RESEARCH COMMUNICATION

Lipid modification of the Cu,Zn superoxide dismutase from *Mycobacterium tuberculosis*

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The leader sequence of *Mycobacterium tuberculosis* Cu,Zn superoxide dismutase (Cu,ZnSOD) contains a prokaryotic membrane lipoprotein attachment site. In the present study, we have found that the protein, which exhibits detectable SOD activity, is lipid-modified and associated with the bacterial membrane when expressed either in *M. tuberculosis* or in *Escherichia coli*. These results provide the first demonstration of lipid modification of a Cu,ZnSOD. An analysis of the *sodC* genes present in available databases indicates that the same signal for lipid modification is also present in the *sodC* gene products from other mycobacteria and Gram-positive bacteria and, uniquely, in two distinct *sodC* gene products from the Gram-negative bacterium *Salmonella typhimurium*. Evidence is also provided for an up-regulation of *M. tuberculosis sodC* in response to phagocytosis by human macrophages, suggesting that Cu,ZnSOD is involved in the mechanisms that facilitate mycobacterial intracellular growth.

Key words: *sodC*, anti-oxidant enzyme, virulence factor, lipoprotein, *Salmonella*.

INTRODUCTION

Mycobacterium tuberculosis is a pathogen able to invade and persist for long periods of time within the highly oxidative macrophage environment [1]. The mechanisms that allow this bacterium to withstand the bactericidal activity of macrophages are not well understood, but it is likely that the enzymes involved in the detoxification of reactive oxygen intermediates contribute significantly to its ability to survive within phagocytic cells. Mycobacteria possess a number of antioxidant enzymes including KatG, a haem-containing catalase/peroxidase, AhpC, an alkyl-hydroperoxide reductase and an Fe-containing superoxide dismutase (SOD), FeSOD. Interestingly, FeSOD, which lacks a signal peptide for protein export, is abundantly secreted in the extracellular milieu from virulent strains of *M. tuberculosis*, but not from non-pathogenic mycobacteria [2–4]. The analysis of the genome sequence of the *M. tuberculosis* strain H37Rv has also revealed the existence of a sequence similar to the *sodC* gene from Gram-negative bacteria, which encodes Cu,ZnSOD [5]. The protein encoded by the *M. tuberculosis sodC* gene is characterized by a hydrophobic N-terminal sequence, typical of a signal peptide for protein export, and is secreted to the periphery of *M. tuberculosis* [6]. Recent observations regarding the contribution of periplasmic Cu,ZnSOD to the virulence of Gram-negative bacteria [7–15] suggest that this gene could be another important component of the antioxidant apparatus of *M. tuberculosis*.

In the present study we report that the protein encoded for by the *M. tuberculosis sodC* gene is characterized by a prokaryotic membrane lipoprotein attachment site, is lipid-modified and anchored to membranes. The analysis of the amino acid sequences of the *sodC* gene products from other bacteria suggests that lipid modification of Cu,ZnSOD could be a feature common to all mycobacteria and Gram-positive bacteria, and also to some Gram-negative bacteria. Moreover, a comparative analysis of *M. tuberculosis sodC* expression in synthetic medium and human macrophages support the hypothesis that this membrane-associated Cu,ZnSOD plays a role in favouring mycobacterial survival in phagocytes.

MATERIALS AND METHODS

Isolation of *M. tuberculosis sodC* and construction of expression vectors

The *sodC* gene from *M. tuberculosis* was isolated by PCR amplification of *M. tuberculosis* H37Rv chromosomal DNA using the oligonucleotides 5′-CCAAGCTTGATGCCAAAGCCCGCCGATCA (which encompasses the ATG starting site) and 5′-GTGAATTCGAGCGCGAACTCCCACTCC (downstream the translation stop codon). The amplified DNA (approx. 800 bp) was digested with *Eco*RI and *Hin*dIII and subcloned in the corresponding sites of pUC19 to obtain pE5MycSOD, where the *M. tuberculosis sodC* gene is under control of the *lacZ* promoter. A plasmid directing expression of a soluble periplasmic form of *M. tuberculosis* Cu,ZnSOD was obtained as follows. *M. tuberculosis sodC* coding sequence was amplified with the oligonucleotides 5′-CACCATGGCCTCGTCGCCGCAGCACGC and 5′-GTGAATTCGAGCGCGAACTCCCACTCC, using pE5MycSOD as a template. The amplified DNA was digested with *Nco*I and *Eco*RI. As there is a *Nco*I site inside the *sodC* gene, the resulting *Nco*I–*Nco*I (430 bp) and *Nco*I–*Eco*RI (260 bp) DNA fragments were separated by agarose-gel electrophoresis and the *Nco*I–*Eco*RI fragment was cloned into plasmid pHEN-1 [16], obtaining pMycShort. Subsequently, the *Nco*I–*Nco*I fragment was inserted into the *Nco*I site of pMycShort, obtaining the plasmid pΔCysMycSOD encoding the mature *M. tuberculosis* protein lacking the leader sequence. In this vector the sequence encoding Cu,ZnSOD is fused to the leader peptide from the *pelB* gene of *Erwinia carotovora* and the amino acid sequence of the exported protein is expected to start from the

Abbreviations used: MOI, multiplicities of infection; RT-PCR, reverse transcriptase-PCR; SOD, superoxide dismutase.

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serine residue immediately downstream of the cysteine residue identified as the putative site of lipid modification in the Lys-Xaa-Yaa-Cys motif (where Xaa and Yaa are generally small neutral amino acids).

[^{3}H]Palmitate labelling of *M. tuberculosis* Cu,ZnSOD expressed in *Escherichia coli* and extraction of lipid-associated proteins from *E. coli* and *M. tuberculosis*

Cellular lipoproteins from *E. coli* 71/18 [17] harbouring pUC19 or pE5MycSOD were labelled with [^{3}H]palmitic acid using a modification of previously described procedures [18,19]. Briefly, 100 μl of overnight cultures were inoculated into 2 ml of Luria–Bertani broth containing ampicillin (100 μg/ml), and the cultures were grown to a D_{600} of 0.5 at 37 °C. Isopropyl β-D-thiogalactoside was then added to a final concentration of 0.1 mM, and the cells were incubated for an additional 30 min at 37 °C. Subseqently, [9,10(n)-^{3}H]palmitate (250 μCi; 52 Ci/mmol; Amersham Pharmacia Biotech) was added and the cells were incubated overnight at 37 °C. Cells were harvested by centrifugation and subsequently washed in 1 ml of ice-cold 10 mM sodium phosphate buffer, pH 7.0. Aliquots of the samples were withdrawn and directly heated to 100 °C in SDS/PAGE sample buffer (2% SDS, 10% glycerol, 10% mercaptoethanol and 0.125 mM Tris/HCl, pH 6.8). Membrane-associated lipoproteins were extracted from the remaining cells by a modification of the method described by Takase and co-workers [19]. Cells were sonicated for 30 s at 20 KHz under cooling with ice/water. Cell debris was removed by centrifugation at 1000 ***g*** for 10 min (repeated twice), and the supernatant was mixed with 40 μl of 4 M NaCl and kept on ice for 10 min. After centrifugation at 14000 ***g*** for 1 h at 4 °C, the supernatant, containing the soluble proteins, was removed and the pellet, containing the bacterial membranes, was washed with 0.2 ml of cold 0.4 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0. After centrifugation, the pellet was suspended with 0.5 ml of chloroform/methanol (2:1, v/v) and shaken for 15 min at room temperature. After centrifugation at 14000 ***g*** for 30 min at 4 °C, the solvent was removed and the pellet was subjected to two further extraction cycles with chloroform/methanol. Finally, the pellet was suspended in 50 μl of 10 mM Tris/HCl, pH 7.0. Proteins were resolved by SDS/PAGE and the labelled proteins were detected by autoradiography.

Lipoprotein extraction procedures using chloroform/ethanol may induce significant denaturation of membrane-associated proteins. Therefore in order to obtain bacterial lipoproteins in native form, the membrane-associated proteins from the *sodAsodB E. coli* strain QC871 [20] harbouring pUC19, pE5MycSOD or pΔCysMycSOD, were extracted by a modification of the procedure described above starting with 6×10^9 cells. In this case, the pellet was washed with cold 0.4 mM NaCl/10 mM sodium phosphate buffer, pH 7.0, suspended in 0.1 ml of 50 mM Tris/HCl (pH 8.0), 0.1 mM EDTA/100 mM NaCl/1% Triton X-100 and kept on ice for 30 min. Finally, the mixture was centrifuged at 13000 ***g*** for 15 min at 4 °C. The supernatant contains the membrane proteins.

M. tuberculosis H37Rv soluble and membrane-associated proteins were extracted from cells grown for 8 weeks on Sauton's medium. In order to catalytically activate Cu,ZnSOD, Sauton's medium was supplemented with 20 μM $CuSO_4$ for 3 h before protein extraction. The mycobacterial surface pellicle (300 mg bacterial mass) was harvested with sterile loops, centrifuged at 6000 ***g*** for 10 min at 4 °C and the pellet was re-suspended in 400 μl of 10 mM Tris/HCl, 1 mM EDTA, 150 mM NaCl, pH 7.5. Cells were incubated for 24 h with 10 mg/ml lysozyme and then lysed by repeated sonication cycles for 60 s at 50 W. Sonicated cells were centrifuged at 25000 ***g*** for 15 min at 4 °C and the supernatant, including membranes and cytosolic proteins, was subjected to Triton-X114 partitioning as described by Bordier [21], with minor modifications. Triton-X114 (Sigma–Aldrich) and NaCl were added at a final concentration of 2% and 150 mM respectively. The suspension was placed on a rotating platform and incubated overnight at 4 °C. Subsequently, insoluble material was removed by centrifuging at 13000 ***g*** for 10 min at 4 °C. The temperature was then raised to 37 °C and the resulting detergent and aqueous phases were separated by centrifugation at 13000 ***g*** for 10 min at room temperature. The upper aqueous and lower detergent phases were both re-extracted once. Proteins from the detergent phase were recovered by acetone precipitation (10 vol.) at −20 °C and resuspended in 50 mM Tris/HCl, 150 mM NaCl and 1% Triton-X100.

Soluble and membrane-associated proteins were analysed by SDS/PAGE as described by Laemmli [22]. Staining of native gels for SOD activity was performed as previously described [23].

Analysis of *M. tuberculosis sodC* expression

M. tuberculosis sodC expression in synthetic medium and infected macrophages were studied by reverse transcriptase-PCR (RT-PCR) as previously described [24]. Monocyte-derived macrophages were obtained from human healthy donors [24]. Macrophages were infected with *M. tuberculosis* at different bacilli/cells multiplicities of infection (MOI), ranging from 1:1 to 50:1. Total RNA was isolated from infected macrophages and mycobacteria grown in Sauton's medium and then reverse transcribed to cDNA with Moloney-murine-leukaemia virus reverse transcriptase (Amersham Pharmacia Biotech) as previously described [24]. Each PCR reaction was carried out using 1 μl of cDNA, corresponding to 0.5–1 μg RNA, or 1 μl of RNA (not treated with reverse transcriptase) as a negative control. PCR amplification of *M. tuberculosis sodC* was obtained using the primers $sodC_{for}$ (5′-ATGCCAAAGCCGCCGATC) and $sodC_{rev}$ (5′-TAGCCGGAACCAATGACACC). PCR products were separated by agarose gel electrophoresis, blotted on to nitrocellulose membranes and hybridized with a 720 bp ^{32}P-labelled *sodC* DNA fragment (obtained by PCR amplification with primers $sodC_{for}$ and $sodC_{rev}$) as previously described [24].

RESULTS AND DISCUSSION

The leader peptides of exported bacterial proteins are cleaved off during protein translocation by two main classes of signal peptidases [25]. Signal peptidase I removes the leader sequence from proteins exported in the periplasmic space or secreted in the extracellular milieu, while signal peptidase II removes the leader peptide from lipid modified proteins, which are targeted to membranes. Although Wu and co-workers [6] have identified a putative signal peptidase I cleavage site in the *M. tuberculosis sodC* gene product, we have observed that a specific feature of this gene product may be highlighted when its sequence is analysed by programs that utilize the PROSITE database of protein families and domains ([26]; http://www.expasy.ch/prosite/). Such analyses indicate that the signal sequence of Cu,ZnSOD contains a typical prokaryotic membrane lipoprotein attachment site (recognized by signal peptidase II), thus suggesting that this enzyme could be lipid modified and anchored to the bacterial membrane [27]. Figure 1 shows that the signal sequence of *M. tuberculosis* Cu,ZnSOD contains the motif Leu-Xaa-Yaa-Cys present in all bacterial lipoproteins, where Xaa and Yaa are generally small neutral amino acids such as alanine,

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M. tuberculosis	mpkpadhrnhaavst-svlsalflgagaal**LSAC**sspqha~
M. leprae	msklaghrnvaavtr-salslsf**VAAC**val**LSAC**iqnqpp~
M. avium	agrrsvrllampkllppvv**LAGC**vva**LGAC**sspqha~
D. radiodurans	mfrtltvvpllalgls**LSAC**a~
B. subtilis	mhrllllmmltalg**VAGC**gqk~
Salmonella sodC1	mkytilslvaga**LISC**sama~
Salmonella sodC2	mkrlslamvt**LLAC**agaa~
Salmonella sodC3	mnkklmtlavflfssaasa~
E. coli	mkrfslailalvvatgaa~

Figure 1 Signal peptide sequences from *sodC* gene products containing the signal peptidase II cleavage site

The reported lengths and boundaries (~) of the signal peptides were determined using the Signal P program ([28], http://www.cbs.dtu.dk/services/SignalP/), which recognizes putative signal peptidase I cleavage sites. A best fit alignment is presented for the leader peptides encoded by the three mycobacterial *sodC* genes. Alignment of mycobacterial leader peptides with those from other bacteria is based on the Leu-Xaa-Yaa-Cys motif. Only the sequence of one (gnl ± TIGR ± M.avium-96 from the Institute of Genomic Research) of the three distinct *sodC* gene products that have been identified from the partial genome sequence of *M. avium* is reported, but all of these gene products contain putative signal peptidase II cleavage sites. The leucine to valine mutation present in one of the two Leu-Xaa-Yaa-Cys motifs of *M. leprae* and in that from *Bacillus subtilis* has previously been observed in other lipoproteins [27]. The leader peptide of periplasmic *E. coli* Cu,ZnSOD is shown for comparison with the highly homologous enzyme encoded by the *Salmonella typhimurium sodC2* gene. The leader peptide of *Deinococcus radiodurans* Cu,ZnSOD is also shown.

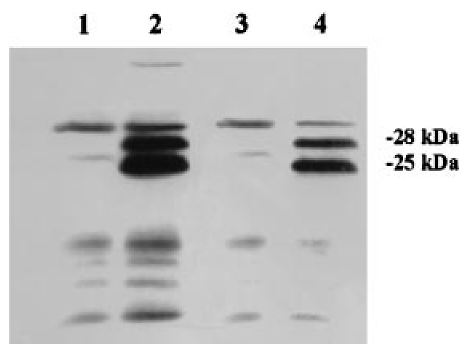

Figure 2 [^{3}H]Palmitic acid labelling of *M. tuberculosis* Cu,ZnSOD

Labelled proteins present in whole cell lysates (lanes 1 and 2) or in the chloroform/methanol-extracted membrane fraction (lanes 3 and 4) were separated by 15% SDS/PAGE and visualized by autoradiography. Labelled proteins from *E. coli* 71/18 (pUC19) are shown in lanes 1 and 3, whereas labelled proteins from *E. coli* 71/18 (pE5MycSOD) are in lanes 2 and 4. Proteins from approx. 5×10^8 bacteria were loaded in each lane.

glycine or serine and the cysteine residue is the first residue of the mature protein and the site where an acylglycerol fatty acid is attached [27].

Signal peptidase II from Gram-positive and Gram-negative bacteria have similar substrate specificity [27]. Therefore we initially attempted to demonstrate lipid modification of *M. tuberculosis* Cu,ZnSOD by expressing the enzyme in *E. coli*. Figure 2 shows that *E. coli* cells harbouring pE5MycSOD expressed two novel and abundant membrane-associated lipoproteins with an apparent molecular mass of approx. 25 and 28 kDa. As lipid attachment proceeds cleavage of the leader sequence by signal peptidase II [25,27], the difference in molecular mass between the two bands suggests that they correspond to mature Cu,ZnSOD and unprocessed Cu,ZnSOD still containing the signal peptide respectively. Attempts to further investigate *M. tuberculosis* Cu,ZnSOD processing in *E. coli* using globomycin, a selective inhibitor of signal peptidase II [27], were unsuccessful due to the rapid induction of cell lysis of bacteria expressing the recombinant enzyme. It should be noted that the apparent molecular mass of mature Cu,ZnSOD determined by SDS/PAGE analysis is higher (25 kDa) than that expected from its amino acid sequence (20.6 kDa in the absence of lipid attachment), a finding already observed for other Cu,ZnSODs, including the enzymes from *E. coli* [29] and *Homo sapiens* [30].

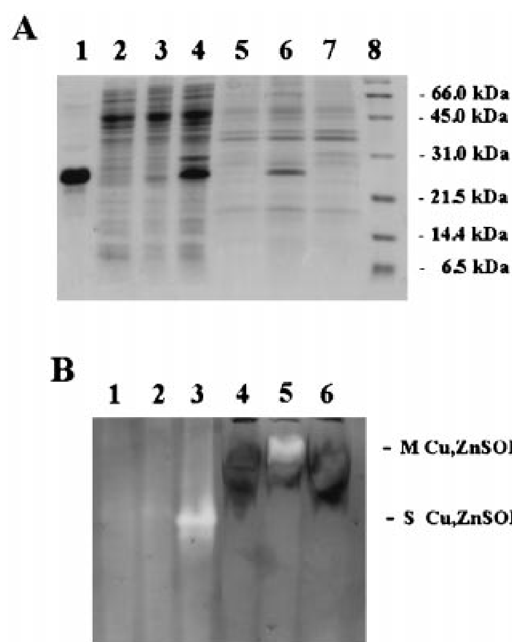

Figure 3 Signal peptide-dependent targeting of *M. tuberculosis* Cu,ZnSOD in *E. coli*

(**A**) SDS/PAGE analysis of soluble (lanes 2–4) and membrane-associated proteins (lanes 5–7) from *E. coli* QC871 bearing pUC19 (lanes 2 and 5), pE5MycSOD (lanes 3 and 6) and pΔCysMycSOD (lanes 4 and 7). Purified soluble *M. tuberculosis* Cu,ZnSOD is in lane 1 and molecular-mass markers in lane 8. Total soluble proteins from 1.5×10^8 cells were loaded in lanes 2–4, whereas membrane proteins from 6×10^8 cells where loaded in lanes 5–7. (**B**) Staining for SOD activity of the same samples shown in (**A**). Soluble proteins from *E. coli* QC871 bearing pUC19, pE5MycSOD and pΔCysMycSOD are shown in lanes 1–3, respectively. Lanes 4–6, membrane-associated proteins from *E. coli* QC871 bearing pUC19, pE5MycSOD and pΔCysMycSOD, respectively. Bands of SOD activity were detected as achromatic zones against a violet background. M Cu,ZnSOD, membrane-associated Cu,ZnSOD; S Cu,ZnSOD, soluble Cu,ZnSOD. Staining with Nitro Blue Tetrazolium led also to the development of a dark-blue area in all samples containing membrane proteins.

To obtain further evidence that the overexpressed protein identified by [^{3}H]palmitate labelling is recombinant *M. tuberculosis* Cu,ZnSOD, and to demonstrate that the Lys-Xaa-Yaa-Cys motif present in its leader sequence is required for lipid attachment, we separated soluble and membrane-associated proteins from *E. coli* cells transfected with plasmids pUC19, pE5MycSOD and pΔCysMycSOD. This latter plasmid contains the sequence encoding mature Cu,ZnSOD lacking the leader sequence. This experiment was carried out in the *E. coli* strain QC871, which is unable to synthesize cytoplasmic SODs, to avoid interference with the activity assay. Figure 3(A) shows that, compared with control *E. coli* (pUC19), *E. coli* (pΔCysMycSOD) accumulated a soluble polypeptide of approx. 25 kDa, whereas *E. coli* (pE5MycSOD) accumulated a polypeptide of comparable molecular mass which was associated with membranes. The same samples were assayed for SOD activity in native gels (Figure 3B). Consistent with the SDS/PAGE analysis, a SOD activity band was present in soluble

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Figure 4 Cu,ZnSOD localization in *M. tuberculosis*

Staining for SOD activity on native PAGE of soluble and membrane-associated proteins from *E. coli* and *M. tuberculosis* H37Rv. Achromatic zones against the dark background indicate the presence of SOD enzymes. Lane 1, soluble proteins from 1.5×10^8 *E. coli* QC871 cells bearing pUC19; lane 2, soluble proteins from 1.5×10^8 *E. coli* QC871 cells bearing pE5MycSOD; lane 3, membrane proteins from 6×10^8 *E. coli* QC871 cells bearing pUC19; lane 4, membrane proteins from 6×10^8 *E. coli* QC871 cells bearing pE5MycSOD; lane 5, purified soluble *M. tuberculosis* Cu,ZnSOD; lane 6, soluble proteins from *M. tuberculosis* H37Rv (corresponding to 12.5 mg cells); lane 7, membrane proteins from *M. tuberculosis* H37Rv (corresponding to 300 mg of cells). The position and size of the bands attributed to *M. tuberculosis* Cu,ZnSOD and FeSOD are indicated at the right (M Cu,ZnSOD, membrane-associated Cu,ZnSOD; S Cu,ZnSOD, soluble Cu,ZnSOD).

proteins from *E. coli* (pΔCysMycSOD) and in membrane proteins from *E. coli* (pE5MycSOD), demonstrating that the *sodC* gene from *M. tuberculosis* encodes an active enzyme. In spite of the similar molecular mass (Figure 3A), the two forms of recombinant SOD exhibited clearly different migration under native conditions, confirming the hypothesis that the enzyme containing its original leader peptide is lipid-modified when it is expressed in *E. coli*.

To demonstrate that *M. tuberculosis* Cu,ZnSOD is also lipid-modified in the natural host, soluble and membrane-associated proteins from *M. tuberculosis* H37Rv grown on Sauton's medium were isolated by Triton-X114 extraction and separated by native PAGE, which were then stained for SOD activity. Figure 4 shows that different SOD activity bands were observed in the soluble and membrane fractions. A strong SOD activity band, due to FeSOD, was observed in soluble proteins, while a fainter band, migrating as lipid-modified recombinant *M. tuberculosis* Cu,ZnSOD produced in *E .coli*, was present only in the membrane fraction. This latter band was clearly observed in extracts from cells incubated with $CuSO_4$ before protein extraction, whereas it was hardly observed in cells cultivated in standard Sauton's medium (results not shown). Moreover, a 20 min preincubation of gels with the copper chelator diethyl dithiocarbamate (5 mM) before staining strongly inhibited the SOD activity band present in the membrane fraction, thus confirming that this activity is due to Cu,ZnSOD. These findings indicate that Cu,ZnSOD is fatty-acylated and also associated with bacterial membranes in *M. tuberculosis*. Although it has already been reported that the extracellular Cu,ZnSOD from *Schistosoma mansoni* is tightly associated with membranes [31], this is the first demonstration of lipid modification of a Cu,ZnSOD. The demonstration of this kind of post-translational modification in a bacterial Cu,ZnSOD confirms our previous observation [32,33] that enzymes belonging to this protein family, which in eukaryotes is characterized by an impressive degree of structural and functional conservation [34], exhibit a considerable structural variability in prokaryotes.

The presence of a putative signal peptidase II cleavage site has also been previously reported in the *S. typhimurium sodC1* gene product [8]. In considering the possibility of a wider distribution of such a lipid modification signal in bacterial Cu,ZnSODs, we have searched for this motif in all the *sodC* gene sequences present in GenBank® and the preliminary *M. avium* sequence database. An interesting finding highlighted by this analysis is that *sodC*-like sequences are present in different mycobacteria species, as well as in other Gram-positive bacteria, including *B. subtilis* and *D. radiodurans*. The *sodC* gene from mycobacteria and Gram-positive bacteria are all characterized by encoding a signal peptidase II cleavage site (in the cases of *M. leprae* and *M. avium* the Leu-X-Y-Cys motif is repeated twice). This feature is probably related to the lack of a proper periplasmic space in Gram-positive bacteria, since lipid modification of periplasmic proteins in Gram-negative bacteria has been observed in other cases [35].

The same analysis also indicates that all the *sodC* genes from Gram-negative bacteria (there are 15 complete genes in GenBank®) encode a classical signal peptidase I cleavage site, indicative of a periplasmic localization of Cu,ZnSODs. The lipoprotein attachment signal is present in only two *sodC* gene products from *S. typhimurium* (Figure 1), although the length of the signal sequences for signal peptidase II encoded by the *sodC2* gene is at the lower limit of those found in other bacterial lipoproteins. It has been observed that the *Salmonella sodC2* gene product is highly homologous with the *E. coli* enzyme [12]. The identity between the two mature enzymes (without their signal peptides) is 85 %, and all the residues important for metal binding, substrate attraction and the global protein fold are completely conserved [32]. In contrast, the identity between the two signal peptides is only 56 %. Although the signal peptide sequences are expected to have lower constraints for sequence variations than the enzyme moieties, the major differences between the signal peptides encoded by the *E. coli* and *S. typhimurium sodC*2 genes are in the Leu-Xaa-Yaa-Cys region (see Figure 1), and involve several mutations of the DNA sequence. This observation suggests that the presence of a signal peptidase II cleavage site in the *S. typhimurium sodC* gene products is not merely chance. Several studies have highlighted the contribution of the *sodC* genes in the virulence of *S. enterica* [8,9,12], and recently a novel *sodC* gene (*sodC3*) has been found in the *S. typhimurium* strain LT2 [36]. The introduction of *sodC3* in an attenuated *S. typhimurium* strain lacking *sodC1* was not sufficient to restore the virulence lost due to the absence of this latter gene [36]. Although a number of different hypotheses could explain these results [36], it is worth noting that the Cu,ZnSOD encoded by *sodC3* does not have mutations in residues important for the enzyme's function, whereas its signal peptide is devoid of the lipoprotein attachment signal which characterizes the two other *Salmonella sodC* gene products (Figure 1). On the basis of these analyses, we suggest that a partitioning between the periplasmic space and the bacterial membranes could be a relevant feature of this *Salmonella* virulence factor, possibly important in gaining increased protection against the macrophage-induced oxidative burst.

The Cu,ZnSOD activity in *M. tuberculosis* grown in Sauton's medium is quite low (see Figure 4). As Cu,ZnSOD has been proposed to modulate bacterial virulence, we wondered whether the expression of this enzyme could be enhanced upon infection of host macrophages. To test this possibility, *sodC* expression in synthetic medium and in human macrophages was analysed by RT-PCR, a technique that we have recently used to demonstrate that some *M. tuberculosis* genes are differentially expressed in the two environments [24]. Figure 5 shows that hybridization with a ^{32}P-labelled *sodC* probe gave comparable, or significantly stronger, signals on RT-PCR products obtained from cDNAs from infected macrophages than from bacteria grown on Sauton's medium. This result is particularly significant considering that

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Figure 5 Comparison of *M. tuberculosis sodC* expression in Sauton's medium and in human macrophages

PCR products were run on a 2% agarose gel, transferred on to a nitrocellulose membrane and hybridized with a PCR-generated *sodC* DNA fragment. PCRs were carried out on 10 ng of *M. tuberculosis* genomic DNA (lane 1) or on cDNAs obtained by reverse transcription of total RNA extracted from the following samples: lane 2, *M. tuberculosis* grown in Sauton's synthetic medium; lane 3, the same sample of lane 2, not treated with RT (negative control); lane 4, *M. tuberculosis* infected macrophages at a MOI of 10:1 taken at day 3 of infection; lane 5, *M. tuberculosis* infected macrophages at a MOI of 10:1 taken at day 6 of infection from the same culture as sample 4; lane 6, *M. tuberculosis* infected macrophages at a MOI of 1:1 taken at day 6 of infection; lane 7, *M. tuberculosis* infected macrophages at a MOI of 50:1 taken at day 6 of infection.

each PCR amplification was carried out with a comparable amount of reverse-transcribed cDNA, and that a very large fraction of the RNA isolated from infected macrophages derives from the expression of host genes [24]. The amount of *sodC* cDNA was higher in samples prepared from macrophages taken 6 days after *M. tuberculosis* infection compared with cells 3 days after infection (compare lanes 4 and 5 of Figure 5), due to the increase in the number of live intracellular mycobateria [24]. The lower hybridization signal in samples from cells infected at a very high bacilli/cell MOI (Figure 5, lane 7) may indicate that *sodC* expression is influenced by *M. tuberculosis* cell density within macrophages. However, we believe that this result more likely reflects the significant cytotoxic effects observed at such a MOI [24]. Although gene expression cannot be accurately quantified by the experimental approach we have used, our results indicate that *M. tuberculosis sodC* expression is significantly enhanced within human macrophages, suggesting that Cu,ZnSOD plays a role in bacterial intracellular survival. This conclusion is in apparent contradiction with the recent results by Doussurget and co-workers [37], who failed to demonstrate a reduction of virulence in a *M. tuberculosis* strain bearing an inactivated *sodC* gene. Our data indicate that *M. tuberculosis* exports two SODs (FeSOD and Cu,ZnSOD) with different subcellular localizations. FeSOD, which is released outside the cell [2–4], could play a primary role in the neutralization of superoxide produced in the phagosome, whereas Cu,ZnSOD, which is bound to the bacterial membrane, could protect specific membrane-associated targets from oxy-radical damage. We suggest that the lack of reduction of virulence upon *sodC* inactivation could be due to the overlapping functions of FeSOD and Cu,ZnSOD, whose combined action could at least partially account for the significant resistance of *M. tuberculosis* against oxidative stress. Further support for the hypothesis that Cu,ZnSOD plays an important role in mycobacterial infections can be inferred from the analysis of the genomes of *M. leprae* and *M. avium*, two bacterial species closely correlated to *M. tuberculosis*. In fact, despite the massive gene decay which characterizes the leprosy bacillus (whose genome contains only a small number of protein-encoding genes and is rich in pseudogenes with intact counterparts in *M. tuberculosis*), the two SOD genes appear to be still able to encode functional proteins [38]. Moreover, analysis of the partial genome sequence for *M. avium* reveals the presence of three distinct *sodC* genes, all characterized by putative lipid-modification signals (results not shown).

This work was partially supported by the Ministero della Sanità, Ricerca Finalizzata, and by the CNR Target Project 'Biotechnology'. Globomycin was generously given by Dr. Masatoshi Inukai (Sankyo Co., Ltd., Tokyo, Japan). *M. avium* preliminary sequence data were obtained from The Institute for Genomic Research (http://www.tigr.org).

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Received 4 May 2001/19 June 2001; accepted 14 August 2001

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