Supplemental material

Roles of Alanine Dehydrogenase and Induction of Its Gene in *Mycobacterium smegmatis* under Respiration-inhibitory Conditions

Ji-A Jeong,[a] Sae Woong Park,[b] Dahae Yoon,[c] Suhkmann Kim,[c] Ho-Young Kang,[a] and Jeong-Il Oh[a]#

[a]Department of Microbiology, Pusan National University, 46241 Busan, Korea

[b]Department of Microbiology and Immunology, Weill Cornell Medical College, New York, USA

[c]Department of Chemistry, Center for Proteome Biophysics and Chemistry Institute for Functional Materials, Pusan National University, 46241 Busan, Korea

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#Corresponding author

Jeong-Il Oh

Telephone: +82-51-510-2593

Fax: +82-51-514-1778

E-mail: joh@pusan.ac.kr

MATERIALS AND METHODS

Construction of mutant strains of *M. smegmatis*.

Deletion mutants of *M. smegmatis* were constructed by allelic exchange mutagenesis using the suicide vector pKOTs containing a temperature-sensitive replication origin (Table 1).

(i) Δ*ald* mutant. To construct an *ald* deletion mutant, two rounds of recombination PCR were conducted. Using the chromosomal DNA of *M. smegmatis* mc^{2}155 as a template, two primary PCR reactions were performed with the primers, F_ald_Mut (5’-GAAAGGATCCAGAACTGCTGCTCAAGGTG-3’) and Rec_ald_R (5’-CAGTAGAAGATCGTGTCGTGGTTGAGATCGAACACCGTCAC-3’), as well as with the primers, Rec_ald_F (5’-GTGACGGTGTTCGATCTCAACCACGACACGATCTTCTACTG-3’) and R_ald_Mut (5’-ATTAAAGCTTGATGGTGCGGACGCGCGG-3’), to generate two 41-bp overlapping DNA fragments (427 and 428 bp, respectively). Both PCR products contained the same 270-bp deletion within *ald* (*MSMEG_2659*) in the overlapping region. In the secondary PCR, an 814-bp DNA fragment with in-frame deletion of *ald* was obtained using both the primary PCR products as templates and the primers, F_ald_Mut and R_ald_Mut. The secondary PCR product was restricted with HindIII and cloned into pKOTs digested with HindIII and EcoRV, yielding pKOTsΔald. The pKOTsΔald plasmid was introduced into *M. smegmatis* mc^{2}155 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 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 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% (wt/vol) sucrose at 37°C.

(ii) Δ*bd* mutant. For the construction of a deletion mutant of *cydA* encoding subunit I of the *bd* quinol oxidase, two primary PCR reactions were performed with the primers, F_cydA_Mut (5'-GGGGAAGCTTCGAGTCCACGTTCATCGG-3') and R_cydA_rec (5'-CCGAACTTCTCCTCGTAGCTCGCGGCGTCGGCGGCCGATTC-3'), as well as with the primers, F_cydA_rec (5'-GAATCGGCCGCCGACGCCGCGAGCTACGAGGAGAAGTTCGG-3') and R_cydA_Mut (5'-AAAAGCGGCCGCCACCAGGCGTAAACC-3'), to generate two 41-bp overlapping DNA fragments (379 and 408 bp, respectively), both encompassing the same 300-bp deletion within *cydA* (*MSMEG_3233*) in the overlapping region. In the secondary PCR, a 746-bp DNA fragment with in-frame deletion of *cydA* was obtained using both the primary PCR products as templates and the primers, F_cydA_Mut and R_cydA_Mut. The secondary PCR product was restricted with NotI and HindIII and cloned into pKOTs digested with the same enzymes, yielding pKOTsΔbd. The allelic exchange using pKOTsΔbd was performed in the same way as construction of the Δ*ald* mutant.

(iii) Δaa_3 mutant. To introduce an in-frame deletion into *ctaC* (*MSMEG_4268*) encoding subunit II of the aa_3 cytochrome *c* oxidase, two primary PCR reactions were performed with the primers, F_ctaC_Mut (5'-GTTTGGATCCGCGCCAGTATGTCTGATC-3') and R_ctaC_rec (5'-GAGTTCAGGACGAACTCGATGAACGGGATGACCGTCAGCG-3'), as well as with the primers, F_ctaC_rec (5'-CGCTGACGGTCATCCCGTTCATCGAGTTCGTCCTGAACTC-3') and R_ctaC_Mut (5'-GTTTAAGCTTGAGCACGGCGTACACCACC-3'), to generate two 40-bp overlapping DNA fragments (497 and 489 bp, respectively), both bearing the same 351-bp deletion

69 within *ctaC* (*MSMEG_4268*) in the overlapping region. In the secondary PCR, a 946-bp DNA
70 fragment with in-frame deletion of *ctaC* was obtained using both the primary PCR products as
71 templates and the primers, F_ctaC_Mut and R_ctaC_Mut. The secondary PCR product was
72 restricted with HindIII and cloned into pKOTs digested with HindIII and EcoRV, yielding
73 pKOTsΔaa_3. The allelic exchange using pKOTsΔaa_3 was conducted as described above.
74 **(iv) Δ*bd*Δ*ald* mutant**. The Δ*bd*Δ*ald* double mutant was constructed using the Δ*ald* mutant and
75 pKOTsΔbd in the same way as the construction of the Δ*bd* mutant.
76 The allelic exchange in the mutants constructed in this study was verified by PCR with isolated
77 genomic DNA (Fig. S3).
78
79 **Construction of plasmids.**
80 **(i) pMV306ctaC and pMV306ald.** pMV306ctaC was constructed for complementation of the
81 Δ*aa₃* mutant. A 1,380-bp DNA fragment containing the *ctaC* gene of *M. smegmatis* was amplified
82 with the primers, F_ctaC_comp (5'-GAAATCTAGACCAGCTGAGATCCGGTTC-3') and
83 R_ctaC_Mut, using chromosomal DNA of *M. smegmatis* mc^2155 as a template. The PCR product
84 was restricted with XbaI and HindIII and cloned into the pMV306 integration vector, yielding
85 pMV306ctaC.
86 To complement the Δ*ald* mutant, pMV306ald carrying a 1,554-bp XbaI-HindIII fragment with
87 the *ald* gene of *M. smegmatis* was constructed in the same way as pMV306ctac, except using the
88 primers, R_279bp (5'-AAAATCTAGAACGCGTCCGTGGCACGTC-3') and R_ald_Mut.
89 **(ii) pNBV1cydA.** To construct pNBV1cydA for complementation of the Δ*bd* mutant, a 1,932-bp
90 DNA fragment containing the *cydA* gene of *M. smegmatis* was amplified with the primers,

91 F_cydA_com (5’-ATTTTCTAGAATCGCTCGCGACGCTGGC-3’) and R_cydA_com (5’-

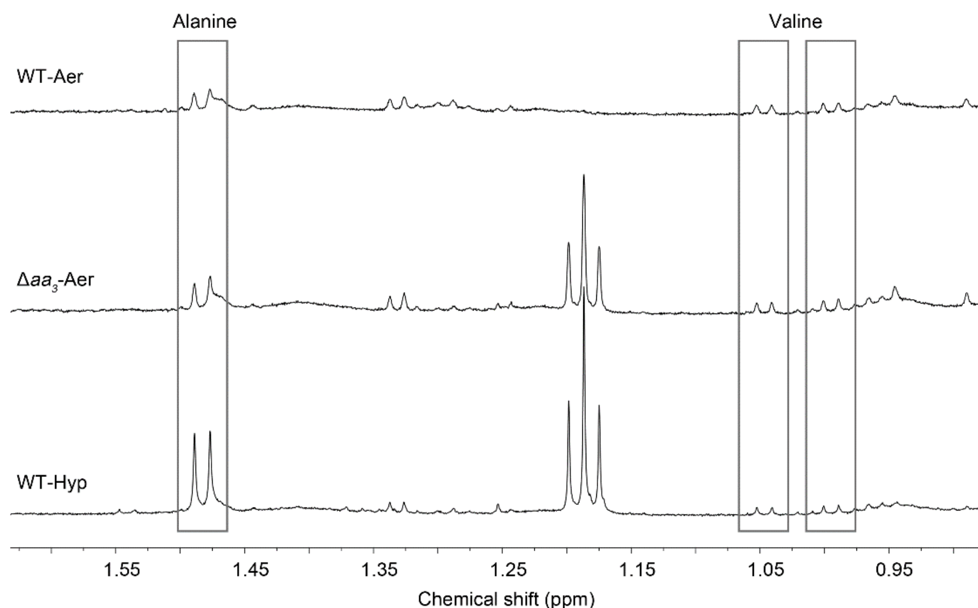
92 ATTTAAGCTTGCCGTCCCACACCGGTCC-3’), using chromosomal DNA of *M. smegmatis*

93 mc^2155 as a template. The PCR product was restricted with XbaI and HindIII and cloned into the

94 pNBV1 shuttle vector, resulting in pNBV1cydA.

95

96 **FIGURES**



97

98 **FIG S1** 600 MHz ^{1}H-NMR spectra of the WT and Δ*aa$_3$* mutant strains of *M. smegmatis* grown
99 either aerobically (Aer) or under hypoxic conditions (Hyp). The regions representing the alanine
100 and valine peaks in NMR spectra (δ 0.9-1.65) are magnified. The peaks of alanine CH_3 signal at
101 1.47 ppm and valine CH_3 signals at 0.98 and 1.03 ppm are boxed.

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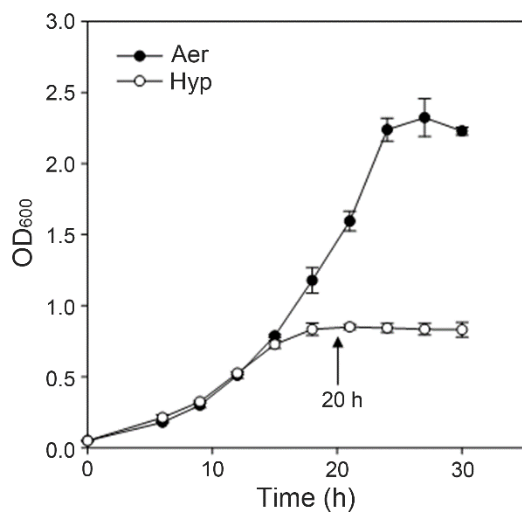

104 **FIG S2** Growth of *M. smegmatis* WT strain under aerobic and hypoxic conditions. The strain was
105 grown in 7H9-glucose medium either aerobically (Aer) or under hypoxic conditions (Hyp) as
106 described in Materials and Methods, and their growth was monitored spectrophotometrically at
107 600 nm at 3 h intervals for 30 h. The arrow indicates the time point of harvest for hypoxic cultures
108 used in this study. All values provided are the averages of the results from three independent
109 determinations. Error bars indicate the standard deviations.

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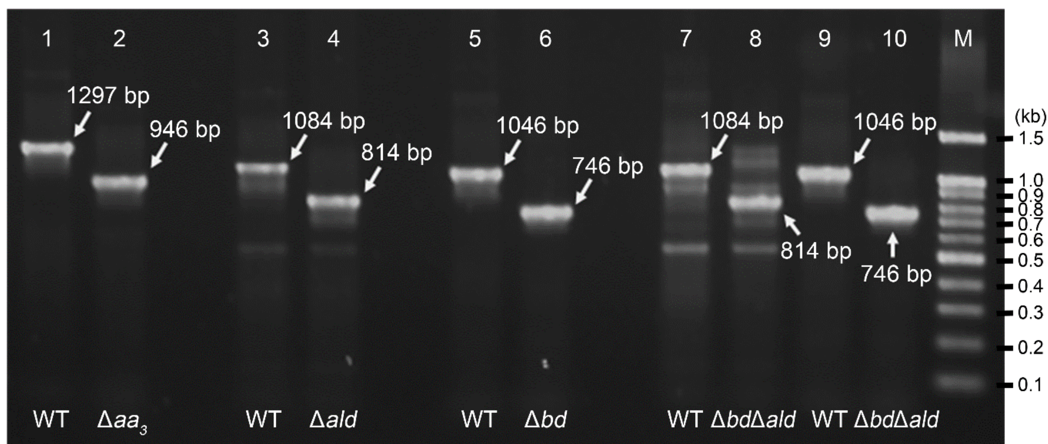

112 **FIG S3** Validation of the Δaa_3, Δ*ald*, Δ*bd*, and Δ*bd*Δ*ald* mutant strains of *M. smegmatis* by PCR.
113 The PCR reactions were carried out using the genomic DNA from the WT, Δaa_3, Δ*ald*, Δ*bd*, and
114 Δ*bd*Δ*ald* mutant strains of *M. smegmatis* with the following primer sets: F_ctaC_Mut/R_ctaC_Mut
115 to confirm deletion of *ctaC* (lanes 1 and 2); F_ald_Mut/R_ald_Mut to confirm deletion of *ald*
116 (lanes 3, 4, 7 and 8); F_cydA_Mut/R_cydA_Mut to confirm deletion of *cydA* (lanes 5, 6, 9, and
117 10). Lane M indicates the DNA size marker. Expected sizes of PCR products are shown with the
118 arrows.

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