Export of Recombinant *Mycobacterium tuberculosis* Superoxide Dismutase Is Dependent upon Both Information in the Protein and Mycobacterial Export Machinery

A MODEL FOR STUDYING EXPORT OF LEADERLESS PROTEINS BY PATHOGENIC MYCOBACTERIA*

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We have investigated the expression and extracellular release of enzymatically active superoxide dismutase, one of the 10 major extracellular proteins of *Mycobacterium tuberculosis*, both in its native host and in the heterologous host *Mycobacterium smegmatis*. We found that the *M. tuberculosis* superoxide dismutase gene, encoding a leaderless polypeptide of $M_\text{r} = 23,000$ representing one of the four identical subunits of the enzyme, is expressed constitutively under normal growth conditions and at a 5-fold increased level under conditions of hydrogen peroxide stress. The highly pathogenic mycobacterium *M. tuberculosis* expresses 93-fold more superoxide dismutase than the nonpathogenic mycobacterium *M. smegmatis*, and it exports a much higher proportion of expressed enzyme (76 versus 21%); taking both expression and export into consideration, *M. tuberculosis* exports $350$-fold more enzyme than *M. smegmatis*. In *M. smegmatis*, recombinant *M. tuberculosis* superoxide dismutase is expressed at 8.4 times the level of the endogenous enzyme and the proportion exported (66%) approaches that in the homologous host; hence *M. smegmatis* exports up to 26-fold more of the recombinant than endogenous enzyme. Interestingly, subunits of the *M. tuberculosis* and *M. smegmatis* enzymes readily and stoichiometrically exchange with each other, forming five different complexes of four subunits, both when the enzymes are expressed in the recombinant host and when the purified enzymes are incubated together; however, each subunit retains its characteristic metal ion, iron for *M. tuberculosis* and manganese for *M. smegmatis*. Compared with the cell-associated enzyme, the supernatant enzyme of recombinant *M. smegmatis* is enriched for *M. tuberculosis* enzyme subunits, consistent with preferential export of the *M. tuberculosis* enzyme. Recombinant *M. tuberculosis* superoxide dismutase transcomplements a superoxide dismutase-deficient *Escherichia coli*, resulting in a reduction of sensitivity of the strain to oxidative stress, but the enzyme is not exported from this nonmycobacterial host. Our findings indicate that the information for export of the *M. tuberculosis* superoxide dismutase is contained within the protein but that export additionally requires export machinery specific to mycobacteria.

*Mycobacterium tuberculosis* secretes or otherwise releases a large number of proteins into its extracellular milieu. Approximately 11 such proteins are released into broth cultures in sufficient abundance so as to merit designation as major extracellular proteins. In all cases examined thus far, these major extracellular proteins are also released into the phagosome of *M. tuberculosis* in human mononuclear phagocytes. The functions of several of these proteins are known, including glutamine synthetase (subunit molecular mass 58 kDa), mycolyl transferase (30/32-kDa complex), and iron-specific superoxide dismutase (subunit molecular mass 23 kDa).

Extracellular proteins of *M. tuberculosis* are of great interest because of the likelihood that these proteins play critical roles in host-pathogen interaction, because these proteins are leading candidates for incorporation into subunit vaccines, and because these proteins are prime targets for the development of new antimycobacterial drugs (1–4).

Most of the extracellular proteins have leader sequences, and their export presumably occurs after processing of the leader peptide by a leader peptide-specific peptidase. However, two proteins, glutamine synthetase and superoxide dismutase, do not have leader sequences. The mechanism by which these proteins or their subunits are exported is unknown.

In previous studies, we have examined the extracellular release of *M. tuberculosis* glutamine synthetase. These studies demonstrated that glutamine synthetase is abundantly exported by pathogenic mycobacteria, including *M. tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium avium* but not by nonpathogenic mycobacteria, including *Mycobacterium smegmatis* and *Mycobacterium phlei*. The studies also demonstrated that the information for export was contained within the protein since recombinant *M. tuberculosis* glutamine synthetase expressed in *M. smegmatis* was abundantly (>250 µg of protein per liter of *M. smegmatis* culture and amounting to >90% of all glutamine synthetase molecules synthesized) exported, whereas the endogenous *M. smegmatis* enzyme was located intracellularly (>95% of all enzyme molecules synthesized) (1, 5).

These findings with respect to the export of glutamine synthetase prompted us to investigate the other leaderless major extracellular protein of *M. tuberculosis*, superoxide dismutase. In particular, we wished to determine if its pattern of export followed the same paradigm as glutamine synthetase. A previous report in the literature that recombinant *M. tuberculosis*
superoxide dismutase is not exported in M. smegmatis suggested that superoxide dismutase follows a different paradigm (6).

Superoxide dismutase (EC 1.15.1.1) catalyzes the dismutation of the superoxide anion. The enzyme is believed to be an integral part of the cell’s defense against toxic oxygen metabolites. Phagocytic cells, including mononuclear phagocytes, the host cells of M. tuberculosis, generate these toxic oxygen metabolites as part of their effort to fend off pathogenic invaders (7). Superoxide dismutase has been characterized at the DNA and protein level in several mycobacterial species in addition to M. tuberculosis. All of these enzymes are members of a family of homologous superoxide dismutases with similar biochemical properties and tertiary structure, combining either two or four subunits to form the active enzyme and coordinating either iron or manganese ions at the catalytic site (4, 8, 9). A feature of the M. tuberculosis superoxide dismutase that distinguishes it from the other mycobacterial enzymes studied is that it binds iron as its metal ion ligand instead of manganese (4).

This study presents our findings on the expression and extracellular release of endogenous and recombinant superoxide dismutases from M. tuberculosis and M. smegmatis. The experiments were designed to answer the following fundamental questions concerning the expression and secretion of this enzyme in mycobacterial species. (i) To what extent is superoxide dismutase exported in these two species? (ii) Is recombinant M. tuberculosis superoxide dismutase expressed and secreted in M. smegmatis? (iii) Is recombinant M. tuberculosis superoxide dismutase capable of transcomplementing a completely superoxide dismutase-deficient Escherichia coli mutant, a species that, when superoxide dismutase-proficient, expresses the enzyme strictly intracellularly? (iv) Most importantly, does the export of superoxide dismutase and glutamine synthetase follow a similar or dissimilar paradigm?

**EXPERIMENTAL PROCEDURES**

**Bacterial Cultures**

M. tuberculosis strain Erdman (ATCC 35801) and M. smegmatis 1-2c (provided by Peadar O’Gara, Imperial College School of Medicine at St. Mary’s, London, UK) (10) were grown in 7H9 medium (Difco) or Sauton’s medium (11) at pH 6.7. M. tuberculosis cultures were maintained in stationary flasks at 37 °C and a mixture of 5% CO₂, 95% air, growing logarithmically for approximately 3 weeks, whereas M. smegmatis cultures were maintained in an environmental shaker at 180 rpm for 3–4 days or in unshaken flasks for 6–8 days at 37 °C and a mixture of 5% CO₂, 95% air. M. smegmatis transformants were initially grown in 7H9 medium supplemented with 2% glucose before being transferred to standard medium containing hygromycin at 50 μg/ml.

E. coli DH5α (12) was grown in Luria-Bertani (LB) medium, and transformants were grown under the appropriate selective conditions.

The superoxide dismutase-relevant E. coli strains QC771, QC772, QC773, and QC774, specifying the parent strain, single superoxide dismutase mutants, and the sodA sodB double mutant (provided by Daniele Touati, Institut Jaques Monod, Université Paris, Paris, France (13), respectively, were cultured in selective medium as recommended (13). For the analysis of physiologic parameters, these strains and the QC774 double mutant transcomplemented with the M. tuberculosis superoxide dismutase were grown under various culture conditions that are specified in the corresponding figure legends.

**Molecular Cloning and DNA Sequence Determination of M. tuberculosis and M. smegmatis Superoxide Dismutase Genes**

High molecular weight genomic DNA of M. tuberculosis Erdman was isolated by hot phenol extraction and used as a template in 40 rounds of amplification at 94-55-72 °C of a region containing the published DNA sequence of the structural gene for superoxide dismutase from M. tuberculosis H37Rv (6). The amplification product with the 5’ and 3’ primers at its respective ends (5’ primer, 5’ → 3’ GTGGCCGAATA-CACCTTGCCAGAC, specifying the first 8 amino acids of superoxide dismutase including the initiator methionine GTG; 3’ primer, 5’ → 3’ CACCTTGCCAGACGGTGGCCGAATATCAACCCCTTGGT, specifying the last 7 amino acids of the enzyme plus the stop codon) was cloned into pCR™ (Invitrogen) and used as a probe for Southern analyses with M. tuberculosis genomic DNA digested with various restriction enzymes. Southern hybridizations were performed on nitrocellulose membranes at 60–65 °C for 24 h in 5× SSC (1× SSC: 150 mM sodium chloride and 15 mM sodium citrate, pH 7.2) or 5× SSPE (1× SSPE: 150 mM sodium chloride, 10 mM sodium phosphate, 1 mM EDTA, pH 7.2) with probes labeled to specific activities of 5 × 10⁷ to 1 × 10⁸ of 32P/μg. Filters were washed at hybridization temperature of 0.2× SSC or SSPE, dried, and autoradiographed on Kodak X-Omat XAR5 or Fuji RX film for various times either at room temperature or at ~70 °C using a Cronex Lightning-Plus film (DuPont).

BamHI restriction fragments of ~6–10 kilobase pairs (kb), containing the hybridizing genomic DNA fragment of ~8 kb, were cut from an agarose gel, eluted by centrifugation through siliconized glass wool, ethanol-precipitated, verified by Southern hybridization to contain the superoxide dismutase-encoding fragment, and digested with various restriction enzymes other than BamHI to produce smaller fragments for rapid directional cloning in the E. coli/mycobacteria shuttle plasmids pSMT3 (10) or pNBV-1 (14). Restriction fragments of ~4 kb generated with the enzymes BamHI and ClaI yielded unambiguous patterns in Southern and colony hybridizations performed as described above as well as in restriction site mapping analyses that verified that the isolated restriction fragment was identical to the published DNA sequence of the canonical T624 encoded sodA locus (15). Based on this sequence and using the same reaction conditions as described above, we employed two pairs of primers (5’ primer 1 (primer 1 in Fig. 1): 5’ → 3’ CAGT (arbitrary nucleotides) -ATCGAT (ClaI site) -GGTTAGTGGTGATGCTTGGCAAG, annealing from 541 to 518 nucleotides upstream of the superoxide dismutase gene’s initiator GTG codon, and 3’ primer 1 (primer 2 in Fig. 1): 5’ → 3’ CAGT (arbitrary nucleotides) -GATCC (BamHI site) -CTAGCTGGATACCAAGCATGCG, annealing to nucleotides 156 to 133 downstream of the gene’s TGA stop codon; and a second pair, 5’ primer 2 and 3’ primer 2, with exactly the same sequences except that the restriction sites were switched) to amplify two fragments of ~1.3 kb that were restricted with ClaI and BamHI and cloned directly into pNBV-1 restricted with the same enzymes. Both plasmid inserts were completely sequenced by the chain termination method (16), and their sequence was identical to the published sodA locus on cosmID T624 (15).

The M. smegmatis 1-2c superoxide dismutase gene sequence was established for an amplification product whose primers were derived from M. tuberculosis and Mycobacterium fortuitum superoxide dismutase gene sequences. Two amplification products were combined to yield a single 3,036-bp fragment of M. smegmatis 5’ primer 1, 5’ → 3’ GTGGCCGAATA-CACCTTGCCAGACGGTGGCCGAATATCAACCCCTTGGT, corresponding to the first 8 amino acids of the M. tuberculosis superoxide dismutase gene, and M. smegmatis 3’ primer 1, 5’ → 3’ CAGTGGCCGAATA-CACCTTGCCAGACGGTGGCCGAATATCAACCCCTTGGT, corresponding to the first 8 amino acids of the M. smegmatis superoxide dismutase gene, and M. smegmatis 3’ primer 2, 5’ → 3’ CAGTGGCCGAATA-CACCTTGCCAGACGGTGGCCGAATATCAACCCCTTGGT, corresponding to the last 7 codons plus stop codon of the M. fortuitum enzyme), yielded an amplification product of ~325 bp. Both amplification products were sequenced completely, and cloned into pCR™.

**Constructs Used for the Expression of Recombinant M. tuberculosis Erdman Superoxide Dismutase in M. smegmatis**

The constructs used to express recombinant M. tuberculosis superoxide dismutase in M. smegmatis were based on the aforementioned amplification products of a stretch of ~1.3 kb of M. tuberculosis DNA spanning the superoxide dismutase coding region plus 5’- and 3’-flanking sequences. One construct contained the amplification product inserted into the ClaI-BamHI sites of the multi-cloning region of pNBV-1 in a 5’ → 3’ direction, thereby allowing transcription of the superoxide dismutase gene to proceed in a direction opposite the direction of transcription of the vector encoded β-galactosidase promoter; the second construct was inserted in the same restriction sites but in opposite orientation, thus aligning the superoxide dismutase and β-galactosidase promoters. Constructs were first established in E. coli DH5α and then electroporated into M. smegmatis 1-2c as described (10). Stable M.
smegmatis 1-2c transformants were selected and characterized for expression of superoxide dismutase as described below.

**Construct Used for the Expression of Recombinant M. tuberculosis Erdman Superoxide Dismutase in E. coli QC774**

For transcomplementation of the *E. coli* sodA sodB double mutant QC774, an amplification product of the *M. tuberculosis* superoxide dismutase gene was ligated in the NcoI-HindIII sites of the high level expression vector pKK233-2 (provided by Andrew Campbell, Brown University, Providence, RI (17)). The amplification reaction was performed under standard conditions (see above) with the cloned *M. tuberculosis* superoxide dismutase gene as a template and the following primer pair: 5' primer (primer 3 in Fig. 1), 5'GATC (arbitrary nucleotides) -CCATGG- (NcoI site, the internal ATG provides the initiator methionine, the second G is part of the alanine codon, the first amino acid of the mature protein) -CCGAATACACCTTGCCAGACCT-GGACTGGGAC (corresponding to codons 1–11) and 3' primer (primer 4 in Fig. 1), 5'CTAG (arbitrary nucleotides) -AAGCTT (HindIII site) -TCAGCCGAATATCAACCCCTTGGTCTGCGA (corresponding to the last 9 codons plus stop codon). The amplification product was first cloned into pCRTM, verified by DNA sequencing, recloned into pKK233-2 as an NcoI-HindIII fragment, maintained in DH5α, and transformed into QC774 under the appropriate selection conditions (13).

**Analysis of Expression of Endogenous and Recombinant Superoxide Dismutases**

Characterization of endogenous and recombinant superoxide dismutases was performed by the following analytical methods. Polyacrylamide Gel Electrophoresis of Cell Pellets and Culture Supernatants—*M. tuberculosis*, *M. smegmatis*, and *E. coli* were cultured in standard medium as described above. Aliquots of the cultures were removed and separated into cell pellets and supernatants by centrifugation. Cell pellets were taken up in a small volume of 1/3 phosphate-buffered saline (50 mM sodium phosphate and 150 mM sodium chloride, pH 7.2) and lysed either by vortexing vigorously with 60-mesh crystalline alumina beads (Fisher) for 5 min at room temperature (mycobacterial species) or by the addition of lysozyme at 100 μg/ml for 20 min on ice (*E. coli*). Insoluble material was collected by centrifugation, and the cell pellet proteins were dialyzed extensively against 1/3 phosphate-buffered saline, centrifuged again, and adjusted to a final volume such that 1 ml contained the supernatant proteins of 1 x 10^8 cells of the original culture. Proteins in the cell pellets and culture supernatants were analyzed by electrophoresis on standard, 10% denaturing polyacrylamide gels, followed by staining with Coomassie Brilliant Blue G-250.
Blue. Protein concentrations in the cell pellets and culture superna-
tants were determined by the bicinchoninic acid reagent (Pierce).

**Immunoreactivity of Endogenous and Recombinant Superoxide Dis-
mutases—** Polyvalent antibodies against the purified *M. tuberculosis*
superoxide dismutase were raised as follows. The enzyme was purified
from cell pellets and culture supernatants of *M. smegmatis* and *M.
Erdman* cultures. The pellets were extracted with ammonium
sulfate precipitation and chromatography on DEAE-CL6B and Superdex
75 to yield a homogeneous enzyme prepa-
ration as judged by denaturing polyacrylamide gel electrophoresis
and staining with Coomassie Brilliant Blue. The superoxide dismutase
band (subunit, 23 kDa) was excised and eluted from the gel. Rabbits
were immunized with an emulsion of 1 mg of enzyme and 1 mg of Syntex
adjuvant formulation (18) containing 100 mg of purified enzyme
and 1.5 mg of N-acetylmuramyl-L-alanyl-D-isoglutamine, fol-
lowed by three booster immunizations at 12-day intervals with 100 mg
of enzyme. Antibody preparations had reciprocal titers of <100 pre-
imunization and ~10^6 post-immunization against the native and
denatured enzyme isolated from *M. tuberculosis* Erdman cell pellets
and culture supernatants. In Western blots, superoxide dismutase
was the only protein of all cellular and extracellular *M. tuberculosis*
Erdman proteins that reacted with the antibodies.

For the detection and identification of endogenous and recombinant
superoxide dismutases, proteins in cell pellets and culture superna-
tants were electrophoresed in 10% denaturing polyacrylamide gels,
transferred to nitrocellulose membranes, and incubated first with pre-
tempts were electrophoresed in 10% denaturing polyacrylamide gels,
proteins that reacted with the antibodies.

**Materials**

All chemicals and enzymes were purchased from Sigma unless indi-
cated otherwise and were of the highest grade available. All oligonu-
cleotides were purchased from Genosys Biotechnologies, Inc.

**RESULTS**

**Endogenous Superoxide Dismutase Is Abundantly Exported from M. tuberculosis but Not M. smegmatis**

We first established a base line for the expression of the endogenous superoxide dismutases from both mycobacterial
cultures and supernatants of unshaken *M. tuberculosis* cultures and both shaken and un-
shaken *M. smegmatis* cultures for enzyme activity. The results
clearly showed that in all cultures enzyme activity increased in
parallel with cell density, and the protein patterns did not
change significantly during the observation period, suggesting
that there are few if any truly early or late released proteins. The only measurable difference was that *M. smegmatis*
cultures in unshaken flask cultures grew with approximately 50% the
growth rate of the shaken cultures. This result further indi-
cated that the proteins in the culture supernatants were stable. This supposition was later confirmed by immunoblot analyses
of cell pellets and culture supernatants, which showed no de-
tectable degradation products of the superoxide dismutases. In
view of these results, we selected the following time points for
assessing the various cultures for the amount, distribution, and
activity of expressed superoxide dismutase: 3 weeks for *M.
tuberculosis* cultures and 3 days for *M. smegmatis* shaken
cultures.

The two mycobacterial species differed dramatically in their
superoxide dismutase expression patterns with regard to both
the level of expression and distribution of the enzyme. In an
analysis in which superoxide dismutase expression was quan-
tified by densitometrically scanning immunoblots (Fig. 2, mid-
dle portion), *M. tuberculosis* expressed a total (cell pellet +
supernatant) of 2.8 fg per cell versus 0.03 fg per cell for *M.
smegmatis*, a 93-fold difference. Moreover, *M. tuberculosis*
exported 76% of the expressed enzyme versus 21% for *M. smeg-
matis* (2.1 fg versus 0.006 fg per cell). Hence, *M. tuberculosis*
exported ~550-fold more endogenous superoxide dismutase
than *M. smegmatis*.

An analysis of enzyme activity in the culture supernatant
confirmed these results. *M. tuberculosis* exported 2,778
nanounits per cell versus 7.6 nanounits per cell for *M. sme-
gmatis*, a 365-fold difference (Fig. 2, bottom portion).

The presence of extracellular superoxide dismutase in both
species was not due to leakage of proteins in the culture me-
dium from dead cells. All cultures were continuously monitored
for the release of the intracellular marker enzyme lactate de-
hydrogenase. For all bacterial cultures, the enzyme was found to
be strictly present and not the culture supernatant. Calculated on the basis of the total de-
detectable lactate dehydrogenase activity, i.e. intracellular plus extracellular lactate dehydrogenase activity, the extracellularly detectable lactate dehydrogenase activity increased over time but did not amount to more than about 0.5% of the total activity by the end of the growth period for any culture including M. smegmatis expressing recombinant M. tuberculosis superoxide dismutase.

**M. tuberculosis and M. smegmatis Superoxide Dismutase Genes**

The finding that the enzyme superoxide dismutase is abundantly released by pathogenic but not by a nonpathogenic mycobacterium (6) and that the pattern of expression and extracellular release of this enzyme mimics that of the other major leaderless extracellular enzyme of M. tuberculosis, glutamine synthetase, prompted us to study the expression of M. tuberculosis superoxide dismutase in a heterologous host. This first required that we clone and characterize the M. tuberculosis Erdman superoxide dismutase gene.

Cloning of the Superoxide Dismutase Gene from M. tuberculosis Erdman—Starting point for the analysis of the M. tuberculosis Erdman superoxide dismutase gene was an amplification product of genomic DNA, which was based on the published DNA sequence of the structural gene for superoxide dismutase from M. tuberculosis H37Rv (6) and which was used for all subsequent Southern and colony hybridizations. All cloned restriction fragments containing the superoxide dismutase gene were quite large, the smallest being a 1.3 kb that included the superoxide dismutase coding region, ~540 bp of 5′-flanking region and ~125 bp of 3′-flanking region (Fig. 1). This strategy allowed us to exclude other potential open reading frames contained in these larger fragments and to utilize the same restriction sites (ClaI and BamHI) that are present as unique sites in the E. coli/mycobacterial shuttle expression vector pNBV-1 (14) for bidirectional cloning and expression of the superoxide dismutase-containing amplification product.

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**Fig. 2.** Expression of endogenous and recombinant M. tuberculosis superoxide dismutase. The top part of the figure depicts schematically the recombinant pNBV-1 construct used to express the M. tuberculosis Erdman superoxide dismutase in the heterologous host M. smegmatis 1-2c. The M. tuberculosis superoxide dismutase gene (gray-shaded box), flanked at its 5′ end by 541 bp and at its 3′ end by 156 bp (lightly and densely patterned boxes), is inserted in the vector DNA at the Clal site (5′ end) and the BamHI site (3′ end). The 5′-flanking region provides the gene’s regulatory regions (unmapped) and a Shine-Dalgalorno sequence (indicated by lightly and densely patterned boxes) immediately the recombinant pNBV-1 construct used to express the M. tuberculosis Erdman superoxide dismutase gene was an amplification product of genomic DNA, which was based on the published DNA sequence of the structural gene for superoxide dismutase from M. tuberculosis H37Rv (6) and which was used for all subsequent Southern and colony hybridizations. All cloned restriction fragments containing the superoxide dismutase gene were quite large, the smallest being a 1.3 kb that included the superoxide dismutase coding region, ~540 bp of 5′-flanking region and ~125 bp of 3′-flanking region (Fig. 1). This strategy allowed us to exclude other potential open reading frames contained in these larger fragments and to utilize the same restriction sites (ClaI and BamHI) that are present as unique sites in the E. coli/mycobacterial shuttle expression vector pNBV-1 (14) for bidirectional cloning and expression of the superoxide dismutase-containing amplification product.
Cloning of the Superoxide Dismutase Gene from M. smegmatis 1-2c—

We developed and pursued a similar strategy for the cloning and expression of the M. smegmatis 1-2c superoxide dismutase gene. However, since we were primarily interested in the elucidation of differences in expression and secretion between the M. tuberculosis and M. smegmatis enzymes, we first focused on establishing the DNA sequence of the coding region of the M. smegmatis enzyme, which is shown in Fig. 3 along with the aligned coding region of the M. tuberculosis enzyme. This DNA sequence was determined for an amplification product of M. smegmatis genomic DNA, which was cloned into pCRTM.

Nucleotide Analysis of the M. tuberculosis and M. smegmatis Superoxide Dismutase Genes—

The results of the nucleotide analysis of the two DNA sequences coding for superoxide dismutase can be summarized as follows. (i) Both the M. tuberculosis and M. smegmatis genome most likely contain only one gene coding for an iron-specific and manganese-specific superoxide dismutase, respectively. If a second gene is present, its DNA sequence must differ significantly from the ones described here. (ii) Both coding regions are 624 bp long, utilize GTG as the initiator methionine codon, and encode a mature protein of 206 amino acids with a theoretical molecular mass of 23,000 Da. This mass is consistent with the migration patterns of purified superoxide dismutases on denaturing polyacrylamide gels (Fig. 2, middle portion). (iii) The coding regions are highly homologous to each other and to other described mycobacterial superoxide dismutases (8, 9) and to a lesser degree to superoxide dismutases from other bacterial species (20, 21). (iv) Although the active site residues, His-28, His-76, His-164, and Asp-160, are conserved between the two sequences, the amino acid residue 145 puts the M. tuberculosis enzyme (His-145) in the category of iron-binding enzymes and the M. smegmatis enzyme (Gln-145) in the category of manganese-binding enzymes, according to a recent structural study on metal ion specificity of superoxide dismutases (4).

(v) The M. tuberculosis superoxide dismutase gene sequence does not code a leader peptide. The absence of a leader peptide for the enzymes of both species was confirmed by analyses of the N termini of both cellular and extracellular superoxide dismutase molecules: AEYTLPDLDW for M. tuberculosis and AEYTLP-DLDY for M. smegmatis.

(vi) Although a very strong ribosome-binding site is present upstream of the initiator GTG in the M. tuberculosis DNA sequence (position 215 to 245: GAAGGAAG-GAA), the positions of the promoter elements, including potential binding sites for factors that may influence the rate of gene transcription under hydrogen peroxide stress, and the mRNA start site remain unclear. Analyses of the 5′-flanking region of the M. smegmatis superoxide dismutase gene are in progress.
Recombinant *M. tuberculosis* Superoxide Dismutase Is Exported Abundantly in a Heterologous Mycobacterial Host

The two recombinant pNBV-1 constructs described above contained the *M. tuberculosis* superoxide dismutase gene and flanking DNA sequences in two orientations (Fig. 2). The plasmids were maintained in *E. coli* DH5α where they were stably propagated without any detectable changes and without any indication of expression of recombinant superoxide dismutase, most likely because the *E. coli* transcription machinery is unable to recognize the mycobacterial promoter. Following transformation of the two recombinant constructs into *M. smegmatis* 1-2c, several transformants for each construct were assayed for expression of recombinant superoxide dismutase as described above for *M. smegmatis* cultures. Since the promoter region contained in the plasmid insert cannot be regulated, any expression of recombinant superoxide dismutase in these cultures is constitutive.

The recombinant *M. tuberculosis* superoxide dismutase was expressed from its own promoter in both pNBV-1 constructs (Fig. 2, top portion). Expression of the correct protein in *M. smegmatis* was verified by sequence analysis of the 15 N-terminal amino acids, which confirmed that the recombinant protein contained a tryptophan residue in position 10 (AEYTLPDLDW), whereas the endogenous *M. smegmatis* enzyme contained a tyrosine residue in that position (AEYTLPDLDY). This difference is very important from an analytical point of view, because the next difference between the two enzymes occurs at position 40. Intra- and extracellular recombinant superoxide dismutases had identical N termini, indicating the lack of a leader peptide.

The recombinant enzymes were expressed at greatly different levels depending on the construct used (Fig. 2, middle portion). The construct containing the superoxide dismutase insert in the same direction of transcription as the vector-encoded β-galactosidase expressed the recombinant enzyme at a level that was only approximately twice that of the endogenous enzyme and the distribution between cell pellet and culture supernatant was similar to that of the endogenous enzyme. In contrast, the construct containing the insert in the opposite direction expressed the recombinant enzyme at a level that was approximately 8.3-fold greater than the endogenous enzyme. Moreover, approximately 66% of the expressed enzyme was exported, a level of export approaching that of the endogenous *M. tuberculosis* enzyme.

Superoxide dismutase in the culture supernatant of the recombinant *M. smegmatis* appeared as a set of five bands with slightly different mobilities on the activity gels (Fig. 2, bottom portion). The top band comigrated with the endogenous *M. tuberculosis* enzyme and the bottom band, showing the least activity, comigrated with the endogenous *M. smegmatis* enzyme, suggesting a possible mixing of *M. tuberculosis* and *M. smegmatis* subunits to form an active tetrameric enzyme. That this was so was confirmed by N-terminal sequence analysis and metal analysis of the five bands. The top and bottom bands contained homogeneous subunits corresponding to the endogenous *M. tuberculosis* and *M. smegmatis* enzymes, respectively. The middle three bands contained subunits of both enzymes, as evidenced by a mixture of tryptophan (M. tuberculosis enzyme) and tyrosine *(M. smegmatis* enzyme) residues at position 10. The ratio of detected picomoles of tryptophan for the *M. tuberculosis* enzyme subunit to tyrosine for the *M. smegmatis* enzyme subunit suggested a composition of the recombinant enzyme as follows: three *M. tuberculosis* subunits plus one *M. smegmatis* subunit for the second band; two *M. tuberculosis* subunits plus two *M. smegmatis* subunits for the third band; and one *M. tuberculosis* subunit plus three *M. smegmatis* subunits for the fourth band. Paralleling these results, the top band contained iron, utilized by the *M. tuberculosis* enzyme, and the bottom band contained manganese, utilized by the *M. smegmatis* enzyme. The calculation of the composition of the middle band is as follows. The protein amount of this band was 1.8 µg, which corresponds to 4.9×10¹³ polyepitides of ~23 kDa. The metal content was 2.5 ng of manganese and 2.2 ng of iron, corresponding to 2.8×10¹³ manganese atoms and 2.4×10¹³ iron atoms or a total of 5.2×10¹³ metal atoms for 4.9×10¹³ enzyme subunits, in close agreement with the prediction of 1 metal atom per subunit. Taken together, the data indicate that the five bands contain in descending order the following ratio of *M. tuberculosis*: *M. smegmatis* subunits, 4:0, 3:1, 2:2, 1:3, and 0:4.

The recombinant *M. smegmatis* strain, of course, expresses its own endogenous superoxide dismutase, but its contribution to the total detectable enzyme activity is minimal, since its expression level does not change dramatically when compared with the parent strain. Although we cannot prove directly that the expression level of endogenous *M. smegmatis* superoxide dismutase remains nearly constant, two observations provide strong support for this assumption. (i) In the activity gel analyses, the level of expression of the *M. smegmatis* enzyme subunits by the parent strain (1 band) was comparable to that of the recombinant strain (predominantly the lower bands of the 5-band set). (ii) More importantly, N-terminal amino acid sequence analysis of the exported recombinant enzyme (1 band with a molecular mass of ~23 kDa on a denaturing polyacrylamide gel and transferred to a polyvinylidene difluoride membrane) demonstrated a >100:1 ratio of *M. tuberculosis*: *M. smegmatis* subunits (presence of tryptophan versus tyrosine at position 10). Since the increase in expression overall was only 8-fold, this result indicated that the increased amount of extracellularly detectable enzyme activity in the recombinant *M. smegmatis* strain is due primarily to the presence of the recombinant *M. tuberculosis* enzyme and not to a major increase in export of the endogenous *M. smegmatis* enzyme.

This disproportionate increase in *M. tuberculosis* enzyme subunits in the extracellular medium of recombinant *M. smegmatis* revealed by N-terminal amino acid analysis indicated that *M. tuberculosis* superoxide dismutase is preferentially exported. To confirm this, we compared the banding pattern of the enzyme in the supernatant and pellet fractions of recombinant *M. smegmatis* (Fig. 4). We reasoned that if the *M. tuber-
Superoxide dismutase enzyme was not preferentially exported, then the pellet and supernatant fractions would have the same banding pattern. On the other hand, if the *M. tuberculosis* enzyme is preferentially exported, then the banding pattern in the supernatant should be enriched for *M. tuberculosis* subunits (upper bands) relative to the banding pattern in the pellet. Consistent with preferential export of *M. tuberculosis* superoxide dismutase, the banding pattern of the enzyme in the supernatant was enriched for *M. tuberculosis* subunits. This was true for recombinant *M. smegmatis* whether the orientation of the superoxide dismutase and β-galactosidase promoters in the expression construct was in the opposite direction or unidirectional.

The subunits of the *M. tuberculosis* and *M. smegmatis* enzymes not only mix to form five different complexes of subunit enzymes during expression of the recombinant *M. tuberculosis* enzyme in *M. smegmatis* but also when the two purified enzymes are incubated together for a short period (2 h) at room temperature (Fig. 5). Mixing experiments suggested that homologous or heterologous subunits associated with each other with comparable affinity. Moreover, when the middle of the five bands, containing two subunits of each enzyme, is eluted from a polyacrylamide gel and immediate re-electrophoresis; and the middle band after excision from a polyacrylamide gel, elution from the gel with ammonium acetate and magnesium acetate, incubation for 24 h in phosphate-buffered saline, and re-electrophoresis, revealing redistribution of the subunits into the five different complexes.

Recombinant *M. tuberculosis* Superoxide Dismutase Transcomplements a Superoxide-deficient Heterologous Host

To characterize further the functional activity and export capacity of the recombinant *M. tuberculosis* superoxide dismutase, we studied its capacity to complement a superoxide dismutase-deficient mutant *E. coli*. The superoxide dismutase proficient parent strain QC771 and the sodA sodB double mutant QC774 were first grown in aerated broth cultures under various conditions to establish critical base-line values required for the expression of a successful transcomplementation of the mutant strain by the mycobacterial enzyme. The parent strain QC771 grew very well in LB and in glucose containing minimal medium (Fig. 6A). The mutant QC774 grew in LB medium, but more slowly than the wild type, and it grew in minimal medium only when the medium was supplemented with 20 amino acids; even then, growth was very slow (Fig. 6A). The superoxide generator paraquat slightly inhibited growth of the wild type, but more markedly inhibited growth of the mutant (Fig. 6B), as previously reported (13).

Both the wild type and mutant were assessed for the expression of superoxide dismutase by immunoblotting and activity assays (Fig. 7, lower portion). The wild type (QC771) showed the presence in the cell pellet but not in the culture supernatant of two very faint bands containing enzymatic activity and corresponding to the iron- (M, ~21,000) and manganese (M, ~23,000)-binding superoxide dismutases. The level of expression was far below that measured even for *M. smegmatis*, amounting to ~0.36 nanounits per cell, consistent with the results of earlier studies of superoxide dismutase from *E. coli* B (22). The mutant strain (QC774) was completely devoid of any enzyme activity.

We explored the capacity of a mycobacterial enzyme to substitute for the *E. coli* enzymes by transforming the recombinant plasmid pKK233-2, containing the gene of the *M. tuberculosis* Erdman superoxide dismutase, into *E. coli* QC774 (Fig. 7, upper portion). The plasmid pKK233-2 was chosen for its very strong promoter, its optimal placement of regulatory regions, allowing the expression of recombinant enzyme by itself rather than as a fusion protein, and the presence of the initiator ATG codon, overlapping the NcoI cloning site.

Upon transformation, the recombinant strain remained stable, maintaining the introduced plasmid over many cell generations, although the strain did not plate uniformly, an issue that could not be resolved satisfactorily. Presence of the plasmid was also verified by isolating the recombinant pKK233-2 construct and DNA sequencing across the vector insert junctions with primers annealing to the cloned *M. tuberculosis* superoxide dismutase gene. After induction of recombinant superoxide dismutase expression, strain QC774 grew very well in LB medium and exhibited a growth pattern in minimal medium that approached that of the noncomplemented mutant in minimal medium supplemented with all 20 amino acids (Fig. 6A). The addition of paraquat at a concentration of ≤50 μM to LB broth cultures had no growth inhibitory effect on the transcomplemented strain. At 50 μM, the difference in colony-forming units between QC774 with and without the recombinant pKK233-2 plasmid amounted to almost 1 log unit by the end of the growth phase (Fig. 6B). At concentrations higher than 100 μM, both the wild type and the transcomplemented strains showed significant growth inhibition.

Whereas treatment with 2.5 and 5 mM hydrogen peroxide had only a minimal effect on the wild-type QC771, it exerted a fast-acting, detrimental effect on the QC774 mutant, whose viability at the end of the 60-min treatment period dropped by 2 log units (2.5 mM hydrogen peroxide) and >3 log units (5 mM hydrogen peroxide), respectively (Fig. 6C). Transcomplementation of the mutant with the *M. tuberculosis* superoxide dismutase gene substantially protected the strain from oxidative stress, indicating that the recombinant enzyme was active in the heterologous host but could not fully substitute for its endogenous enzymes. Even under the harshest conditions, 5

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**Fig. 5.** Exchange of subunits of *M. tuberculosis* and *M. smegmatis* superoxide dismutase. The three lanes on the left show, from left to right, purified endogenous *M. tuberculosis* superoxide dismutase (M.tb. SOD, ~10 units); purified endogenous *M. smegmatis* superoxide dismutase (M.s. SOD, ~50 units); and the mixing of subunits of both enzymes after being incubated together for 2 h at room temperature in phosphate-buffered saline. Enzymes were electrophoresed on a native 15% polyacrylamide gel and stained for enzyme activity (19). The three lanes on the right show, from left to right, recombinant *M. tuberculosis* superoxide dismutase (rM.tb. SOD, ~100 units), expressed in *M. smegmatis* as a tetramer exhibiting the five different possible combinations of *M. tuberculosis* and *M. smegmatis* enzyme subunits; the middle band (Band 3) of the five bands, containing two subunits of each enzyme, after excision from a polyacrylamide gel and immediate re-electrophoresis; and the middle band after excision from a polyacrylamide gel, elution from the gel with ammonium acetate and magnesium acetate, incubation for 24 h in phosphate-buffered saline, and re-electrophoresis, revealing redistribution of the subunits into the five different complexes.
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**FIG. 6.** Analysis of expression of recombinant *M. tuberculosis* superoxide dismutase in the nonmycobacterial host *E. coli* QC774. 

A shows the growth curves of *E. coli* QC771 (superoxide dismutase-proficient) and QC774 (superoxide dismutase-deficient) without and with supplements in M9 minimal medium containing 0.4% glucose and 1 μg/ml thiamin. Strain QC774 was supplemented with either all 20 amino acids or recombinant *M. tuberculosis* superoxide dismutase, expressed from the *E. coli* specific “ATG vector” pKK233-2. All broth cultures were started at a cell density of 1 × 10^6 cells per ml and grown at 37 °C for 12–72 h with vigorous shaking. Changes in cell density were monitored by removing aliquots of the cultures every 2 h mm hydrogen peroxide, the transcomplemented strain’s viability differed by <1 log unit from that of the wild type (Fig. 6C).

**Recombinant Superoxide Dismutase Is Not Abundantly Exported from a Nonmycobacterial Host**

In the mycobacterial host *M. smegmatis*, the recombinant *M. tuberculosis* superoxide dismutase was not only expressed, it was abundantly exported, as in *M. tuberculosis*. In this case, the hosts were relatively closely related and the enzymes relatively similar. To determine if the recombinant *M. tuberculosis* enzyme could be abundantly exported in a more distantly related nonmycobacterial host, and one whose endogenous enzymes are even more dissimilar, we investigated directly the expression and export of the recombinant *M. tuberculosis* superoxide dismutase in *E. coli*.

For the expression of the mycobacterial enzyme, there were two requirements. First, there was an absolute requirement for the presence of an *E. coli* promoter. The two constructs used to express the *M. tuberculosis* enzyme in *M. smegmatis* are maintained in *E. coli* QC774 under proper selection but did not express any superoxide dismutase molecules at all. Second, there was a requirement for the presence within the plasmid’s NcoI site of an ATG start codon. The *M. tuberculosis* superoxide dismutase gene uses GTG as its initiator codon, as does the *M. tuberculosis* glutamine synthetase, but this codon is not accepted as the initiator codon in this plasmid in *E. coli* (5).

Both immunoblotting and activity assays showed that the recombinant *M. tuberculosis* enzyme is strictly expressed intracellularly. No protein or activity was detected in the culture supernatant (Fig. 7, lower portion). The expression level was well below that observed for *M. smegmatis* and *M. tuberculosis* cultures but ~5.3-fold greater than that measured for wild-type *E. coli* QC771, amounting to ~1.9 nanounits of superoxide dismutase activity per cell. This is approximately 1,500-fold below its activity in *M. tuberculosis*. The recombinant enzyme was of the correct size. It migrated as one band with a molecular mass of ~23,000 Da on denaturing polyacrylamide gels, and it migrated with the same mobility as the endogenous *M. tuberculosis* enzyme on native activity gels. Analysis of the first 7 N-terminal amino acid residues revealed the sequence AEYTLPD, identical to the sequence of the endogenous *M. tuberculosis* enzyme.

**DISCUSSION**

Export of Endogenous Superoxide Dismutase—Our study demonstrates that the enzyme superoxide dismutase is abundantly exported in active form from a pathogenic mycobacte-
Export of Leaderless M. tuberculosis Superoxide Dismutase

FIG. 7. Expression of recombinant M. tuberculosis superoxide dismutase (SOD) in E. coli. The upper portion of the figure depicts schematically the recombinant pKK233-2 construct used to transcomplement E. coli QC774. The M. tuberculosis superoxide dismutase gene (gray-shaded box) is inserted in the vector DNA at the NcoI site (5' end and also providing the ATG start codon) and HindIII site (3' end, immediately following the T2A stop codon). Marked features of the vector include the tet B gene, conferring tetracycline resistance; the hybrid trp-lac promoter pTrc; a Shine-Dalgarno sequence, S.D.; the gene encoding the 5 S rRNA, and the two transcription terminators T1 and T2; the amp B gene, conferring ampicillin resistance; and ori B, the colEI origin of replication. The lower portion of the figure shows the results of assaying the transcomplemented strain QC774 for expression of recombinant M. tuberculosis superoxide dismutase (rM.tbc SOD). Included in the analyses are the wild-type E. coli strain QC771 and the strain QC774 prior to its transcomplementation. The analyses of the cell pellets (P) and culture supernatants (S) include, from left to right, (i) immunoblotting with anti-M. tuberculosis superoxide dismutase-specific antibodies at a dilution of 1:5,000; pre-immune antibodies gave negative results in all cases and are not included; (ii) visualization of enzymatic activity by electrophoresis of aliquots of cell pellets in nondenaturing polyacrylamide gels followed by a superoxide dismutase-specific staining procedure (19); and (iii) quantitation of the observed activity of the recombinant M. tuberculosis enzyme. On the left, the molecular weights of marker proteins (M) are indicated at their positions in M, × 10^2.

rrium, the highly virulent Erdman strain of M. tuberculosis, but not from the nonpathogenic mycobacterium M. smegmatis. M. tuberculosis not only expresses approximately 100-fold more enzyme than M. smegmatis, it exports most (76%) of what it makes, whereas M. smegmatis retains most (79%) of what it makes. Consequently, the amount of enzyme exported by M. tuberculosis is enormous, 350-fold more than M. smegmatis.

Why superoxide dismutase, a typically intracellular enzyme, is abundantly exported by M. tuberculosis is not clear. Other major extracellular enzymes exported by M. tuberculosis have been implicated in cell wall construction. These include the mycolyl transferases of the 30/32-kDa (Antigen 85) complex and glutamine synthetase (1, 3). Superoxide dismutase, however, has not been implicated in cell wall construction. Rather, this enzyme is typically involved in protection against oxidative stress. Superoxide may not only cause damage by itself, it can participate in a chemical reaction with hydrogen peroxide and iron (the Fenton mechanism) that can produce an even more toxic molecule, hydroxyl radical. The host cells of M. tuberculosis are known to produce toxic oxygen metabolites, and it is tempting to speculate that M. tuberculosis releases superoxide dismutase to neutralize these metabolites in the phagosome or lung cavity, the two sites in which the pathogen multiplies in the host. In this regard, superoxide dismutase has been documented in the phagosome of M. tuberculosis in human mononuclear phagocytes. The release of superoxide dismutase in the vicinity of M. tuberculosis might have the advantage of neutralizing toxic oxygen molecules even before they reach the outer cell wall of the organism. Exported superoxide dismutase might thus protect external cell wall structures of the pathogen that are not protected by its intracellular superoxide dismutase. If exported superoxide dismutase serves this purpose, this would constitute the second mechanism by which M. tuberculosis protects itself from toxic oxygen metabolites. By entering mononuclear phagocytes via complement receptors (23), which when ligated by C3 do not trigger an oxidative burst (24), M. tuberculosis avoids much of the toxic consequences of this burst.

It is of interest that the M. tuberculosis superoxide dismutase enzyme binds iron. This may reflect the greater availability of iron than manganese in the human host. It may also be pertinent that M. tuberculosis has evolved an elaborate system for acquiring iron involving the abundant export of an extracellular high affinity iron-binding siderophore, the exochelins (25). The binding of iron by superoxide dismutase may allow the enzyme to counteract hydroxyl radical production via the Fenton mechanism in two ways, by scavenging iron and by neutralizing superoxide. In this regard, it is noteworthy that exochelins themselves block hydroxyl radical production via the Fenton mechanism both in tissue culture in vitro and in isolated rabbit hearts (26).

Despite differences in their expression and export, the superoxide dismutases of M. tuberculosis and M. smegmatis display a high degree of identity or similarity at the nucleotide and amino acid level. These enzymes are also highly homologous to the superoxide dismutases of other mycobacteria including Mycobacterium marinum, Mycobacterium kansasi, and M. fortuitum (8, 9, 27, 28).

Export of Recombinant Superoxide Dismutase—Three lines of evidence support the concept that the information for abundant export of M. tuberculosis superoxide dismutase is contained within the protein. First, an unusually large amount of the leaderless protein is exported by M. tuberculosis, whereas other abundantly expressed proteins, e.g. certain heat shock proteins, remain cell-associated. Second, the recombinant enzyme is abundantly exported from M. smegmatis, whereas the homologous endogenous enzyme in M. smegmatis is not abundantly exported. Although the M. tuberculosis enzyme is not expressed as abundantly in the heterologous host as in the homologous host, possibly reflecting different “set points” for expression of this enzyme, expression of the recombinant enzyme in M. smegmatis is nevertheless 8-fold that of the M. tuberculosis outside the host. This supports the concept that the information for abundant export is contained within the superoxide dismutase gene. Third, the leaderless superoxide dismutase gene is abundantly exported by M. tuberculosis, whereas the homologous endogenous enzyme in M. smegmatis is not abundantly exported. Although the M. tuberculosis enzyme is not expressed as abundantly in the heterologous host as in the homologous host, possibly reflecting different “set points” for expression of this enzyme, expression of the recombinant enzyme in M. smegmatis is nevertheless 8-fold that of the M. tuberculosis enzyme in the host.
M. smegmatis endogenous superoxide dismutase. Moreover, 66% of the recombinant enzyme is exported versus 21% for the endogenous enzyme. The net result is that M. smegmatis exports 26-fold more of the recombinant enzyme than its own endogenous enzyme. Third, N-terminal amino acid analysis of exported recombinant superoxide dismutase and a comparison of the banding patterns of exported and retained recombinant superoxide dismutase indicated that recombinant M. smegmatis preferentially exports M. tuberculosis superoxide dismutase. A theoretically possible alternative explanation for these results is that M. tuberculosis superoxide dismutase is more stable than M. smegmatis superoxide dismutase extracellularly, whereas M. smegmatis superoxide dismutase is more stable than M. tuberculosis superoxide dismutase intracellularly. However, we know of no reason why one of these highly related enzymes whose subunits indiscriminately complex with each other would be more stable in one setting and less stable in the other. Taken together, the evidence strongly indicates that an intrinsic characteristic of M. tuberculosis superoxide dismutase instructs its export.

The high correlation between expression and export of superoxide dismutase raises the possibility that export is additionally dependent on the level of expressed enzyme. However, while expression dependence could be a factor in the high level export of M. tuberculosis superoxide dismutase both in the endogenous host and in recombinant M. smegmatis and the low level export of M. smegmatis superoxide dismutase in the parent strain, this mechanism would not account for either (a) the abundant export of superoxide dismutase by M. tuberculosis but not other proteins expressed in even greater abundance or (b) the preferential export of M. tuberculosis superoxide dismutase versus the endogenous superoxide dismutase in recombinant M. smegmatis.

We do not have an explanation for the different ratio of intracellular to exported recombinant superoxide dismutase in the M. smegmatis strains with the unidirectional orientation of the superoxide dismutase and β-galactosidase promoters. One possibility, alluded to in the previous paragraph, is that a threshold level of superoxide dismutase must be reached before the bacterial cell is able to export significant amounts of the enzyme.

Our finding that the recombinant M. tuberculosis superoxide dismutase is abundantly exported from M. smegmatis is in contrast to the result of a previously published study (6). Although the investigators detected superoxide dismutase in the culture supernatant of M. tuberculosis by immunoblotting and activity assays, they could not detect any enzyme activity in supernatants of M. smegmatis nor in M. smegmatis cultures transformed with the M. tuberculosis superoxide dismutase gene.

Interestingly, some M. smegmatis clones that express recombinant M. tuberculosis superoxide dismutase export more endogenous enzyme than the parent M. smegmatis strain. Since M. smegmatis and M. tuberculosis subunits freely exchange with each other, one possible explanation for this observation is that endogenous enzyme subunits are exported in association with recombinant enzyme subunits, i.e., the more export-prone recombinant subunits in effect carry the endogenous subunits across the wall with them.

The recombinant M. tuberculosis superoxide dismutase successfully transcomplemented a superoxide dismutase-deficient E. coli, proving that the recombinant enzyme is active. As in the case of the heterologous mycobacterial host M. smegmatis, the level of expression of the recombinant enzyme in E. coli was much lower than in its homologous host but still 5-fold greater than the level of expression of the endogenous superoxide dismutase in the parent E. coli strain. Again, the lower expression of recombinant enzyme in the heterologous than homologous host may reflect a lower set point for superoxide dismutase expression in E. coli. Additionally, it may reflect differences between the two species in codon usage, protein folding, or association of subunits.

Although the recombinant M. tuberculosis superoxide dismutase is abundantly exported from a mycobacterial host, it is not abundantly exported from the nonmycobacterial host E. coli. One possible explanation for this is that the E. coli export machinery does not recognize the mycobacterial protein. Although the superoxide dismutases from E. coli exhibit the same 4 metal ion coordinating amino acid residues at His-26, His-73/81 (iron/manganese-binding enzyme), His-160/171, and Asp-156/167 (20) as the mycobacterial enzymes, the variation between the E. coli and the M. tuberculosis enzymes is greater than that between different mycobacterial species. Another possible explanation for the lack of export in E. coli is that E. coli lacks a nonclassical export pathway present in mycobacteria.

Superoxide Dismutase and Glutamine Synthetase: Parallel Models for Study of Export of Leaderless Multimeric Proteins—A common theme appears to govern the expression and export of superoxide dismutase and glutamine synthetase. Both are leaderless proteins that are abundantly expressed and exported by M. tuberculosis. Indeed, these two enzymes are among the 10 major extracellular proteins in M. tuberculosis. Both enzymes are exported abundantly in a heterologous mycobacterial host, M. smegmatis, whereas the endogenous counterparts in M. smegmatis are not abundantly exported. Finally, both enzymes are expressed in active form but not exported from the nonmycobacterial host E. coli. The similarities with respect to the expression and export of M. tuberculosis superoxide dismutase and glutamine synthetase in the homologous and heterologous host make these two enzymes excellent models for studying export mechanisms of M. tuberculosis proteins across its complex cell wall, including differences in export machinery between pathogenic and nonpathogenic mycobacteria. Possible export mechanisms specific to pathogenic mycobacteria include chaperone-guided docking to a membrane-bound transporter or exit via specific porin tunnels.

With respect to dissecting such mechanisms of export, another pertinent similarity between superoxide dismutase and glutamine synthetase is that both enzymes are multimeric complexes of identical subunits, 4 in the case of superoxide dismutase and 12 in the case of glutamine synthetase. However, one interesting difference between the two enzymes is that in the case of superoxide dismutase but not glutamine synthetase, heterologous subunits of the enzyme from M. tuberculosis and M. smegmatis readily exchange with each other. In either case, whether these proteins are exported as a multimeric complex or whether the individual subunits are first exported and then assembled on the extracellular aspect of the cell surface remains to be determined.

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Export of Recombinant Mycobacterium tuberculosis Superoxide Dismutase Is Dependent upon Both Information in the Protein and Mycobacterial Export Machinery: A MODEL FOR STUDYING EXPORT OF LEADERLESS PROTEINS BY PATHOGENIC MYCOBACTERIA

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