Mycobacterium bovis BCG Vaccines Exhibit Defects in Alanine and Serine Catabolism

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Mycobacterium bovis BCG is the only accepted vaccine for the prevention of tuberculosis (TB) in humans. BCG is a live vaccine, and induction of immunity to TB requires productive infection of the host by BCG. However, BCG is not a satisfactory vaccine, because it fails to protect against pulmonary TB in adults. In this study, we found that BCG strains cannot utilize many naturally occurring amino acids as the sole nitrogen source for growth. This defect is caused, at least partially, by the lack of functional metabolic enzymes. All BCG strains are unable to catabolize L-alanine or D-alanine due to a frameshift mutation in the L-alanine dehydrogenase gene (ald). Some BCG strains, such as BCG-Pasteur and BCG-Frappier, cannot catabolize L-serine, apparently due to inadequate expression of L-serine deaminase (sdaA). We also found that undegraded alanine and serine inhibit the growth of BCG through blockage of glutamine synthetase. These results suggest that BCG strains are limited in nitrogen metabolic capacity and predict defects that may restrict multiplication and persistence of the live vaccine within the host.

Bacille Calmette-Guérin (BCG), an attenuated strain of Mycobacterium bovis isolated in 1921, is currently administered to over 100 million newborns per year as the only available vaccine against tuberculosis (TB) (13). BCG is only efficacious as a live vaccine; killed BCG does not provide protection in animal models, and killed Mycobacterium tuberculosis in human trials provided only weak and transient protection. In clinical trials of BCG vaccination, the observed efficacy has ranged from no protection to 80% fewer cases of TB (11, 13). A better understanding of this variable efficacy should help guide BCG programs and suggest avenues for the development of novel anti-TB vaccines.

Several hypotheses have been generated to explain the vaccine trial data (3). The most prominent is that exposure to environmental mycobacteria partially sensitizes the host against mycobacteria and thereby provides heterologous immunity that obscures the potential benefits of BCG vaccination (14, 15). To support this, a recent study showed that the multiplication of the Danish strain of BCG was inhibited in animals previously sensitized with environmental mycobacteria. Consequently BCG vaccination elicited only a transient immune response and afforded no protection against a subsequent TB challenge (8). A second hypothesis involves differences in the vaccine strains used in clinical trials (9). After their introduction in 1921, BCG vaccines were maintained by in vitro passage in a variety of vaccine laboratories for 4 to 5 decades, resulting in phenotypic and genetic differences between BCG strains (6, 27). The capability of each of these BCG vaccines to protect against TB is unknown, because most clinical trials were performed with vaccine strains that have not been preserved. Notably, among genetic changes in BCG strains after 1921, one observes deletions in regulatory genes (which are postulated to govern the capacity to survive in the host) and antigenic proteins (which are known to elicit a host immune response) (5).

Survival of members of the M. tuberculosis complex, including BCG, within host tissues appears to depend on the ability of the bacteria to reorient their metabolism and utilize any available source of carbohydrate, nitrogen, and energy (4). As an example, a recent study revealed that fatty acids serve as a source of carbon and are required for persistence of M. tuberculosis in both mice and activated macrophages (26). In another study, a BCG strain lacking anaerobic nitrate reductase, an enzyme essential for nitrate respiration, failed to persist in mice (16). Thus for understanding mycobacterial pathogenesis, it is important to determine the metabolic capacity and limitations of members of the M. tuberculosis complex, including BCG.

In this study, we found that BCG strains cannot utilize L-alanine, D-alanine, or L-serine as the sole nitrogen source for growth and that the growth of some BCG strains is severely inhibited by these amino acids, even when ammonium chloride is provided as an additional nitrogen source. We show that the absence of functional L-alanine dehydrogenase and L-serine deaminase is responsible for the failure of BCG to catabolize alanine and serine, respectively. We further show that alanine and serine inhibit the growth of BCG through blockage of glutamine synthetase (GS).

MATERIALS AND METHODS

Materials. All chemicals and amino acids were purchased from Sigma. Monoclonal antibody (HBF10) against L-alanine dehydrogenase of M. tuberculosis H37Rv was kindly provided by A. B. Andersen and P. Andersen (Statens Serum Institut, Copenhagen, Denmark).

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**Bacterial strains and culture conditions.** Twelve *M. bovis* BCG strains were used in this study: BCG-Japan, BCG-Russia, BCG-Moreau, BCG-Sweden, BCG-Birkhaug, BCG-Frappier, BCG-Pasteur, BCG-Blasco, BCG-Phipps, BCG-Tice, BCG-Denmark, and BCG-Prague. The identities and histories of these strains have been described in detail previously (6). Middlebrook 7H9 medium (Difco) contains the following (per liter): 0.5 g of ammonium sulfate, 0.5 g of l-glutamate, 0.1 g of sodium citrate, 1 mg of pyridoxine, 0.5 mg of biotin, 2.5 g of disodium phosphate, 1 g of monopotassium phosphate, 40 mg of ferric ammonium citrate, 50 mg of magnesium sulfate, 0.5 mg of calcium chloride, 1 mg of zinc sulfate, 1 mg of copper sulfate, and 2 ml of glycerol. The medium was supplemented with 10% ADC (5 g of bovine albumin [fraction V], 2 g of dextrose), with 0.05% Tween 80 added after sterilization. Sauton medium contains the following (per liter): 4 g of l-asparagine, 0.5 g of monopotassium phosphate, 0.5 g of magnesium sulfate, 50 mg of ferric ammonium citrate, 2 g of citric acid, 1 mg of zinc sulfate, and 60 ml of glycerol (with 0.05% Tween 80 added after sterilization). For amino acid growth experiments, a basal Sauton medium was prepared by omitting l-asparagine. Glycerol-alanine-salts (GAS) medium contains (per liter) 2 g of ammonium chloride, 1 g of l-alanine, 0.3 g of Bacto Casitone (Difco), 4 g of dibasic potassium phosphate, 2 g of citric acid, 50 mg of ferric ammonium citrate, 1.2 g of magnesium chloride hexahydrate, 0.6 g of potassium sulfate, 1.8 ml of 10 M sodium hydroxide, and 10 ml of glycerol. Tween 80 was added to 0.05% after sterilization. An l-alanine-free version of GAS medium was prepared by omitting the l-alanine. BCG cultures were grown at 37°C with constant shaking for 2 to 4 weeks. *Escherichia coli* DH5α, which was used for routine manipulation and amplification of plasmid DNA, was grown in Luria-Bertani (LB) broth or agar (Difco). When necessary, the medium was supplemented with kanamycin at 25 μg/ml for mycobacteria and 50 μg/ml for *E. coli.*

**Molecular cloning.** An ordered BAC library of the *M. tuberculosis* H37Rv genome (obtained from S. Cole, Institut Pasteur, Paris, France) was used as the DNA template for cloning. Standard protocols were used for manipulation of DNA. Cloning of ald (Rv2780) was accomplished in two steps. First, a 4.5-kb Scal fragment of BAC415 was ligated to Eco13HII-linearized pUC19 to generate pUC-ald. Then mycobacterial plasmid pALD was created by ligating the 1.9-kb KpnI fragment containing the ald gene to KpnI-linearized pMD31. To clone sdaA (Rv0096C), a 9.5-kb BamHI fragment of BAC60 was ligated to BamHI-linearized pMD31 to generate pSDA1. Plasmid pSDA2 was generated by cleavage of pSDA1 with PstI, followed by self-ligation of the 10.9-kb PstI fragment. To clone ald (Rv0096C), a 9.5-kb NheI fragment of BAC214 was first inserted into shuttle vector pMD31 linearized with XbaI to generate pGln, which contains the glnA1, glnA2, and glnE genes. To generate pGLN1A, pGLN was cut with KpnI and religated, which removes all of glnA2 and the N-terminal portion of glnE, but leaves the entire glnE gene intact.

The constructed plasmids and control vectors were introduced by electroporation into *M. bovis* BCG, and recombinant *M. bovis* was selected on Middlebrook 7H11 agar (Difco) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment and 25 μg of kanamycin per ml.

**DNA sequence analysis of ald and sdaA.** The ald and sdaA genes of BCG-Japan, -Pasteur, and -Frappier were amplified by PCR. Template genomic DNA was prepared by established protocol. Oligonucleotide primers ALD-F (5'-TG AAGCGTACAGTATCGAGAGGGGTA-3') and ALD-R (5'-CGCTCTACCCGGAACTGCAC-3') were used for PCR amplification of ald. Oligonucleotide primers SDAA-F (5'-CGCTCTACCCGGAACTGCAC-3') and SDAA-R (5'-CGTGTGCTCAAGCAACCGTGGTTCGCTC-3') were used for PCR of sdaA. The ald and sdaA PCR products were purified and sequenced from both 5' and 3' ends by using the 5' and 3' PCR primers as well as internal primers. DNA sequencing was carried out at the Core Sequencing Facility of York University, Toronto, Canada, and confirmed by independent DNA sequencing at the Montreal General Hospital, Montreal, Canada.

**Western blot analysis of l-alanine dehydrogenase.** Culture filtrate proteins (CFP) and cell lysate (CL) proteins of *Mycobacterium marinum, BCG-Japan, -Frappier, and -Pasteur* were prepared from cultures grown in Sauton (containing l-asparagine) or Sauton basal medium with 5 or 96 mM ammonium chloride. To prepare CFP, bacterial cultures were centrifuged, and the supernatant was passed through a 0.45-μm-pore-diameter filter. The cell-free CFP was dialyzed against double-distilled water and lyophilized. Cell lysates were prepared as follows. Cell pellets were washed and resuspended with 50 mM Tris-HCl (pH 7.5)–10 mM β-mercaptoethanol, and then they were homogenized by a Mini-Beadbeater cell disruptor (Biospec Products, Bartlesville, Okla.). The homogenate was centrifuged twice, and the cell lysates were obtained. For Western blotting, equivalents of CFP or CL proteins (5 μg per lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were then probed with anti-Ald (1:1,000 dilution) antibody. The secondary antibodies (Sigma) used were anti-mouse antibodies conjugated to alkaline phosphatase.

**Nucleotide sequence accession number.** The sequences of ald of *M. bovis* BCG-Japan, -Frappier, and -Pasteur have been submitted to the GenBank database under accession no. AF531175 to AF531177.

**RESULTS**

**Growth of BCG strains in GAS medium.** During the course of our studies, we found that the BCG-Japan strain was able to grow in GAS medium, albeit more slowly than in 7H9 medium, whereas BCG-Frappier and BCG-Pasteur did not show any visible growth, despite prolonged incubation (2 months). The growth capabilities of other BCG strains in GAS, 7H9, and Sauton media were also examined. The Sauton and 7H9 media supported the growth of all BCG strains, but only BCG-Japan, -Russia, -Moreau, -Sweden, and -Birkhaug strains could grow in GAS medium (Table 1). The chemical compositions of GAS, 7H9, and Sauton media were compared to identify ingredients that might account for this differential growth. GAS supplemented with ZnSO4 (1 mg/liter), which is present in 7H9 and Sauton media, but not in GAS medium, or sodium pyruvate (0.5%), which is required for growth of large colonies of *M. bovis,* did not improve the growth of BCG strains in GAS (data not shown). Next, nitrogen sources were compared. l-Asparagine (27 mM) is the main nitrogen source in GAS. When l-asparagine (at 27 mM) was added to GAS medium, BCG-Frappier, -Pasteur, -Glaxo, -Phipps, -Tice, -Denmark, and -Prague strains grew rapidly (Table 1). GAS medium supplemented with l-aspartate, l-glutamine, or l-glutamate also supported the growth of these BCG strains, but no other amino acids did (Table 1). These results suggest that BCG strains vary in their ability to catabolize amino acids.

**Amino acids as the nitrogen source for growth of BCG strains.** Next, we examined the ability of BCG strains to utilize each amino acid as the sole nitrogen source for growth. Since GAS medium contains a small amount of Bacto Casitone (0.3 g/liter), which is a complex mixture of various amino acids and peptides, we chose Sauton medium, which is a defined medium, for this study. The original Sauton medium requires 27 g/liter, which is a complex mixture of various amino acids and peptides, we chose Sauton medium, which is a defined medium, for this study. The original Sauton medium requires 27
BCG strains grew rapidly when L-asparagine, L-aspartate, L-glutamine, or L-glutamate was the sole nitrogen source. BCG-Japan was also able to grow on cationic amino acids (e.g., L-arginine and L-lysine), whereas BCG-Pasteur and -Frappier could not. Interestingly, none of the BCG strains grew when L-alanine, L-serine, L-leucine, L-isoleucine, L-methionine, or L-arginine and L-lysine), whereas BCG-Pasteur and -Frappier could not. Interestingly, none of the BCG strains grew when L-alanine, L-serine, L-leucine, L-isoleucine, L-methionine, or L-phenylalanine were utilized and degraded, as measured by removal of these amino acids (supplied at 1 g/liter) from the culture supernatant of M. tuberculosis (23). In particular, L-serine, L-alanine, L-asparagine, and L-aspartate were depleted by more than 50%, while the other amino acids were utilized approximately 40% (23). Our results indicated that the BCG strains exhibit defects in amino acid catabolism that are not found in (or distinguish them from) other mycobacteria.

L-Alanine, D-alanine, and L-serine inhibit the growth of BCG. One surprising result from the study described above is that all BCG strains can grow on ammonium chloride as the sole nitrogen source at either low (1 mM) or high (27 mM) concentrations (Table 2). This is contrary to the results obtained with GAS medium, in which L-alanine replaced by D-alanine (hatched bar) (A) or GAS in which L-alanine replaced by L-alanine (open bar), GAS without L-alanine (closed bar) and, GAS containing D-alanine (hatched bar) supplemented with 27 mM L-asparagine (B). Cultures were incubated at 37°C with constant shaking for 16 days, and then 2-ml aliquots of cell culture were centrifuged and the cell pellet was lyophilized to determine cell dry weight.

![FIG. 1. Inhibition of BCG growth by L-alanine and D-alanine in GAS. BCG-Japan, -Frappier, and -Pasteur, grown to stationary phase in 7H-ADC-glycerol-Tween 80 liquid medium, were inoculated to a cell density of 2 x 10⁷ cells per ml into triplicate 5-ml volumes of GAS medium (open bar), GAS medium without L-alanine (solid bar), and GAS supplemented with 27 mM L-asparagine (hatched bar) (A) or GAS in which L-alanine replaced by D-alanine (open bar), GAS without L-alanine (closed bar) and, GAS containing D-alanine (hatched bar) supplemented with 27 mM L-asparagine (B). Cultures were incubated at 37°C with constant shaking for 16 days, and then 2-ml aliquots of cell culture were centrifuged and the cell pellet was lyophilized to determine cell dry weight.](http://iai.asm.org/...)

### TABLE 2. Growth of M. bovis BCG and other mycobacteria in Sauton medium with an amino acid as the single nitrogen source

<table>
<thead>
<tr>
<th>Nitrogen source</th>
<th>BCG-Japan</th>
<th>BCG-Frappier</th>
<th>M. marinum</th>
<th>M. smegmatis</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Asn</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Asp</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Glu</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Gln</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Cys</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Arg</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-His</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Lys</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Pro</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Ala</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Ser</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Leu</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Ile</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Met</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gly</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Phe</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Tyr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Val</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L-Thr</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Each amino acid at a final concentration of 27 mM was used to supplement Sauton basal medium (containing no asparagine). NH₄Cl was tested at 1 and 27 mM.

** Growth studies with M. marinum were performed at 30°C, and studies with the other bacteria were performed at 37°C.

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mM L-asparagine, which we replaced with the same concentration of each type of amino acid. Ammonium chloride at 27 or 1 mM as the sole nitrogen source in Sauton medium was also tested. Table 2 summarizes the results for three representative BCG strains: BCG-Japan, -Pasteur, and -Frappier. Consistent with the results summarized in Table 1, these three BCG strains grew rapidly when L-asparagine, L-aspartate, L-glutamine, or L-glutamate was the sole nitrogen source. BCG-Japan was also able to grow on cationic amino acids (e.g., L-arginine and L-lysine), whereas BCG-Pasteur and -Frappier could not. Interestingly, none of the BCG strains grew when L-alanine, L-serine, L-leucine, L-isoleucine, L-methionine, or glycine was the sole nitrogen source. Other Mycobacterium species, including the pathogenic species M. avium (25) and M. marinum, as well as the nonpathogenic species M. smegmatis, were able grow on most of these amino acids as the sole nitrogen source (Table 2). A previous study showed that M. tuberculosis was able to grow in Sauton medium containing L-alanine, L-asparagine, L-aspartate, L-glutamate, or L-glutamine as the sole nitrogen source (24). Although the use of other amino acids as the sole nitrogen source for the growth of M. tuberculosis has not been formally studied, L-serine, L-arginine, L-glycine, L-isoleucine, L-leucine, and L-phenylalanine were utilized and degraded, as measured by removal of these amino acids (supplied at 1 g/liter) from the culture supernatant of M. tuberculosis (23). In particular, L-serine, L-alanine, L-asparagine, and L-aspartate were depleted by more than 50%, while the other amino acids were utilized approximately 40% (23). Our results indicated that the BCG strains exhibit defects...
L-alanine completely inhibited the growth of BCG and the growth of BCG-Japan, -Frappier, and -Pasteur was containing NH4Cl (96 mM) with increasing concentrations of L-alanine in Sauton medium containing NH4Cl (96 mM). BCG-Pasteur, -Frappier, and -Japan, grown to stationary phase in 7H9-ADC-L-asparagine was replaced by ammonium chloride (96 mM), L-alanine in Sauton medium containing NH4Cl (96 mM). BCG-Pasteur and -Frappier strains were inoculated into triplicate 5-ml volumes of Sauton medium containing NH4Cl (96 mM) with increasing concentrations of L-alanine. BCG-Pasteur and -Frappier strains expressing ald grew rapidly in GAS medium containing L-alanine or D-alanine. BCG-Japan transformed with either L-alanine dehydrogenase. To test this, the ald gene of M. tuberculosis was cloned into shuttle vector pMD31 and transformed into the BCG-Frappier, -Pasteur, and -Japan strains. Expression of ald in BCG relieves the growth inhibition by L-alanine and D-alanine. L-Alanine can be used as the sole nitrogen source for growth of most Mycobacterium species, including M. tuberculosis, M. avium, and M. smegmatis (24, 25). L-Alanine can be broken down to ammonium and pyruvate by L-alanine dehydrogenase. D-Alanine can be converted via racemization to L-alanine and then acted upon by L-alanine dehydrogenase. The L-alanine dehydrogenase of M. tuberculosis H37Rv is encoded by the ald gene (Rv2780). The biochemical activity of the M. tuberculosis enzyme has been demonstrated in vitro. Ald converts L-alanine to pyruvate and ammonium, and the activity is highly specific for L-alanine (22). Interestingly, this enzyme was detected in the culture supernatant fraction of M. tuberculosis, but not in M. bovis BCG-Japan nor BCG-Copenhagen, even though DNA hybridization (Southern blotting) showed that the ald gene is present in both BCG strains (2). To determine if the ald gene is disrupted in BCG, the ald alleles of BCG-Japan, -Pasteur, and -Frappier were amplified by PCR, and the DNA sequences were determined. DNA sequencing revealed that all three BCG alleles contain an identical point deletion (nucleotide A at 266) in ald, which results in a frameshift mutation and prevents expression of a full-length L-alanine dehydrogenase protein (data not shown). Genome sequence data obtained from the Sanger Center revealed that the M. bovis type strain AF2122/97 (spoligotype 9) contains the same point deletion. This suggests that the ald mutation was already present in the M. bovis strain employed by Calmette and Guérin to derive the BCG vaccine.

We postulated that the failure of BCG strains to catabolize L- or D-alanine is caused by the absence of a functional L-alanine dehydrogenase. To test this, the ald gene of M. tuberculosis was cloned into shuttle vector pMD31 and transformed into the BCG-Frappier, -Pasteur, and -Japan strains. Western blot analysis confirmed that the L-alanine dehydrogenase protein was produced in the recombinant strains (see below). These recombinant BCG strains were tested for their ability to grow in GAS medium containing L-alanine or D-alanine. BCG-Pasteur and -Frappier strains expressing M. tuberculosis ald grew rapidly in GAS medium containing either L-alanine or D-alanine, whereas control strains containing pMD31 did not grow more rapidly (Fig. 3). These results confirm that the inhibitory effects of alanine are due to the absence of a functional alanine dehydrogenase and indicate that the accumulation of undegraded alanine inhibits growth of BCG strains in GAS medium.

Expression of sdaA in BCG relieves the growth inhibition by L-serine. L-Serine is a good nitrogen source for the growth of M. tuberculosis and M. smegmatis, but not M. bovis BCG (Table 2). We hypothesized that BCG lacks a functional enzyme for the degradation of L-serine. In E. coli, two L-serine deaminases, SDA and SDA2, are involved in the catabolism of L-serine (23). We postulated that SDA is encoded by ald and SDA2 by another gene. To determine if this is the case, we cloned the ald gene of M. tuberculosis into shuttle vector pMD31 and transformed it into the BCG-Frappier, -Pasteur, and -Japan strains. Expression of sdaA in BCG relieves the growth inhibition by L-serine.

L-asparagine was replaced by ammonium chloride (96 mM), and the growth of BCG-Japan, -Frappier, and -Pasteur was examined (Fig. 2). Strikingly, very low concentrations (0.3 mM) of L-alanine completely inhibited the growth of BCG-Frappier and BCG-Pasteur. The effect on BCG-Japan was somewhat less severe, but growth was significantly reduced upon addition of L-alanine at 0.5 mM and completely inhibited by 8 to 16 mM (Fig. 2). Taken together, these results demonstrate that L-alanine does not simply fail to support the growth of M. bovis BCG—it actually inhibits the growth of these vaccine strains.

We further found that D-alanine and L-serine also inhibit the growth of BCG strains. The presence of D-alanine (11 mM) in the aforementioned L-alanine-free GAS medium inhibited the growth of BCG-Pasteur and BCG-Frappier and significantly reduced the growth of BCG-Japan (Fig. 1B). Similarly, addition of L-serine (11 mM) to L-alanine-free GAS medium inhibited the growth of BCG-Frappier, BCG-Pasteur, and, to a lesser degree, BCG-Japan (data not shown).

Expression of sdaA in BCG relieves the growth inhibition by L-serine. L-Serine is a good nitrogen source for the growth of M. tuberculosis and M. smegmatis, but not M. bovis BCG (Table 2). We hypothesized that BCG lacks a functional enzyme for the degradation of L-serine. In E. coli, two L-serine deaminases,
encoded by the sdaA and sdaB genes, catalyze the deamination of L-serine to pyruvate and ammonia (30). The genome of M. tuberculosis H37Rv contains a homolog to sdaA (Rv0069c), but not sdaB. The sdaA gene of M. tuberculosis was cloned into pMD31 and electroporated into BCG-Frappier, -Pasteur, and -Japan. The recombinant BCG-Pasteur and -Frappier strains containing pSDAA grew rapidly in alanine-free GAS medium containing L-serine, whereas the vector control strains did not grow (Fig. 4). BCG-Japan strain carrying the sdaA gene also grew more rapidly than a strain containing pMD31 vector alone (Fig. 4). This result suggests that later BCG strains contain mutations that affect either the production or level of expression of L-serine deaminase. The absence of active L-serine deaminase would explain why BCG-Pasteur and BCG-Frappier cannot catabolize L-serine. To test this, we amplified the sdaA alleles of BCG by PCR and determined the DNA sequences. Sequence analysis revealed that the sdaA alleles of all three BCG strains are identical to the M. tuberculosis sdaA gene (and also the M. bovis sdaA sequence obtained from the Sanger Center [data not shown]). Thus, it appears that the failure of BCG-Pasteur and BCG-Frappier to catabolize L-serine is caused by inadequate L-serine deaminase expression, but not a defect in the sdaA gene.

L-Alanine, D-alanine, and L-serine inhibit GS of BCG. We have shown that BCG-Pasteur and BCG-Frappier lack functional L-alanine dehydrogenase and L-serine deaminase activities and cannot catabolize alanine or L-serine. We now suggest that this undegraded alanine and serine prevent the growth of BCG strains by inhibiting GS activity. GS plays a central role in nitrogen metabolism in bacteria (29). Working in tandem with glutamate synthase (GOGAT), GS catalyzes the synthesis of glutamine and glutamate. GS assimilates nitrogen by aminating L-glutamate in an ATP-dependent reaction. The L-glutamine formed by GS is in turn used by GOGAT to generate L-glutamate, which together provide nitrogen for almost all amino acids, proteins, and nucleotides.

In gram-negative bacteria, such as E. coli, Salmonella enterica, and Klebsiella aerogenes, GS is under feedback inhibi-
Purified GS is inhibited in vitro by L-alanine, L-serine, and glycine, which bind to the substrate site for glutamate (29). In *M. tuberculosis* and *M. bovis* BCG, GS has been identified as an extracellular protein encoded by *glnA1* (Rv2220) (18). An inhibitor of GS, L-methionine-S-sulfoximine, blocked the multiplication of pathogenic mycobacteria, including *M. tuberculosis* and *M. bovis* BCG, demonstrating that GS is essential in these bacteria (17). This raised the possibility that undegraded alanine and serine could inhibit GS and subsequently prevent the growth of BCG. Therefore, we hypothesized that overexpression of *glnA1* in BCG should alleviate the sensitivity of BCG to alanine and serine.

To test this possibility, the *M. tuberculosis* *glnA1* gene was cloned into pMD31 and transformed into BCG-Frappier and BCG-Pasteur. Consistently, the recombinant strains containing *glnA1* grew rapidly in alanine-free GAS medium supplemented with high concentrations of (11 mM) L-serine or D-alanine, whereas the vector control strains did not (Fig. 5A). However, the recombinant strains were still unable to grow in the original GAS medium, which contains L-alanine (Fig. 5A). It is possible that L-alanine has stronger inhibitory effect on GS than D-alanine and L-serine and that the level of GS is still not high enough to relieve this inhibition. Others have shown that in *M. tuberculosis* and *M. avium*, the ammonium concentration in the culture medium profoundly influences the GS activity: an increase in the concentration of ammonium from 7.6 to 76 mM results in a 10-fold decrease in GS activity (1, 18). The standard GAS medium contains a high concentration of ammonium (38 mM) that may decrease GS activity. Indeed, when the ammonium concentration in GAS medium was lowered to 5 mM, the BCG-Frappier strain overexpressing *glnA1* was able to grow in the presence of L-alanine (Fig. 5B). Taken together, these results indicate that undegraded alanine and serine inhibit BCG growth through blockage of GS.

**Regulation of ald and sdaA expression by ammonium.** We found that the expression of *ald* is regulated by the ammonium concentration of the culture medium. Western blotting showed that L-alanine dehydrogenase was detected in recombinant BCG strains carrying the *M. tuberculosis* *ald* gene grown in Sauton medium, but not in Sauton medium in which asparagine was replaced by ammonium (96 mM) (Fig. 6). A similar result was obtained with *M. marinum*, a fish pathogen that...
contains a functional chromosomal \textit{ald} gene (Fig. 6). These results suggest that expression of \textit{ald} is suppressed by a high ammonium concentration, which would explain why, even in the presence of low concentrations of alanine, recombinant BCG containing \textit{ald} was still unable to grow in Sauton medium containing ammonium (96 mM) (similar to Fig. 2 [data not shown]). Alternatively, GS may be posttranslationally modified and have very low activity at 96 mM ammonium, such that only a small amount of t-alanine is required for total inhibition of GS. Similarly, recombinant BCG strains containing \textit{sdaA} did not grow in Sauton medium containing ammonium (96 mM) when t-serine was present (data not shown), presumably for the same reasons.

**DISCUSSION**

In this study, we found that unlike other mycobacteria, \textit{M. bovis} BCG strains have a limited ability to utilize amino acids as the sole nitrogen source (Table 2). In addition, early and late BCG strains differ in their ability to catabolize amino acids. Late BCG strains, such as BCG-Pasteur and BCG-Frappier, can grow only on five types of amino acids (Table 2). These differences may be important determinants of mycobacterial pathogenicity and may contribute to variations in BCG vaccine efficacy. Our results show that defects in metabolic
enzymes, including L-alanine dehydrogenase and L-serine deaminase, are responsible for the failure of BCG strains to catabolize amino acids. However, introduction of ald and sdaA into BCG strains, which enables them to break down alanine and serine, respectively, does not restore their ability to grow on these amino acids as the sole nitrogen source (data not shown), suggesting additional unknown mechanisms are involved. More studies are required to elucidate these mechanisms.

L-Alanine dehydrogenase, encoded by ald, catalyzes the oxidative deamination of L-alanine or, in the reverse reaction, the reductive amination of pyruvate. This enzyme is known to be required for normal sporulation in Bacillus subtilis (33) and for normal development of Myxococcus xanthus (35). In mycobacteria, L-alanine dehydrogenase is associated with bacterial persistence during infection. Expression of ald is upregulated in M. tuberculosis upon nutrient starvation (7) and in M. marinum during persistence within the granulomas of infected frogs (10). L-Alanine dehydrogenase production and activity were also increased when M. tuberculosis and M. smegmatis were shifted from aerobic to anaerobic growth (21, 31, 34). In contrast, Western blotting has failed to detect L-alanine dehydrogenase in BCG, even though the ald gene has been identified by DNA hybridization (2). We confirmed that strains of BCG do not express L-alanine dehydrogenase and determined that this absence is due to a single nucleotide deletion within the ald gene that causes a frameshift mutation and disrupts the full-length protein. A functional copy of ald was able to complement this metabolic defect. Unlike the control strains, recombinant BCG strains, expressing the ald gene from M. tuberculosis, produced L-alanine dehydrogenase (as detected by Western blotting [Fig. 6]) and were able to grow in GAS medium containing L-alanine or D-alanine. We also confirmed that expression of M. tuberculosis ald, as expressed in BCG, and the native chromosomal ald of M. marinum increases with decreasing ammonium concentration. Consistent with this, a recent study of a mycobacterial strain, HE5, showed that L-alanine dehydrogenase activity was not detectable in a bacterium grown on pyruvate and ammonium (5.6 mM), but was at its highest level when the bacterium was grown on L-alanine as the nitrogen source (32). These findings suggest that the primary role of L-alanine dehydrogenase is the oxidative deamination of L-alanine under nitrogen-limiting conditions. This is in contrast to an in vitro analysis of the purified mycobacterial enzyme that indicated the pH optimum is 10 to 11 for the oxidative deamination reaction and 7 to 7.5 for the reductive amination reaction (22). Because these data suggested that the deamination reaction occurred outside the physiological range, it was assumed that in vivo, L-alanine dehydrogenase exclusively catalyzed the formation of L-alanine. Our study demonstrates that the alanine deamination reaction catalyzed by Ald predominates in vivo.

Our growth experiments indicated that early derivatives of M. bovis BCG, such as BCG-Japan, were able to catabolize L-serine, while later strains, including BCG-Pasteur and BCG-Frappier, could not (Fig. 4). However, the sdaA alleles of BCG-Japan, -Pasteur, and -Frappier are identical to each other and to the published sdaA genes of M. bovis and M. tuberculosis and are predicted to encode a functional L-serine deaminase. Thus, the failure of BCG-Pasteur and BCG-Frappier to catabolize L-serine is not due to a defect in sdaA, but likely results from inadequate L-serine deaminase expression. Consistent with this, we found that the growth defect in BCG-Pasteur and BCG-Frappier is complemented by overexpression of M. tuberculosis sdaA.

Although defects in the ald gene and the expression of sdaA impair catabolism of some amino acids, the mutations themselves are not lethal. The inhibitory effects are only manifested upon addition of alanine or L-serine. Our studies suggest that accumulation of undegraded alanine or serine impairs activity of GS. Others have shown that potent inhibitors of GS, such as L-methionine-5-sulfoximine, inhibit M. tuberculosis and M. bovis BCG growth both in vitro and in vivo (17). Antisense RNA to gna1 mRNA also prevents M. tuberculosis growth (19). These data indicate that GS is an essential enzyme. In this work, growth of BCG was impaired by addition of alanine or L-serine, amino acids known to inhibit GS activity. Moreover, expression of M. tuberculosis ald or sdaA in BCG, which should complement the metabolic defect and restore amino acid catabolism such that GS activity is no longer impaired, did relieve the growth inhibition by alanine or L-serine. We also found that addition of L-glutamine, L-glutamate, L-asparagine, and L-aspartate enabled the growth of BCG strains in the alanine-rich GAS medium (Table 1). Glutamine circumvents the GS pathway. Asparagine and aspartate can be catabolized to yield glutamate, which at increased intracellular concentration can outcompete alanine and serine for the glutamate binding site on GS, relieving the inhibition. Finally, we found that expression of M. tuberculosis gna1 in BCG also alleviated the growth inhibition by alanine and L-serine. Although expression of gna1 should not prevent the accumulation of inhibitory amino acids, it is likely that complementation increases GS to a level at which some activity is not inhibited.

Survival and persistence of the live BCG vaccine are necessary to elicit protective immunity. It has been shown that early treatment of infected mice with isoniazid, which inhibits BCG growth, prevents the development of effective acquired resistance (12). Similarly, killed BCG strains elicit an immune response that is weak and transient (28). A recent study also showed that, in a mouse model of infection, prior exposure to live environmental mycobacteria blocked the multiplication of BCG, and consequently, BCG failed to provide protective immunity against a TB challenge (8). The continuous secretion of many different antigens by live BCG is likely important for the induction of protective immunity and is a key advantage over subunit vaccines or DNA vaccines that transiently produce a few antigens. Indeed, more than 200 different anti-TB vaccines have been tested to date, and the only vaccine candidate that has shown greater protection than BCG itself is a modified BCG strain expressing one antigen, Ag85 (20). Our findings—that BCG vaccine strains utilize limited types of amino acids as the nitrogen source for growth and that the growth of BCG is inhibited by naturally occurring L-alanine and L-serine—suggest that the ability of BCG to grow and persist within the vaccinated host is restricted. The concentration of L-alanine that is available to BCG growing in humans is estimated to be 0.33 to 0.42 mM (4), which is sufficient to inhibit the growth of BCG-Pasteur or BCG-Frappier and significantly reduce the growth of BCG-Japan (Fig. 2). Such inhibition by the host may prevent the development of long-term protective immunity.
and hence the lack of protection against pulmonary TB in adults. Further in vivo studies are required to test this hypothesis.

The novel findings presented in this paper demonstrate that there are differences in metabolic capacity between *M. bovis* BCG and other mycobacteria. A mutation in *ald*, which may have originated in *M. bovis*, distinguishes BCG from *M. tuberculosis, M. smegmatis*, and *M. marinum* and prevents the use of alanine as the sole nitrogen source for growth. In addition, we have identified differences among the BCG vaccine strains. Later derivatives of BCG appear to be more restricted in their ability to catabolize amino acids. These findings suggest new avenues for vaccine development. By complementing the reported deficits, such that amino acid utilization is no longer restricted, it may be possible to produce an improved BCG strain that survives better within the vaccinated host and so provides better protection against TB.

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