

Mycobacterium smegmatis L-Alanine Dehydrogenase (Ald) Is Required for Proficient Utilization of Alanine as a Sole Nitrogen Source and Sustained Anaerobic Growth†

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NAD(H)-dependent L-alanine dehydrogenase (EC 1.4.1.1) (Ald) catalyzes the oxidative deamination of L-alanine and the reductive amination of pyruvate. To assess the physiological role of Ald in *Mycobacterium smegmatis*, we cloned the *ald* gene, identified its promoter, determined the protein expression levels, and analyzed the combined effects of nutrient supplementation, oxygen availability, and growth stage on enzyme activity. High Ald activities were observed in cells grown in the presence of L- or D-alanine regardless of the oxygen availability and growth stage. In exponentially growing cells under aerobic conditions, supplementation with alanine resulted in a 25- to 50-fold increase in the enzyme activity. In the absence of alanine supplementation, 23-fold-higher Ald activities were observed in cells grown exponentially under anaerobic conditions. Furthermore, *M. smegmatis ald* null mutants were constructed by targeted disruption and were shown to lack any detectable Ald activity. In contrast, the glycine dehydrogenase (EC 1.4.1.10) (Gdh) activity in mutant cells remained at wild-type levels, indicating that another enzyme protein is responsible for the physiologically relevant reductive amination of glyoxylate. The *ald* mutants grew poorly in minimal medium with L-alanine as the sole nitrogen source, reaching a saturation density 100-fold less than that of the wild-type strain. Likewise, mutants grew to a saturation density 10-fold less than that of the wild-type strain under anaerobic conditions. In summary, the phenotypes displayed by the *M. smegmatis ald* mutants suggest that Ald plays an important role in both alanine utilization and anaerobic growth.

NAD(H)-dependent L-alanine dehydrogenase (EC 1.4.1.1) (Ald) catalyzes the oxidative deamination of L-alanine to pyruvate and ammonia (catabolic reaction) or, in the reverse direction, the reductive amination of pyruvate to L-alanine (biosynthetic reaction). Ald has been extensively studied in many microorganisms since it was first discovered in *Bacillus subtilis* (51). Ald plays a key role in the utilization of carbon and nitrogen sources in various microorganisms, thus providing a link between carbohydrate and amino acid metabolic pathways (6). In addition, Ald is required for spore formation in *B. subtilis* (37) and fruiting body development in *Myxococcus xanthus* (44). The specific functions of Ald are different in different bacterial species and include a catabolic role in *B. subtilis* for energy production during spore germination (37) and a biosynthetic role in *Bradyrhizobium japonicum* (38, 39), *Rhizobium leguminosarum* (1), and *Pseudomonas* sp. strain MA (4) for nitrogen assimilation. However, its primary functions in many other microorganisms remain unknown.

Ald activity was discovered in *Mycobacterium tuberculosis* H37Ra in 1959 (17). In the early 1990s, studies with monoclonal antibodies against *M. tuberculosis* led to identification of a 40-kDa antigen recognized by HBT-10. This protein was demonstrated to be a functional L-alanine dehydrogenase (2). Ald

is one of the predominant antigens in *M. tuberculosis* culture filtrate, although it lacks a signal peptide, like several other *M. tuberculosis* extracellular proteins. It has been suggested that Ald may play a role in peptidoglycan biosynthesis since both L-alanine and D-alanine are important components of mycobacterial peptidoglycan. However, immunoblot analysis failed to detect Ald in *Mycobacterium bovis* BCG strains (2). Similar results were obtained by proteomic analysis (24). Thus, the physiological role of Ald in mycobacteria has not been elucidated.

Information about the regulation of Ald and its possible role in mycobacterial pathogenesis is limited. In the saprophytic organism *Mycobacterium smegmatis*, Ald is a cytosolic protein and is overproduced under anaerobic conditions which mimic the dormancy state of *M. tuberculosis* (14, 22, 32, 43). This result has led to the hypothesis that Ald contributes to maintenance of the NAD pool (i.e., recycling of NADH) (22). By microarray analysis, the *M. tuberculosis ald* transcript was recently shown to be overproduced under hypoxic conditions (36), under nutrient starvation conditions (5), or by sodium dodecyl sulfate (SDS) treatment in a *sigE* mutant (26). On the basis of biochemical evidence, Usha et al. (43) suggested that reductive amination of pyruvate and reductive amination of glyoxylate are catalyzed by the same protein in *M. smegmatis*, presumably Ald. Transcription induction of the *ald* gene has been observed upon infection of leopard frogs with *Mycobacterium marinum* (9). The role of Ald in the adaptation of pathogenic and saprophytic mycobacteria to physiological stresses needs to be investigated. In this study, we characterized the expression and regulation of the *M. smegmatis ald*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference and/or source
Strains		
<i>E. coli</i> XL10-GOLD	Tet ^r Δ (<i>mcrA</i>)183183 Δ (<i>mcrCB</i> - <i>hsdSMR</i> - <i>mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> Hte [F' <i>proAB lacI^qZ</i> Δ M15 Tn10 (Tet ^r) Amy Cam ^r]	Stratagene
<i>M. smegmatis</i> mc ² 155	High-transformation mutant of <i>M. smegmatis</i> mc ² 6	40
<i>M. smegmatis</i> GPM258	mc ² 155(pBUN246); overproducing Ald	This study
<i>M. smegmatis</i> GPM267	mc ² 155 Δ <i>ald::aph</i> ; <i>ald</i> null mutant	This study
<i>M. smegmatis</i> GPM268	mc ² 155 Δ <i>ald::aph</i> ; <i>ald</i> null mutant	This study
<i>M. smegmatis</i> GPM269	mc ² 155 Δ <i>ald::aph</i> ; <i>ald</i> null mutant	This study
<i>M. smegmatis</i> GPM270	mc ² 155 Δ <i>ald::aph</i> ; <i>ald</i> null mutant	This study
<i>M. smegmatis</i> GPM276	GPM267(pBUN279); <i>M. smegmatis ald</i> mutant complemented with the wild-type <i>ald</i> gene integrated at the mycobacteriophage L5 <i>attB</i> site	This study
<i>M. smegmatis</i> GPM282	mc ² 155(pBUN286a); wild-type <i>M. smegmatis</i> carrying an additional copy of the <i>ald</i> promoter at the mycobacteriophage L5 <i>attB</i> site	This study
Plasmids		
pBluescript II KS+	Standard <i>E. coli</i> cloning vector	Stratagene
pCR2.1	TA cloning vector	Invitrogen
pUC4K	Plasmid containing an excisable aminoglycoside 3'-phosphotransferase gene from Tn903	Amersham Pharmacia (28)
pMV262	<i>E. coli</i> - <i>Mycobacterium</i> shuttle plasmid	MedImmune (41)
pPR27	<i>E. coli</i> - <i>Mycobacterium</i> shuttle plasmid with <i>sacB</i> and the temperature-sensitive mycobacterial <i>ori</i>	31
pYUB178	<i>E. coli</i> - <i>Mycobacterium</i> integration-proficient vector; integrates at the mycobacteriophage L5 <i>attB</i> site	29
pYUB412	<i>E. coli</i> - <i>Mycobacterium</i> integration-proficient vector; integrates at the mycobacteriophage L5 <i>attB</i> site	30
pBUN246	Recombinant plasmid isolated from an <i>M. smegmatis</i> genomic library which hybridized with the <i>M. tuberculosis ald</i> gene; 8.0-kb insert	This study
pBUN253	pCR2.1 with 1.5-kb PCR fragment containing complete <i>M. smegmatis ald</i> gene amplified with SMALDCF1 and SMALDCR	This study
pBUN261	pCR2.1 with 0.4-kb PCR fragment containing the <i>M. smegmatis ald</i> promoter amplified with SMALDCF1 and SMALDPE	This study
pBUN268	pBluescript II KS+ with 1.5-kb <i>EcoRI</i> fragment from pBUN253 in the <i>EcoRI</i> site	This study
pBUN270a	pBUN268 carrying the 1.2-kb blunted <i>EcoRI</i> fragment of pUC4K containing <i>aph</i> gene for the 0.7-kb <i>NruI</i> fragment internal of the <i>ald</i> gene	This study
pBUN272	pPR27 with 2.2-kb <i>EcoRV/XbaI</i> fragment from pBUN270a in the <i>BamHI</i> (polished)/ <i>XbaI</i> site	This study
pBUN279	pYUB412 with 1.5-kb <i>EcoRI</i> fragment from pBUN253 in the <i>EcoRI</i> site	This study
pBUN286a	pYUB178 with 0.4-kb <i>EcoRI</i> fragment from pBUN261 in the <i>EcoRI</i> site	This study

gene. Increased Ald activity was observed in cells grown with alanine supplementation and under anaerobic conditions. Analysis of a null mutant demonstrated that Ald is necessary for proficient alanine utilization and sustained anaerobic growth.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown at 37°C in Luria-Bertani broth or agar. *M. smegmatis* strains were grown at 37°C with shaking (200 rpm; Innova 4300 incubator-shaker; New Brunswick Scientific, Edison, N.J.) in Middlebrook 7H9 broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with albumin-dextrose complex (ADC) and 0.05% Tween 80 (M-ADC-TW). When required, L-alanine was added at a concentration of 25 mM and D-alanine was added at a concentration of 45 mM as these concentrations support optimal growth of *M. smegmatis* (7). For analysis of nitrogen source utilization, *M. smegmatis* was grown in a minimal medium as previously described (8). Anaerobic conditions were achieved by using a GasPack system (BBL Microbiology Systems) with AnaeroPack (Mitsubishi Gas Chemical America, Inc., New York, N.Y.) as recommended by the manufacturer. To monitor the maintenance of anaerobic conditions in the system, indicator strips were placed in the jar and parallel cultures were grown in the presence of 1.5 μ g of methylene blue (AMRESCO Inc., Solon, Ohio) per ml. The dye faded by 24 h and became colorless by 48 h, indicating that oxygen was gradually depleted. The system remained anaerobic during sample manipulation and thereafter. Trans-

formations of *E. coli* and *M. smegmatis* were performed as described previously (15). The following antibiotics were added to the media when necessary: kanamycin (Sigma Chemical Co., St. Louis, Mo.) at a concentration of 50 μ g ml⁻¹, gentamicin (Sigma) at a concentration of 20 μ g ml⁻¹, or carbenicillin (Sigma) at a concentration of 50 μ g ml⁻¹ for *E. coli* strains; and kanamycin at a concentration of 10 μ g ml⁻¹, gentamicin at a concentration of 5 μ g ml⁻¹, or hygromycin B (Roche Molecular Biochemicals, Indianapolis, Ind.) at a concentration of 100 μ g ml⁻¹ for *M. smegmatis* strains.

Oligonucleotide primers, PCR, and primer extension analysis. The oligonucleotide primers (Integrated DNA Technologies, Inc., Coralville, Iowa) used for PCR amplification of the complete *ald* gene from *M. tuberculosis* were TBALDF (5'-TAT CCC GAC AGT GTC CGC TAA C-3') and TBALDR (5'-ACA TCA TCG CTT CCC TTA CTC C-3'). The following primer pairs were used for amplification of the *M. smegmatis ald* gene: SMALDF (5'-CGA TCA TGC TCG TCG GAA T-3') and SMALDR (5'-CGA TCA CGG TTC GGT TGT TA-3'), which amplify a 1.1-kb fragment used in Southern blot analysis; and SMALDCF1 (5'-CGG ATC ACA CCG ATC TCC-3') and SMALDCR (5'-CCT CAT CGG GGA ACT CAC-3'), which amplify a 1.5-kb fragment used for construction of mutant and complemented strains. Primer SMALDPE (5'-GTC AGC TCT GCC ACT CCA G-3') was used in primer extension analysis. PCR amplifications were performed with a Perkin-Elmer GeneAmp 9600 thermal cycler (Roche Molecular Systems, Branchburg, N.J.) by using the Expand High Fidelity PCR system (Roche) as recommended by the manufacturer. For Southern blot analysis, restriction digestion, ligation, and agarose gel electrophoresis, standard procedures were followed as previously described (34). Total RNA from *M. smegmatis* strains was isolated by using RNeasy (Qiagen, Crawfordsville, Ind.) with minor

modifications, as previously described (3). Primer extension analysis of the *ald* mRNA was carried out as described previously (12).

***M. smegmatis* genomic library construction and cloning of the *M. smegmatis ald* gene.** Chromosomal DNA from *M. smegmatis* mc²155 was prepared (50) and partially digested with *Sau*3AI, and 3.0- to 4.0-kb fragments were ligated into the *E. coli*-*Mycobacterium* shuttle plasmid pMV262 (41). The ligation mixture was transformed into *E. coli* XL10-GOLD (Stratagene, La Jolla, Calif.), and approximately 6,000 recombinants were obtained for a theoretical representation of $P > 99\%$ of the *M. smegmatis* genome. Since only the *M. tuberculosis* genomic database was available at the start of these experiments, the *M. smegmatis ald* gene was isolated from a genomic library by using a probe from *M. tuberculosis*. The *M. tuberculosis ald* gene was amplified from H37Rv genomic DNA by using primers TBALDF and TBALDR and was radiolabeled by using the Rediprime II labeling system (Amersham Pharmacia Biotech, Piscataway, N.J.). After three rounds of library screening, recombinant plasmid pBUN246 was obtained and confirmed to contain the complete coding region of the *ald* gene. Based on the partial sequence obtained from The Institute for Genomic Research (TIGR) website (<http://www.tigr.org>), the full-length sequence of the *ald* gene in pBUN246 was obtained by primer walking at the DNA Sequencing & Synthesis Facility of Iowa State University (Ames, Iowa).

Two-dimensional gel electrophoresis and protein N-terminal sequencing. Cell crude extract from *M. smegmatis* strains was prepared as previously described (8). Two-dimensional gel electrophoresis was performed by using a PROTEAN IEF cell (Bio-Rad) with immobilized pH gradient strips that were 7 cm long and had a pH range of 4 to 7 (Bio-Rad). Crude extracts containing approximately 600 μ g of protein were precipitated with 10% trichloroacetic acid and resuspended in rehydration buffer containing 8 M urea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM dithiothreitol, and 0.2% Bio-Lytes 3/10. The strips were actively rehydrated at 50 V for 12 h and focused at 4,000 V for a total of 60,000 V · h. Subsequently, the strips were equilibrated in a solution containing 6 M urea, 275 mM Tris-HCl (pH 6.8), 2% SDS, 0.5 mM Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP), and 20% glycerol and placed on the second dimension (10 to 20% linear gradient of polyacrylamide; Bio-Rad). The gel was electrophoresed at 125 V in 0.3% SDS-Tris-glycine buffer (pH 8.3), and polypeptide spots were visualized by Coomassie blue staining. Proteins from a parallel preparation were electroblotted for 2 h at 45 V onto polyvinylidene difluoride membranes in electrotransfer buffer (10% methanol, Tris-glycine; pH 8.3) by using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). Protein spots on the membranes were visualized by amido black staining, and the proteins of interest were excised from the membranes for automated Edman sequencing. Protein sequencing was performed with an ABI-Procise 494 sequencer (Applied Biosystems, Foster City, Calif.) by using manufacturer-recommended protocols.

Enzyme assays. L-Alanine dehydrogenase activity was measured by using a spectrophotometric method in the direction of oxidative deamination coupled to the production of NADH as described previously (23). All samples were measured in triplicate. Specific activities were expressed in micromoles of L-alanine (substrate) consumed per milligram per minute. No net changes in absorbance were detected when L-alanine was omitted from the reaction mixtures containing active extracts. The reductive amination reaction could not be measured in crude cell extracts due to interference by the lactate dehydrogenase enzyme that uses pyruvate and NADH as substrates. Glycine dehydrogenase activity was determined based on optical measurement of the rate of oxidation of NADH coupled with the reductive amination of glyoxylate as described previously (18). All samples were measured in triplicate. Specific activities were expressed in micromoles of sodium glyoxylate (substrate) consumed per milligram per minute. No net changes in absorbance were detected when sodium glyoxylate was omitted from the reaction mixtures containing active extracts. Consistent with previous observations (18, 43, 48), the oxidative deamination activity could not be detected.

Construction of *M. smegmatis ald* null mutants. To construct *M. smegmatis ald* null mutants, the internal 0.7-kb *Nru*I fragment of the *ald* gene in pBUN268 was replaced by the 1.3-kb kanamycin-resistant determinant from pUC4K (28), yielding pBUN270a. The inactivated allele was cloned into pPR27, a plasmid carrying a gentamicin resistance marker, a temperature-sensitive mycobacterial origin of replication, and a *sacB* gene as a counterselectable marker (31). The resulting plasmid, pBUN272, was electroporated into *M. smegmatis* mc²155, and recombinants were selected at 32°C on medium containing 10 μ g of kanamycin per ml. One of these recombinants was grown in M-ADC-TW to saturation at 32°C, and approximately 10⁹ cells were plated onto Middlebrook 7H9-ADC agar containing kanamycin and 10% sucrose and incubated at 39°C. Five days later, kanamycin- and sucrose-resistant (Kan^r Suc^r) colonies appeared, and they were screened individually for gentamicin sensitivity (Gen^s), as the Kan^r Suc^r Gen^s

phenotype was expected if there was allelic exchange and the plasmid vector was lost. Of the 476 Kan^r Suc^r colonies obtained, 4 were confirmed to be Gen^s. The remaining colonies were Kan^r Suc^r Gen^r and probably resulted from inactivation of the *sacB* gene and/or the leaky temperature-sensitive phenotype of the plasmid. The four Kan^r Suc^r Gen^s colonies were chosen as putative mutants for further study and were designated GPM267, GPM268, GPM269, and GPM270.

Nucleotide sequence accession number. The sequence of the *M. smegmatis* L-alanine dehydrogenase gene cloned in pBUN246 has been deposited in the GenBank database under accession number AF304867.

RESULTS

Cloning and sequence analysis of the *M. smegmatis ald* gene.

A genomic library of mc²155 was constructed in the *E. coli*-*Mycobacterium* replicating vector pMV262, and *E. coli* recombinant clones were screened by colony hybridization. This screening analysis yielded a positive clone carrying a recombinant plasmid with an 8.0-kb insert, which was designated pBUN246. This multicopy plasmid was introduced into wild-type strain mc²155 to generate recombinant strain GPM258. To confirm that pBUN246 carries a functional full-length *ald* gene, a cell extract was prepared and assayed for enzymatic activity. As expected, GPM258 displayed a higher level of Ald activity (approximately 90-fold higher) than the wild-type strain (data not shown).

Sequence analysis of the insert in pBUN246 revealed four open reading frames coding for a putative AsnC family transcriptional regulator homologous to the *M. tuberculosis* Rv2779c, alanine dehydrogenase (Ald), a putative β -lactamase homologous to *Mycobacterium fortuitum* BlaF (42), and a putative peptidase homologous to *M. tuberculosis* PepR (Rv2782c). The *ald* gene appears to comprise a monocistronic message since the flanking open reading frames are encoded on the strand opposite the strand containing *ald*. Consistent with this hypothesis, primer extension analysis of RNA isolated from cells grown in Middlebrook medium under aerobic conditions revealed the presence of a promoter immediately upstream from the *ald* gene (Fig. 1). A transcriptional start site was identified at a guanosine residue, 27 nucleotides upstream from the start codon (ATG) followed by a putative ribosome binding site (GAAGGAG) five nucleotides upstream. No alternative transcriptional start site was observed when primer extension was performed with RNA isolated from cells grown in minimal medium and shifted to anaerobic conditions (data not shown). The coding region of the *M. smegmatis ald* gene contains 1,116 nucleotides, and it encodes a 39.0-kDa protein consisting of 371 amino acids. A putative rho-independent transcriptional terminator (ATCGCCCGCCGGAA and TTCCGGCCGGGCGAT) was located 13 nucleotides downstream of the stop codon (TAA).

Alignment of the amino acid sequences of several bacterial L-alanine dehydrogenases showed that the *M. smegmatis* Ald polypeptide exhibits 79, 80, and 76% amino acid identity to the homologous polypeptides of *Mycobacterium leprae* (11), *M. tuberculosis* (10), and *Mycobacterium avium* (TIGR), respectively. It also exhibits approximately 60% identity to isozymes of *Streptomyces coelicolor* (33) and *B. subtilis* (37). The multiple alignment revealed two highly conserved regions with the consensus sequences K⁷³VKEP⁷⁷ and v¹⁷³iGgGtaGyNAARiA-GmGa-VTvlDvn²⁰⁰, which correspond to the enzyme active site

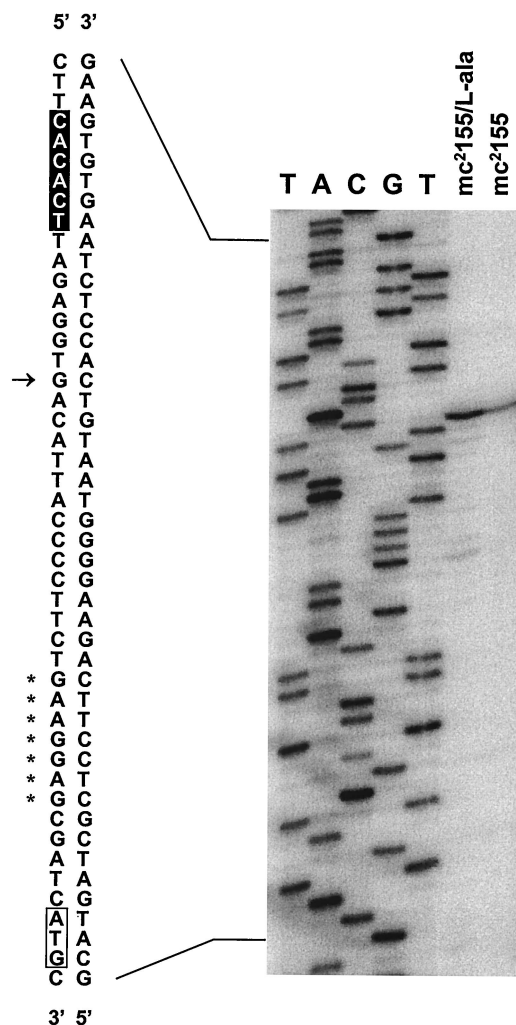


FIG. 1. Primer extension analysis of the *ald* transcript from *M. smegmatis*. Total RNA (50 μ g) from mc²155 cells, grown in either M-ADC-TW or M-ADC-TW supplemented with L-alanine, was annealed with primer SMALDPE and extended as described in Materials and Methods. Lanes A, C, G, and T show a dideoxy sequencing ladder of the *ald* gene generated with the same primer. The transcriptional start site is indicated by an arrow. The putative -10 box deduced from the consensus of mycobacterial promoter sequences is highlighted (19). The start codon (ATG) is enclosed in a box, and the putative ribosome binding site (GAAGGAG) is indicated by asterisks.

for pyruvate binding (13) and the NAD cofactor binding region (2), respectively.

Ald activity is regulated by nutrient supplementation, oxygen availability, and growth stage. Regulation of Ald has been studied in various microorganisms. In *Bacillus* spp. Ald is produced at high levels upon induction by alanine, during sporulation, and at the onset of the stationary phase (16, 27, 37). In *M. smegmatis* and *M. tuberculosis*, expression of the *ald* gene has been shown to be up-regulated under limiting oxygen conditions (22, 36, 43). These findings suggest that multiple factors are involved in the regulation of Ald. However, previous studies of mycobacterial Ald have not investigated the combined effects of nutrient supplementation, oxygen availability, and growth stage. To gain further insight into the function and

regulation of Ald, we analyzed the expression of the *M. smegmatis ald* gene in exponentially growing or stationary cells in the presence of L- or D-alanine under aerobic or anaerobic conditions. We first analyzed the effect of alanine on the expression of the *ald* gene. A dramatic increase in the transcription of the *ald* gene was observed when cells were grown in the presence of L-alanine (Fig. 1). The increase in the mRNA level corresponded to a similar level of overproduction of a polypeptide spot (molecular weight, 42,000; pI 5.8) induced by L- or D-alanine, as observed in two-dimensional gels (Fig. 2). We confirmed that this differentially expressed spot corresponded to Ald since N-terminal sequencing analysis yielded a sequence (MLVGIPTEIK) identical to that deduced from translation of the *M. smegmatis ald* gene.

To quantify the increase in Ald enzyme activity in cells grown with L- or D-alanine under aerobic conditions, crude cell extracts were prepared and assayed (Fig. 3). In exponentially growing cells, addition of either L- or D-alanine to the growth medium resulted in an approximately 50-fold increase in enzyme activity. In stationary cells, Ald was produced at a level that was 25-fold greater than the basal level in cells grown without alanine. The basal level of Ald activity in stationary cells was approximately fourfold higher than the level in exponentially growing cells. Under anaerobic conditions, the basal level of Ald activity in exponentially growing cells was 23-fold higher than the level in cells grown under aerobic conditions. Addition of L- or D-alanine increased Ald activity by twofold, and the activity reached approximately the same level as the level in exponentially growing cells under aerobic conditions. When anaerobically grown cells reached the stationary phase, the basal level of Ald activity decreased by threefold, while in the presence of alanine the Ald activity increased to a level similar to that in exponentially growing cells. In summary, high levels of Ald activity were observed in cells grown in the presence of L- or D-alanine regardless of the oxygen availability and growth stage. However, in the absence of alanine, high basal levels of Ald activity were observed in cells growing exponentially under anaerobic conditions.

Isolation and characterization of *M. smegmatis ald* null mutants. To further analyze the physiological role of Ald, *M. smegmatis ald* null mutants were generated by allelic exchange (Fig. 4A) as described in Materials and Methods. Four putative mutants (GPM267 to GPM270) were analyzed further. To determine whether the mutant strains carry the inactivated *ald* allele, genomic DNA was isolated and amplified by PCR with primers SMALDCF1 and SMALDR. As expected for inactivation of the *ald* gene, genomic DNA from all four mutant strains yielded a single 2.2-kb product distinct from the 1.5-kb fragment from mc²155 DNA (data not shown). In addition, Southern blot analysis was used to verify the occurrence of homologous recombination events in these putative *ald* mutants. Genomic DNA was isolated, digested with either *Apa*I or *Pst*I, transferred to a membrane, and hybridized with the wild-type *ald* gene fragment as a probe. After digestion with *Apa*I, wild-type strain mc²155 gave a 3.0-kb band (Fig. 4B, lane 1) and all of the mutants yielded a 3.6-kb band (Fig. 4B, lanes 2 to 5); after digestion with *Pst*I, mc²155 gave a 2.7-kb band (Fig. 4B, lane 6) and all of the mutants yielded two bands at approximately 0.9 and 1.1 kb (Fig. 4B, lanes 7 to 10). These patterns were expected for the predicted recombinational

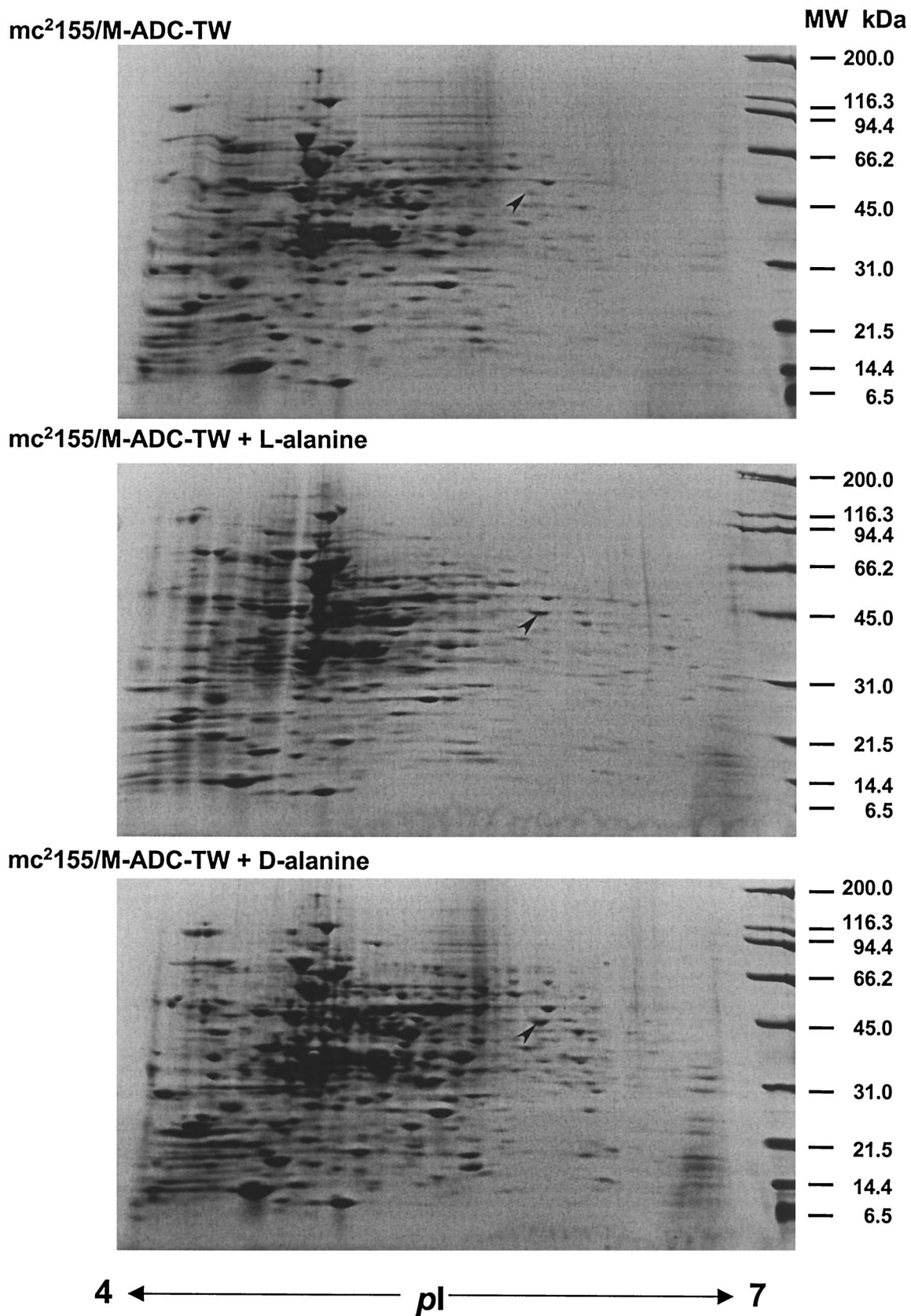
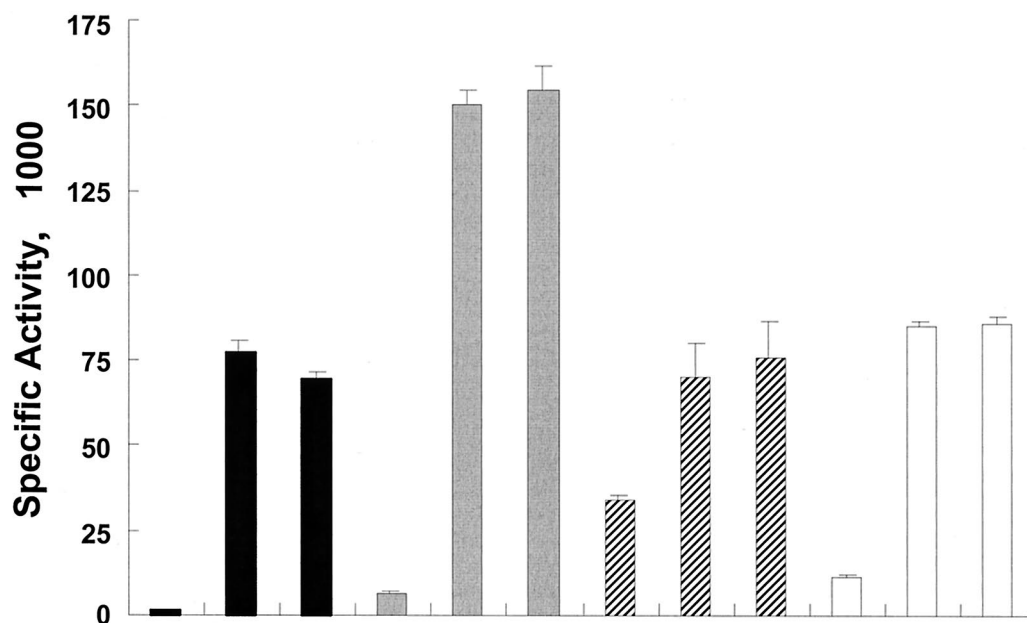


FIG. 2. Two-dimensional gel electrophoresis analysis of *M. smegmatis* mc²155 cellular proteins. Cells were grown in M-ADC-TW or M-ADC-TW supplemented with L- or D-alanine as indicated. Cell extracts were prepared as described in Materials and Methods. Approximately 600 μ g of total proteins was resolved by two-dimensional gel electrophoresis and stained with Coomassie blue. Isoelectric points (pI) are indicated at the bottom, and the positions of molecular mass size markers are indicated on the right. The location of the Ald enzyme (42 kDa, pI 5.8) in each gel is indicated by an arrowhead.



Presence of Alanine	-	L	D	-	L	D	-	L	D	-	L	D
Presence of Oxygen		+			+			-			-	
Phase of Growth	Exponential			Stationary			Exponential			Stationary		
Relative Ald sp act	1.0	52	47	4.2	102	105	23	48	51	7.7	58	58

FIG. 3. Analysis of *M. smegmatis* L-alanine dehydrogenase activities. Cells were grown under either aerobic or anaerobic conditions to the exponential or stationary phase in M-ADC-TW or M-ADC-TW supplemented with L- or D-alanine as indicated. The relative Ald specific activities were calculated by assigning a value of 1.0 to the specific activity of the extract prepared from exponentially growing cells incubated under aerobic conditions without alanine. The specific activities are expressed in micromoles of L-alanine per milligram per minute. The values are means \pm standard deviations of triplicate measurements.

events, validating the construction of the *ald* null mutants. No differences in colony morphology were observed between mc²155 and the *ald* null mutants when the organisms were plated on Middlebrook 7H9-ADC agar. Likewise, no differences in growth rate and saturation density were observed in M-ADC-TW broth (data not shown). Therefore, the *ald* gene of *M. smegmatis* can be inactivated and is nonessential.

***M. smegmatis ald* mutants have no detectable L-alanine dehydrogenase activity.** To determine whether *M. smegmatis ald* mutants are or are not devoid of Ald activity, crude cell extracts from wild-type strain mc²155, *ald* mutant GPM267, and the complemented strain GPM276 were prepared and assayed for Ald activity (Fig. 4C). Crude extracts prepared from GPM267 cells grown in the presence or absence of L-alanine lacked any detectable Ald activity. These extracts yielded background levels of Ald activity that were not significantly different from the levels obtained by substituting bovine serum albumin for GPM267 extracts in the reaction mixture. In complemented strain GPM276, which resulted from integration of a single copy of the wild-type *ald* gene into the GPM267

chromosome at the mycobacteriophage L5 *attB* site, the Ald activity was restored. When the organisms were grown in M-ADC-TW supplemented with L-alanine, the Ald activity of complemented strain GPM276 was slightly lower than that of the wild-type strain. This activity was about sixfold higher than that of the wild-type strain when the organisms were grown without L-alanine. The Ald activity of recombinant strain GPM282, which carried an additional copy of the *ald* promoter at the mycobacteriophage L5 *attB* site, was increased by approximately twofold. This suggests that the presence of two copies of the *ald* promoter and positional effects within the chromosome may moderately alter expression of the gene. We concluded that inactivation of the *ald* gene results in no detectable Ald activity, indicating that Ald is the only protein responsible for this activity in *M. smegmatis*.

Inactivation of the *ald* gene does not affect glycine dehydrogenase activity. Increased glycine dehydrogenase (EC 1.4.1.10) (Gdh) activity has been found in *M. tuberculosis* and *M. bovis* BCG during anaerobic growth, suggesting that Gdh contributes to maintenance of the NAD pool (25, 45, 47). Based on

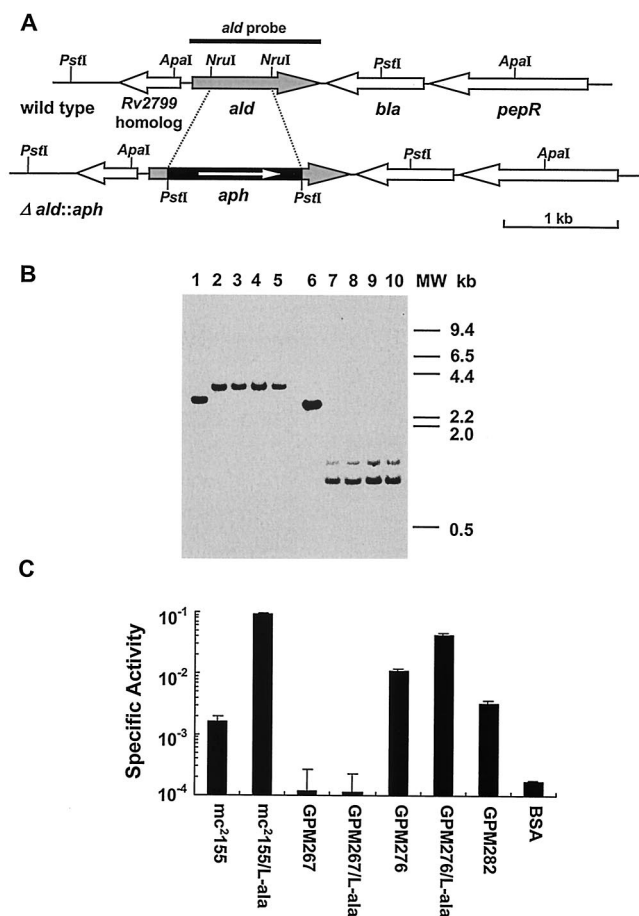


FIG. 4. Construction and characterization of *M. smegmatis* *ald* null mutants. (A) Open reading frame organization at the *ald* locus in both strain mc^2155 (wild type) and the *ald* null mutant strain ($\Delta ald::aph$). Relevant restriction enzyme sites and the PCR fragment used as the *ald* probe are indicated. (B) Southern blot analysis of genomic DNA from wild-type and *ald* null mutant strains. Genomic DNA was digested with *Apa*I (lanes 1 to 5) or *Pst*I (lanes 6 to 10). The strains examined are wild-type strain mc^2155 (lanes 1 and 6) and *ald* mutants GPM267 (lanes 2 and 7), GPM268 (lanes 3 and 8), GPM269 (lanes 4 and 9), and GPM270 (lanes 5 and 10). Blots were hybridized with the radiolabeled 1.1-kb PCR fragment containing the *ald* gene and washed under stringent conditions as described in Materials and Methods. MW, molecular weight. (C) Analysis of L-alanine dehydrogenase activities in *M. smegmatis* wild-type, mutant, and recombinant strains. Extracts from organisms grown in both M-ADC-TW and M-ADC-TW supplemented with L-alanine were prepared and analyzed. Ald specific activities are expressed in micromoles of L-alanine per milligram per minute. The values are means \pm standard deviations of triplicate measurements. A mock assay was also carried out with bovine serum albumin (BSA) in place of equivalent amounts of the cell extracts.

biochemical evidence, two recent reports suggested that reductive amination of pyruvate and reductive amination of glyoxylate are catalyzed by the same protein in both *M. smegmatis* (43) and *M. tuberculosis* (V. Usha, B. C. Elias, R. Jayaram, S. Ravishankar, and K. S. Das, Abstr. Tuberculosis Past, Present, Future, abstr. 151, 2000). If the physiological Gdh activity is carried by Ald, the *M. smegmatis* *ald* null mutant cells would be expected to lack Gdh activity. To test this hypothesis, we assayed Ald and Gdh activities in crude extracts of the wild-type,

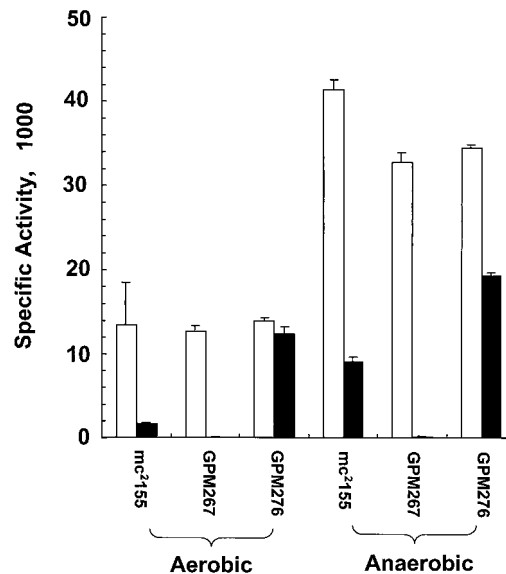


FIG. 5. Analysis of L-alanine dehydrogenase and glycine dehydrogenase activities in wild-type, *ald* null mutant, and complemented strains. *M. smegmatis* was grown in M-ADC-TW to an optical density at 600 nm of approximately 0.8 under aerobic conditions. Approximately one-half of each culture was harvested, and the remaining cells were shifted to anaerobic growth for 24 h. Crude cell extracts were prepared and assayed for Ald (solid bars) and Gdh (open bars) activities as described in Materials and Methods. Specific activities are expressed in micromoles of substrate per milligram per minute. The values are means \pm standard deviations of triplicate measurements.

ald mutant, and complemented strains by using cells grown either under aerobic conditions or following an anaerobic shift (Fig. 5). Ald activity was significantly higher under anaerobic conditions for the wild-type and complemented strains, while it was undetectable for the mutant strain either under aerobic conditions or following an anaerobic shift. However, all of the strains displayed similar Gdh activities under either aerobic or anaerobic conditions, with an approximately fourfold increase following an anaerobic shift, as previously observed (43). Since the Gdh activity in the *ald* null mutant was not affected, we concluded that another enzyme protein is responsible for the physiological Gdh activity in *M. smegmatis*.

***M. smegmatis* *ald* mutants are impaired in the utilization of alanine as a sole nitrogen source.** To further characterize the role of the Ald enzyme in nitrogen metabolism, the wild-type, *ald* null mutant, and complemented strains were tested for the ability to utilize various nitrogen sources. When ammonium chloride or L-asparagine was used as the sole nitrogen source, the *ald* null mutant utilized either source as proficiently as wild-type strain mc^2155 and the complemented strain (Fig. 6A and data not shown). However, the mutant grew poorly on L-alanine as the sole nitrogen source, while the wild-type and complemented strains displayed similar growth rates and saturation densities on both L-alanine and ammonium chloride (Fig. 6B). With L-alanine as the sole nitrogen source, the mutant reached a saturation cell density of 2.5×10^7 CFU ml^{-1} , which was approximately 10-fold greater than the density of the initial inoculum but 100-fold less than the densities of the wild-type and complemented strains. Similar results were obtained when D-alanine was used as the sole nitrogen source in

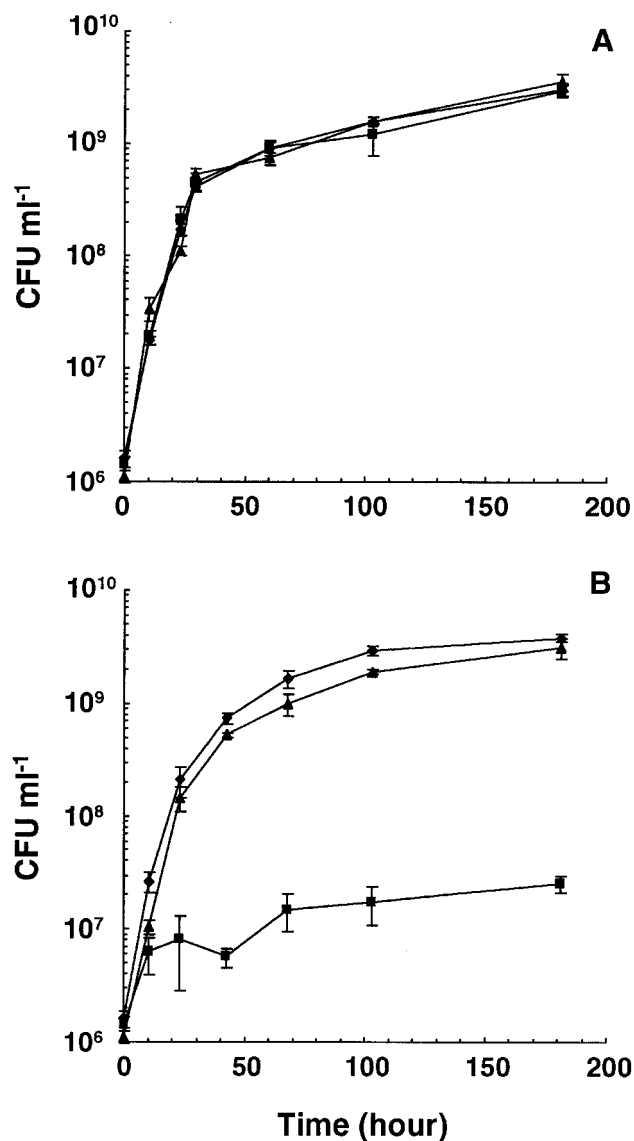


FIG. 6. Utilization of nitrogen sources by *M. smegmatis* wild-type, *ald* null mutant, and complemented strains. Exponentially growing cells of mc²155 (◆), GPM267 (▲), and GPM276 (■) were harvested, washed, and inoculated into minimal medium containing ammonium chloride (A) or L-alanine (B) as the sole nitrogen source at an initial density of approximately 2.0×10^6 CFU ml⁻¹. At various times, the number of viable cells was determined.

broth, as well as when cells were grown on solid agar with either L-alanine or D-alanine as the sole nitrogen source (data not shown). Therefore, *M. smegmatis* Ald is required for proficient utilization of alanine as a nitrogen source.

***M. smegmatis ald* mutants are impaired for anaerobic growth.** Since mycobacterial Ald is overproduced during growth under low-oxygen conditions (22, 36, 43; also see above), we tested the effect of inactivation of the *M. smegmatis ald* gene on anaerobic growth. The growth of the wild-type strain was substantial during the first 7 days and then continued at a lower rate, and the cell density reached approximately 2×10^8 CFU ml⁻¹ at 20 days postinoculation. The *ald* null

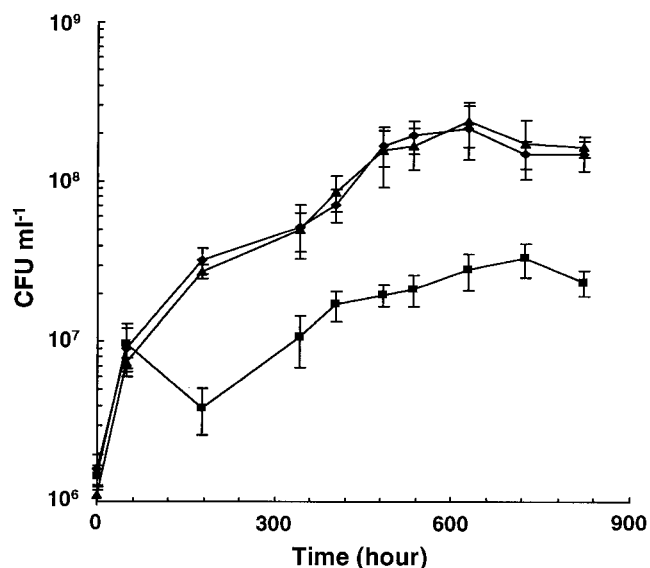


FIG. 7. Growth of *M. smegmatis* wild-type, *ald* null mutant, and complemented strains under anaerobic conditions. Exponentially growing cells of mc²155 (◆), GPM267 (▲), and GPM276 (■) were inoculated into M-ADC-TW at an initial density of approximately 2.0×10^6 CFU ml⁻¹ in a GasPack system as described in Materials and Methods. At various times, the number of viable cells was determined.

mutant was able to reach a cell density similar to that of the wild-type strain 2 days after inoculation. However, at later time points, its growth was minimal, and the saturation density was 10-fold lower than that of the wild-type strain (Fig. 7). No differences were observed between the complemented and wild-type strains. We concluded that the *M. smegmatis* Ald is necessary for optimal growth under anaerobic conditions.

DISCUSSION

Regulation of L-alanine dehydrogenase is determined by multiple factors. In *Bacillus* spp., L-alanine dehydrogenase is repressed by glucose and is induced by alanine and other amino acids (16), entry into the stationary phase, or spore germination (27, 37). In mycobacterial species, Ald is overproduced during anaerobic growth of *M. smegmatis* (22, 43). Likewise, the *M. tuberculosis ald* gene is up-regulated 10-fold under hypoxic conditions (36). Ald from *Mycobacterium* strain HE5 is stimulated by L-alanine and the xenobiotic compound morpholine (35). In our studies of *M. smegmatis* Ald, we found that the presence of either stereoisform of alanine led to a 50-fold increase in Ald activity for exponentially growing cells under aerobic conditions and a 25-fold increase for stationary cells, reflecting the catabolic role of Ald when alanine is abundant. The fourfold induction of the basal Ald activity in the absence of alanine by entry into the stationary phase may be due to protein turnover with the concomitant increase in alanine in the intracellular amino acid pool. The elevated Ald activity observed when wild-type cells are supplemented with alanine under aerobic conditions is consistent with the impaired utilization of alanine as a sole nitrogen source in the *ald* mutant. These data suggest that oxidative deamination by Ald is the main route by which *M. smegmatis* utilizes alanine.

Under anaerobic conditions, the Ald activity was elevated 23-fold under basal conditions, and addition of alanine resulted in only a twofold increase. This indicates that Ald may play an important role in growth under anaerobic conditions since this increase is observed in exponentially growing cells. This hypothesis is consistent with our observation that there was a threefold decrease in the basal Ald activity upon entry into the stationary phase under anaerobic conditions. At physiological pH, *M. tuberculosis* Ald seems to play a biosynthetic role in catalyzing the reductive amination of pyruvate, which is the thermodynamically favored conversion ($K_{eq} = 5.5 \times 10^{-11}$) (17, 23). In addition to generating alanine for protein and peptidoglycan biosynthesis, this reaction may also contribute to the recycling of NADH, which is necessary for growth under anaerobic conditions when oxygen as the terminal electron acceptor becomes depleted. Consistent with this biosynthetic role of Ald under anaerobic conditions, we found that the *M. smegmatis* *ald* null mutants displayed a defective phenotype during anaerobic growth. However, the mutant multiplied to a cell density approximately 10 times greater than that of the initial inoculum, suggesting that another enzyme(s) may also participate in the recycling of NADH under anaerobic conditions. In the Wayne dormancy culture model, the 10-fold increase in *M. tuberculosis* Gdh activity has been proposed to contribute to maintenance of the NAD pool by reductive amination of glyoxylate (47, 48). However, the corresponding glycine dehydrogenase gene has not yet been identified. In the *M. tuberculosis* genome, one gene (*gcvB*) codes for a putative glycine dehydrogenase, but its product probably catalyzes the decarboxylation of glycine instead of the reductive amination of glyoxylate (10, 48). Purified Ald from either *Bacillus megaterium* or *Mycobacterium* strain HE5 has been reported to catalyze the reductive amination of glyoxylate (20, 35). Two recent reports have also suggested that the same enzyme protein, presumably Ald, from either *M. smegmatis* or *M. tuberculosis* is responsible for the physiological Ald and Gdh activities (43; also see above). In contrast, our data showed wild-type levels of Gdh activity in the *ald* mutant under either aerobic or anaerobic conditions. Consistently, *M. bovis* BCG lacks a functional Ald enzyme (2, 24) as a consequence of a frameshift mutation in the *ald* gene (21), while increased Gdh activity was observed under anaerobic conditions (25). Therefore, the identities of the Gdh enzyme and the corresponding gene in mycobacteria remain unknown.

In summary, the phenotypes displayed by the *M. smegmatis* *ald* mutants suggest that Ald plays an important role in both alanine utilization and anaerobic growth. In strains of the *M. tuberculosis* complex, the roles of Ald and other enzymes involved in adaptation to anaerobiosis are less clear. *M. bovis* BCG (Ald⁻) (2, 24) and *M. tuberculosis* (Ald⁺) (2) behave in similar ways in the Wayne model of adaptation to anaerobiosis (25, 45). In *M. tuberculosis*, increased nitrate reduction by nitrate reductase encoded by the *narGHIIJ* cluster was observed during shift-down from aerobic to anaerobic growth (46). Although homologous genes have been identified in the *M. smegmatis* genome (TIGR), it was observed that *M. smegmatis* mc²155 does not display this enzyme activity even under anaerobic conditions (49). The recently constructed *M. tuberculosis* *ald* mutant should be a useful tool to further assess the role of Ald in pathogenesis (S. C. Woolwine, C. Ko, S. Tyagi,

and W. R. Bishai, Abstr. 102nd Gen. Meet. Am. Soc. Microbiol., abstr. U-53, 2002). Comparative analysis of *M. smegmatis* and *M. tuberculosis* *ald* mutants may reveal whether there are important differences in the adaptations of saprophytic and pathogenic mycobacteria to anaerobic growth conditions.

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