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Regulation of the *ald* Gene Encoding Alanine Dehydrogenase by AldR in *Mycobacterium smegmatis*

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The regulatory gene *aldR* was identified 95 bp upstream of the *ald* gene encoding L-alanine dehydrogenase in *Mycobacterium smegmatis*. The AldR protein shows sequence similarity to the regulatory proteins of the Lrp/AsnC family. Using an *aldR* deletion mutant, we demonstrated that AldR serves as both activator and repressor for the regulation of *ald* gene expression, depending on the presence or absence of L-alanine. The purified AldR protein exists as a homodimer in the absence of L-alanine, while it adopts the quaternary structure of a homohexamer in the presence of L-alanine. The binding affinity of AldR for the *ald* control region was shown to be increased significantly by L-alanine. Two AldR binding sites (O1 and O2) with the consensus sequence GA-N_2-ATC-N_2-TC and one putative AldR binding site with the sequence GA-N_2-GTT-N_2-TC were identified upstream of the *ald* gene. Alanine and cysteine were demonstrated to be the effector molecules directly involved in the induction of *ald* expression. The cellular level of L-alanine was shown to be increased in *M. smegmatis* cells grown under hypoxic conditions, and the hypoxic induction of *ald* expression appears to be mediated by AldR, which senses the intracellular level of alanine.

Alanine dehydrogenase (EC 1.4.1.1; Ald) catalyzes the reversible oxidative deamination of L-alanine to pyruvate with the concomitant reduction of oxidized nicotinamide adenine dinucleotide (NAD^+) to nicotinamide adenine dinucleotide phosphate (NADH). Its forward reaction appears to be necessary for the aerobic utilization of alanine as a nitrogen source in *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*, and *Mycobacterium bovis* BCG (1–3). The reverse reaction of Ald was proposed to play a role in recycling NADH under respiration-inhibitory conditions, such as hypoxia, by oxidizing NADH to NAD^+ (2, 4, 5). Ald proteins from *M. tuberculosis* and *M. smegmatis* have glyoxylate-reductive aminase activity, which converts glyoxylate to glycine, but do not catalyze the reverse reaction, in which glycine is converted to glyoxylate by oxidative deamination (3, 6). The quaternary structure of Ald is a homohexamer consisting of three dimers, and each subunit is composed of an N-terminal catalytic domain and C-terminal NADH (NAD^+) binding domain (7, 8).

It was reported that expression of the *ald* genes encoding Ald was upregulated in *M. tuberculosis* under nutrient starvation and energy-limiting conditions, as well as in *Mycobacterium marinum* during long-term granulomatous infection in its host (5, 9, 10). The synthesis and activity of Ald as well as expression of *ald* were shown to be induced when *M. tuberculosis* and *M. smegmatis* were shifted from aerobic to hypoxic growth conditions (4, 6, 11–13). Furthermore, the addition of alanine to aerobic cultures of *M. tuberculosis* and *M. smegmatis* led to a strong induction of *ald* expression (2, 3). Although the induction conditions of the *ald* gene were well known, the regulatory mechanism, which underlies upregulation of the gene under the different conditions mentioned above, remained unsolved. Here, we report that expression of the *ald* gene of *M. smegmatis* is under the control of its upstream gene product (AldR), which acts as both activator and repressor depending on the presence or absence of alanine, and that the hypoxic induction of *ald* is a result of increased levels of alanine in *M. smegmatis* cells grown under hypoxic conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *M. smegmatis* strains were grown in Middlebrook 7H9 medium (Difco, Sparks, MD) supplemented with 0.2% (wt/vol) glucose as a carbon source and 0.02% (vol/vol) Tween 80 as an anticlumping agent at 37°C. *M. smegmatis* strains were grown aerobically or hypoxically as described previously (14). For various stress conditions, except hypoxic conditions, *M. smegmatis* strains were grown to an optical density at 600 nm (OD_{600}) of 0.5 to 0.6 on a gyratory shaker (200 rpm). Following the addition of chemicals to the cultures, the strains were further grown for 1 h. The treatment concentrations of the chemicals were 25 mM L-amino acids, 5 mM diamide, 15 mM hydrogen peroxide, and 5 mM sodium nitroprusside (SNP). For heat stress conditions, the aerobic cultures grown to an OD_{600} of 0.5 to 0.6 were further grown at 45°C for 1 h. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C. Ampicillin (100 μg/ml for *E. coli*), hygromycin (200 μg/ml for *E. coli* and 50 μg/ml for *M. smegmatis*), and kanamycin (50 μg/ml for *E. coli* and 30 μg/ml for *M. smegmatis*) were added to the growth medium when required.

DNA manipulation and electroporation. Standard protocols or manufacturer's instructions were followed for recombinant DNA manipulations (15). The introduction of plasmids into *M. smegmatis* strains was carried out by electroporation as described elsewhere (16).

Construction of plasmids. (i) pALDLACZ, pNC218bp, pNC153bp, and pNC109bp. pALDLACZ, pNC218bp, pNC153bp, and pNC109bp are the *lacZ* transcriptional fusion plasmids that contain the 5′ portion (105 bp) of *ald*, as well as 410-, 218-, 153-, and 109-bp DNA sequences upstream of the *ald* start codon, respectively. For the construction of pALD-

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Reference or source
Strains		
M. smegmatis		
mc^{2}155	High-transformation-efficiency mutant of *M. smegmatis* ATCC 607	16
Δ*devR*	*devR* (*MSMEG_5244*) deletion mutant derived from *M. smegmatis* mc^{2}155	20
Δ*aldR*	*aldR* (*MSMEG_2660*) deletion mutant derived from *M. smegmatis* mc^{2}155	22
E. coli		
DH5α	φ80d*lacZ*ΔM15 Δ*lacU169 recA1 endA1 hsdR17 supE44 thi1 gyrA96 relA1*	48
BL21(DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *dcm gal* λ (DE3)	Promega
Plasmids		
pBluescript II KS +	Amp^r; *lacPOZ′*	14
pNC	Hyg^r; promoterless *lacZ*	49
pT7-7	Amp^r; T7 promoter, ribosome binding site, and translation start codon overlapping NdeI site	50
pNBV1	Hyg^r; 5.8-kb plasmid derived from p16R1	51
pALDLACZ	pNC with 0.52-kb XbaI-ClaI fragment containing the *ald* promoter region	This study
pBSMS2659	pBluescript II KS+ with 0.52-kb XbaI-ClaI fragment from pALDLACZ	This study
pBSPM1	pBSMS2659 with point mutations (TC→CT) in the O1 region	This study
pBSPM2	pBSMS2659 with point mutations (AT→GC) in the O1 region	This study
pBSPM3	pBSMS2659 with point mutations (AC→GT) in the O1 region	This study
pNC218bp	pNC with 0.33-kb XbaI-ClaI fragment containing 218 bp of the *ald* promoter region	This study
pNC153bp	pNC with 0.26-kb XbaI-ClaI fragment containing 153 bp of the *ald* promoter region	This study
pNC109bp	pNC with 0.22-kb XbaI-ClaI fragment containing 109 bp of the *ald* promoter region	This study
pM1	pNC with 0.52-kb XbaI-ClaI fragment from pBSPM1	This study
pM2	pNC with 0.52-kb XbaI-ClaI fragment from pBSPM2	This study
pM3	pNC with 0.52-kb XbaI-ClaI fragment from pBSPM3	This study
pT7MS2660His	pT7-7 with 0.54-kb NdeI-HindIII fragment containing *aldR* with 6 His codons before its stop codon	This study
pMV306lacZald	pMV306lacZ with 0.51-kb EcoRI-HindIII fragment containing the *ald* promoter region	22
pNBV1aldR	pNBV1 with 1.11-kb BamHI-HindIII fragment containing *aldR* of *M. smegmatis* mc^{2}155	This study

LACZ, a 530-bp DNA fragment including the *ald* gene upstream region was amplified with F_MS2659 (5′-TTATTCTAGAAGCTGCGGATCTTGCCGC-3′) and R_MS2659 (5′-ATTGATCGATGATCACCTCGTGACCTCT-3′), using *M. smegmatis* mc^{2}155 genomic DNA as the template and *Pfu* DNA polymerase. The PCR product was restricted with ClaI and XbaI and cloned into the promoterless *lacZ* vector pNC, which had been digested with the same restriction enzymes. In order to construct the other plasmids, the *ald* upstream regions of the corresponding lengths were amplified by PCR using the forward primers with an XbaI restriction site and the R_MS2659 primer with a ClaI restriction site. pALDLACZ was used as the template for PCR. The PCR products were restricted with ClaI and XbaI and cloned into pNC, resulting in the plasmids pNC218bp, pNC153bp, and pNC109bp.

(ii) pT7MS2660His. A 551-bp DNA fragment including the *aldR* gene and the six histidine codons immediately before its stop codon was amplified with F_MS2660_Histag (5′-CCCCCATATGAGTGAAGGATCATCGATCAC-3′) and R_MS2660_Histag (5′-TTTTAAGCTTTCAGTGATGGTGATGGTGATGCAGCGGCGACGCGCCCCGCA-3′) using *M. smegmatis* mc^{2}155 genomic DNA as the template and *Pfu* DNA polymerase. The PCR product was restricted with NdeI and HindIII and cloned into pT7-7 digested with same restriction enzymes, yielding pT7MS2660His.

(iii) pNBV1aldR. To construct pNBV1aldR, used for complementation, a 1,110-bp BamHI-HindIII fragment containing *aldR* was amplified with MSMEG_2660_F (5′-AACGGGATCCGACCAGACCTGGTCGGCG-3′) and MSMEG_2660_R (5′-AACGAAGCTTCCAAGCCCACGCTGACGA-3′) using *M. smegmatis* mc^{2}155 genomic DNA as the template and *Pfu* DNA polymerase. The PCR product was restricted with BamHI and HindIII and cloned into pNBV1 digested with the same restriction enzymes, yielding pNBV1aldR.

Site-directed mutagenesis. To construct pM1, pM2, and pM3, site-directed mutagenesis was performed using the plasmid pBSMS2659 as the template and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). pBSMS2659 was constructed by cloning of a 518-bp XbaI-ClaI fragment from pALDLACZ into pBluescript II KS+. Synthetic oligonucleotides 33 to 34 bases long and containing mutated nucleotides in the middle of their sequences were used to mutagenize a putative AldR binding site (O1) located upstream of *ald*. Mutations were verified by DNA sequencing. The 518-bp XbaI and ClaI fragment from the mutated pBSMS2659 was cloned into pNC, resulting in the plasmids pM1, pM2, and pM3, which had the same construct as pALDLACZ except for the point mutations.

2D electrophoresis. The mutant *M. smegmatis* Δ*devR* strain was grown under either hypoxic or aerobic conditions as described above, and cells were harvested. Bacterial pellets were resuspended in 2.5 ml of buffer (20 mM Tris-HCl [pH 8.0], 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride [PMSF]), and cells were disrupted by five passages through a French pressure cell at 800 lb/in^2. Following the DNase/RNase treatment (5 U DNase I and 5 U RNase I/ml in the presence of 10 mM $MgCl_2$) for 30 min on ice, soluble fractions were obtained by ultracentrifugation at 100,000 × *g* for 90 min at 4°C, and proteins were precipitated by treat-

ment with trichloroacetic acid (TCA) to a final concentration of 10% (wt/vol) for 1 h on ice. TCA was removed by acetone treatment. The sample was resuspended in 250 μl of the sample preparation solution, composed of 8 M urea, 4% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 2% (vol/vol) IPG buffer (pH 4 to 7; GE Healthcare, Piscataway, NJ), and 40 mM dithiothreitol (DTT). The protein concentration was determined by using a bicinchoninic acid (BCA) protein assay kit (Thermo, Milford, MA) and a two-dimensional (2D) Quant kit (GE Healthcare). 2D gel electrophoresis was conducted with a MultiTemp III system (GE Healthcare) according to the manufacturer's instructions. Thirteen-cm Immobiline DryStrip gels (pH 4 to 7; GE Healthcare) were rehydrated for 10 h in rehydration buffer (8 M urea, 2% [wt/vol] CHAPS, 0.002% [wt/vol] bromophenol blue) with IPG buffer at a final concentration of 0.5% (vol/vol). One hundred to 120 μg of proteins was loaded on the rehydrated gel strips, and proteins were electrofocused on an Ettan IPGphor II manifold (GE Healthcare). Isoelectrofocusing started at 100 V, and the applied voltage was increased stepwise to 8,000 V for 5 h and then maintained at 8,000 V to reach a total of 65,000 V · h, as described in the manufacturer's manual (GE Healthcare). Subsequently, the isoelectrofocused gel strips were equilibrated for 15 min in 10 ml of the equilibration solution (1% [wt/vol] DTT, 75 mM Tris-HCl [pH 8.8], 6 M urea, 29.3% [vol/vol] glycerol [87%, wt/wt], 2% [wt/vol] SDS, 0.002% [wt/vol] bromophenol blue) and for another 15 min in 10 ml of the equilibration solution with 2.5% (wt/vol) iodoacetamide (IAA). The 2D separation was performed by using an SE 600 Ruby electrophoresis system (GE Healthcare) in 12.5% (wt/vol) SDS-PAGE at 10 mA for 1 h and then at 50 mA for 4 h. Proteins were stained by Coomassie brilliant blue. The protein spots were excised from the gels and digested with trypsin for identification by matrix-assisted laser desorption ionization–time of flight mass spectroscopy (MALDI-TOF MS) as previously described (17).

RT-PCR and qRT-PCR. RNA isolation from *M. smegmatis* strains, preparation of cDNA, reverse transcription-PCR (RT-PCR), and quantitative RT-PCR (qRT-PCR) were carried out as described elsewhere (14). To synthesize cDNA, the primers RT-16sr(−) (5′-ACAACGCTCGGACCCTAC-3′) for the 16S rRNA gene, Rihsp− (5′-CGCCCGTTGGTCTCCTTCTTC-3′) for the *hspX* gene, and MSMEG_2659_R (5′-TGGCCGTGGCGTCCTGATGG-3′) for the *ald* gene were used. For RT-PCR, the primers RT-16sr(+) (5′-CTGGGACTGAGATACGGC-3′) and RT-16sr(−) for the 16S rRNA gene, Rihsp+ (5′-GGGTCTGCCGTCGTGGGCCTC-3′) and Rihsp− for the *hspX* gene, and MSMEG_2659_F (5′-GAGTAGCGGGTCTCGATGCG-3′) and MSMEG_2659_R for the *ald* gene were employed.

β-Galactosidase assay and determination of the protein concentration. The β-galactosidase activity was assayed spectrophotometrically as described previously (18). The protein concentration was determined by using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard protein.

Purification of AldR and determination of its molecular weight in the native form. The *E. coli* BL21(DE3) strain carrying pT7MS2660His was grown aerobically at 37°C in LB medium containing 100 μg/ml ampicillin to an OD_{600} of 0.4 to 0.5. The *aldR* gene was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, and the cells were further grown for 4 h at 30°C. After 700 ml culture was harvested, cells were resuspended in 10 ml of buffer A (20 mM Tris-HCl [pH 8.0] containing 100 mM NaCl) and disrupted by two passages through a French pressure cell. Following DNase I treatment (10 U/ml) in the presence of 10 mM $MgCl_2$ for 30 min on ice, cell-free crude extracts were obtained by centrifugation two times at 20,000 × *g* for 20 min. After addition of imidazole to a final concentration of 5 mM, 0.6 ml of 80% (vol/vol) Ni-Sepharose high-performance resin (Amersham Biosciences, Piscataway, NJ) was added to the crude extracts and mixed gently by shaking for 2 h on ice. The protein-resin mixture was loaded into a column, and the column was washed with 20 volumes of buffer A containing 5 mM imidazole, followed by 20 volumes of buffer A containing 50 mM imidazole. The His_6-tagged AldR protein was eluted with buffer A containing 250 mM imidazole. Imidazole and NaCl were removed from purified AldR by means of a PD-10 desalting column (GE Healthcare) equilibrated with 20 mM Tris-HCl (pH 8.0).

Gel filtration chromatography was performed with a Superose 12 10/300 GL (GE Healthcare) column equilibrated with 20 mM Tris-HCl (pH 8.0) and calibrated with the standard proteins of known molecular masses (alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; carbonic anhydrase, 29 kDa; Sigma). When required, L-alanine or L-cysteine was added to the 20 mM Tris-HCl (pH 8.0) buffer and samples to a final concentration of 10 mM.

EMSA. Electrophoretic mobility shift assay (EMSA) was carried out by using an EMSA kit (Invitrogen, Carlsbad, NJ) according to the manufacturer's instructions. A 243-bp DNA fragment encompassing the putative AldR binding site (O1, O2, and O3), a 216-bp DNA fragment with the deletion of O1, and a 234-bp DNA fragment with the deletion of both O1 and O2 were used in EMSA. The 243-bp DNA fragment was generated by PCR using pALDLACZ as the template and the primer sets F_EMSA (5′-AACCATCGATAGCATGATCGCTCCTTCAGAAG-3′) and R_218bp (5′-ATTGTCTAGATATGCGTGCATCGTCGTGCAAC-3′). To delete the O1 sequence, a 128-bp DNA fragment containing the upstream region of O1 was amplified by PCR using pALDLACZ as the template and the primer set R_EMSA_dI (5′-GAAAATTCGTTAGGATTGTGGCGTCCAAACGCCCGGTTCG-3′) and R_218bp (5′-ATTGTCTAGATATGCGTGCATCGTCGTGCAAC-3′). A 128-bp DNA fragment containing the downstream region of O1 was amplified by PCR using pALDLACZ as the template and the primer set F_EMSA and F_EMSA_dI (5′-CGAACCGGGCGTTTGGACGCCACAATCCTAACGAATTTTC-3′). In the secondary PCR, a 216-bp DNA fragment deleted of O1 was obtained by using both the primary PCR products as the templates and the F_EMSA and R_218bp primers. The same strategy was used for the synthesis of the 234-bp DNA fragment. A 146-bp DNA fragment containing the upstream region of O2 was amplified by PCR using pALDLACZ as the template and the primer set R_279bp (5′-AAAATCTAGAACGCGTCCGTGGCACGTC-3′) and R_EMSA_dI2 (5′-GAAAATTCGTTAGGATTGTGGACCTCGACGACATCGACCG-3′). A 128-bp DNA fragment containing the downstream region of O1 was amplified by PCR using pALDLACZ as the template and the primer set F_EMSA and F_EMSA_dI2 (5′-CGGTCGATGTCGTCGAGGTCCACAATCCTAACGAATTTTC-3′). The secondary PCR was performed with the primary PCR products and the F_EMSA and R_279bp primers, resulting in a 234-bp DNA fragment with the deletion of both O1 and O2. DNA binding reaction mixtures were composed of the appropriate amounts of DNA (2 μl), purified AldR (5 μl), 1 μl of H_2O, and 2 μl of 5× binding buffer (included in the kit). L-Amino acids (alanine, serine, cysteine) were added, when necessary, to a final concentration of 20 mM. The binding reaction mixtures were incubated for 20 min at room temperature. After the addition of 2 μl of 6× loading buffer (included in the kit), the samples were subjected to the nondenaturing PAGE (6% [wt/vol] acrylamide) in buffer (pH 8.3) containing 83 mM Tris-borate and 1 mM EDTA at 60 V for 5 h at 4°C. The gels were stained with SYBR green staining solution (included in the kit). Bands were visualized by using a SYBR photographic filter (Invitrogen).

Quantification of intracellular alanine. The cell pellets of *M. smegmatis* strains were resuspended in 3 ml of H_2O and centrifuged to wash the cells. The cells were resuspended in 2 ml of H_2O and disrupted by five passages through a French pressure cell. Cell-free crude extracts were obtained by centrifugation at 20,000 × *g* for 20 min. Determination of the alanine concentration in the crude extracts was conducted by measuring spectrophotometrically the reduction of NAD^+ to NADH at 340 nm for 90 min in an Ald coupled enzyme assay. A 1.5-ml aliquot of the reaction mixture was composed of the crude cell extracts (100 μg of protein), 40 mM hydrazine monohydrate, 50 mM glycine, 2.67 mM NAD^+, and 0.5 U/ml Ald of *Bacillus subtilis* (Sigma, St. Louis, MO). The obtained absorbance values were normalized to those of the controls without *B. subtilis* Ald. The standard curve was generated using standard solutions with different concentrations of L-alanine (0 to 120 nmol).

RESULTS

Hypoxic induction of *ald* is independent of the DevSR two-component system. The DevSR two-component system (TCS) (DevS, MSMEG_5241; DevR, MSMEG_5244) is a major regulatory system that is responsible for hypoxic induction of gene expression in *M. smegmatis* (19). There is another set of genes for DevS (MSMEG_3941) and DevR (MSMEG_3944) in *M. smegmatis*. However, they appear not to be functional for hypoxic induction of the genes that are under the control of DevR, since the *hspX* gene, which is regulated by the DevSR TCS, was shown not to be induced in either *MSMEG_5241* or *MSMEG_5244* mutants grown under hypoxic conditions (14, 20). To identify regulatory systems other than the DevSR TCS which are involved in hypoxic induction of genes in *M. smegmatis*, we first looked for proteins whose levels were increased by more than 5-fold in an *M. smegmatis* Δ*devR* (*MSMEG_5244*) mutant strain subjected to the gradual depletion of oxygen for 20 h (i.e., 20 h of hypoxic conditions) compared to the same strain grown aerobically. As shown in Fig. 1A, 2D electrophoresis and subsequent MALDI-TOF MS led to the identification of three proteins, L-alanine dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and D-3-phosphoglycerate dehydrogenase, encoded by *MSMEG_2659* (*ald*), *MSMEG_3084* (*gap*), and *MSMEG_2378* (*serA*), respectively. Since the synthesis of alanine dehydrogenase (Ald) was increased the most (11.8-fold) among these three proteins in the strain grown under hypoxic conditions, we further studied the regulation of *ald* expression. To examine the hypoxic induction of *ald* at the transcriptional level, the Δ*devR* mutant strain of *M. smegmatis* was grown either aerobically or under hypoxic conditions for 10, 15, and 20 h, and the expression level of the *ald* gene was determined by RT-PCR (Fig. 1B). RT-PCR for the 16S rRNA gene was performed to ensure that the same amounts of total RNA were used in the experiment. The *hspX* gene under the control of DevR was used as the control. As expected, expression of *hspX* was not induced in the Δ*devR* mutant strain grown under hypoxic conditions, whereas it was strongly induced in the wild-type strain grown under the same conditions (10, 15, and 20 h of hypoxic conditions). Expression of *ald* was strongly induced under 10 h of hypoxic conditions, and its expression level was drastically reduced thereafter. qRT-PCR showed that the expression level of *ald* in the Δ*devR* mutant strain grown under hypoxic conditions for 10 h was 6.3-fold higher than that in the mutant grown aerobically. The discrepancy in the levels of *ald* mRNA and Ald protein in *M. smegmatis* grown under 20 h of hypoxic conditions can be explained by the slow turnover of the Ald protein.

The mechanism by which expression of *ald* is induced in mycobacteria under hypoxic conditions remained undetermined. A possible regulatory mechanism is that an alternative sigma factor, like SigH, is involved in the regulation of *ald*. To examine this possibility, we measured *ald* expression in the *M. smegmatis* Δ*devR* mutant exposed to various stress conditions (diamide, H_2O_2, SNP, and heat), as well as to hypoxic and alanine-supplemented conditions, by mean of an *ald*::*lacZ* transcriptional fusion plasmid, pALDLACZ (Fig. 2). Expression of *ald* in the Δ*devR* mutant strain grown under hypoxia and with L-alanine was increased by 3.7- and 9.1-fold, respectively, compared to that in the strain grown aerobically (control). Expression of *ald* in the Δ*devR* strain exposed to SNP and heat stress conditions was approximately 1.6- and 1.4-fold increased, respectively, relative to that of the control.

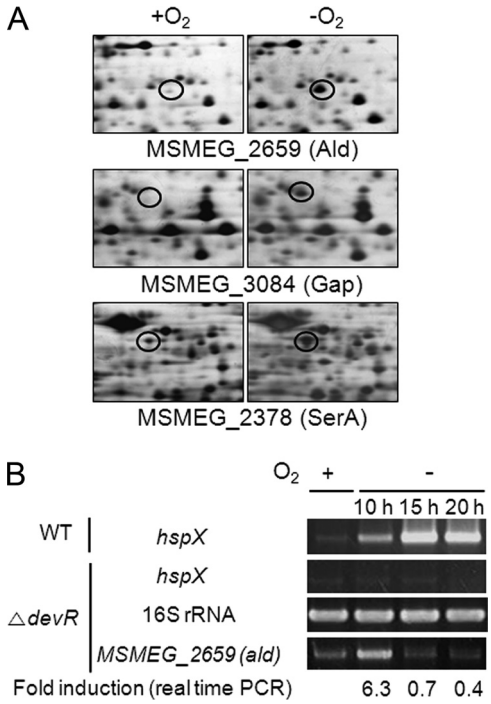

FIG 1 Identification of the *ald* gene whose expression was upregulated in a Δ*devR* mutant strain of *M. smegmatis* grown under hypoxic conditions. (A) Comparative 2D electrophoresis analysis of soluble proteins of the *M. smegmatis* Δ*devR* strain grown under hypoxic ($-O_2$) or aerobic ($+O_2$) conditions for 20 h. The spots indicated by the open circles represent three proteins whose synthesis was increased more than 5-fold under hypoxic conditions compared to their levels under aerobic conditions. By means of MALDI-TOF MS, the proteins were identified as MSMEG_2659 (Ald), with a molecular mass of 38 kDa and pI of 5.9, MSMEG_3084 (Gap), with a molecular mass of 32 kDa and pI of 5.2, and MSMEG_2378 (SerA), with a molecular mass of 51 kDa and pI of 4.9. (B) Determination of the transcription levels of *ald* under hypoxic conditions. The wild-type (WT) and Δ*devR* strains were grown either aerobically ($+O_2$) or under hypoxic conditions ($-O_2$) for 10, 15, and 20 h. The expression levels of *ald* and 16S rRNA genes were determined by RT-PCR. The *hspX* gene, whose expression was known to be induced by DevR under hypoxic conditions, was included in the experiment as a control. The levels of mRNA specific for *ald* were also determined by qRT-PCR and normalized to those of 16S rRNA. Fold induction of *ald* expression indicates the level of *ald* mRNA in hypoxic cultures relative to that in aerobic culture and is given below the RT-PCR results.

In contrast, when the Δ*devR* mutant strain was treated with diamide or H_2O_2, which are the signals activating expression of the SigH regulon (21), the expression level of *ald* was similar to that of the control, indicating that the hypoxic induction of *ald* expression is not caused by SigH.

Identification of the *aldR* gene. The open reading frame designated *MSEMG_2660* was identified 95 bp upstream of *ald* and in the opposite transcriptional orientation (Fig. 3). Its deduced protein product consists of 171 amino acid residues with a calculated molecular mass of 18.6 kDa. We named *MSMEG_2660 aldR* (for alanine dehydrogenase regulator). AldR is a member of the family of Lrp/AsnC transcriptional regulators. The genetic organization of the genes encoding the Ald and AldR homologs is also conserved in *M. tuberculosis* (Fig. 3A). The amino acid sequences of Ald and AldR of *M. smegmatis* exhibit 80 and 75% overall identity, respectively, to their homologs in *M. tuberculosis*.

AldR acts as both activator and repressor in the regulation of *ald* expression. To gain further insights into the function of AldR

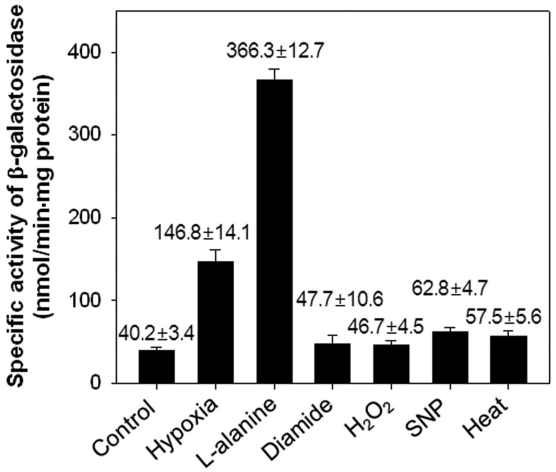

FIG 2 Expression of *ald* in the *M. smegmatis* Δ*devR* strain exposed to various conditions. The *M. smegmatis* Δ*devR* strain, carrying the *ald*::*lacZ* transcriptional fusion plasmid pALDLACZ, was grown either aerobically (control), under hypoxic conditions for 10 h (hypoxia), or exposed to various conditions as described in Materials and Methods. Cell-free crude extracts were used to determine β-galactosidase activity. The β-galactosidase activity is expressed as nanomoles per minute per milligram of protein. All values provided are the averages of the results from two independent determinations. Error bars indicate the deviations from the means.

on *ald* expression in the *M. smegmatis* strain, a mutant carrying a deletion within *aldR* (Δ*aldR*) was constructed (22). No difference in the growth rate between the wild-type and Δ*aldR* mutant strains of *M. smegmatis* was observed under aerobic conditions (data not shown). The possible role of AldR in the regulation of *ald* expression was investigated by determining the activity of β-galactosidase in the wild-type and Δ*aldR* mutant strains with pALDLACZ grown in the presence or absence of L-alanine (Fig. 4). The wild-type strain of *M. smegmatis* grown in the presence of L-alanine showed a significantly higher expression level of *ald* than the same strain grown in the absence of L-alanine, indicating that alanine is a strong inducer of *ald* expression. In contrast, expression of *ald* in the Δ*aldR* mutant was derepressed in the absence of L-alanine to a level corresponding to ~35% of that in the wild type grown in the presence of L-alanine, and the addition of L-alanine to the culture did not alter the expression level of *ald*. When the *aldR* gene on pNBV1aldR was introduced into the Δ*aldR* mutant *in trans*, the regulation pattern of *ald* was restored to that observed in the wild type (data not shown).

Identification of *cis* control elements upstream of the *ald* gene. The transcriptional start point of *ald* was previously determined by Feng et al. (2). The putative promoter of *ald* resembling the mycobacterial −35 and −10 regions was identified on the basis of the position of the transcriptional start point (Fig. 3B). A conspicuous inverted repeat sequence (O1) was identified 93 bp

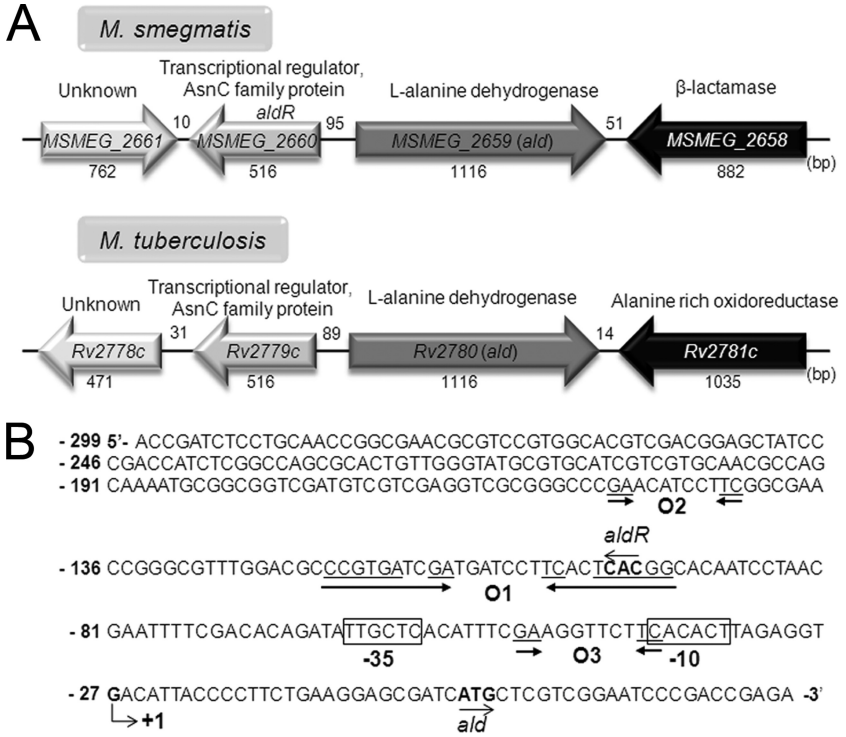

FIG 3 Genetic organization of the *ald* locus and the putative *cis*-acting elements for *ald* expression. (A) The *ald* genes and their flanking genes in *M. smegmatis* mc^2155 and *M. tuberculosis* H37Rv. The genes of the putative transcriptional regulators, *MSMEG_2660* (*aldR*) and *Rv2779c*, are divergently located upstream of the *ald* genes. The lengths of genes and intergenic regions are given as the nucleotide numbers below and above their names, respectively. (B) The transcription start site (+1) of the *ald* gene of *M. smegmatis* was previously reported to be a guanosine residue that is located 27 nucleotides upstream of the start codon of *ald* (2). The −10 and −35 regions of the putative promoter for *ald* deduced from the transcription start point are boxed. The putative AldR binding site (O1), which shows an interrupted inverted repeat sequence ($CCGTGAN_2GA\text{-}N_7\text{-}TCN_2TCACGG$), is marked by two head-facing arrows. The O2 and O3 sequences ($GA\text{-}N_7\text{-}TC$) exhibiting partial sequence similarity to O1 are indicated by two arrows. The start codons of *ald* and *aldR* are highlighted in boldface and by the arrows indicating the transcriptional direction. The numbers on the left of the nucleotide sequence indicate the positions of the leftmost nucleotides relative to the *ald* gene.

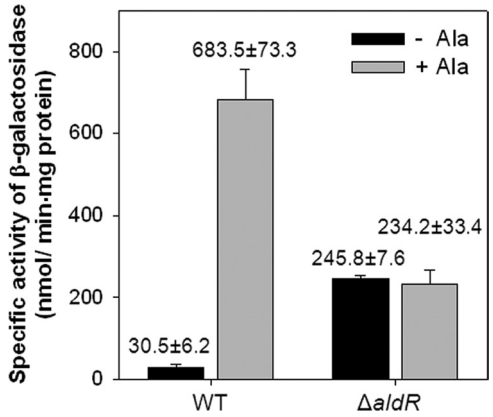

FIG 4 Expression of the *ald* gene in the wild-type and Δ*aldR* mutant strains of *M. smegmatis*. *M. smegmatis* wild-type (WT) and Δ*aldR* strains containing the *ald*::*lacZ* transcriptional fusion plasmid (pALDLACZ) were grown aerobically in 7H9-glucose medium to an OD_{600} of 0.5 to 0.6. Following the addition of 25 mM L-alanine to the cultures, the strains were further grown for 1 h (+Ala). For the control, the *M. smegmatis* strains were grown aerobically without the addition of L-alanine (−Ala). The *ald* promoter activities were measured by determining the β-galactosidase activity. All values provided are the averages of the results from two independent determinations. Error bars indicate the deviations from the means.

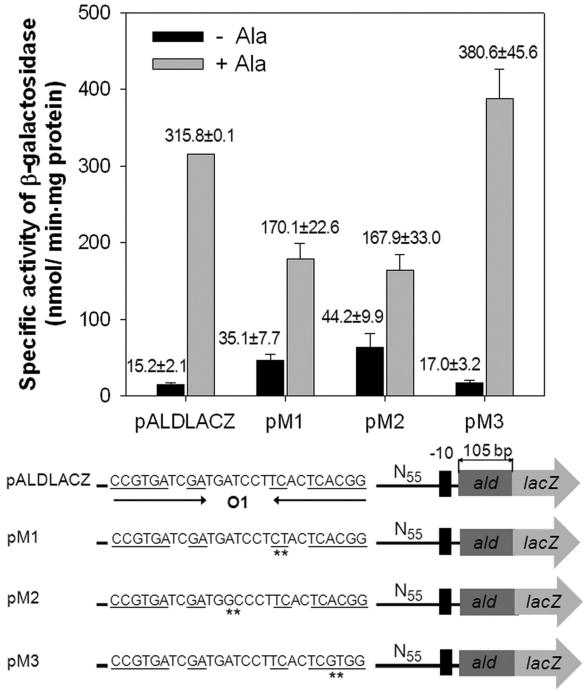

FIG 5 Effect of base substitution mutations within the O1 site on *ald* expression. The *ald* promoter activities were determined by using the *ald*::*lacZ* transcriptional fusions with point mutations in the O1 region (pM1, pM2, and pM3), which are derivatives of pALDLACZ. Transition mutations within the O1 site are indicated by the asterisks. As the positive control, pALDLACZ was included in the experiment. *M. smegmatis* wild-type strains harboring the transcriptional fusion plasmids were grown aerobically in the presence (+Ala) or absence (−Ala) of L-alanine, and β-galactosidase activities were determined. All values provided are the averages of the results from two independent determinations. Error bars indicate the deviations from the means.

upstream of *ald*, and it completely overlaps with the start codon of *aldR* (Fig. 3B). The O1 site contains a long, interrupted inverted repeat sequence, namely, CCGTGATCGATG**ATC**CTTCACTC ACGG (the dyad symmetry sequences are underlined, and the central sequence is indicated in boldface). Using several *ald*::*lacZ* transcriptional fusions (pM1, pM2, and pM3, which are derivatives of pALDLACZ) containing point mutations within the O1 region, we examined the role of the identified O1 region in *ald* expression and which sequences within the O1 region are important for the regulation of *ald* expression (Fig. 5). As a positive control, the wild-type strain harboring pALDLACZ was included in the experiment. As shown in Fig. 5, base substitution mutations (pM3, AC to GT) within the hexameric dyad (CCGTGA-N_{15}-TC ACGG [the mutated nucleotides are underlined]) did not affect the regulation of *ald* expression, whereas those (pM1, TC to CT; pM2, AT to GC) within the internal dimeric dyad (GA-N_7-TC) and the central sequence (ATC) led to partial deregulation of *ald* expression in the presence and absence of L-alanine. This result indicates that the internal dyad and the central ATC sequence (GA-N_2-ATC-N_2-TC) are important for the regulation of *ald* expression. The consensus DNA sequence for *E. coli* Lrp, *Pseudomonas putida* MdeR, and *Pyrococcus* sp. strain OT3 F11 is known as GA-N_2-WWW-N_2-TC (W is A or T) (23–25), which is very similar to the O1 sequence, except for the C in the central sequence. Using the criterion of GA-T_7-TC and two W nucleotides in the central sequence, we identified two additional putative AldR binding sites (O2 and O3) in the region upstream of *ald*. The O2 sequence perfectly matches the corresponding sequence of O1 (GA-N_2-ATC-N_2-TC) and is located upstream of O1, with an interval of 40 bp between the T nucleotides of the central sequences of O1 and O2. The O3 site has the sequence GA-N_2-GTT-N_2-TC, and its location overlaps that of the promoter of *ald* (Fig. 3B).

To investigate the role of O1 and O2 in the regulation of *ald* expression, a series of *ald*::*lacZ* transcriptional fusions with 5′-nested deletions of the *ald* upstream region were employed to study the expression of *ald* in the presence and absence of L-alanine (Fig. 6). The wild-type strain of *M. smegmatis* carrying pNC218bp with O1, O2, and O3 showed strong induction of *ald* expression in the presence of L-alanine. The deletion of the 5′ half of O2 (pNC153bp) severely affected the induction of *ald* expression by L-alanine, indicating that both O1 and O2 are required for the full induction of *ald*. When the *ald* upstream region was further deleted to remove the 5′ half of O1 (pNC109bp), the *ald* gene was no longer expressed in the presence and absence of L-alanine, indicating that the upstream region between −153 and −109 with regard to the start codon of *ald* is essential for *ald* expression.

To ascertain whether the identified sites were required for AldR binding *in vitro*, we performed EMSA with purified AldR and the 243-bp DNA fragment containing the O1, O2, and O3 sequences in the presence or absence of L-alanine (Fig. 7A). The binding affinity of AldR for the DNA fragment was considerably enhanced by L-alanine, indicating that L-alanine serves as a positive effector for AldR. The binding was proportional to the amount of applied AldR, with higher concentrations of the protein causing multiple retarded DNA-protein complexes. The multiple retarded complexes imply that at least two AldR binding sites exist in the DNA fragment. For more detailed analysis of the role of O1, O2, and O3 in the binding of AldR, we carried out EMSA with purified AldR and three different DNA fragments (243, 216, and 234 bp) in the presence of L-alanine (Fig. 7B). The 216- and 234-bp DNA frag-

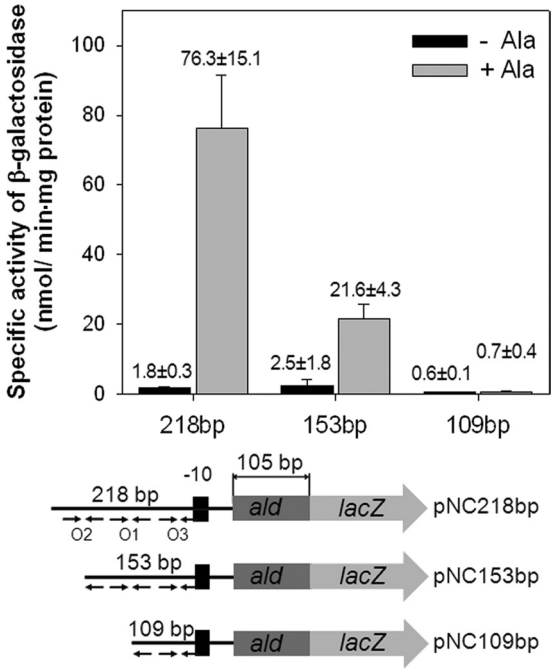

FIG 6 Promoter activities of the serially deleted upstream regions of *ald* in *M. smegmatis*. The *ald* promoter activities were determined by using *ald*::*lacZ* transcriptional fusions with 5′-nested deletions of the *ald* upstream region. The DNA fragments cloned into pNC consist of a shared 105-bp 5′ portion of *ald* and *ald* upstream regions in the different lengths denoted above the schematic diagrams (218, 153, and 109 bp). The relative positions and the presence or absence of the O1, O2, and O3 sites in the transcriptional fusions are indicated by the arrows. *M. smegmatis* wild-type strains harboring the transcriptional fusions were grown aerobically in the presence (+Ala) or absence (−Ala) of L-alanine, and β-galactosidase activities were determined. All values provided are the averages of results from two independent determinations. Error bars indicate the deviations from the means.

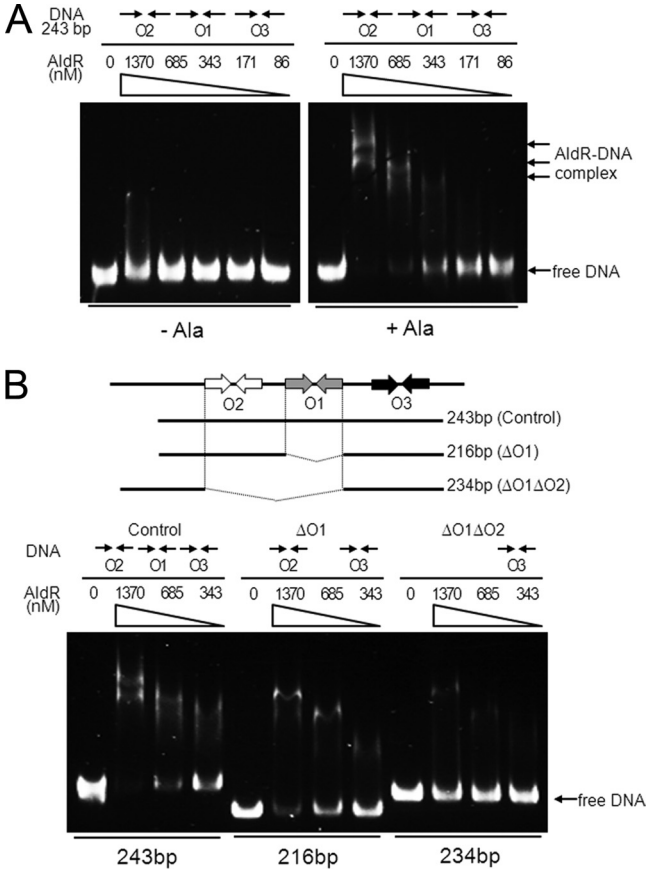

FIG 7 Binding of purified AldR to the *ald* control region. (A) The 243-bp DNA fragment (10 ng, corresponding to 6.7 nM) encompassing the O1, O2, and O3 sequences was incubated with various concentrations of purified AldR in the presence (+Ala) or absence (−Ala) of 20 mM L-alanine. The concentrations of AldR used are given above the lanes. Native PAGE was run in the presence of 83 mM Tris-borate buffer (pH 8.3) containing 1 mM EDTA, and the gels were stained with SYBR green EMSA gel staining solution. The arrows indicate bands of free DNA and retarded AldR-DNA complexes. (B) Ten ng of each of the DNA fragments (10 ng corresponds to 6.7 nM for control, 7.5 nM for ΔO1, and 6.9 nM for ΔO1O2), which contain O1, O2, and O3 (control), both O2 and O3 (ΔO1), and only O3 (ΔO1O2), were incubated with various concentrations of purified AldR in the presence of 20 mM L-alanine and subjected to native PAGE.

ments contained both O2 and O3 (ΔO1) and only O3 (ΔO1O2), respectively. The 243-bp DNA fragment containing O1, O2, and O3 was included in the experiment as a control. A smaller amount of DNA was retarded when O1 was deleted from the DNA fragment (ΔO1) than with the control DNA. When both O1 and O2 were deleted (ΔO1O2), the formation of retarded bands was almost abolished. This result indicates that the O1 and O2 sites are required for AldR binding, that O1 and O2 are more favored sites for AldR binding than O3, and that any combination of two binding sites is needed for effective binding of AldR to the *ald* control region.

Some Lrp/AsnC family regulators, like *E. coli* Lrp and *M. tuberculosis* LrpA, are known to recognize a broad range of amino acids as effector molecules (26, 27). This fact led us to determine the effect of various amino acids on both *ald* expression and binding of AldR to the *ald* control region (Fig. 8). In addition to L-leucine and L-asparagine, we examined the L-amino acids (cysteine, serine, and valine) that have structures close to that of alanine. As a positive control, L-alanine was included in the experiment. Consistent with previous results, the addition of L-alanine to the culture of *M. smegmatis* led to a significant increase in *ald* expression compared to that of the control without amino acid treatment. Interestingly, expression of *ald* was 6.3-fold induced by L-cysteine relative to that of the control, while the addition of L-serine did not induce *ald* expression. The addition of neither L-leucine nor L-asparagine affected expression of *ald*. Furthermore, EMSA was performed to investigate the effect of L-alanine, L-serine, and L-cysteine on the binding of AldR to the 243-bp DNA fragment containing O1, O2, and O3 (Fig. 8B). Interestingly, the presence of L-cysteine resulted in the formation of AldR-DNA complexes. However, the band pattern of the AldR-DNA complexes in the presence of L-cysteine was different from that in the presence of L-alanine. When L-serine was used in the experiment, the DNA fragment was retarded only with a high concentration of AldR, indicating that L-serine can bind to AldR and alter the binding affinity of AldR for the *ald* control region, and that the binding affinity or conformation of the serine-bound form of AldR is not enough to induce *ald* expression *in vivo*.

Quaternary structure of AldR. Changes in the quaternary structure of AldR by L-alanine and L-cysteine were examined by means of gel filtration chromatography (see Fig. S1 in the supplemental material). Although the theoretical molecular mass of the

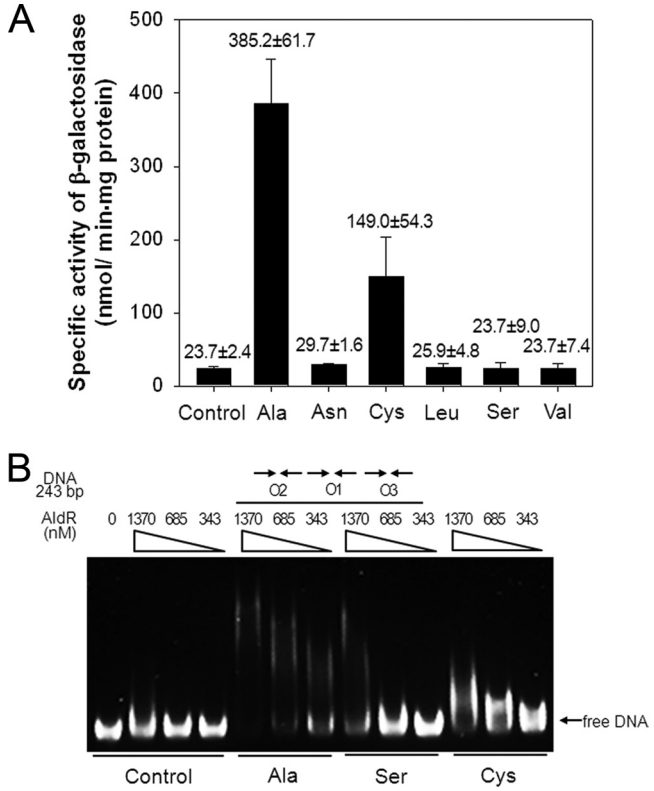

FIG 8 Effect of various amino acids on *ald* expression and binding of AldR to the *ald* control region. (A) *M. smegmatis* wild-type strain containing the *ald*::*lacZ* transcriptional fusion plasmid pALDLACZ was grown aerobically in 7H9 glucose medium to an OD_{600} of 0.5 to 0.6. Following the addition of 25 mM L-amino acids, the strain was further grown for an additional 1 h. The *ald* promoter activities were measured by determining the β-galactosidase activity. All values provided are the averages of results from two independent determinations. Error bars indicate the deviations from the means. (B) EMSA with the 243-bp DNA fragment (6.7 nM) encompassing O1, O2, and O3 and purified AldR in the presence of 20 mM L-alanine (Ala), L-serine (Ser), or L-cysteine (Cys). The concentrations of AldR used are given above the lanes. Native PAGE was run in the presence of 83 mM Tris-borate buffer (pH 8.3) containing 1 mM EDTA, and the gels were stained with SYBR green EMSA gel staining solution.

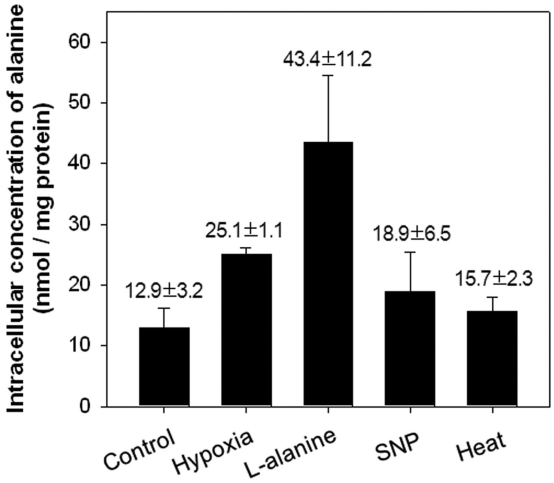

FIG 9 Determination of the intracellular alanine concentration in the *M. smegmatis* Δ*devR* mutant strain grown under various conditions. To measure the intracellular alanine levels, the *M. smegmatis* Δ*devR* mutant strain was grown either aerobically (control), under hypoxic conditions for 10 h (hypoxia), or exposed to various conditions (L-alanine, SNP, and 45°C heat) as described in Materials and Methods. The concentration of alanine in *M. smegmatis* Δ*devR* cells was determined by spectrophotometrically measuring the amounts of NADH produced in a *B. subtilis* L-alanine dehydrogenase-linked enzyme assay. All values provided are the averages of the results from two independent determinations. Error bars indicate deviations from the means.

N-terminally His_6-tagged AldR protein is 19.4 kDa, the molecular mass of purified AldR was found to be 17 kDa in SDS-PAGE (see Fig. S1A). On the basis of the elution volumes in gel filtration chromatography, the molecular mass of native AldR in the absence and presence of L-alanine was estimated to be 41 and 107 kDa, respectively. Considering the molecular mass of the AldR monomer (19.4 kDa), AldR exists as a homodimer in amino acid-free solution. The presence of L-alanine appears to change the oligomerization state of AldR from homodimer to homohexamer. In the presence of L-cysteine, the AldR protein was eluted at the position corresponding to 90 kDa, implying that AldR dimers assemble into homotetramers.

Intracellular concentration of alanine is increased under hypoxic conditions. The fact that expression of *ald* was increased under various stress conditions, such as hypoxic, SNP, and heat stress conditions, led us to speculate that the intracellular concentration of alanine is increased under these conditions, thereby resulting in the induction of *ald* expression by AldR. To explore this hypothesis, we determined the intracellular concentration of L-alanine in the *M. smegmatis* Δ*devR* strain grown under various conditions (hypoxia, L-alanine, SNP, and heat stress). The same strain grown aerobically without any treatment was included in the experiment as a control. As shown in Fig. 9, the *M. smegmatis* Δ*devR* strain grown with the supplementation of L-alanine showed the highest intracellular concentration of L-alanine. The strain grown under hypoxic conditions exhibited an intermediate value between those of the control and L-alanine-supplemented strains. A slightly increased level of L-alanine was detected in the strains grown under SNP and heat conditions. The concentration of L-alanine under various conditions correlated well with the expression levels of *ald* under the corresponding conditions (Fig. 2), implying that induction of *ald* expression under hypoxic, SNP, and heat conditions is the result of increased levels of alanine under these conditions.

DISCUSSION

Regulation of *ald* expression in *M. smegmatis*: *trans*- and *cis*-regulatory elements. The *hspX* gene, which is under the control of the DevSR TCS, was strongly upregulated in the wild-type strain of *M. smegmatis* subjected to the gradual depletion of oxygen for 15 and 20 h (15 and 20 h of hypoxic conditions), while the three identified genes (*ald*, *gap*, and *serA*) that are regulated in a DevSR-independent way were strongly induced under 10 or 15 h of hypoxic conditions, and their expression levels were reduced thereafter (data not shown for *gap* and *serA*). This result indicates that expression of *ald*, *gap*, and *serA* is induced under early hypoxic conditions and is distinct from that of the genes under the control of DevR. A common feature of the enzymes encoded by *ald*, *gap*, and *serA* is that they are $NADH/NAD^+$-dependent dehydrogenases, implying that the change in the ratio of NADH to NAD^+ in the cells under early hypoxic conditions is a direct or indirect signal for the upregulation of these genes.

Since the hypoxic induction of *ald* was the strongest of the three genes and a gene (*MSMEG_2660*; *aldR*) encoding an Lrp/AsnC family regulator is located immediately upstream of *ald*, we first chose the *ald* gene for further study of gene regulation. Expression of *ald* was far more induced by exogenous L-alanine than by hypoxic stress, and the intracellular level of L-alanine was increased in the cells grown under hypoxic conditions relative to those grown aerobically. These findings led us to hypothesize that the hypoxic induction of *ald* in *M. smegmatis* is mediated through an increase in alanine levels in the cells under hypoxic conditions.

A BLAST search revealed that the *aldR* gene product is a member of the Lrp/AsnC family of regulators. Like other members of this family, AldR is a small DNA binding protein (18.6 kDa) composed of two domains, the N-terminal DNA binding domain (amino acids 41 to 71) containing a helix-turn-helix (HTH) motif and the C-terminal ligand binding domain (amino acids 92 to 163), called RAM (regulation of amino acid metabolism) (28, 29). The C-terminal domain is also known to be involved in the oligomerization of the proteins in this family (29). AldR shows more than 75% sequence identity to its homologs in mycobacteria, and the genetic organization in the gene loci encoding the AldR homologs is well conserved, i.e., the *aldR* gene is located upstream of and adjacent to *ald*. AldR also exhibits an overall amino acid sequence identity of 22 to 28% to other Lrp/AsnC family members, e.g., 27% to Lrp of *E. coli*, 22% to AsnC of *E. coli*, 22% to LrpC of *B. subtilis*, 23% to FL11 of *Pyrococcus* sp. strain OT3, 24% to LrpA of *Pyrococcus furiosus*, 25% to LrpA of *M. tuberculosis* H37Rv, and 28% to MdeR of *P. putida*. Since most Lrp/AsnC family regulators are known to be involved in the regulation of the genes with related amino acid metabolism (30), we assumed the involvement of AldR in *ald* regulation. Using a Δ*aldR* mutant of *M. smegmatis*, we demonstrated that induction of *ald* expression by exogenous L-alanine and hypoxic stress was abolished in the mutant (data not shown for hypoxic stress). Intriguingly, the *ald* gene was partially derepressed in the mutant in the absence of L-alanine compared to the wild type and was constitutively expressed in the mutant regardless of the presence or absence of L-alanine. This regulation pattern provided evidence for a dual role of AldR in the control of *ald*. AldR serves as both an activator for *ald* expression in the presence of L-alanine and a repressor in the absence of L-alanine. Furthermore, this finding indicates that the *ald* gene has a promoter that drives a basal level of transcription without help of *trans*-acting regulatory protein(s). This novel type of positive and negative regulation by a single regulator is known for the regulation of the *dadAX* operon by Lrp of *E. coli* in the Lrp/AsnC family (31), and it has been well studied for AraC, which regulates the *araBAD* operon of *E. coli* (32). The FdsR regulator belonging to the LysR-type transcriptional regulator family was also reported to act as both activator and repressor to regulate expression of the *fdsGBACD* operon encoding the soluble formate dehydrogenase of *Ralstonia eutropha* (33).

The basic assembly unit of AldR appears to be the homodimer, as in other members of the Lrp/AsnC family. The purified AldR protein exists as a homodimer in the absence of L-alanine and appears to assemble into a higher-order structure of homohexamer in the presence of L-alanine, unlike the ring-like octamer structure consisting of four dimers that most members of the Lrp/AsnC family adopt (24, 25, 27, 29, 34–40). Among the Lrp/AsnC regulators, the oligomerization state of the FL11 protein of *Pyrococcus* sp. strain OT3, LrpA of *M. tuberculosis*, and Lrp of *E. coli* was reported to be affected by their cognate ligands, lysine, phenylalanine, and leucine, respectively (25, 27, 38). In the case of FL11, the presence of lysine brings about the assembly of FL11 dimer into homooctamer, while LrpA and Lrp change their quaternary structure from hexadecamer to octamer in the presence of their ligands. Purified Grp of *Sulfolobus tokodaii* and AsnC of *Neisseria meningitides* were demonstrated to exist as homooctamers irrespective of the presence or absence of their ligands (glutamine for Grp and leucine for AsnC), but the association of the ligands with the proteins was shown to increase the stability for their octamer formation (37, 39). The increased binding affinity of AldR for the control region of *ald* and homohexamer formation of AldR in the presence of L-alanine indicate that the higher order form of AldR has a higher binding affinity for the *ald* control region than the dimeric form. The fact that AldR serves as the repressor for *ald* expression in the absence of L-alanine indicates that the dimer form of AldR can also bind to the *ald* control region with a low binding affinity in a manner that represses expression of *ald*. In this study, we identified two AldR binding sites (O1 and O2) and one putative AldR binding site (O3) upstream of *ald* (Fig. 3B). The O1 and O2 sites have a consensus sequence of GA-N_2-ATC-N-$_2$-TC and are located upstream of the *ald* promoter. The O1 and O2 sites are separated by 40 bp between the T nucleotides of the central sequences of O1 and O2. The O3 site has the sequence of GA-N_2-GTT-N_2-TC, and its location overlaps that of the *ald* promoter. The location of a *cis*-regulatory DNA sequence determines its function in many cases. Transcriptional activators bind predominantly to positions between −80 and −30 relative to the transcriptional start points in the case of σ^{70}-dependent promoters to recruit RNA polymerase or to promote open complex formation, while many repressor binding sites overlap the promoters to occlude the binding of RNA polymerase to the promoters (41, 42). In this regard, we assume that O1 and O2 are involved in activation of *ald* expression in the presence of L-alanine, whereas O3 might be responsible for repression of *ald* expression in the absence of L-alanine. If AldR dimers are arranged in a 3-fold axis to form a ring-like hexamer with the HTH DNA binding domains facing out, the rotational angle between two neighboring dimers would be 120°. It was predicted that the two adjacent dimers bind to the target DNA sequences that are separated by ~30 bp between the centers of dyad symmetry in the case of the Lrp octamer with an angle of 90°, and that the two neighboring dimers with an angle of 122.5° simultaneously bind to the target DNA sequences separated by ~40 bp (35, 43), which implies that a molecule of AldR hexamer simultaneously binds to O1 and O2, which are separated by 40 bp, instead of binding to O1 and O3, which are separated by 61 bp. Since the simultaneous binding of AldR to both O1 and O2 seems to be thermodynamically more stable than the binding of AldR to O3 alone, binding of AldR hexamer to O1 and O2 might be favored. When O1 and O2 are occupied by the AldR hexamer and DNA bending occurs as a result, the access of another AldR hexamer to O3 might be occluded by steric hindrance. The AldR hexamer bound to O1 and O2 in the presence of L-alanine results in the recruitment of RNA polymerase to the *ald* promoter or the promotion of open complex formation at the promoter to activate the transcription of *ald*. In the absence of L-alanine, each AldR dimer might bind to O1, O2, and O3, but with a much less binding affinity than the AldR hexamer. When the O3 site is occupied by the AldR dimer, *ald* transcription is repressed. Binding of the AldR dimer to the O1,

O2, and O3 binding sites might be cooperative, like many Lrp/AsnC regulators binding to the multiple target sites (44). Therefore, it is possible that mutations in O1 led to partial derepression of *ald* in the absence of L-alanine through compromised binding of AldR dimer to O3 (Fig. 5).

Exogenous L-cysteine, which has an additional sulfhydryl group at the side chain of L-alanine, also induced the expression of *ald in vivo*, although it caused a substantially lower level of induction than L-alanine. Furthermore, L-cysteine appears to increase the binding affinity of AldR for the target DNA (Fig. 8). Intriguingly, EMSA analysis and gel filtration chromatography revealed that the quaternary structure of AldR in the presence of L-cysteine (tetramer) is different from that in the presence of L-alanine (Fig. 8; also see Fig. S1 in the supplemental material). It is currently unknown whether L-cysteine plays a role in the regulation of *ald in vivo*. Further study is required to address this and to confirm the quaternary structure of AldR in the presence of L-cysteine.

Proposed model for hypoxic induction of *ald*. Hypoxic induction of *ald* expression is mediated by AldR, which recognizes the cellular level of L-alanine. When the culture of *M. smegmatis* undergoes a gradual transition from aerobic conditions to hypoxic conditions, the depletion of oxygen leads to a reduced functionality of the respiratory electron transport chain (ETC). As a result, the redox state of the NADH/NAD^+ pool in the cell is shifted toward the more reduced state, and the cellular metabolism producing NADH might be slowed. Under these circumstances, the reductive amination reaction by Ald converting pyruvate to alanine, with the concomitant oxidation of NADH to NAD^+, might be accelerated due to an increase in the reactants, thereby producing more alanine, which in turn promotes the transcription of the *ald* gene through AldR. This positive feedback of *ald* gene expression helps mycobacteria regenerate NAD^+ to maintain the redox state of NADH/NAD^+ under hypoxic conditions. In good agreement with this model, it was reported that the growth of an *ald* knockout mutant of *M. smegmatis* was compromised under hypoxic conditions (2). The treatment of *M. smegmatis* cells with SNP, a generator of NO and CN^- (45), led to increased expression of *ald*. We also observed that expression of *ald* was increased by 27% relative to that of the control when *M. smegmatis* cells were treated with 500 μM CN^- (data not shown). There are two functional terminal oxidases in the ETC of *M. smegmatis*, aa_3 cytochrome *c* oxidase and *bd* quinol oxidase (46, 47). The activity of aa_3 cytochrome *c* oxidase is inhibited by NO and CN^-, while *bd*-type quinol oxidase is insensitive to CN^- (46). The induction of *ald* expression by SNP and heat might be a consequence of partial inhibition of the respiratory ETC. Since the intracellular level of alanine reflects the functional state of the respiratory ETC, AldR can recognize not only alanine directly but also respiration-inhibitory signals, such as hypoxia, SNP, heat, etc.

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