegmatis*, which were generated by a single recombination event, were selected for their hygromycin resistance on 7H9-glucose agar plates at 42°C (replication-nonpermissive temperature). The selected heterogenotes were grown on 7H9-glucose medium without antibiotics for 3 - 5 days at 37°C. Isogenic homogenotes were obtained from the heterogenotes after a second recombination by selecting them for sucrose resistance on 7H9-glucose agar plates containing 10% (w/v) sucrose at 37°C. The allelic exchange in the *crp2* mutant was verified by PCR with isolated genomic DNA. The *crp2* mutant is a deletion mutant in which a 250-bp internal fragment is deleted from the *crp2* gene. The deletion results in a frameshift, leading to the complete inactivation of Crp2 including the C-terminal DNA-binding domain.

**Construction of plasmids**

**(i) pKOTscrp2.** To construct pKOTscrp2, PCR was conducted with the F\_crp2mut and R\_crp2mut primers and the chromosomal DNA of *M. smegmatis* as a template. The amplified 1,086-bp DNA fragment was restricted with HindIII and KpnI and cloned into pBSII KS+ digested with the same enzymes, yielding pBSIIcrp2. The 251-bp DNA fragment within *crp2* was excised from pBSIIcrp2 by restriction with SalI, and the linear plasmid was self-ligated, resulting in pBSIIcrp2. Using pBSIIcrp2as a template, an 835-bp DNA fragment was obtained by PCR with *Pfu* DNA polymerase and the primers F\_crp2mut and R\_crp2mut. The amplified PCR product was restricted with HindIII and cloned into pKOTs digested with HindIII and EcoRV, yielding pKOTscrp2.

**(ii) pNCIIcydA, pNCIISD1, pNCIISD2, pNCIISD3, and pNCIISD4.** pNCIIcydA, pNCIISD1, pNCIISD2, pNCIISD3, and pNCIISD4 are *cydA*::*lacZ* translational fusion plasmids that contain the 5’ portion (54 bp) of *cydA*, as well as 199-, 163-, 143-, 123-, and 83-bp serially deleted DNA sequences upstream of *cydA*, respectively. For the construction of pNCIIcydA, a 253-bp DNA fragment was amplified with the primers F\_cydAlacZ and R\_cydAlacZ. The PCR product was digested with ClaI and XbaI and cloned into pBSII KS+, resulting in pBSIIcydA. pBSIIcydA was restricted with ClaI and XbaI, and the 253-bp fragment was cloned into pNCII, yielding the pNCIIcydA. To construct pNCIISD1, pNCIISD2, pNCIISD3, and pNCIISD4, the *cydA* upstream regions of the corresponding lengths were amplified by PCR using the forward primers (F\_cydASD1, F\_cydASD2, F\_cydASD3, and F\_cydASD4, respectively) and the reverse primer R\_cydAlacZ. pBSIIcydA was used as a template for PCR. The PCR products were restricted with ClaI and XbaI and cloned into pNCII, resulting in pNCIISD1, pNCIISD2, pNCIISD3, and pNCIISD4.

**(iii) pNCIIM1, pNCIIM2, and pNCIIM3.** Using pBSIIcydA as a template, PCR-based site-directed mutagenesis was carried out to mutagenize CBS1 and CBS2, resulting in pBSIIM1\_1 with mutations in the right half sequence of CBS1 and pBSIIM2\_1 with mutations in the left half sequence of CBS2. Using pBSIIM1\_1 and pBSIIM2\_1, further site-directed mutagenesis was performed to generate pBSIIM1\_2 and pBSIIM2\_2 with additional mutations in the left half sequence of CBS1 and the right half sequence and CBS2, respectively. For the construction of pBSIIM3\_2, the procedure for the construction of pBSIIM1\_2 was followed using pBSIIM2\_2 as a template. Mutation were verified by DNA sequencing. The 253-bp ClaI-XbaI fragments from pBSIIM1\_2, pBSIIM2\_2, and pBSIIM3\_2 were cloned into pNCII, resulting in the plasmids pNCIIM1, pNCIIM2, and pNCIIM3, respectively.

**(iv) pMV306crp1\_2B8 and pMV306crp2\_2B8.** To express the C-terminally 2B8 epitope-tagged Crp1 and Crp2 proteins from their own promoters in *M. smegmatis*, the *crp1* and *crp2* genes with the upstream regions encompassing their own promoters and regulatory sequences were cloned into the integration vector pMV306. A 1,006-bp DNA fragment containing the *crp1* gene was amplified using the chromosomal DNA of *M. smegmatis* as a template and the primers F\_crp1 and R\_crp1 containing the epitope-coding sequence. The PCR product was restricted with KpnI and HindIII, and the restricted DNA fragment was cloned into pMV306, yielding pMV306crp1\_2B8. To construct pMV306crp2\_2B8, PCR was conducted with the primers F\_crp2 and R\_crp2 containing the epitope-coding sequence. The amplified 1,006-bp DNA fragment was digested with XbaI and HindIII and cloned into pMV306, resulting in pMV306crp2\_2B8.

**(v) pUC19cydAFootR.** The plasmid was used as a template for the generation of fluorescence-labeled DNA fragments for DNase I footprinting analysis. A 220-bp DNA fragment encompassing two Crp-binding sites (CBS1 and CBS2) was amplified by PCR with the primers F\_cydAFootR and R\_cydAFootR using the chromosomal DNA of *M. smegmatis* as a template. The PCR product was restricted with EcoRI and HindIII and cloned into pUC19, yielding pUC19cydAFootR.



**FIGURE S1. Expression levels of *sigA* in the WT, *crp1*, *crp2*,and *aa3* mutant strains of *M. smegmatis*.** Transcript levels of *sigA* were extrapolated from the reads per kilo base pair per million mapped reads (RPKM) values obtained from RNA sequencing analysis on the WT,*crp1*, and *crp2* mutant strains that were grown aerobically to an OD600 of 2.0-2.1 (A), as well as the WT and *aa3* mutant strains that were grown aerobically to an OD600 of 0.45-0.5 (B) (Y. Oh and J. I. Oh, in press).



**FIGURE S2. SDS-PAGE analysis of purified Crp1 (MSMEG\_6189).** C-terminally His6-tagged Crp1 was overexpressed in the *E. coli* BL21 (DE3) strain with pT7-7crp1. The overexpressed Crp1 protein was purified by affinity chromatography using Ni-Sepharose high-performance resin (GE Healthcare, Piscataway, NJ). Lane M, molecular weight marker. Lane 1, lysates of *E. coli* BL21 (DE3) harboring the empty vector pT7-7. Lane 2, lysates of *E. coli* BL21 (DE3) harboring pT7-7crp1. Lane 3, eluents during the washing step with 20 mM Tris-HCl (pH 8.0) containing 60 mM imidazole. Lane 4, purified C-terminally His6-tagged Crp1.

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**FIGURE S3. Effect of NO and H2S treatment on *cydA* expression in the WT and *crp1* mutant strains of *M. smegmatis*.** (A)When the strains were grown aerobically to an OD600 of 0.45-0.5, sodium nitroprusside (SNP, NO generator) was added to the cultures with a final concentration of 5 mM and the cultures were further grown under the illumination of light for 30 min (+SNP). As controls, the strains without SNP treatment were included in the experiment (-SNP). (B) The strains were grown aerobically to an OD600 of 0.45-0.5 and treated with 200 M NaHS (H2S donor) for 30 min (+NaHS). As controls, the strains without NaHS treatment were included in the experiment (-NaHS). The expression level of *cydA* was quantitatively determined by qRT-PCR and normalized to *sigA* expression. The expression level of the *cydA* gene in the SNP-untreated (A) or NaHS-untreated (B) WT strain is set at 1, and the relative values are expressed for the other strains. All values provide are the averages of the results from three independent experiments. The error bars indicate the standard deviations. Statistical significance was determined by two-tailed Student’s *t* test. \*, *p* < 0.05.



**FIGURE S4. Effect of KCN on aerobic growth of the WT,*crp1*, and *crp2* mutant strains of *M. smegmatis*.** *M. smegmatis* strains were grown aerobically in 7H9-glucose medium to an OD600 of 0.5, and the cultures were treated with 100 M KCN (+KCN). As controls, the *M. smegmatis* strains without treatment of KCN were used in the experiment (-KCN). The cultures were further grown for 21 h, and the growth of the strains was monitored spectrophotometrically at 600 nm at 3-h intervals. All values provided are averages of the results from three independent determinations. The error bars indicate the standard deviations.

**TABLE S1.** Strains and plasmids used in this study

|  |  |  |
| --- | --- | --- |
| Strain/plasmid | Relevant phenotype/genotype\* | Reference |
| Strains |  |  |
| *E.coli* DH5 | 80d*lacZ*M15 *lacU169* *recA1 endA1 hsdR17 supE44 thi1 gyrA96 relA1* | Jessee, 1986 |
| *E.coli* BL21 (DE3) | F-, *ompT hsdSB* (rB-, mB-) *dcm gal* (DE3) | Promega |
| *M.* *smegmatis* mc2155 | High-transformation-efficiency mutant  of *M. smegmatis* ATCC 607 | Snapper et al., 1990 |
| *M.* *smegmatis* *aa3* | *MSMEG\_4268* (*ctaC*) deletion mutant derived from *M. smegmatis* mc2155 | Jeong et al., 2018 |
| *M.* *smegmatis* *aa3**sigF* | *MSMEG\_1804* (*sigF*) deletion mutant derived from *M. smegmatis* mc2155 | Oh et al., 2020 |
| *M.* *smegmatis* *crp1* | *MSMEG\_6189* (*crp1*) deletion/insertion mutant derived from *M. smegmatis* mc2155; Hygr;Previously this mutant was named *crp*. To distinguish *crp1* and *crp2* mutants, we renamed the *crp* mutant to *crp1* | Lee et al., 2014 |
| *M.* *smegmatis* *crp2* | *MSMEG\_0539* (*crp2*) deletion mutant derived from *M. smegmatis* mc2155 | This study |
| plasmids |  |  |
| pBSII KS+ | Ampr; *lacPOZ’* | Stratagene |
| pT7-7 | Ampr; T7 promoter, ribosome binding site, and translation start codon overlapping with NdeI site | Tabor and Richardson, 1985 |
| pNCII | Hygr; promoterless *lacZ* | Oh et al., 2020 |
| pUC19 | Ampr; *lacPOZ’* | Yanisch-Perron et al., 1985 |
| pKOTs | Hygr; pKO-based vector constructed by inserting the HindIII-KpnI fragment containing pAL500Ts and pUC ori derived from pDE | Jeong et al., 2013 |
| pMV306 | Kmr; integration vector containing *int* and the *attP* site of mycobacteriophage L5 for integration into the mycobacterial genome | Stover et al., 1991; Brown et al., 2007 |
| pNCIIcydA | pNC2::0.253-kb XbaI-ClaI fragment containing the *cydA* promoter region | This study |
| pNCIISD1 | pNC2::0.217-kb XbaI-ClaI fragment containing the *cydA* promoter region | This study |
| pNCIISD2 | pNC2::0.197-kb XbaI-ClaI fragment containing the *cydA* promoter region | This study |
| pNCIISD3 | pNC2::0.177-kb XbaI-ClaI fragment containing the *cydA* promoter region | This study |
| pNCIISD4 | pNC2::0.137-kb XbaI-ClaI fragment containing the *cydA* promoter region | This study |
| pBSIIcydA | pBSII KS+::0.253-kb XbaI-ClaI fragment containing the *cydA* operon promoter region | This study |
| pBSIIM1\_1 | pBSIIcydA with three point mutations (GTG→CCC) in CBS1 | This study |
| pBSIIM1\_2 | pBSIIM1\_1 with three point mutations (CAC→TTT) in CBS1 | This study |
| pBSIIM2\_1 | pBSIIcydA with three point mutations (GTG→CCC) in CBS2 | This study |
| pBSIIM2\_2 | pBSIIM2\_1 with three point mutations (CAC→TTT) in CBS2 | This study |
| pBSIIM3\_1 | pBSIIM1\_2 with three point mutations (GTG→CCC) in CBS2 | This study |
| pBSIIM3\_2 | pBSIIM3\_1 with three point mutations (CAC→TTT) in CBS2 | This study |
| pNCIIM1 | pNC2::0.253-kb XbaI-ClaI fragment from pBSIIM1\_2 | This study |
| pNCIIM2 | pNC2::0.253-kb XbaI-ClaI fragment from pBSIIM2\_2 | This study |
| pNCIIM3 | pNC2::0.253-kb XbaI-ClaI fragment from pBSIIM3\_2 | This study |
| pT7-7crp1 | pT7-7::0.693-kb NdeI-HindIII fragment containing *crp1* (*MSMEG\_6189*) with 6 His codons before its stop codon | Bong et al., 2019 |
| pMV306crp | pMV306::1.239-kb ClaI-HindIII fragment containing *crp1* | Lee et al., 2014 |
| pUC19cydAFootR | pUC19::0.219-kb EcoRI-HindIII fragment containing the *cydA* promoter region | This study |
| pKOTscrp2 | pKOTs::0.815-kb EcoRV-HindIII fragment containing *crp2* | This study |
| pMV306crp1\_2B8 | pMV306::1.006-kb KpnI-HindIII fragment containing *crp1* | This study |
| pMV306crp2\_2B8 | pMV306::1.006-kb XbaI-HindIII fragment containing *crp2* | This study |

\*Abbreviations: Ampr, ampicillin resistance; Hygr, hygromycin resistance; Kmr, kanamycin resistance.

**TABLE S2.** Oligonucleotides used in this study

|  |  |  |
| --- | --- | --- |
| **Oligonucleotide** | **Nucleotide sequences (5'→3')** | **Purpose** |

|  |  |  |
| --- | --- | --- |
| F\_cydAlacZ | ATATTCTAGATCGGCGTCTTCGAGGTCG | *cydA*::*lacZ* fusion |
| R\_cydAlacZ | ATATATCGATGTGGTAGACCGTGGTGATTCCG | *cydA*::*lacZ* fusion |
| F\_crp2mut | ATATGGTACCGACGAAGTGCTGGCGC | Δ*crp2* construction |
| R\_crp2mut | ATATAAGCTTGCTTGAGGGTGAGGCCACAAC | Δ*crp2* construction |
| F\_cydASD1 | ATATTCTAGAGCAGCGGGGTCAGGAAGC | *cydA*::*lacZ* fusion |
| F\_cydASD2 | ATATTCTAGACTCACCATCGGCGCCTCC | *cydA*::*lacZ* fusion |
| F\_cydASD3 | ATATTCTAGAGGCGCCGCCTTGTCGACC | *cydA*::*lacZ* fusion |
| F\_cydASD4 | ATATTCTAGACCTGCGTCGATCTCCTGGC | *cydA*::*lacZ* fusion |
| F\_cydAM1\_1 | CTTGTCGACCGGCTTTTCATCGGCGCCTCCC | Site-directed mutagenesis (CBS1) |
| R\_cydAM1\_1 | GGGAGGCGCCGATGAAAAGCCGGTCGACAAG | Site-directed mutagenesis (CBS1) |
| F\_cydAM1\_2 | CTCGGCGCCGCCTTCCCGACCGGCTTTTCAT | Site-directed mutagenesis (CBS1) |
| R\_cydAM1\_2 | ATGAAAAGCCGGTCGGGAAGGCGGCGCCGAG | Site-directed mutagenesis (CBS1) |
| F\_cydAM2\_1 | CATCGGCGCCTCCCCCCAGCTAACCCACCGC | Site-directed mutagenesis (CBS2) |
| R\_cydAM2\_1 | GCGGTGGGTTAGCTGGGGGGAGGCGCCGATG | Site-directed mutagenesis (CBS2) |
| F\_cydAM2\_2 | CCCCCCAGCTAACCTTTCGCGCTACCTGCGT | Site-directed mutagenesis (CBS2) |
| R\_cydAM2\_2 | ACGCAGGTAGCGCGAAAGGTTAGCTGGGGGG | Site-directed mutagenesis (CBS2) |
| F\_crp1 | ATATGGTACCCCCGCGAGCAGGCACCA C | Δ*crp1* complementation |
| R\_crp1 | ATATAAGCTTTCAGGGGGGGAAGGCGGGCAGCGGGTCGCGGCGGGCGCGGCGGGCCAG | Δ*crp1* complementation |
| F\_crp2 | ATATTCTAGAGTCGTAGAGCAACGGCAGCG | Δ*crp2* complementation |
| R\_crp2 | ATATAAGCTTTCAGGGGGGGAAGGCGGGCAGGGGGTCGCGGTTCGCGCGCCGCGCGAG | Δ*crp2* complementation |
| F\_cydAFootR | ATATAAGCTTCGTCCTCGGCGTCTTCG | DNase I footprinting |
| R\_cydAFootR | ATATGAATTCGTCCAGAGCGTCCATCTGACC | DNase I footprinting |
| F\_TAMRA\_pUC19 | TAMRA-GTTTTCCCAGTCACGACGTTGTA | DNase I footprinting |
| F\_cydAEMSA | GCAGCGGGGTCAGGAAGC | EMSA (specific DNA) |
| R\_cydAEMSA | GCCAGGAGATCGACGCAGG | EMSA (specific DNA) |
| F\_80\_EMSA | CATCCCCCTTTCGCCAGC | EMSA (control DNA) |
| R\_80\_EMSA | CCATTCAGGCTGCGCAAC | EMSA (control DNA) |
| F\_sigA\_RT | CTTGAGGTGACCGACGATCT | qRT-PCR |
| R\_sigA\_RT | AGCTTCTTCTTCCTCGTCCT | qRT-PCR |
| F\_cydA\_RT | CGGTGGCAGTTCGGAATCAC | qRT-PCR |
| R\_cydA\_RT | CAGAAAAAGTTTGCCGAAGAAACG | qRT-PCR |
| F\_3680\_RT | GTTTGCCGCAGCGCTCGC | qRT-PCR |
| R\_3680\_RT | CCCCACCCGGTTCACCAC | qRT-PCR |
| F\_crp2\_RT | GGCATCTTCCAGGGGGTGC | qRT-PCR |
| R\_crp2\_RT | CGGCCGTCCACCGACTTG | qRT-PCR |

**TABLE S3.** Summarized statistics of RNA sequencing alignment

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **WT\_1** | **WT\_2** | **WT\_3** | ***crp1*\_1** | ***crp1*\_2** | ***crp1*\_3** |
| Read length (bp) | 101 | 101 | 101 | 101 | 101 | 101 |
| Total reads | 44,006,244 | 58,518,714 | 42,824,056 | 44,214,864 | 48,894,924 | 39,772,582 |
| No. of reads after trimming | 42,632,572 | 56,994,762 | 40,838,124 | 42,556,276 | 47,698,382 | 38,517,206 |
| No. of processed readsa | 21,316,286 | 28,497,381 | 20,419,062 | 21,278,138 | 23,849,191 | 19,258,603 |
| No. of mapped reads | 11,876,302 | 19,947,198 | 6,274,069 | 6,827,931 | 12,209,518 | 6,256,176 |
| No. of failed-to-align readsb | 4,045,381 | 4,090,121 | 3,376,054 | 3,501,038 | 4,944,196 | 3,536,126 |
| No. of suppressed reads by multiple mappingc | 5,394,603 | 4,460,062 | 10,768,939 | 10,949,169 | 6,695,477 | 9,466,301 |

aThe number of the processed reads indicates the number of the reads that remained after preprocessing of the total reads, e.g., removal of adapter sequences and artifacts such as contaminant DNA and PCR duplicates, etc. The processed reads were used for mapping to the reference genome using the program Bowtie v1.1.2.

bThe number of failed-to-align reads indicates the number of the reads that were not mapped to the reference genome due to the sequence mismatch.

cThe number of suppressed reads by multiple mapping indicates the number of the reads that were not mapped to the reference genome due to multiple mapping of the reads to two or more genes.

**TABLE S4.** The genes that are upregulated in the *aa3* mutant of *M. smegmatis* relative to the WT strain in a SigF-independent way and belong to the Crp1 regulon

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | |  | ***aa3*/WT** | | ***crp1*/WT** | |
| **Locus tag** | **Description** | | **log2FC** | ***p*-value** | **log2FC** | ***p*-value** |
| MSMEG\_0265 | UdgX family uracil-DNA binding protein | | 3.2 | 0.024 | -1.0 | 0.000 |
| MSMEG\_0430 | | ISL3-like element ISMsm4 family transposase | 2.8 | 0.023 | -0.9 | 0.005 |
| MSMEG\_0450 | | Hypothetical protein | 2.9 | 0.029 | -0.8 | 0.000 |
| MSMEG\_0637 | | FAD-dependent oxidoreductase | 2.7 | 0.046 | -1.2 | 0.000 |
| MSMEG\_0651 | | Hypothetical protein | 2.7 | 0.000 | -0.8 | 0.001 |
| MSMEG\_1097 | | Glycosyltransferase | 3.0 | 0.044 | -1.0 | 0.000 |
| MSMEG\_1315 | | Mechanosensitive ion channel family protein | 2.3 | 0.046 | -0.7 | 0.000 |
| MSMEG\_1605 | | Phosphate signaling complex protein PhoU | 2.8 | 0.028 | -0.9 | 0.000 |
| MSMEG\_1773 | | Iron-containing redox enzyme family protein | 2.7 | 0.035 | -1.2 | 0.000 |
| MSMEG\_1970 | | GAF domain-containing protein | 3.1 | 0.007 | -0.8 | 0.000 |
| MSMEG\_3232 | | Cytochrome *d* ubiquinol oxidase subunit II | 3.0 | 0.000 | -0.7 | 0.000 |
| MSMEG\_3233 | | Cytochrome ubiquinol oxidase subunit I | 2.8 | 0.000 | -0.9 | 0.000 |
| MSMEG\_3418 | | SRPBCC family protein | 2.5 | 0.042 | -1.5 | 0.000 |
| MSMEG\_3543 | | Protein disulfide oxidoreductase | 2.6 | 0.028 | -0.8 | 0.000 |
| MSMEG\_3680 | | Hypothetical protein | 2.1 | 0.004 | -3.0 | 0.000 |
| MSMEG\_3865 | | Hypothetical protein | 2.5 | 0.006 | -2.1 | 0.000 |
| MSMEG\_4195 | | Hypothetical protein | 2.2 | 0.026 | -0.8 | 0.000 |
| MSMEG\_4465 | | Cutinase family protein | 2.8 | 0.002 | -3.2 | 0.000 |
| MSMEG\_4618 | | Cysteine hydrolase | 2.5 | 0.030 | -0.6 | 0.000 |
| MSMEG\_5559 | | Sugar porter family MFS transporter | 2.3 | 0.029 | -1.0 | 0.000 |
| MSMEG\_5722 | | Hypothetical protein | 3.4 | 0.015 | -0.6 | 0.000 |
| MSMEG\_6233 | | Hypothetical protein | 2.5 | 0.038 | -1.0 | 0.002 |
| MSMEG\_6345 | | Hypothetical protein | 2.1 | 0.009 | -0.6 | 0.000 |
| MSMEG\_6498 | | Hypothetical protein | 2.1 | 0.000 | -2.7 | 0.000 |
| MSMEG\_6610 | | DUF58 domain-containing protein | 2.6 | 0.049 | -1.4 | 0.000 |

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