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High Extracellular Levels of *Mycobacterium tuberculosis* Glutamine Synthetase and Superoxide Dismutase in Actively Growing Cultures Are Due to High Expression and Extracellular Stability Rather than to a Protein-Specific Export Mechanism

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Glutamine synthetase (GS) and superoxide dismutase (SOD), large multimeric enzymes that are thought to play important roles in the pathogenicity of *Mycobacterium tuberculosis*, are among the bacterium's major culture filtrate proteins in actively growing cultures. Although these proteins lack a leader peptide, their presence in the extracellular medium during early stages of growth suggested that they might be actively secreted. To understand their mechanism of export, we cloned the homologous genes (*glnA1* and *sodA*) from the rapid-growing, nonpathogenic *Mycobacterium smegmatis*, generated *glnA1* and *sodA* mutants of *M. smegmatis* by allelic exchange, and quantitated expression and export of both mycobacterial and nonmycobacterial GSs and SODs in these mutants. We also quantitated expression and export of homologous and heterologous SODs from *M. tuberculosis*. When each of the genes was expressed from a multicopy plasmid, *M. smegmatis* exported comparable proportions of both the *M. tuberculosis* and *M. smegmatis* GSs (in the *glnA1* strain) or SODs (in the *sodA* strain), in contrast to previous observations in wild-type strains. Surprisingly, recombinant *M. smegmatis* and *M. tuberculosis* strains even exported nonmycobacterial SODs. To determine the extent to which export of these large, leaderless proteins is expression dependent, we constructed a recombinant *M. tuberculosis* strain expressing green fluorescent protein (GFP) at high levels and a recombinant *M. smegmatis* strain coexpressing the *M. smegmatis* GS, *M. smegmatis* SOD, and *M. tuberculosis* BfrB (bacterioferritin) at high levels. The recombinant *M. tuberculosis* strain exported GFP even in early stages of growth and at proportions very similar to those of the endogenous *M. tuberculosis* GS and SOD. Similarly, the recombinant *M. smegmatis* strain exported bacterioferritin, a large (~500-kDa), leaderless, multimeric protein, in proportions comparable to GS and SOD. In contrast, high-level expression of the large, leaderless, multimeric protein malate dehydrogenase did not lead to extracellular accumulation because the protein was highly unstable extracellularly. These findings indicate that, contrary to expectations, export of *M. tuberculosis* GS and SOD in actively growing cultures is not due to a protein-specific export mechanism, but rather to bacterial leakage or autolysis, and that the extracellular abundance of these enzymes is simply due to their high level of expression and extracellular stability. The same determinants likely explain the presence of other leaderless proteins in the extracellular medium of actively growing *M. tuberculosis* cultures.

Mycobacterium tuberculosis, the primary etiologic agent of tuberculosis, is one of the world's leading causes of death, killing 2 million persons annually worldwide (23). New modalities to combat *M. tuberculosis* and a greater understanding of the biology and immunology of this pathogen are high priorities of tuberculosis research.

The extracellular proteins of *M. tuberculosis* have been the focus of many studies investigating their role in host immunity, their potential as vaccine candidates and diagnostic reagents, and more recently as drug targets (4, 5, 7, 31, 32, 46, 50). Although sensitive techniques have identified hundreds of proteins in culture filtrates of *M. tuberculosis* (36, 56, 62), approximately 12 proteins are released in relatively large amounts (accounting for ~90% of total extracellular protein with a molecular mass of ≥10 kDa) and have been designated as the major extracellular proteins (reference 32 and unpublished data). Although most of the major extracellular proteins have typical leader peptides, the multimeric enzymes glutamine synthetase (GS) and superoxide dismutase (SOD) do not (29, 30, 71). GS and SOD are found in the extracellular medium of *M. tuberculosis* cultures in the early stages of growth, suggesting that they might be actively secreted by the bacterium. Both of these enzymes are considered to be strictly intracellular in most other bacteria.

GS is a dodecamer of identical 53-kDa subunits that has a central role in nitrogen metabolism, catalyzing the synthesis of L-glutamine from L-glutamate, ammonia, and ATP. Our group has identified GS as a major component of *M. tuberculosis* culture filtrates (28). We have proposed that GS may alter the ammonia level (and pH) of the host cell phagosome, possibly aiding the bacilli in preventing phagosome-lysosome fusion. We have also proposed that GS may be necessary for synthesis of poly-L-glutamate-glutamine

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Promoter[a]	Reference or source
Strains			
E. coli DH5α			Gibco BRL
M. smegmatis 1-2c	Wild-type strain with increased electroporation efficiency		76
M. smegmatis 1-2c *glnA1*	Insertionally inactivated *glnA1* locus, Km^r		This study
M. smegmatis 1-2c *sodA*	Insertionally inactivated *sodA* locus, Km^r		This study
M. tuberculosis Erdman	Wild-type strain		ATCC 35801
Plasmids			
pUC18	*E. coli* cloning vector		48
pUC19	*E. coli* cloning vector		75
pUC18-*Ms-glnA1*	*M. smegmatis glnA1* genomic locus		This study
pUC18-*Ms-sodA*	*M. smegmatis sodA* genomic locus		This study
pPR27	Mycobacterial allelic exchange vector, t.s. mycobacterial origin, *sacB*, Gm^r		51
pPR27-*Ms-glnA1*::Km^r	*glnA1* allelic exchange construct		This study
pPR27-*Ms-sodA*::Km^r	*sodA* allelic exchange construct		This study
pUC19-Km^r	Km^r cassette		This study
pNBV1	*E. coli*-mycobacterial shuttle vector, Hyg^r		33
pNBV1-*Ms*GS	*M. smegmatis glnA1*	Native	This study
pNBV1-*Ms*SODA	*M. smegmatis sodA*	Native	This study
pNBV1-*Mtb*GS	*M. tuberculosis glnA1*	Native	This study
pNBV1-*Mtb*SODA	*M. tuberculosis sodA*	Native	29
pNBV1-*Ec*SODA	*E. coli sodA*	*Mtb glnA1*	This study
pNBV1-*Ec*SODB	*E. coli sodB*	*Mtb glnA1*	This study
pNBV1-*Bs*SODA	*B. subtilis sodA*	*Mtb glnA1*	This study
pNBV1-*St*GS	*S. enterica* serovar Typhimurium *glnA*	*Mtb glnA1*	This study
pNBV1-GFPuv	UV-optimized GFP	BCG *hsp60*	This study
pNBV1-MDH	*M. tuberculosis mdh*	*Mtb glnA1*	This study
pNBV1-BFRB	*M. tuberculosis bfrB*	*Mtb glnA1*	This study
pNBV1-*Ms*GS-*Ms*SODA-MDH	*M. smegmatis glnA1, M. smegmatis sodA*, and *M. tuberculosis mdh*	Native, native, *Mtb glnA1*	This study
pNBV1-*Ms*SODA-*Ms*GS-BFRB	*M. smegmatis glnA1, M. smegmatis sodA*, and *M. tuberculosis bfrB*	Native, native, *Mtb glnA1*	This study
pSMT3	Source of *M. bovis* BCG *hsp60* promoter		49
pGFPuv	Source of UV-optimized *Aequorea victoria* GFP gene		Clontech
pCR2.1	TA cloning vector; template DNA for Tn*5* kanamycin resistance gene (*aphA-2*)		Invitrogen

[a] For expression constructs, the gene from which the promoter is derived is listed. The term "native" indicates that transcription is driven from the gene's own native promoter.

present in the cell wall of pathogenic, but not nonpathogenic, mycobacteria.

SOD is ubiquitous in aerobes and is part of the mechanism to protect the cell from oxidative stress, catalyzing the dismutation of superoxide to oxygen and hydrogen peroxide (27). In bacteria, there are two families of SODs: the FeSOD/MnSOD family, whose members do not contain leader peptides, and the Cu,ZnSOD family, whose members do contain leader peptides (11). The two enzyme families do not share sequence similarities and are likely a result of convergent evolution. *M. tuberculosis* contains both a tetrameric FeSOD (SodA) and a less well characterized Cu,ZnSOD (SodC) (22, 39, 74, 76). Kusunose et al. first observed that *M. tuberculosis* exports large amounts of its FeSOD (SodA) (39). The Cu,ZnSOD is surface associated; however, SOD activity detectable in culture filtrates is mainly due to the presence of SodA. In several gram-negative pathogens, the periplasmic Cu,ZnSOD (SodC) has been associated with virulence (18, 25, 58, 72). However, a recent report by Dussurget et al. has shown that a SodC-deficient *M. tuberculosis* mutant is not attenuated in a guinea pig model (22). In humans, SOD may be important to *M. tuberculosis* survival in the phagocyte, where the bacterium may encounter host-generated superoxide and other reactive oxygen species.

Previous studies from this laboratory of recombinant *M. tuberculosis* GS and SOD in *M. smegmatis* found that a large percentage of the recombinant enzymes (>95% GS and 66% SOD) but a smaller percentage of the *Mycobacterium smegmatis* endogenous enzymes (1% GS and 21% SOD) were exported, suggesting that export of the two *M. tuberculosis* enzymes relied on information in the protein sequence and/or structure (29, 30). In this study we now provide evidence that strongly suggests that GS and SOD are not actively secreted but rather are released into the extracellular medium as a result of bacterial leakage or autolysis and that their extracellular abundance is simply dependent upon their high expression and extracellular stability.

MATERIALS AND METHODS

Materials. Phenol, phenol-$CHCl_3$-isoamyl alcohol (25:24:1), Superdex-75, and Sephacryl 300HR were purchased from Amersham Pharmacia Biotech. Reactive Red-120 Sepharose, xanthine oxidase, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT), and gamma-glutamic acid hydroxamate were purchased from Sigma. RNase (bovine pancreas) and proteinase K were purchased from Boehringer Mannheim.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. *M. smegmatis* strains were grown on Middlebrook 7H11 agar (Difco) containing 10% (vol/vol) OADC (Becton Dickinson) and 0.5% (vol/vol) glycerol at 37°C or as shaken cultures in Middlebrook 7H9 broth (Difco) supplemented only with 0.2% (vol/vol) glycerol at 28 or 37°C. *M. tuberculosis* strains were grown on 7H11 agar containing 10% (vol/vol) OADC and 0.5% (vol/vol) glycerol or as unshaken cultures in 7H9 broth supplemented only with 0.2% (vol/vol) glycerol at 37°C in an atmosphere of 5% CO_2–95% air. Hygromycin (50 μg ml^{-1}) and/or kanamycin (20 μg ml^{-1}) were included as

TABLE 2. Recombinant mycobacterial strains used in this study

Strain and plasmid
M. smegmatis 1-2c
pNBV1
pNBV1-*Ec*SODA
pNBV1-*Ec*SODB
pNBV1-*Bs*SODA
M. smegmatis 1-2c *glnA1*
pNBV1
pNBV1-*Ms*GS
pNBV1-*Mtb*GS
pNBV1-*St*GS
pNBV1-*Ms*GS-*Ms*SODA-MDH
pNBV1-*Ms*SODA-*Ms*GS-BFRB
M. smegmatis 1-2c *sodA*
pNBV1
pNBV1-*Ms*SODA
pNBV1-*Mtb*SODA
pNBV1-*Ec*SODA
pNBV1-*Ec*SODB
pNBV1-*Bs*SODA
M. tuberculosis Erdman
pNBV1
pNBV1-*Ms*SODA
pNBV1-*Ec*SODB
pNBV1-GFPuv

appropriate. For the cultures shown in Fig. 12, glucose and/or additional $(NH_4)_2SO_4$ was added to our standard 7H9 medium, which contains 0.2% (vol/vol) glycerol and 3.8 mM $(NH_4)_2SO_4$. Other supplements are described in the text.

Escherichia coli DH5α, *Salmonella enterica* serovar Typhimurium (ATCC 13311), and *Bacillus subtilis* SB168 (ATCC 27689) were grown on Luria-Bertani agar, Luria-Bertani broth, or Terrific broth at 37°C. Ampicillin (100 μg ml^{-1}), gentamicin (20 μg ml^{-1}), hygromycin (250 μg ml^{-1}), and kanamycin (50 μg ml^{-1}) were included as appropriate.

Recombinant DNA methods. Plasmid DNA was isolated using Quantum Prep (Bio-Rad) or Wizard Plus SV (Promega) miniprep kits. Genomic DNA was isolated from *M. smegmatis, M. tuberculosis, E. coli*, *S. enterica* serovar Typhimurium, and *B. subtilis* by extraction with hot phenol (65°C, Tris-equilibrated, pH ≥ 8) and ethanol precipitation. The DNA was further purified by sequential digestion with RNase and proteinase K, additional extractions with phenol and phenol-$CHCl_3$-isoamyl alcohol (25:24:1), and a final ethanol precipitation.

Electroporation of mycobacteria. Cells were washed two or three times with 10% (vol/vol) glycerol and resuspended in 10% (vol/vol) glycerol to a density of 10^9 to 10^{10} cells ml^{-1}. *M. smegmatis* cells were kept at 0 to 4°C throughout the washing and electroporation procedures while *M. tuberculosis* cells were maintained at room temperature (68). DNA (1 to 5 μg) was mixed with 400 μl of cells, transferred to a 0.2-cm-gap electroporation cuvette (Bio-Rad), and a single electrical pulse was delivered (2.5 kV, 25 μF, 1,000 Ω). For *M. smegmatis*, the cells were immediately transferred to 2 ml of SOC medium (57) (with 5 mM L-glutamine for the *glnA1* mutant) and incubated at 37°C with vigorous shaking for 4 to 6 h before being plated on selective media. For cells electroporated with temperature-sensitive pPR27-derived plasmids, the incubation was done at ≤32°C. For *M. tuberculosis*, 600 μl of SOC medium was added directly to the cells in the cuvette and the cells were incubated without shaking at 37°C for 18 to 24 h before being plated on selective media.

Southern and colony hybridizations. Restriction fragments of genomic DNA or plasmids were electrophoresed in agarose gels, transferred to positively charged nylon membranes (Hybond-N+; Amersham Pharmacia Biotech) in 0.4 M NaOH, and hybridized to specific probes. Hybridizations were done at 60°C in 6× SSPE–2% skim milk for 15 to 20 h (1× SSPE is 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA [pH 7.4]). Membranes were washed very stringently at 65°C for 15 to 20 min once with 1× SSPE–1% skim milk and twice with 0.1× SSPE–1% skim milk before being exposed to X-ray film for various times. Hybridization and washing conditions were identical for colony hybridizations. Probes were radiolabeled to a specific activity of 1×10^8 to 2×10^8 dpm μg^{-1} with [α-^{32}P]dCTP by random priming using the Multiprime DNA Labeling Kit (Amersham Pharmacia Biotech).

Cloning and sequencing of the *M. smegmatis glnA1* and *sodA* genes. Primers were designed based on the *M. tuberculosis glnA1* sequence (30) and used to amplify a 1-kb target region encoding ~70% of the *M. smegmatis glnA1* gene from *M. smegmatis* 1-2c genomic DNA. The amplification product was cloned into pCR2.1 (Invitrogen), sequenced, and found to be highly similar to the *M. tuberculosis glnA1* gene. The *M. smegmatis glnA1* gene fragment was released from pCR2.1 by *Eco*RI digestion, gel purified, and used as a probe for the identification and isolation of its genomic locus in Southern and colony hybridizations. Likewise, a 0.65-kb *Eco*RI fragment containing the entire coding region of the *M. smegmatis* 1-2c *sodA* gene from a PCR amplification product (29) was used as a probe for the genomic *M. smegmatis* 1-2c *sodA* locus.

M. smegmatis 1-2c genomic DNA was completely digested with both *Bam*HI and *Eco*RI and subjected to Southern analysis. The *glnA1* probe hybridized to a 2.2-kb fragment, and the *sodA* probe hybridized to a 1.1-kb fragment. Restriction fragments from 1.7 to 2.4 kb and 0.8 to 1.2 kb were isolated and ligated into pUC18 digested with *Bam*HI and *Eco*RI. The ligation was transformed into *E. coli* DH5α, and clones were verified for both genes by colony hybridizations, restriction analysis, and Southern hybridizations. The plasmids were designated pUC18-*Ms-glnA1* and pUC18-*Ms-sodA*. For both plasmids, the entire insert was sequenced in both directions by cycle sequencing with ABI PRISM dye terminators (Davis Sequencing). One region of *M. smegmatis* 1-2c *sodA* exhibited particularly strong secondary structure and the sequence was determined with the Fidelity DNA Sequencing System (Oncor) with reaction products resolved on a 40% formamide–6 M urea gel. The sequences were assembled using the programs Pregap4 (version 1.0) and Gap4 (version 4.4) from the Staden Package (13). The sequences were scanned against the GenBank database using the BLAST programs (3) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Construction of *M. smegmatis glnA1* and *sodA* mutants. A promoterless, nonpolar kanamycin resistance (Km^r) cassette was constructed using an approach similar to the one described previously (43), using a different kanamycin resistance gene, replacing the gram-negative ribosomal binding site with the *M. tuberculosis dnaK* ribosomal binding site and including convenient restriction sites for exchanging resistance genes (Fig. 1). The cassette was constructed by amplification of the Tn*5* kanamycin resistance gene (*aphA-2*) using pCR2.1 as the template DNA and contains stop codons in all three reading frames immediately upstream of the ATG start codon of the *aphA-2* gene. Immediately downstream of the *aphA-2* gene stop codon is the sequence for the ribosomal binding site and ATG start codon of the *M. tuberculosis dnaK* gene to allow for translation of the 3′ portion of the disrupted gene. The blunt-end PCR product was cloned into the *Sma*I site of pUC19. Clones resistant to ampicillin and kanamycin were selected and were confirmed by restriction analysis. The cassette was released from pUC19 as a 0.8-kb fragment by *Ava*I digestion, and the 5′ overhangs were filled with T4 DNA polymerase.

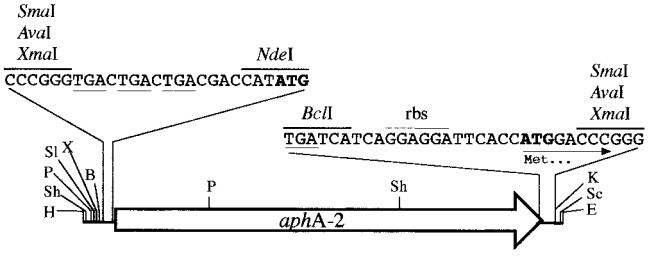

FIG. 1. Nonpolar kanamycin resistance cassette cloned into the *Sma*I site of pUC19. The cassette contains the kanamycin resistance gene (*aphA-2*) coding region but lacks both a promoter and a transcriptional terminator. The stop codons in all three frames immediately upstream of *aphA-2* are underlined, as is the stop codon of the gene. The start codon of *aphA-2* and the start codon provided at the 3′ end of the cassette are shown in boldface type. The 15 nucleotides immediately downstream of the *Bcl*I site are identical to the sequence directly upstream of the *M. tuberculosis dnaK* gene (the ribosomal binding site [rbs] is indicated). *Nde*I and *Bcl*I sites were included to facilitate cloning of other resistance genes into the cassette. Abbreviations for restriction sites: H, *Hind*III; Sh, *Sph*I; P, *Pst*I; Sl, *Sal*I; X, *Xba*I; B, *Bam*HI; K, *Kpn*I; Sc, *Sac*I; E, *Eco*RI.

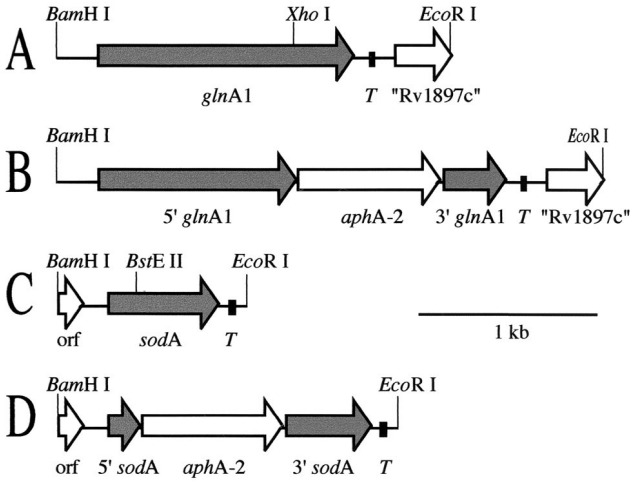

FIG. 2. Maps of the *M. smegmatis* 1-2c *glnA1* and *sodA* genomic loci. Both the *glnA1* (A) and the *sodA* (C) loci were isolated as *Bam*HI→*Eco*RI (5′-to-3′) genomic fragments. Both genes have putative transcriptional terminators (*T*) directly downstream of their coding regions. The *glnA1* locus contains a truncated open reading frame (orf) with a high degree of similarity to the *M. tuberculosis* Rv1897c gene. The *sodA* locus contains a truncated open reading frame upstream of *sodA* with a predicted protein sequence that is highly similar (75% identity) to a truncated open reading frame in the same location in the *M. fortuitum sodA* locus (44). The *glnA1* and the *sodA* loci were cloned into the multiple cloning site of pNBV1 for expression of GS and SOD in *M. smegmatis* and *M. tuberculosis*. The Km^r cassette (Fig. 1) was inserted into the unique *Xho*I site of *glnA1* (B) and the *Bst*EII site of *sodA* (D) to generate the disrupted loci used for allelic exchange.

Unique sites in the coding regions of the *M. smegmatis glnA1* and *sodA* genomic loci were identified (*Xho*I in pUC18-*Ms-glnA1* and *Bst*EII in pUC18-*Ms-sodA*). The plasmids were digested, and the 5′ overhangs were filled with T4 DNA polymerase. The Km^r cassette was blunt end ligated into the vectors such that the 3′ portion of *glnA1* or *sodA* was in frame with the ATG start codon provided at the 3′ end of the Km^r cassette (Fig. 2B and D). Clones were identified by restriction analysis that had the Km^r cassette in the same orientation as *glnA1* or *sodA*, allowing for transcription of the kanamycin resistance gene from the *glnA1* or *sodA* promoter. The disrupted genes were released from pUC18 by digestion with *Bam*HI and *Eco*RI. The *Eco*RI sites were modified by ligation to an adapter with multiple restriction sites (designated ECHXB) (Fig. 3) and the fragments were redigested with *Bam*HI and cloned into the *Bam*HI site of the temperature-sensitive allelic exchange vector, pPR27 (51). Constructions were verified by restriction analysis and designated pPR27-*Ms-glnA1*::Km^r and pPR27-*Ms-sodA*::Km^r.

The two allelic exchange constructs were electroporated into *M. smegmatis* 1-2c, and transformants were selected on 7H11 with kanamycin (20 μg ml^{-1}) at 32°C. Transformants were inoculated into 7H9 with kanamycin (20 μg ml^{-1}) and 5 mM L-glutamine and incubated at 32°C for 8 h to allow allelic exchange to take place. Cells were then plated on 7H11 with kanamycin (20 μg ml^{-1}) supplemented with 10% (wt/vol) sucrose and 5 mM L-glutamine and incubated at 39°C for 4 days to counterselect against those cells that retained the plasmid. Out of a total of 1,500 clones (each), six *sodA* clones and three *glnA1* clones were selected for further analysis. Four of the six *sodA* clones no longer expressed SodA activity. All three *glnA1* clones grew well on plates with added L-glutamine

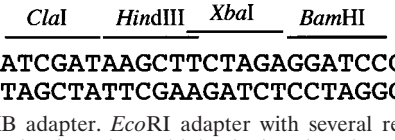

FIG. 3. ECHXB adapter. *Eco*RI adapter with several restriction sites useful for cloning into the multiple cloning site of pNBV1 and other vectors.

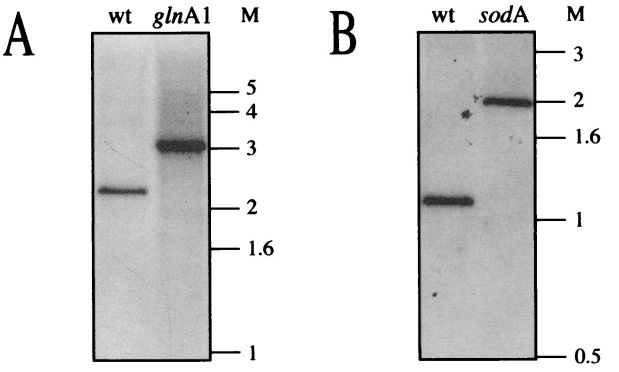

FIG. 4. Southern analysis of *M. smegmatis glnA1* and *sodA* mutants. Genomic DNA from *M. smegmatis* 1-2c (wild type [wt]), *M. smegmatis glnA1*, and *M. smegmatis sodA* strains was digested with *Bam*HI and *Eco*RI and probed with the entire *glnA1* locus (Fig. 2A) (A) or the entire *sodA* locus (Fig. 2C) (B). M, molecular mass markers in kilobases.

(5 mM) but did not grow at all on regular 7H11 plates. Genomic DNA was isolated from one *sodA* and one *glnA1* clone, digested to completion with both *Bam*HI and *Eco*RI, and subjected to Southern analysis (Fig. 4) using the following radiolabeled DNA probes: the *M. smegmatis glnA1* genomic locus (Fig. 2A), the *M. smegmatis sodA* genomic locus (Fig. 2C), vector (pPR27), and the Km^r cassette. For the *glnA1* mutant, the 2.2-kb band that is present in the wild-type strain was replaced by a 3-kb band indicating insertion of the 0.8 kb Km^r cassette. Likewise, for the *sodA* mutant, the 1.1-kb band that is present in the wild-type strain was replaced by an ~2-kb band. When probed with just the Km^r cassette, only the 3- and 2-kb bands (*glnA1* and *sodA* mutants, respectively) hybridized, and nothing hybridized in the wild-type DNA (data not shown). Also, the vector (pPR27) did not hybridize to DNA from the wild-type or mutant strains (data not shown).

Construction of recombinant mycobacterial strains. In previous studies, the *M. tuberculosis glnA1* locus was cloned into pSMT3 for expression in the *M. smegmatis* 1-2c wild-type strain (30). This 1.8-kb locus was transferred as a *Bam*HI→*Hin*dIII fragment (5′ to 3′) into the multiple cloning site of pNBV1. The *M. tuberculosis sodA* locus was previously cloned into pNBV1 as a *Cla*I→*Bam*HI PCR product (5′ to 3′), and its expression was studied in *M. smegmatis* 1-2c (29). This plasmid was used without modification.

The *M. smegmatis glnA1* and *sodA* genomic loci cloned into pUC18 likely contained sufficient upstream DNA sequence to encode all of the necessary promoter elements for expression in a mycobacterial host. Therefore, the loci were released by digestion with *Bam*HI and *Eco*RI and the *Eco*RI site was modified by ligation to the ECHXB adapter (Fig. 3). The DNA was digested with *Xba*I or *Hin*dIII and cloned into the mycobacterial shuttle vector pNBV1 cut with *Bam*HI and *Hin*dIII or *Bam*HI and *Xba*I so as to obtain the genes in both orientations on the plasmid. Prior to electroporation into mycobacteria as described above, all constructs were confirmed by restriction analysis. The *glnA1* plasmids were electroporated into the *glnA1* mutant, and the *sodA* plasmids were electroporated into the *sodA* mutant. Transformants expressed high levels of GS and SOD for both orientations of the *glnA1* and *sodA* loci, respectively. One clone of each with the gene cloned (5′ to 3′) in the *Bam*HI and *Hin*dIII sites was chosen for all further studies (designated pNBV1-*Ms*GS and pNBV1-*Ms*SODA).

The gene encoding a UV-optimized green fluorescent protein (GFPuv) was amplified by PCR from pGFPuv (Clontech), modifying the upstream region to contain an *Nde*I site at the ATG start codon and adding a *Hin*dIII site downstream of the stop codon. The PCR product was digested with *Nde*I and *Hin*dIII and cloned into a pNBV1 construct that contains a modified *M. bovis* BCG *hsp60* promoter (amplified from pSMT3) in which the sequence upstream of the ATG start codon was modified to contain an *Nde*I site. The gene contains an internal *Nde*I site which required first cloning the 3′ portion of the gene into the vector as an *Nde*I→*Hin*dIII (5′ to 3′) fragment. A clone was isolated and digested with *Nde*I, and the 5′ portion of the gene was cloned as an *Nde*I fragment.

The remaining genes (*M. tuberculosis mdh, M. tuberculosis bfrB, E. coli sodA, E. coli sodB, B. subtilis sodA*, and *S. enterica* serovar Typhimurium *glnA*) were amplified from genomic DNA using the Advantage-GC2 PCR kit (Clontech) and cloned downstream of the *M. tuberculosis glnA1* promoter. For all of the genes except for *bfrB* the same strategy was employed, cloning into the *Bsm*I site at the

5′ end (immediately upstream of the *M. tuberculosis glnA1* GTG start codon) and the *Hin*dIII site at the 3′ end. The 5′ forward primers started with the sequence 5′-GCTAGCATTCT*GTG* with 18 to 20 additional nucleotides corresponding to codons 2 through 8 of the gene being amplified. The *Bsm*I site is underlined, and the GTG start codon is shown in italics. The 3′ reverse primers incorporated a *Hin*dIII site (underlined) immediately downstream of the gene's stop codon, 5′-CCCAAGCTT, with 21 or 22 additional nucleotides complementary to the 3′ end of the gene. Because a *Bsm*I site is present in pNBV1, the 1.8-kb *M. tuberculosis glnA1* locus was first transferred into pUC19. The PCR-amplified genes were cloned downstream of the *glnA1* promoter, replacing the *glnA1* coding region, and then the gene plus promoter was transferred to pNBV1 as a *Bam*HI→*Hin*dIII fragment. The *B. subtilis sodA* gene has an internal *Hin*dIII site that was first removed by PCR mutagenesis.

One of the genes (*bfrB*) was cloned downstream of an *M. tuberculosis glnA1* promoter modified to contain an *Nde*I site at the start codon. There are no *Nde*I sites in pNBV1, which allowed for the PCR product to be cloned downstream of the promoter directly in pNBV1, avoiding the initial cloning into pUC19 required for the earlier constructs described above. The sequence upstream of and including the GTG start codon, TTCTGTG, was modified to TTCaTaTG in otherwise identical promoters (start codons are underlined and lowercase letters indicate added or modified nucleotides). The forward *bfrB* primer incorporated an *Nde*I site at the ATG start codon and the reverse *bfrB* primer incorporated a *Hin*dIII site as described above.

Recombinant plasmids carrying both *sodA* and *glnA1* as well as a third gene were constructed by first cloning the *M. smegmatis sodA* locus into the *Bam*HI and *Xba*I (5′-to-3′) sites of pNBV1-*Ms*GS to yield a plasmid (designated pNBV1-*Ms*GS-*Ms*SODA) containing both *M. smegmatis glnA1* and *sodA* in opposite orientations. A 3.3-kb *Hin*dIII fragment from pNBV1-*Ms*GS-*Ms*SODA, containing the entire sequence of both loci, was cloned into the *Hin*dIII site of pNBV1-MDH and pNBV1-BFRB (Fig. 5).

Plasmids were electroporated into mycobacteria as described above. Recombinant *M. smegmatis* and *M. tuberculosis* strains are listed in Table 2.

Analysis of *M. smegmatis* and *M. tuberculosis* recombinant strains. Cultures were typically inoculated with cells from 7H9 cultures in late log phase or early stationary phase to give a calculated A_{550} of 0.01 to 0.02 (100-fold dilution). When the cultures were intended for analysis of early-log-phase extracellular proteins, the cells were washed in fresh media before inoculation to avoid carryover of extracellular proteins. Because some proteins were expressed at higher levels at 28 than 37°C in *M. smegmatis*, particularly malate dehydrogenase (MDH), all *M. smegmatis* cultures were grown at 28°C except where indicated. When single time points of a culture were analyzed, the cultures were grown to late log phase or early stationary phase prior to harvest. Aliquots (10, 20, 40, or 100 ml) were removed for analysis and centrifuged. The supernate was filtered (Acrodisc PF 0.8/0.2 μm filter; Pall Corporation) and concentrated to 100 to 200 μl with a Centricon Plus-20 concentrator (5000 or 8000 MWCO; Millipore), and the concentrate was then diluted to 0.5 or 1.0 ml with phosphate-buffered saline (PBS). The cell pellet was washed by resuspending in 5 ml of PBS, centrifuging, and decanting the supernatant fluid. The cells were stored frozen at −80°C or used immediately. Cells were lysed by resuspending in 5 ml of PBS and sonicating twice for 2 min each (*M. smegmatis*) or once for 4 min (*M. tuberculosis*) on ice with a Heat Systems Ultrasonics W-375 sonicator (50% pulse; maximum setting with a microtip). Cellular debris was removed by centrifugation and the cleared lysate was filtered (pore size, 0.2 μm).

Enzyme assays. GS was assayed by the transfer reaction (73). Enzyme (100 μl) was added to 900 μl of assay mix (prewarmed to 37°C) to give the following concentrations of components: 30 mM L-glutamine, 60 mM NH_2OH, 20 mM arsenate, 20 mM imidazole, 1 mM $MnCl_2$, and 0.4 mM ADP at pH 7.0. The reaction was incubated at 37°C for 10 to 120 min (depending on activity) and stopped by the addition of 250 μl of 8% (wt/vol) trichloroacetic acid, 2 N HCl, and 3.3% (wt/vol) $FeCl_3$. The samples were centrifuged for 1 min to remove precipitated protein, and the absorbance was read at 540 nm. Blank reactions, which substituted PBS for enzyme, were subtracted. A standard curve was prepared from gamma-glutamic acid hydroxamate. A unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of gamma-glutamic acid hydroxamate per min under the assay conditions.

SOD was assayed using a modified version of the method described by Ukeda et al. (66). Enzyme (100 μl) was added to 800 μl of assay mix, and the reaction was initiated by the addition of xanthine oxidase (100 μl; 19 mU ml^{-1} in PBS containing 1 mg of bovine serum albumin ml^{-1}) to give the following concentration of components: 50 mM NaH_2PO_4 (pH 8.0), 200 mM NaCl, 0.1 mM EDTA, 0.1 mM xanthine, 25 μM XTT, and 1.9 mU of xanthine oxidase per ml. The reaction was monitored continuously at 470 nm at room temperature (25°C) and was linear for 12 min. One unit is defined as the amount of SOD that inhibits by 50% the reduction of XTT by superoxide.

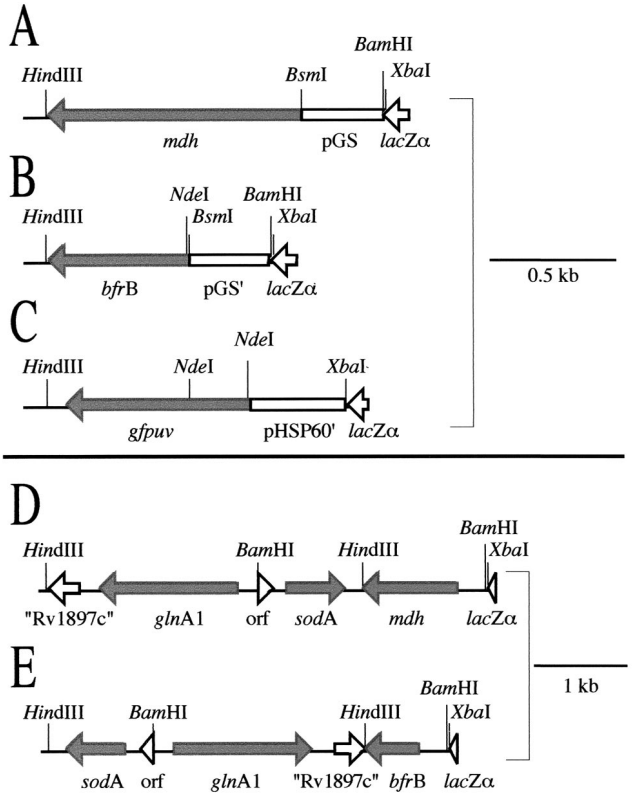

FIG. 5. Maps of the mycobacterial expression constructs. All of the constructs were cloned into the *Hin*dIII and *Bam*HI or *Xba*I sites of the multiple cloning site of pNBV1. (A) The *M. tuberculosis mdh* gene was cloned downstream of the *M. tuberculosis glnA1* promoter (pGS). The following genes were cloned in the same way as *mdh*: *E. coli sodA, E. coli sodB, B. subtilis sodA*, and *S. enterica* serovar Typhimurium *glnA*. (B) The *M. tuberculosis bfrB* gene was cloned downstream of an *M. tuberculosis glnA1* promoter (pGS′) modified to have an *Nde*I site at the start codon. (C) The UV-optimized GFP gene (*gfpuv*) was cloned downstream of an *M. bovis* BCG *hsp60* promoter (pHSP60′) modified to contain an *Nde*I site at the start codon. (D and E) A 3.3-kb *Hin*dIII fragment containing the *M. smegmatis glnA1* and *sodA* loci was cloned into the *Hin*dIII site of pNBV1-MDH (A) or pNBV1-BFRB (B). The *M. smegmatis glnA1* and *sodA* genes were transcribed from their own promoters.

MDH was assayed by adding 100 μl of enzyme to 900 μl of assay mix to give the following concentrations of components: 50 mM NaH_2PO_4 (pH 7.5), 200 mM NaCl, 1 mM KCN, 0.2 mM NADH, and 2 mM oxaloacetate. The reaction was monitored continuously at 340 nm for 2 to 3 min during which time the reaction was linear. Blank reactions in which oxaloacetate was omitted were subtracted. KCN at 1 mM was useful in inhibiting respiratory chain oxidation of NADH (17). For *M. smegmatis* strains overexpressing the *M. tuberculosis* MDH, lysates were diluted 100-fold in PBS immediately before assay. A unit is defined as the amount of enzyme that catalyzes the consumption of 1 μmol of NADH per min under the assay conditions (ε = 6,300 M^{-1} cm^{-1}).

SDS-polyacrylamide gel electrophoresis (PAGE) analysis. Culture filtrates and cell lysates were analyzed on sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels. The gels were stained with colloidal Coomassie brilliant blue G-250 (47).

SOD activity gels. Culture filtrates and cell lysates were electrophoresed on 15% nondenaturing polyacrylamide gels at 80 V for 16 to 20 h at room temperature. Gels were negatively stained for SOD activity as described in reference 10.

Immunoblotting. Culture filtrates and cell lysates of *M. tuberculosis* pNBV1-GFPuv were electrophoresed on an SDS–12.5% polyacrylamide gel and transferred to a nitrocellulose membrane. Strips containing GS, SOD, and GFPuv

TABLE 3. Identity matrix of bacterial superoxide dismutases

Protein[a]	% Identity[b] with:					Accession no.
	M. fortuitum SodA	*M. tuberculosis* SodA	*E. coli* SodA	*E. coli* SodB	*B. subtilis* SodA	
M. smegmatis SodA (Mn)[a]	96	81	43	39	44	AF061031
M. fortuitum SodA (Mn)		81	42	38	44	X70914
M. tuberculosis SodA (Fe)			41	40	45	AF061030
E. coli SodA (Mn)				45	60	X03951
E. coli SodB (Fe)					53	J03511
B. subtilis SodA (Mn)						D86856

[a] The metal cofactor used by the SOD is given in parentheses.
[b] The percentage of identical residues is given for each pair of proteins based on a multiple alignment of all the proteins using the program Clustal X (version 1.8) (64).

antigens were probed separately with rabbit polyclonal antibodies specific for the *M. tuberculosis* GlnA1 (diluted 1:2,500) (28), the *M. tuberculosis* SodA (diluted 1:2,500) (29), and GFP (diluted 1:1,000) (Molecular Probes). The membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit antibodies (diluted 1:10,000; Bio-Rad), a chemiluminescent substrate (SuperSignal West Pico; Pierce) was added, and the proteins were visualized on X-ray film.

Gel and immunoblot analysis. Gels were photographed wet and the negatives were scanned to obtain an 8-bit gray-scale image. Developed X-ray film from chemiluminescent immunoblotting was scanned directly. Protein bands were quantitated using the public domain NIH Image (version 1.62) program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Metabolic labeling of *M. tuberculosis* pNBV1-GFPuv. An actively growing culture of *M. tuberculosis* pNBV1-GFPuv (10 ml) was diluted into 40 ml of fresh medium (7H9 with hygromycin at 50 μg ml^{-1}) to give an initial A_{550} of ~0.06 and grown for 4 days at 37°C to an A_{550} of 0.18. Then, a mixture of [^{35}S]L-methionine and [^{35}S]L-cysteine (Promix; Amersham Pharmacia Biotech) was sterile filtered and added to the culture at a final concentration of 50 μCi ml^{-1}. The culture was incubated at 37°C for 3 more days, reaching a final A_{550} of 0.28, during which time 10-ml aliquots were removed at 7, 23, 48, and 72 h. Culture filtrates and cell lysates were prepared as described above and analyzed by SDS-PAGE. After staining with Coomassie brilliant blue, the gel was dried and exposed to X-ray film for various times (16 to 160 h).

Purification and stability of MDH. *M. smegmatis* cells overexpressing the *M. tuberculosis* MDH (*M. smegmatis glnA1* pNBV1-*Ms*GS-*Ms*SODA-MDH) were grown to stationary phase in 7H9 and harvested by centrifugation. The supernate was sterilized by filtration (pore size, 0.2 μm) and saved at 4°C. The cells were resuspended in PBS, lysed by sonication, and centrifuged, and the cleared lysate was filtered (pore size, 0.2 μm). The lysate (15 ml) was loaded on a Reactive Red-120 Sepharose column (20 ml, 2.6 by 3.5 cm) and eluted by gravity flow. The column was eluted with 90 ml of PBS, 60 ml of PBS–1 mM AMP, 30 ml of 0.1× malate buffer, 90 ml of 0.1× malate buffer–1 mM AMP, and finally with 90 ml of 1× malate buffer–1 mM AMP (1× malate buffer is 50 mM NaH_2PO_4, 100 mM L-malic acid, and 500 mM NaCl [pH 7.5]). Fractions were assayed for MDH activity, which was found only in the final wash with 1× malate buffer. The active fractions were concentrated with Centricon Plus-20 concentrators (MWCO 8000) and loaded on a Superdex-75 column (1.6 by 55 cm) equilibrated in PBS. The column was eluted at 1 ml min^{-1} with PBS and MDH was collected as a single, symmetrical peak that eluted very close to the void volume.

Purified MDH (~500 μg in 5 ml) was sterile filtered (pore size, 0.2 μm), added to 195 ml of the sterile culture filtrate (see above), and incubated at 28°C with shaking (same conditions as for the *M. smegmatis* cultures). Aliquots of 20 ml were removed immediately after adding MDH and 1, 2, 3, and 7 days later. The samples were concentrated to 100 to 200 μl with Centricon Plus-20 concentrators (8000 MWCO) and then diluted to 1.0 ml with PBS. The concentrated samples were stored at 4°C until day 3, at which point all of the samples were assayed for MDH and GS activity. On day 7, all of the samples were analyzed by SDS-PAGE.

Nucleotide sequence accession numbers. The nucleotide sequences have been deposited in the GenBank database (*glnA1*, GenBank accession number AY008693; *sodA*, GenBank accession number AF061031).

RESULTS

The proportion of GS and SOD exported from *M. smegmatis* and *M. tuberculosis* is not dependent upon the species from which the protein is derived. Previously, we found a high level of expression and export of recombinant *M. tuberculosis* GS and SOD compared with the endogenous *M. smegmatis* homologues in a wild-type *M. smegmatis* host (29, 30). To explore export of these large, leaderless, multimeric proteins further, we cloned the *M. smegmatis glnA1* and *sodA* genes and constructed *glnA1* and *sodA* mutants (see Materials and Methods), allowing for high-level expression of the *M. tuberculosis* and *M. smegmatis* proteins in a null background. *The M. smegmatis* SodA was found to be highly similar (>80% identity) to many mycobacterial SodA proteins as well as the SodA from *Nocardia asteroides* (*Mycobacterium fortuitum*, accession no. X70914; *Mycobacterium avium*, accession no. U11550; *Mycobacterium lepraemurium*, accession no. D13288; *M. leprae*, accession no. X16453; *N. asteroides*, accession no. U02341; *M. tuberculosis*, accession no. AF061030). The *M. smegmatis* GlnA1 was most similar (83 to 84% identity) to the *M. tuberculosis* GlnA1 (accession no. U87280) and *M. leprae* GlnA1 (accession no. CAC31306), with the next most similar GlnA proteins (70 to 73% identity) being from *Streptomyces coelicolor* (accession no. M23172), *Corynebacterium glutamicum* (accession no. Y13221), and *Amycolatopsis mediterranei* (accession no. AF050112). In contrast, the mycobacterial SodA proteins share only 39 to 45% identity with *E. coli* and *B. subtilis* SODs (Table 3), and the GlnA1 proteins share ~50% identity with *S. enterica* serovar Typhimurium GlnA (accession no. M14536).

Expression and export of homologous and heterologous GSs from the *M. smegmatis* wild-type and *glnA1* strains. The *M. smegmatis glnA1* strain has an absolute requirement for L-glutamine. Complementation of the *glnA1* strain was achieved by supplying either a copy of the *M. smegmatis glnA1* or *M. tuberculosis glnA1* in *trans* on pNBV1 with expression driven from their own promoters. Because genes are often poorly expressed from *E. coli* promoters in *M. smegmatis* (8), expression of *S. enterica* serovar Typhimurium *glnA* was driven from the strong *M. tuberculosis glnA1* promoter (Fig. 5A). This construct was also capable of complementing the *glnA1* mutant. The *M. smegmatis* wild type, *glnA1* mutant, and complemented strains were analyzed for expression and export (extracellular accumulation) of GS by measuring enzyme activity (Table 4) and by SDS-PAGE analysis (Fig. 6). The *M. smegmatis* wild-type strain expressed GS activity at 94 mU per ml of

TABLE 4. Expression and export of GS from *M. smegmatis*

M. smegmatis strain	Activity (mU ml^{-1})[a]		Extracellular activity (%)	Relative activity[b]
	Culture filtrate	Cell lysate		
pNBV1	6.3	87.9	6.7	1.00
pNBV1 (10 mM L-Gln)	0.4	17.9	2.3	0.19
*gln*A1 pNBV1 (10 mM L-Gln)	0.0	0.1	0.0	0.00
*gln*A1 pNBV1-*Ms*GS	62.3	1,238.4	4.8	13.81
*gln*A1 pNBV1-*Mtb*GS	13.1	520.6	2.5	5.67
*gln*A1 pNBV1-*St*GS	0.1	3.9	2.7	0.04

[a] Activity is expressed in milliunits per milliliter of the original culture volume.
[b] Based on the total GS activity in the culture filtrate and lysate. Values are relative to the activity of the wild-type strain (pNBV1).

original culture volume, while virtually no GS activity (0.1 mU ml^{-1}) was detected for the *glnA1* mutant (grown with 10 mM L-glutamine). Growth of the wild-type strain with 10 mM L-glutamine did decrease the GS activity to approximately 20% compared to growth in regular 7H9 but still gave clearly detectable activity. The level of GS activity in the mutant strain complemented with the *M. smegmatis glnA1* (*Ms glnA1* pNBV1-*Ms*GS) or *M. tuberculosis glnA1* (*Ms glnA1* pNBV1-*Mtb*GS) was 6- to 13-fold higher than in the wild-type strain. Consistent with this, bands of much greater intensity (compared with the wild-type strain) were observed on SDS-PAGE analysis. Even though the *S. enterica* serovar Typhimurium *glnA* was expressed from a strong mycobacterial promoter, the mutant strain complemented with this gene (*Ms glnA1* pNBV1-*St*GS) had very low levels of GS activity (4 mU ml^{-1}), but the levels were clearly detectable above background and no protein band for GS could be detected by SDS-PAGE analysis. However, this strain grew just as well as the strains complemented with the mycobacterial *glnA1* genes.

For all of the strains, the proportion of total GS activity detected in culture filtrates was quite low (2 to 7%), and no significant difference in the proportion of the *M. smegmatis* and *M. tuberculosis* GSs exported was observed.

Expression and export of homologous and heterologous SODs from *M. smegmatis*, *M. smegmatis sodA*, and *M. tuberculosis*. The *sodA* mutation in *M. smegmatis* did not appear to result in any growth defect (i.e., the mutant grew well in 7H9 medium). Strains were constructed from the *sodA* mutant that expressed the *M. smegmatis* SodA, *M. tuberculosis* SodA, *E. coli* SodA, *E. coli* SodB, or *B. subtilis* SodA. Transcription of the mycobacterial *sodA* genes was from their own promoters. Nonmycobacterial SOD genes were all transcribed from the *M. tuberculosis glnA1* promoter (Fig. 5A), as was done for the *S. enterica* serovar Typhimurium *glnA*. The plasmids containing the nonmycobacterial SOD genes were also transformed into the wild-type *M. smegmatis* to investigate whether the nonmycobacterial SOD subunits could mix with the *M. smegmatis* SodA subunits.

The strains were grown in 7H9 medium, and the culture filtrates and lysates were analyzed by SDS-PAGE and SOD activity gels (Fig. 7A to C and Table 5). By SDS-PAGE analysis, the 23-kDa band for the *M. smegmatis* SodA was absent from the *sodA* strain, as was the major band on the activity gel. However, there was a small amount of SOD activity in the *sodA* mutant that ran slightly higher than the *M. smegmatis* SodA on the activity gel. This band was not evident in the wild-type strain or the *sodA* strain expressing the *M. smegmatis* SodA from a plasmid (*Ms sodA* pNBV1-*Ms*SODA). However, it may have run differently in the presence of the *M. smegmatis* SodA, perhaps forming hybrids, and been hidden by the larger band. A nearly identical phenotype was previously observed for an *M. smegmatis sodA* mutant resulting from a single crossover event (21). All of the recombinant SODs were expressed at levels comparable to, or greater than, the endogenous *M. smegmatis* SodA as judged by SDS-PAGE and SOD activity (Table 5).

All SODs, recombinant and endogenous, were present in the culture filtrates of *M. smegmatis* strains at relatively low percentages (3 to 15%) of the total expressed. *M. tuberculosis* and *M. smegmatis* SodA subunits are known to mix, forming hybrid enzymes (29, 76). Also, the *E. coli* SodA and SodB subunits form a hybrid SOD and the *B. subtilis* SodA subunits are capable of forming hybrid SODs with both the *E. coli* SodA and SodB subunits (11, 35). However, no mixing of the *E. coli* and *B. subtilis* SOD subunits with *M. smegmatis* SodA subunits was detected (Fig. 7C).

The recombinant *M. tuberculosis* strain expressing the *M. smegmatis* SodA (*Mtb* pNBV1-*Ms*SODA) produced the recombinant enzyme at levels even greater than its own SodA (Fig. 7D and E and Table 5). The *M. smegmatis* SodA subunits formed hybrids with the endogenous *M. tuberculosis* SodA subunits as observed previously for *M. smegmatis* expressing the recombinant *M. tuberculosis* SodA (29, 76). The recombinant *M. tuberculosis* strain expressing the *E. coli* SodB (*Mtb* pNBV1-*Ec*SODB) produced the recombinant SOD at levels comparable to its endogenous SodA. Mixing of the endogenous *M. tuberculosis* SodA subunits with the *E. coli* SodB subunits was not detected (Fig. 7E), as was previously observed for the *M. smegmatis* SodA and *E. coli* SodB subunits in an *M. smegmatis* host (Fig. 7C).

All three SODs (*M. tuberculosis* SodA, *M. smegmatis* SodA, and *E. coli* SodB) were present in the culture filtrate of *M. tuberculosis* at similar proportions (14 to 23%) of the total expressed.

The results presented thus far showed that while the absolute amounts of GS and SOD exported were high relative to

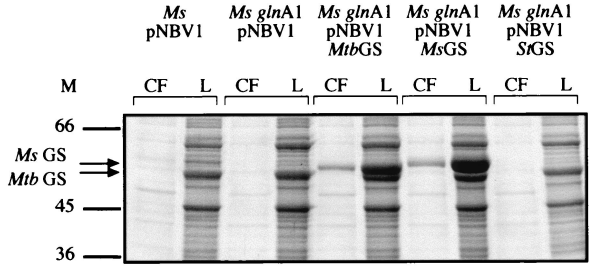

FIG. 6. GS expression and export in *M. smegmatis* wild-type and *M. smegmatis glnA1* strains. SDS-PAGE analysis of culture filtrates (CF) and lysates (L). Tenfold more CF than L was loaded on the gel (the equivalent of 2 ml versus 0.2 ml of the original culture volume). The results are representative of three independent cultures. Arrows indicate the positions of the *M. smegmatis (Ms)* and *M. tuberculosis (Mtb)* GSs. M, molecular mass markers in kilodaltons.

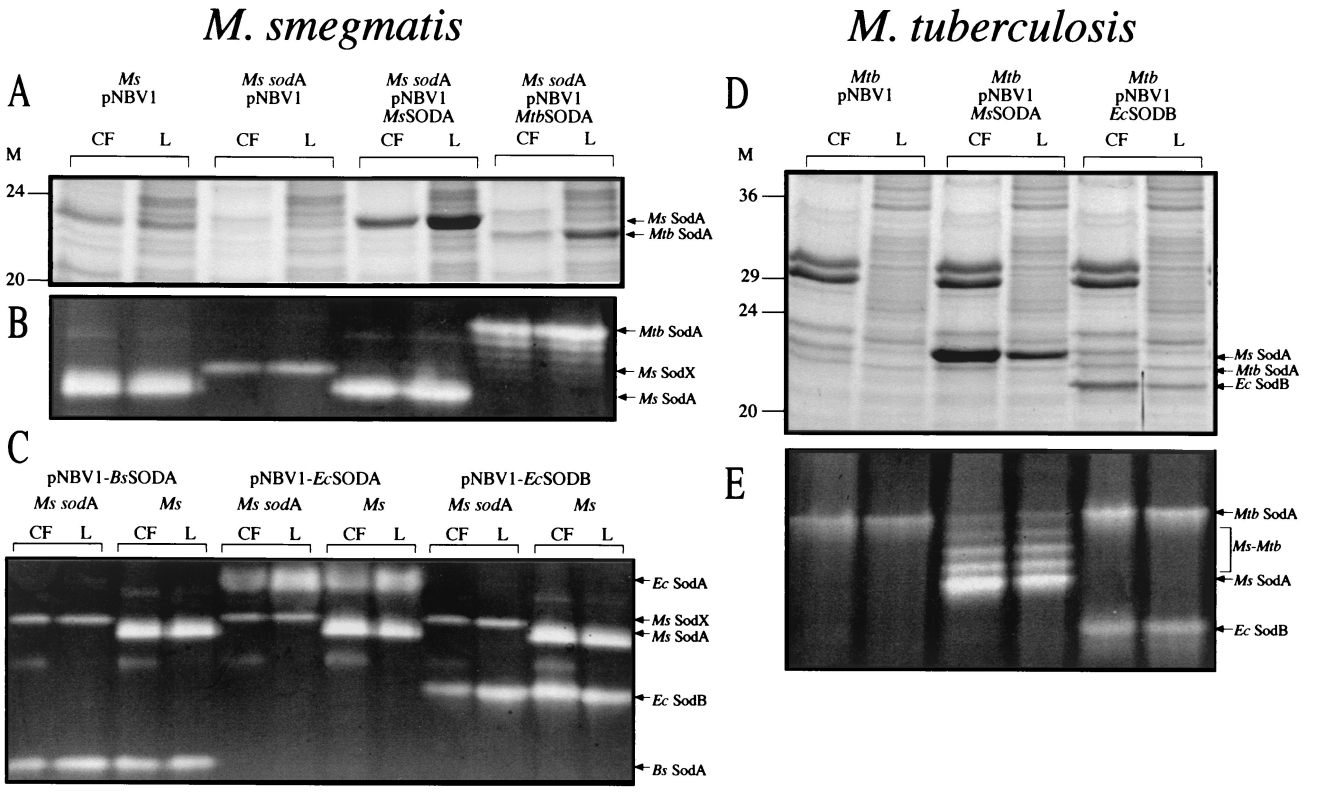

FIG. 7. SOD expression and export in *M. smegmatis* wild type, *M. smegmatis sodA*, and *M. tuberculosis* wild-type strains. *M. smegmatis* strains were analyzed for SOD expression by SDS-PAGE (A) and SOD activity gel (B and C). *M. tuberculosis* strains were also analyzed for SOD expression by SDS-PAGE (D) and SOD activity gel (E). Tenfold more culture filtrate (CF) than lysate (L), based on the original culture volume, was loaded on the gels for each analysis (4 ml of CF and 0.4 ml of L [A and D], 6 ml of CF and 0.6 ml of L [B and C], and 2 ml of CF and 0.2 ml of L [E]). The results are representative of two or three independent cultures. Arrows indicate the positions of the *M. smegmatis* (*Ms*) SodA, *M. tuberculosis (Mtb)* SodA, *M. smegmatis-M. tuberculosis* hybrid SODs (*Ms-Mtb*), *E. coli (Ec)* SodA, *E. coli* SodB, *B. subtilis (Bs)* SodA, and the *M. smegmatis* SOD activity in the *sodA* mutant (*Ms* SodX). M, molecular mass markers in kilodaltons.

other culture filtrate proteins, the proportion exported represented only a minority of the total expressed. Moreover, there was no significant difference in the proportion of GS and SOD exported depending upon whether they were native to pathogenic mycobacteria, nonpathogenic mycobacteria, or nonmycobacteria. This suggested that export was largely expression dependent rather than protein specific. To explore this possibility further, we prepared recombinant strains of *M. smegmatis* and *M. tuberculosis* expressing large amounts of three other leaderless proteins, all typically intracellular, and studied their expression and export.

The presence of large amounts of GS, SOD, and other leaderless proteins in the culture filtrate of mycobacteria reflects their high expression and extracellular stability. (i) Expression of MDH in *M. smegmatis*. The cytoplasmic enzyme lactate dehydrogenase is frequently assayed as a marker for lysis of bacterial and eukaryotic cells (41). However, we chose MDH, a Krebs cycle enzyme that is also expected to be strictly intracellular, as a marker for autolysis in *M. smegmatis* primarily because *M. smegmatis* does not express an MDH activity (54) while *M. tuberculosis* expresses a high level of activity. This suggested that overexpression of the *M. tuberculosis* MDH in *M. smegmatis* would be successful and analysis would not be complicated by the presence of endogenous enzyme. MDH forms either dimers or tetramers of identical 30- to 35-kDa subunits and has a three-dimensional structure similar to LDH (45). The *M. tuberculosis mdh* gene was cloned downstream of the *M. tuberculosis glnA1* promoter in pNBV1 (Fig. 5A), and a plasmid was constructed containing the *M. smegmatis glnA1, M. smegmatis sodA*, and *mdh* as separate transcriptional units (Fig. 5D) in order to follow MDH activity in recombinant bacteria overexpressing GS and SOD. This plasmid was transformed into the *M. smegmatis glnA1* mutant, and expression and export of GS, SOD, and MDH were analyzed by SDS-PAGE and enzyme activity as a function of growth (Fig. 8). The strain expressed large amounts of all three enzymes. The percentage of GS and SOD activity in the culture filtrate increased during growth and reached a maximum of 15 and 25% after the cells were well into stationary phase. During the earlier stages of growth (39 to 88 h), the percentages (means ± standard deviations) of extracellular GS protein (5.6% ± 1.4%; $n = 5$) and GS activity (6.2% ± 1.9%; $n = 5$) were similar to previous results (Fig. 6 and Table 4). The percentages of extracellular SOD protein (8.5% ± 0.6%; $n = 5$) and SOD activity (13.6% ± 0.6%; $n = 5$) were also similar to previous results (Fig. 7 and Table 5). In contrast, although there was a readily detectable quantity of MDH in the culture filtrate at all time points (10 to 70 mU per ml of original culture

TABLE 5. Expression and export of SOD from *M. smegmatis* and *M. tuberculosis*

Species and strain	SOD[a]	% Extracellular[b]		Relative amount[c]	
		Activity gel	SDS-PAGE	Activity gel	SDS-PAGE
M. smegmatis					
pNBV1	*M. smegmatis* SodA	10	12	1.0	1.0
*sod*A pNBV1	*M. smegmatis* SodX	6	—[d]	0.3	—
*sod*A pNBV1-*Ms*SODA	*M. smegmatis*SodA	8	4	1.1	5.6
*sod*A pNBV1-*Mtb*SodA	*M. tuberculosis* SodA	7	4	1.4	1.7
*sod*A pNBV1-*Bs*SODA	*M. smegmatis* SodX	7	—	0.3	—
	B. subtilis SodA	6	5	0.6	0.7
pNBV1-*Bs*SODA	*M. smegmatis* SodA	9			
	B. subtilis SodA	9	11	0.5	—
*sod*A pNBV1-*Ec*SODA	*M. smegmatis* SodX	7	—	0.2	—
	E. coli SodA	4	7	1.1	1.6
pNBV1-*Ec*SODA	*M. smegmatis* SodA	12	14		
	E. coli SodA	7	14	1.0	2.4
*sod*A pNBV1-*Ec*SODX	*M. smegmatis* SodX	6	—	0.3	—
	E. coli SodB	5	3	0.9	0.8
pNBV1-*Ec*SODB	*M. smegmatis* SodA	10	13		
	E. coli SodB	12	15	0.8	1.0
M. tuberculosis					
pNBV1	*M. tuberculosis* SodA	15	20	1.0	1.0
pNBV1-*Ms*SODA	*M. tuberculosis* SodA	15	23		
	M. smegmatis SodA	20	20	3.2	7.9
pNBV1-*Ec*SODB	*M. tuberculosis* SodA	14	20		
	E. coli SodB	16	19	0.5	2.2

[a] Many of the strains have two SOD activities which often could be analyzed separately. The SOD activity present in the *M. smegmatis sodA* mutant, which was not evident in the wild-type strain, is designated SodX.

[b] The percentages of extracellular SOD, from both SOD activity gels and SDS-polyacrylamide gels (Fig. 7), were determined with NIH Image software (version 1.62). Because *B. subtilis* SodA and *M. smegmatis* SodA have nearly identical mobilities on SDS-polyacrylamide gels, the extracellular percentage is based on the combination of the two SODs.

[c] For *M. tuberculosis* and *M. smegmatis* wild-type strains, relative amount refers to the amount of recombinant SOD compared with the endogenous *M. tuberculosis* or *M. smegmatis* SodA, respectively (i.e., an internal comparison). For the *M. smegmatis sodA* strains, "relative amount" refers to the amount of recombinant SOD compared with the amount of *M. smegmatis* SodA produced by a culture of the *M. smegmatis* pNBV1 wild-type strain grown under the same conditions.

[d] —, unable to analyze because a band could not be identified or was not clearly resolved.

volume), this extracellular MDH activity represented less than 0.5% of the total MDH activity. The SDS-PAGE results were consistent, showing no accumulation of MDH in the culture filtrate.

However, after 3 weeks in stationary phase, by which time the enzyme would have been expected to have been released extracellularly by autolysis, only 0.2% of the total MDH activity was present in the culture filtrate, while intracellular MDH activity was at 96% of its maximum value (data not shown). This suggested that MDH was not stable in the extracellular medium. Therefore, we examined the stability of the recombinant *M. tuberculosis* MDH in culture filtrate. We partially purified MDH from a cell lysate of *M. smegmatis glnA1* pNBV1-*Ms*GS-*Ms*SODA-MDH, added the purified MDH to the sterile culture filtrate from the same strain, and incubated the mixture under the same conditions as a growing culture. Aliquots were removed at various times, analyzed by SDS-PAGE, and assayed for MDH and GS activity (Fig. 9). MDH was clearly unstable in 7H9 culture filtrate, while the amounts of other proteins such as GS and SOD remained constant over the course of 1 week at 28°C. Further experiments showed that MDH had a half-life of 2.3 to 3 h in culture filtrate or fresh 7H9 medium at 28°C, and the addition of a combination of protease inhibitors (2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μM pepstatin A) or bovine serum albumin (10 mg ml^{-1}) did little to improve stability (data not shown).

(ii) Expression of bacterioferritin in *M. smegmatis*. Because of the significant and unexpected instability of MDH in culture filtrates, we sought a more stable protein for use as an intracellular marker for *M. smegmatis*. Ferritins and bacterioferritins are large (~450-kDa), highly stable, multimeric, iron storage proteins composed of 24 subunits of ~19 kDa (6). *M. tuberculosis* has two bacterioferritin genes (*bfrA* and *bfrB*) that do not encode leader peptides. One of the bacterioferritin genes, *bfrB*, was cloned downstream of the *M. tuberculosis glnA1* promoter (Fig. 5B), and a plasmid was constructed containing the *M. smegmatis glnA1, M. smegmatis sodA*, and *M. tuberculosis bfrB* as separate transcriptional units (Fig. 5E), as was done for *mdh*. This plasmid was transformed into the *glnA1* mutant, and expression and export of GS, SOD, and BfrB were analyzed by SDS-PAGE as a function of growth (Fig. 10). In contrast to MDH, BfrB was detected in the culture filtrate at levels comparable to GS and SOD at all time points analyzed, even in early log phase. Furthermore, BfrB in the culture filtrate likely exists as a functional 24-subunit multimer based on analysis of culture filtrate proteins in which BfrB largely coeluted with GS (molecular mass, ~640 kDa) on a Sephacryl-300HR size exclusion column (data not shown). Thus, in addition to GS, another very large and typically intracellular protein was released by *M. smegmatis* cultures into the growth medium.

(iii) Expression of GFP in *M. tuberculosis*. We chose the nonbacterial GFP as an intracellular marker for *M. tuberculosis*

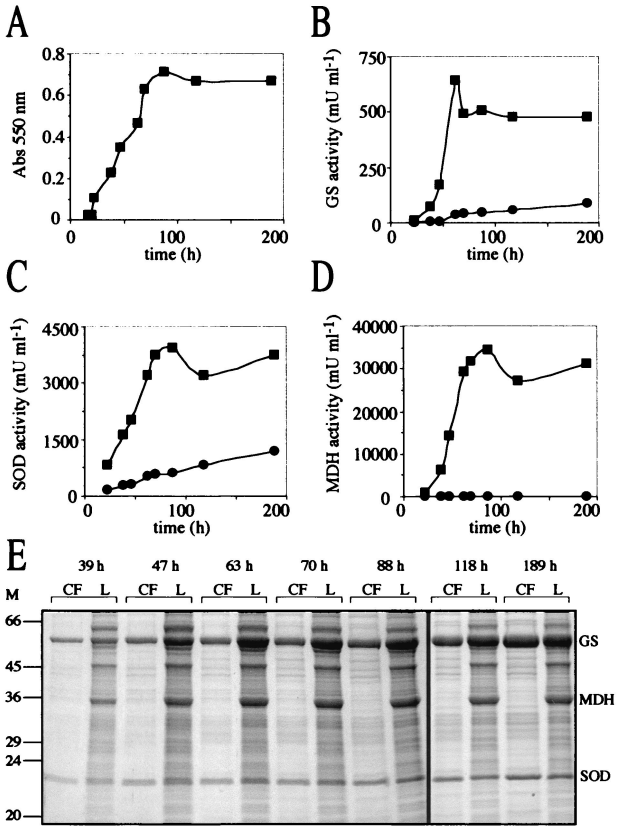

FIG. 8. Expression and export of GS, SOD, and MDH by *M. smegmatis glnA1* pNBV1-*Ms*GS-*Ms*SODA-MDH. (A) Growth of *M. smegmatis glnA1* pNBV1-*Ms*GS-*Ms*SODA-MDH. (B, C, and D) Enzyme activity of GS, SOD, and MDH in the culture filtrate (●) and lysate (■) over time. (E) SDS-PAGE analysis of culture filtrates (CF) and lysates (L). Tenfold more CF than L, based on the original culture volume, was loaded on the gels (at 39 and 47 h, 4 ml of CF and 0.4 ml of L; at 63 to 189 h, 2 ml of CF and 0.2 ml of L). The positions of the *M. smegmatis* GS, *M. smegmatis* SOD, and the *M. tuberculosis* MDH are indicated. M, molecular mass markers in kilodaltons.

because we anticipated that (i) it would be expected to remain strictly intracellular since it lacks a leader peptide, (ii) it would not be exported by *M. tuberculosis* based on similarities to endogenous proteins since it is not a bacterial protein, and (iii) it would persist in the culture medium if released since it is highly stable (14). *M. tuberculosis* was transformed with a plasmid containing the gene for GFPuv downstream of the *M. bovis* BCG *hsp60* promoter (Fig. 5C), a strong mycobacterial promoter that has been successfully used to express GFP in mycobacteria (20, 37). High level expression of GFPuv was obtained, and the cells were bright green when examined under long-wavelength UV light. Expression and export of the recombinant GFPuv along with the endogenous *M. tuberculosis* GlnA1 and *M. tuberculosis* SodA was analyzed by SDS-PAGE and immunoblotting as a function of growth (Fig. 11). All three proteins were clearly present in the *M. tuberculosis* culture filtrate during all stages of growth and at similar percentages (GS, 8 to 14%; GFPuv, 11 to 18%; SOD, 8 to 15%). Expression of GFPuv had no noticeable toxicity on the cells, and the export of SodA by the GFPuv-expressing strain was consistent with previous results obtained with three different *M. tuberculosis* strains not expressing GFPuv (Fig. 7 and Table 5) (SodA protein, 21.0% ± 1.7%; SodA activity, 14.7% ± 0.6%).

Because all three proteins appeared in the culture filtrate even during the early stages of growth, the kinetics of export were investigated by metabolic labeling of *M. tuberculosis* pNBV1-GFPuv with [^{35}S]L-methionine and [^{35}S]L-cysteine. Export of GFPuv and the endogenous *M. tuberculosis* GlnA1 was analyzed over a 3-day period (Fig. 11F). By 7 h, the cell lysate proteins were fairly heavily labeled, whereas the culture filtrate was nearly devoid of labeled proteins with the exception of the 30/32-kDa complex (antigen 85 complex) and 23.5-kDa (MPT64) major secretory antigens. These antigens all contain N-terminal signal peptides and have high localization indexes (70). Radiolabeled GFPuv and GlnA1 in the 7-h culture filtrate were close to, or below, detection limits and accounted for ≤0.5% of the total metabolically labeled GFPuv and GlnA1. By 72 h, only 5% of GFPuv and 3% of GlnA1 were present in the culture filtrate. The 30/32-kDa and 23.5-kDa antigens were very heavily labeled at this time, and many other proteins were evident in the culture filtrate. SodA could not be analyzed by this technique because it is poorly labeled, possessing only two methionine residues (one of which is cleaved off [29]) and no cysteine residues. GlnA1 and GFPuv have 13 and 7 total methionine and cysteine residues, respectively.

These experiments on expression and export of MDH and

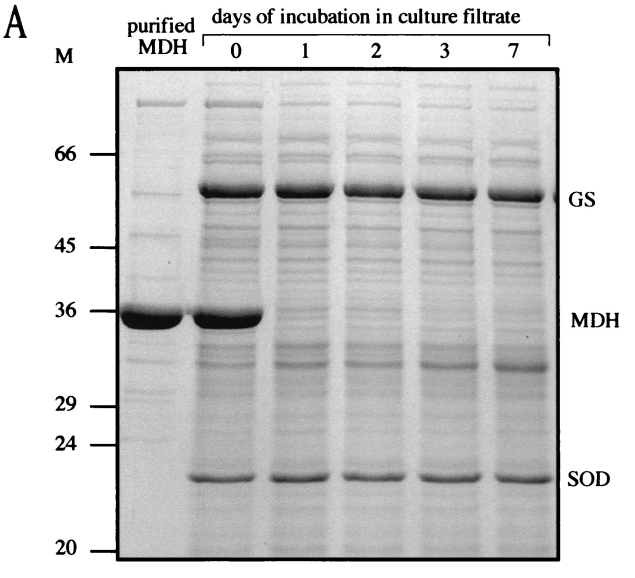

FIG. 9. Stability of MDH in 7H9 culture filtrate. (A) SDS-PAGE analysis of purified MDH (lane 1) and its stability after addition to culture filtrate. An amount equivalent to 2 ml of the original culture volume was loaded for each culture filtrate time point. The positions of the *M. smegmatis* GS, *M. smegmatis* SOD, and the *M. tuberculosis* MDH are indicated. The purified MDH was judged to be 85% pure by analysis with NIH Image software (version 1.62). (B) The percentage of initial activity of MDH and GS is listed beneath each lane, with the activity at day zero defined to be 100%. Abbreviations: ND, not determined; M, molecular mass markers in kilodaltons.

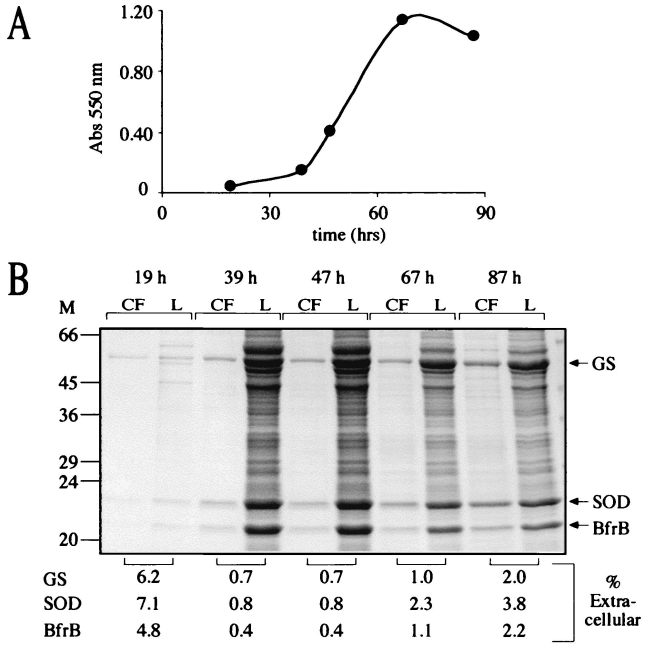

FIG. 10. Expression and export of GS, SOD, and BfrB by *M. smegmatis glnA1* pNBV1-*Ms*SODA-*Ms*GS-BFRB. (A) Growth of *M. smegmatis glnA1* pNBV1-*Ms*GS-*Ms*SODA-BFRB. (B) SDS-PAGE analysis of culture filtrates (CF) and lysates (L). Tenfold more CF than L, based on the original culture volume, was loaded on the gels (at 19 and 39 h, 8 ml of CF and 0.8 ml of L; at 47 h, 4 ml of CF and 0.4 ml of L; at 67 and 87 h, 2 ml of CF and 0.2 ml of L). The percentages of extracellular GS, SOD, and BfrB (listed below the gel) were determined with NIH Image software (version 1.62). Arrows indicate the positions of the *M. smegmatis* GS, *M. smegmatis* SOD, and the *M. tuberculosis* BfrB (BfrB). M, molecular mass markers in kilodaltons.

bacterioferritin by *M. smegmatis* and GFPuv by *M. tuberculosis* demonstrate that proteins accumulate extracellularly in large amounts if they are abundantly expressed and resistant to degradation in the extracellular milieu. Also, the metabolic labeling showed that the export is very likely not an active process because of the very slow release of GS and GFPuv compared to actively secreted mycobacterial proteins.

The role of carbon and nitrogen on release of proteins from *M. smegmatis* and evidence that autolysis plays a role in the accumulation of extracellular proteins. To investigate the potential role of carbon and nitrogen in protein export, we cultured *M. smegmatis glnA1* pNBV1-*Ms*SODA-*Ms*GS-BFRB for 4 days in 7H9 medium in the presence of various amounts of added glucose and $(NH_4)_2SO_4$. The addition of both $(NH_4)_2SO_4$ and glucose to 7H9 medium resulted in a dramatic decrease in extracellular proteins (Fig. 12A). In fact, rather modest absolute amounts of the two substances [12.4 mM $(NH_4)_2SO_4$ and 0.25% (wt/vol) Glc] were capable of preventing the accumulation of extracellular proteins (Fig. 12A, culture 2). Growth in 7H9 medium with additional $(NH_4)_2SO_4$ but without glucose did not affect the profile of extracellular proteins (Fig. 12B, culture 6). However, growth in 7H9 medium with 1% (wt/vol) glucose and no additional $(NH_4)_2SO_4$ resulted in a much greater level of extracellular proteins than growth in standard 7H9 (Fig. 12C, culture 10). The increase in extracellular proteins was most likely due to autolysis, because

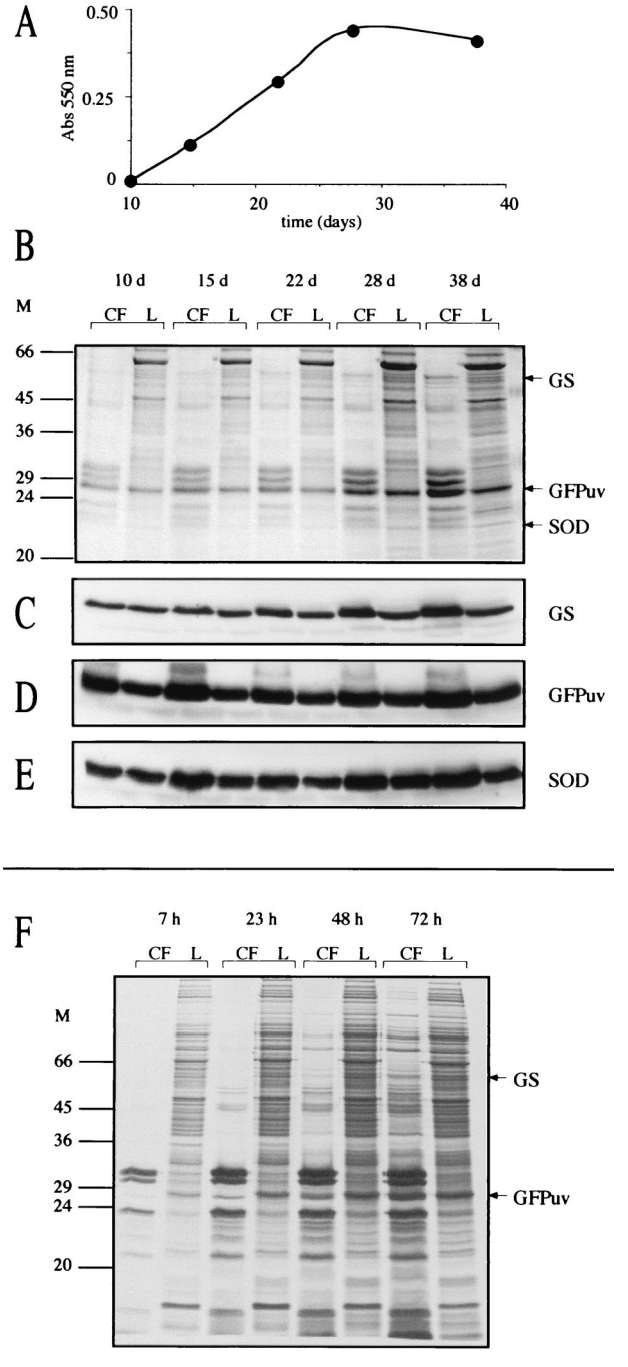

FIG. 11. Expression and export of GS, SOD, and GFPuv by *M. tuberculosis* pNBV1-GFPuv. (A) Growth of *M. tuberculosis* pNBV1-GFPuv. (B) SDS-PAGE analysis of culture filtrates (CF) and lysates (L). Tenfold more CF than L, based on the original culture volume, was loaded on the gels (at 10 days; 15 ml of CF and 1.5 ml of L; at 15 days, 6 ml of CF and 0.6 ml of L; at 22 to 38 days, 2 ml of CF and 0.2 ml of L). (C to E) Immunoblot analysis of GS, GFPuv, and SOD. (F) Autoradiogram of CF and L from *M. tuberculosis* pNBV1-GFPuv metabolically labeled with [^{35}S]L-methionine and [^{35}S]L-cysteine. The bacteria were grown for 4 days, radiolabel was added, and aliquots were removed for analysis 7, 23, 48, and 72 h later. The culture was actively growing over the 72-h period, increasing in turbidity (A_{550}) from 0.18 to 0.28. Twentyfold more CF than L was loaded on the gel (the equivalent of 2 ml versus 0.1 ml of original culture volume). Arrows indicate the positions of the *M. tuberculosis* GS, *M. tuberculosis* SOD, and GFPuv. M, molecular mass markers in kilodaltons.

cultures analyzed after 2 days rather than after 4 days of growth showed no difference in culture filtrate proteins compared with growth in standard 7H9 medium (data not shown). Glycerol (1%, vol/vol) could substitute for glucose (1% [wt/vol]), and NH_4Cl could substitute for $(NH_4)_2SO_4$. $MgCl_2$, $MgSO_4$, and NaCl could not substitute for a source of NH_4^+ (data not shown).

After prolonged incubation of the *M. smegmatis* strain in stationary phase (10 days) in 7H9 medium containing 12.4 mM $(NH_4)_2SO_4$ and 0.25% (wt/vol) glucose, extracellular proteins again accumulated, resulting in a culture filtrate protein profile similar to growth for 4 days in standard 7H9 medium. This suggested that the added nutrients were eventually consumed after which a portion of the culture lysed (data not shown). However, after prolonged incubation of the *M. smegmatis* strain in stationary phase (10 days) in 7H9 containing 38 mM $(NH_4)_2SO_4$ and 1% (wt/vol) glucose, almost no extracellular proteins were present, presumably because an excess of carbon and nitrogen was maintained. It was also observed that high-molecular-weight DNA (≥12 kb) was present in culture filtrates and was highly correlated with the presence of extracellular proteins (i.e., DNA was readily detected in culture filtrates when proteins were present but not when proteins were absent [data not shown]), providing further evidence that autolysis was occurring and that increased levels of glucose and nitrogen could prevent it.

When *M. smegmatis glnA1* pNBV1-*Ms*SODA-*Ms*GS-BFRB was grown under the same conditions as *M. tuberculosis* (37°C unshaken), additional glucose and NH_4^+ still prevented the release of GS, SOD, and BfrB into the growth medium (data not shown). However, when *M. tuberculosis* was grown under the four extreme culture conditions (cultures 1, 5, 6, and 10 in Fig. 12D) the extracellular protein profiles of the cultures were very similar; i.e., additional carbon and nitrogen in the medium did not prevent the release of proteins as it did for *M. smegmatis*. DNA was readily detected in culture filtrates under both standard conditions and with additional glucose and NH_4^+ in the medium (data not shown), suggesting that autolysis was also occurring in *M. tuberculosis* cultures but that it could not be prevented by the addition of glucose and NH_4^+.

DISCUSSION

The culture filtrate proteins of *M. tuberculosis* have been the subject of numerous studies (see, for example, references 4, 5, 7, 31, 32, 46, and 50). One often-discussed topic is to what degree autolysis contributes to the presence of antigens in the culture filtrate (for a recent discussion, see reference 69). Culture filtrate proteins have largely been defined as secreted based on their presence in early culture filtrates and/or the lack of an intracellular marker (enzyme activity or antigen) in the culture filtrate, but only a few studies have determined a localization index and/or an extracellular percentage for selected culture filtrate proteins (2, 5, 28–30, 46, 55, 62, 70, 71). Occasionally, when Hsp65 (GroEL, antigen 82) was not present in the culture filtrate, it was assumed that minimal autolysis had occurred (2, 62, 76). However, Wiker et al. noted that this antigen is not very stable (70). In the present study, under conditions in which substantial amounts of recombinant GFPuv were released by *M. tuberculosis*, Hsp65 was virtually undetectable in culture filtrates despite a high intracellular level (Fig. 11). This suggests that Hsp65 should not be used as an intracellular marker to measure autolysis. There is consensus, however, that most of the major extracellular proteins of *M. tuberculosis* are encoded by genes containing leader peptide sequences, and they have been shown to be greatly enriched in the culture filtrate (70, 71).

With respect to export of leaderless proteins by *M. tuberculosis*, we previously showed that *M. tuberculosis* and other pathogenic mycobacteria export abundant amounts of GS, whereas *M. smegmatis* and *Mycobacterium phlei* export small amounts (28). Moreover, recombinant *M. tuberculosis* GS was abundantly exported by *M. smegmatis* while very little of the endogenous *M. smegmatis* GS was released (30). Others have also identified *M. tuberculosis* GS in culture filtrates (55, 56, 62, 69). In this study, we found a somewhat lower percentage of total GS (8 to 14%) in *M. tuberculosis* culture filtrates compared with our previous work (33%) and that of Raynaud et al. (17%) (55). We also found, in contrast to our earlier work, that abundantly expressed and active *M. tuberculosis* GS is not released in large proportions by *M. smegmatis*. In fact, the *M. smegmatis* wild-type strain as well as *glnA1* strains expressing high levels of the *M. smegmatis* or *M. tuberculosis* GS, all exported a similar, relatively low, percentage of total GS (3 to 7%). The large, quantitative differences between these and previous results may be due to the different growth conditions used, as previously strains were grown with added glucose (2%, wt/vol). In this study, we have shown that inclusion of high levels of glucose in 7H9 medium may well result in increased autolysis of *M. smegmatis* (Fig. 12C, culture 10). It is known that *M. tuberculosis* undergoes autolysis under low-nitrogen conditions when glucose is present in excess (59), and *M. smegmatis* may suffer a similar fate. For all conditions and strains examined, the origin of GS was not a major determinant of export.

There are conflicting reports on the export of the *M. tuberculosis* SodA. Kusunose et al. first reported that *M. tuberculosis* SodA could be found in substantial amounts in the culture filtrate (39). *M. tuberculosis* SodA was later identified as a major component of an early culture filtrate by Andersen et al. (5). Also, we previously found that 76% of SodA was extracellular, and Raynaud et al. found 92% of SOD activity to be extracellular (29, 55). However, Nagai et al. noted that SOD was released during a late stage of growth, which they attributed to autolysis, and Alito et al. found low levels of SOD in culture filtrates compared to lysates (2, 46). In the present study, we found a substantially lower amount of SodA (~20%) in *M. tuberculosis* culture filtrates compared with our previous work (76%). Several other bacterial pathogens have also been reported to release leaderless SOD into the extracellular environment. *N. asteroides*, a phylogenetically close relative of the mycobacteria, produces abundant amounts of SodA (2 to 5% of total extractable protein) and releases SodA as a major component of the extracellular medium (1, 9). The *M. avium* SodA has also been reported to be surface associated and extracellular (19, 24, 42). The *Helicobacter pylori* SodB is abundantly expressed and has been found to be surface associated and extracellular as well (60, 63).

Several investigators have studied export of recombinant SodAs in *M. smegmatis* hosts and assessed the proportion of

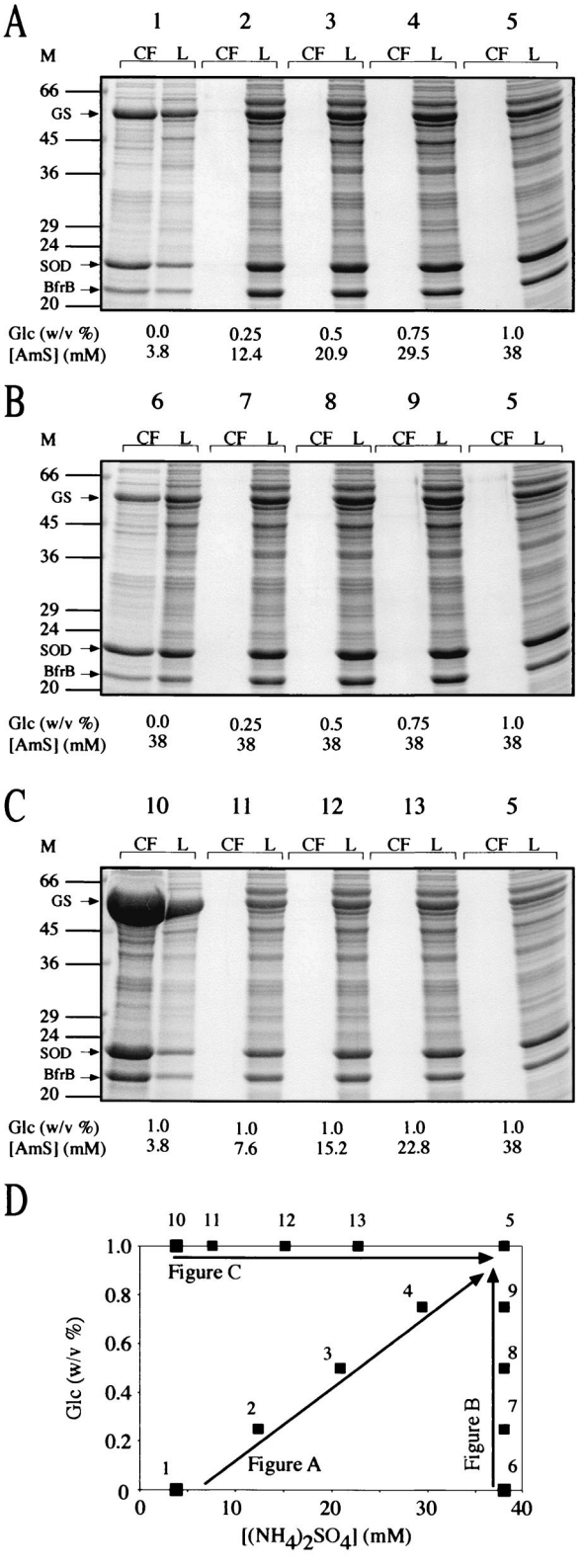

FIG. 12. Expression and export of GS, SOD, and BfrB by *M. smegmatis glnA1* pNBV1-*Ms*SODA-*Ms*GS-BFRB under various growth conditions. (A to C) *M. smegmatis glnA1* pNBV1-*Ms*GS-*Ms*SODA-BFRB was grown for 4 days (cultures reached stationary phase by 2 to 3 days) in 7H9 medium containing 0.2% (vol/vol) glycerol with various amounts of glucose and/or additional $(NH_4)_2SO_4$, and culture filtrates (CF) and lysates (L) were analyzed by SDS-PAGE. Tenfold more CF than L was loaded on the gels (the equivalent of 2 ml versus 0.2 ml of original culture volume). Arrows indicate the positions of the *M. smegmatis* GS, *M. smegmatis* SOD, and the *M. tuberculosis* BfrB (BfrB). Two to four independent cultures were grown and analyzed for each of the four extreme culture conditions (1, 5, 6, and 10) as well as for culture condition 2, and the results shown are representative. (D) Graph indicating the glucose and $(NH_4)_2SO_4$ concentrations of the media that were tested in panels A to C. The standard 7H9 medium (culture 1) contains 82 mM carbon (as 0.2% [vol/vol] glycerol) and 11 mM nitrogen [as 3.8 mM $(NH_4)_2SO_4$ and 3.4 mM L-glutamate]. The $(NH_4)_2SO_4$ (AmS) concentration is stated as the total amount present in the medium for each culture condition. M, molecular mass markers in kilodaltons.

endogenous and recombinant SOD exported. Here too, there have been conflicting reports on export. In our previous study, we found that recombinant *M. smegmatis* exported a larger proportion of *M. tuberculosis* SodA than the endogenous enzyme (66 versus 21%) (29). Although Escuyer et al. observed release of recombinant *M. avium* SodA from *M. smegmatis*, they did not detect any of the endogenous *M. smegmatis* enzyme in the culture filtrate (24). Also, Zhang et al. did not detect release of either the endogenous *M. smegmatis* enzyme or the recombinant *M. tuberculosis* SodA (76).

Thus, while it was clear from previous studies that the *M. tuberculosis* SodA was abundantly expressed and sometimes found at high levels in the extracellular medium, it was not clear what the mechanism of export was and whether or not the origin of SOD itself was a major determinant of export, i.e., whether SOD native to a pathogenic species had a greater inherent propensity for export than SOD native to a nonpathogenic species. To explore these issues more definitively, we expressed *M. tuberculosis* and *M. smegmatis* SodA, as well as three nonmycobacterial SODs, in an *M. smegmatis sodA* mutant, and we expressed *M. smegmatis* SodA and *E. coli* SodB in wild-type *M. tuberculosis*. We found that a similar proportion of both recombinant and endogenous mycobacterial SODs were exported in both *M. smegmatis* and *M. tuberculosis*. Moreover, nonmycobacterial SODs were released into the medium in proportions similar to those of mycobacterial SODs. This was especially surprising considering that the nonmycobacterial SODs are only 39 to 45% identical to the mycobacterial SODs and their structure is different enough that is precludes mixing of nonmycobacterial and mycobacterial subunits. As for GS, the origin of SOD was not a major determinant of export.

Our finding that the proportion of GS and SOD exported was independent of the bacterial species from which the protein was derived indicated that export was not protein specific. To explore this issue further, we studied three additional leaderless proteins as intracellular markers. We first examined expression and export in *M. smegmatis*, choosing the *M. tuberculosis* MDH and coexpressing it at a high level with the recombinant *M. smegmatis* GlnA1 and SodA. SDS-PAGE and enzymatic analysis clearly showed preferential export of GS and SOD compared with MDH. However, this was due to the unexpected instability of MDH in the culture medium. Although MDH activity was present in the culture filtrate along with GS and SOD at all time points, it was always less than 0.5% of the total MDH activity. Given the very short half-life of MDH in the culture medium, much greater amounts must

have leaked out but were degraded. Therefore, a protein noted for its stability (bacterioferritin) was used as an intracellular marker, and in contrast to MDH, it was present in the culture filtrate at levels comparable to those of GS and SOD at all stages of growth, even in early log phase. The amount of extracellular GS and SOD in this experiment was somewhat less than that in the experiment where GS and SOD were coexpressed with MDH. This was typical of the variability observed between cultures and highlights the importance of examining multiple proteins in the same culture when determining intracellular and extracellular location as previously described (70). For the analysis of expression and export in *M. tuberculosis*, we chose GFP, a nonbacterial protein that lacks a leader peptide, so that there should be no mechanism for its release. GFP, being quite stable, was expected to accumulate in the culture medium if it was released (14). Indeed, GFP was found in the culture filtrate, even in the early stages of growth, and at levels very similar to the endogenous GS and SOD.

Although several export and secretion pathways have been identified in bacteria, including mechanisms to secrete large, folded proteins, export of GS and SOD by *M. tuberculosis* does not seem to fit any of the models (12, 15, 34, 53, 61, 65). Type I secretion is *sec* independent (no N-terminal signal peptide) and involves direct secretion of a protein to the extracellular space by an ABC transporter (12). Three proteins are required, and their genes are usually clustered with the gene for the secreted protein. The secretion signal is believed to reside in the C-terminal ~60 amino acids of the protein, which is preceded by a glycine-rich domain. *M. tuberculosis* does have many ABC transporters, but *sodA* and *glnA*1 are not clustered with any, and SodA and GlnA1 do not contain glycine-rich domains. The general export pathway (type II) is *sec* dependent and requires an N-terminal signal peptide that is cleaved off as the unfolded protein is exported across the inner membrane (53). In gram-negative bacteria, some specific proteins exported to the periplasm in this manner are then secreted in a folded state across the outer membrane, requiring a large cluster of genes for their secretion. As stated before, GS and SOD do not have N-terminal signal peptides. Autosecreted proteins such as the *Neisseria gonorrhoeae* immunoglobulin A protease (sometimes referred to as type IV secretion, but different from the type IV secretion discussed below) are exported to the periplasm by the type II pathway and then pass through the outer membrane with no assistance from other proteins (26). In contrast to GS and SOD, autosecreted proteins have N-terminal signal peptides, and only the N-terminal portions of the proteins are translocated across the gram-negative outer membrane; their C-terminal portions contain transmembrane domains that form a pore for translocation (26). Type III secretion has also been identified in gram-negative bacteria and involves the direct transfer of a protein from the bacterial cytoplasm to the cytoplasm of a host cell, although these proteins may accumulate in culture medium as well (34). Secretion is *sec* independent and requires a cluster of approximately 20 genes. Finally, type IV secretion is similar to type III secretion in that it transports macromolecules (DNA and/or proteins) from the bacterial cytoplasm to a target cell by a contact-dependent mechanism which requires a large cluster of genes (15). No clusters of genes necessary for type III or type IV secretion are present in the *M. tuberculosis* genome (16).

While the presence of GS and SOD in early culture filtrates from actively growing *M. tuberculosis* cultures initially suggested a protein-specific export mechanism, the following observations strongly suggest that GS and SOD are released into the culture medium as a result of bacterial leakage or autolysis. (i) The percentage of GS and SOD found in *M. tuberculosis* and *M. smegmatis* culture filtrates was quite low, typically less than 20%. (ii) Heterologous SODs, bacterioferritin, and GFP all accumulate in culture filtrates essentially in parallel with the endogenous mycobacterial GSs and SODs. (iii) Metabolic labeling of *M. tuberculosis* pNBV1-GFPuv showed that GFPuv and GS were slowly released after synthesis, in stark contrast to actively secreted proteins with N-terminal signal peptides. (iv) The presence of high-molecular-weight DNA in culture filtrates was highly correlated with the presence of extracellular proteins. (v) In the case of *M. smegmatis*, a richer growth medium prevented the release of both proteins and DNA. Recently, studies utilizing highly sensitive two-dimensional PAGE techniques have detected hundreds of proteins in *M. tuberculosis* culture filtrates, many of them regarded as cytoplasmic (e.g., ribosomal proteins, transcriptional regulators, and elongation factors [36, 56, 62]). It is likely that many of these proteins are also present due to bacterial leakage or limited autolysis of the cultures.

Our study cannot differentiate between autolysis and leakage as a mechanism by which GS and SOD are released. Thus, as an alternative to lysis it is possible that the proteins leak out of viable bacteria as they remodel their cell wall and divide. One observation favoring this is that, in human macrophages infected with *M. tuberculosis* and stained for GS by the cryosection immunogold technique, extracellular GS is observed in the phagosome of intact healthy-appearing mycobacteria (28). However, it is also a possibility that the extracellular GS came from autolyzed bacteria and was absorbed onto the cell wall of the healthy bacterium before infection of the macrophage. Whether released by autolysis or leakage from intact, viable cells, the extracellular abundance of *M. tuberculosis* GS and SOD is readily explained by their high expression and extracellular stability.

The presence of leaderless proteins in the culture supernatant fluids of the gram-negative pathogen *H. pylori* has also been the subject of several papers recently, with some papers claiming that the proteins are specifically secreted and others claiming that the proteins are released by autolysis (38, 52, 60, 67). Interestingly, SodA was one of the *H. pylori* culture supernatant proteins identified. Also, SodA, as well as some of the other leaderless, extracellular proteins, is expressed in high amounts by *H. pylori*. Phadnis et al. have proposed that cytoplasmic proteins from *H. pylori* are released by autolysis, some of which become adsorbed to the outer membrane of intact bacteria (52). They suggest that the amount of protein is variable and depends upon the culture age and subculture status, accumulating on repeatedly subcultured cells. It is possible that a similar phenomenon also occurs in *M. tuberculosis* cultures.

While autolysis no doubt occurs during the late stages of bacterial growth, the issue here is to what extent it occurs in the early growth phase and under physiologic conditions. Clearly, autolysis can play physiologically important roles in develop-

ment, genetic exchange, and elimination of defective cells in bacterial cultures and is not necessarily simply the result of a prolonged period in stationary phase (40). Interestingly, Krishnamurthy et al. have proposed that *H. pylori* undergoes altruistic suicide to release its abundantly expressed urease, which then coats neighboring cells and is protective against an acidic environment (38). While autolysis of a limited proportion of *M. tuberculosis* bacilli in an otherwise multiplying culture may reflect suboptimal in vitro growth conditions, it is also possible that limited autolysis occurs under physiologic conditions. Hence, we should allow for the possibility that release of proteins by autolysis from a small proportion of the bacteria at a site of bacterial invasion in the tissues may contribute to the overall success of the infection. Viewed in this way, limited autolysis may result in the release of mycobacterial proteins which function as biologically important virulence factors.

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