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The Iron Superoxide Dismutase of *Legionella pneumophila* Is Essential for Viability

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***Legionella pneumophila*, the causative agent of Legionnaires' disease, contains two superoxide dismutases (SODs), a cytoplasmic iron enzyme (FeSOD) and a periplasmic copper-zinc SOD. To study the role of the FeSOD in *L. pneumophila*, the cloned FeSOD gene (*sodB*) was inactivated with Tn*903*dII*lacZ*, forming a *sodB*::*lacZ* gene fusion. By using this fusion, expression of *sodB* was shown to be unaffected by a variety of conditions, including several that influence *sod* expression in *Escherichia coli*: aeration, oxidants, the redox cycling compound paraquat, manipulation of iron levels in the medium, and the stage of growth. A reproducible twofold decrease in *sodB* expression was found during growth on agar medium containing charcoal, a potential scavenger of oxyradicals, in comparison with growth on the same medium without charcoal. No induction was seen during growth in human macrophages. Additional copies of *sodB*$^+$ in *trans* increased resistance to paraquat. Construction of a *sodB* mutant was attempted by allelic exchange of the *sodB*::*lacZ* fusion with the chromosomal copy of *sodB*. The mutant could not be isolated, and the allelic exchange was possible only if wild-type *sodB* was present in *trans*. These results indicate that the periplasmic copper-zinc SOD cannot replace the FeSOD. The data strongly suggest that *sodB* is an essential gene and that FeSOD is required for the viability of *L. pneumophila*. In contrast, Sod$^-$ mutants of *E. coli* and *Streptococcus mutans* grow aerobically and SOD is not required for viability in these species.**

During aerobic growth, virtually all organisms generate toxic by-products of respiration like hydroxyl radical (OH·), hydrogen peroxide (H_2O_2), and superoxide radical (O_2^-) (16, 20). These organisms, however, possess mechanisms to combat potentially destructive reactive oxygen intermediates (ROIs). The principal enzymatic mechanisms in procaryotes and eucaryotes are superoxide dismutases (SODs) to protect against the harmful effects of O_2^- and catalases and peroxidases that protect organisms from the effects of H_2O_2 and other peroxides.

SODs are metalloenzymes that decompose superoxide radicals into hydrogen peroxide and molecular oxygen (28). Three types of SOD exist in procaryotes and eucaryotes, and each contains a different metal cofactor for activity, i.e., iron (Fe), manganese (Mn), and copper and zinc (CuZn) (6, 15, 49). The FeSOD is most commonly found in procaryotes, whereas the MnSOD has been found in both procaryotes and the mitochondria of eucaryotes (43). With some exceptions, the CuZnSOD is primarily found in the cytosol of eucaryotes (1). Exceptions include CuZnSOD in the procaryotes *Photobacterium leiognathi* (41), *Caulobacter crescentus* (48), *Haemophilus influenzae* (25), *Brucella abortus* (3), *Pseudomonas diminuta* and *Pseudomonas maltophilia* (50), and *Legionella pneumophila* (29, 51).

L. pneumophila, the causative agent of Legionnaires' disease, is a facultative intracellular pathogen of humans that infects alveolar macrophages and other phagocytes, causing an acute and sometimes fatal pneumonia (58). *L. pneumophila* requires O_2 for growth and has been shown to have ROI-scavenging enzymes that include peroxidase, SOD, and possibly catalase (22, 38, 51, 56). In addition to its periplasmic CuZnSOD, *L. pneumophila* contains an FeSOD located in the bacterial cytoplasm (47a). The gene encoding the *L. pneumophila* FeSOD, *sodB*, was recently cloned by its ability to confer paraquat resistance to an *Escherichia coli* Δ*sodA* Δ*sodB* strain (51). Paraquat (methyl viologen) is a redox cycling agent that generates O_2^- in the bacterial cytoplasm (8, 10).

In addition to the important detoxifying function of bacterial SOD during aerobic growth, SOD has also been established as a virulence factor for some bacterial pathogens (2, 21). For example, SOD has been shown to protect *Nocardia asteroides* against the bactericidal effects of neutrophils during infection (2). Similarly, FeSOD mutants of *Shigella flexneri* were shown to be extremely sensitive to the killing effects of host macrophages and polymorphonuclear leukocytes (14). Because *L. pneumophila* does not trigger the respiratory burst upon entry into phagocytes (4, 36), it is not known whether the FeSOD is important for *L. pneumophila's* ability to grow within and/or kill host cells.

To determine the role that the *L. pneumophila* FeSOD plays during growth of the organism under aerobic conditions and within host macrophages, the cloned *sodB* gene was mutagenized with Tn*903*dII*lacZ*, a derivative of the transposon Tn*903*. The transposon insertion generated a *sodB*::*lacZ* fusion that was used in attempts to generate a *sodB* mutant of *L. pneumophila* and to study *sodB* gene expression. Our results indicate that like that of *E. coli*, the *L. pneumophila sodB* gene is largely constitutive and not induced by oxidative stress. We were unable to generate a *sodB* mutant strain by allelic exchange of the fusion with chromosomal *sodB*, suggesting that FeSOD is essential for *L. pneumophila* viability. This contrasts with other bacterial species in which Sod$^-$ strains are viable aerobically.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and features	Source or reference
Strains		
L. pneumophila		
JR32	Homogeneous salt-sensitive isolate of AM511	42
AM511	Wild-type *L. pneumophila* Philadelphia-1 Sm^r (r^- m^+)	27
AB1156	AM511 *pig*::Tn*903*dII*lacZ*	57
AB1267	JR32 with pAB1	This study
AB1688	JR32 with pAB100 *sodB*::*lacZ* integrated at *sodB* locus	This study
AB1701	JR32 with pAB100	This study
AB1703	AB1688 with pAB101	This study
AB1704	AB1688 with pAM6	This study
AB1716	*sodB*::*lacZ* mutant of JR32 with pAB101	This study
AB1717	*sodB*::*lacZ* mutant of JR32 with pAB101	This study
AB1778	pAB102 in wild-type JR32	This study
AB1782	pBC SK(+) in wild-type JR32	This study
JW1028	JR32 with pJW26	This study
E. coli		
OX326A	*E. coli* Δ*sodA*Δ*sodB*	51
CAR	OX326A with pCAR1	H. Steinman
DH5α	F^- *endA1 hsdR17* (r^- m^+) *supE44 thi-1* λ^- *recA1 relA1*Δ(*argF-lacZYA*)U169 ϕ80d*lacZ*Δ*M15 deoR gyrA96* Nal^r	59
LE392Δ*lac*	e14$^-$ (r^- m^+) *hsdR514 supE44 supF58* Δ(*lacIZY*)6 *galK2 galT22 metB1 trpR55*λ^-	26
LW211	LE392 with an integrated RP4 (ΔCb^r Tc^r::Mu) transferred by P1 transduction from SM10, Mob^+ Km^r	L. Wiater
LW252	LW211 (Km^r::Tn*7*) Km^s Tri^r Spc^r Sm^r	L. Wiater
SM10	*thi thr leu tonA lacY supE recA* with an integrated RP4-2-(ΔCb^r Tc^r::Mu) Km^r	46
Plasmids		
pBluescript II KS(+)	*oriR*(*f1*) MCS *oriR*(ColE1) Cb^r	44
pBC SK(+)	*oriR*(f1) MCS *oriR*(ColE1) Cm^r	Stratagene
pAM6	2.9-kb *Hin*dIII fragment (encoding Gm^r) from pLB41 cloned into *Hin*dIII site of pBSK II KS(+)	A. Marra
pLB41	2.9-kb *Hin*dIII DNA Gm^r DNA fragment on pBR322	L. Babiss
pAB1	*trpA'-lacZ*$^+$ on 3.9-kb *Eco*RI-*Xmn*I DNA from pMLB1109 in *Eco*RI-*Sma*I site of pMMB207	This study
pMLB1109	pBR322 with *rrnB* T1 term-(4X)-*trpA'-lacZ*$^+$	M. Berman
pAB100	*sodB*::*lacZ* on 9-kb *Eco*RI DNA fragment in *Eco*RV site of pLAW344 (Cm^r Cb^r Km^r Lac^+)	This study
pAB101	*sodB* on 4.5-kb *Eco*RI DNA cloned in *Eco*RI site of pAM6 (Gm^r)	This study
pAB102	*sodB* on 4.5-kb *Eco*RI DNA fragment in *Eco*RI site of pBC (Cm^r)	This study
pAB104	*orf1*::*lacZ* on 9-kb *Eco*RI DNA fragment in *Eco*RV site of pLAW344 (Cm^r Cb^r Km^r Lac^+)	This study
pJW2	*sodB* on 4.5-kb *Eco*RI DNA fragment in *Eco*RI site of pMMB207 (Cm^r)	This study
pJW26	*sodB*::*lacZ* on 9-kb *Eco*RI DNA fragment in *Eco*RI site of pMMB207 (Cm^r)	This study
pJW28	*orf1*::*lacZ* 9-kb *Eco*RI DNA fragment in *Eco*RI site of pMMB207 (Cm^r Km^r Lac^+)	This study
pCAR1	*sodB* contained on 29-kb DNA fragment in *Eco*RI site of pLAFR1 (Tc^r)	H. Steinman
pKD368	*tnpA* Tn*903*dII*lacZ* ('*lacZ* Km^r) *oriR*(ColE1) Ap^r	K. Derbyshire
pLAW344	*sacB* MCS *oriT*(RK2) Cm^r *loxP oriR*(ColE1) Cb^r *loxP*	57
pLAFR1	21.6-kb pRK290 derivative, Tc^r (*mob*$^+$ *tra* mutant)	17
pMMB207	RSF1010 derivative, IncQ *lacI*^{q} *cat*(Cm^r) *Ptac*	32

MATERIALS AND METHODS

Bacterial strains, plasmids, cloning, and DNA manipulations. The bacterial strains and plasmids employed in this work are listed in Table 1. Preparation of plasmid DNA and DNA cloning procedures were performed as described elsewhere (26). Bacterial mating was performed by the method of Miller (30).

Media and reagents. *E. coli* strains were grown overnight in Luria-Bertani broth (LB) or on LB agar plates as described previously (30). *L. pneumophila* was grown in ABYE broth (23) or on ABCYE medium (12). Reagents, chemicals, and fetal calf serum were purchased from Sigma. Iron was added as ferric nitrate to ABYE and ABCYE medium at 250 μg/ml, and L-cysteine was added at 400 μg/ml. For cell culture, RPMI 1640 was obtained from JRH Biosciences and L-glutamine (Gln) was obtained from Cellgro. Normal human serum was obtained from healthy volunteers and stored in 5-ml aliquots at −80°C. DNA restriction enzymes were supplied by New England Biolabs or Boehringer Mannheim Biochemicals. M63 and M9 media were prepared as described by Miller (30). Fructose was sterilized by filtration and added to M9 medium at a final concentration of 0.2% (wt/vol). The substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used as an indicator of β-galactosidase activity. The antibiotic concentrations used for *E. coli* were as follows: tetracycline, 20 μg/ml; kanamycin, 50 μg/ml; carbenicillin and ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; and gentamicin, 15 μg/ml. Antibiotics used for *L. pneumophila* were as follows: streptomycin, 50 μg/ml; kanamycin, 25 μg/ml; chloramphenicol, 5 μg/ml; and gentamicin, 5 μg/ml.

Determination of stress conditions in ABCYE medium. The chemical compounds H_2O_2 and 1,1'-dimethyl-4,4'-bipyri-

dinium dichloride (paraquat; Sigma) were added in increasing amounts (50 μM to 10 mM) to ABCYE medium prior to the addition of wild-type *L. pneumophila* JR32. After 3 days at 37°C, the extent of bacterial growth on the stress plates was compared with that of strain JR32 grown on ABCYE medium alone (data not shown). The amount of each compound that resulted in a slight decrease in the growth of wild-type strain JR32 was used as the stress concentration for measuring effects on *sodB* expression. These concentrations were 5 mM for H_2O_2 and 0.5 mM for paraquat.

Transposon mutagenesis and screen for SOD activity. The *L. pneumophila sodB* gene was mutagenized with the Tn*903* derivative, Tn*903*dII*lacZ*. The transposon contains a gene encoding resistance to kanamycin (Km^r) and carries a 5′ truncated *lacZ* gene that is capable of generating *lacZ* fusions. The plasmid pJW2 encodes *L. pneumophila* FeSOD activity on the 4.5-kb *Eco*RI insert DNA cloned within the RSF1010 derivative pMMB207. Plasmid pMMB207 encodes chloramphenicol resistance (Cm^r) and is mob^+. pJW2 was introduced into strain LW252, with selection for Cm^r, to generate strain JW1030. LW252 contains the mobilization (*tra*) genes of RK2 on an integrated copy of RK2. Tn*903*dII*lacZ* was introduced into strain JW1030 on the pBR322 derivative pKD368, with selection for Cm^r (encoded by pMMB207), carbenicillin resistance (Cb^r) (encoded by pKD368 vector sequences), and Km^r (encoded by Tn*903*dII*lacZ* on pKD368). Independent Cm^r Cb^r Km^r colonies were isolated and subsequently used as donors to transfer pJW2 into the nalidixic acid-resistant (Nal^r) recipient DH5α by bacterial mating. One hundred Lac^+ Nal^r Cm^r Km^r transconjugants were isolated, along with a few Lac^- Nal^r Cm^r Km^r transconjugants. Plasmid DNA was prepared from each of these strains and introduced into the *E. coli* Δ*sodA*Δ*sodB* strain OX326A, with selection for growth on LB containing chloramphenicol. Cm^r colonies were purified once on LB containing chloramphenicol and then streaked onto M9 containing chloramphenicol plus 0.2% fructose. After a 2-day incubation at 37°C, the growth of the bacterial transformants was compared with that of OX326A containing either pJW2 or pMMB207. Unlike pMMB207 (Sod^-), pJW2 (Sod^+) demonstrated healthy colony formation on M9 medium containing fructose after 2 days. For 28 isolates, the sites of Tn*903*dII*lacZ* insertion were determined by restriction analysis.

Allelic exchange. Allelic exchange was accomplished in *L. pneumophila* as previously described (57) by using the vector pLAW344, which contains a Cm^r marker and the counterselectable *sacB* gene from *Bacillus subtilis*. The *sodB*::*lacZ* fusion contained on pJW26 and the downstream open reading frame (*orf1*) *orf1*::*lacZ* fusion contained on pJW28, both marked by the Km^r gene in Tn*903*dII*lacZ*, were isolated on 9-kb *Eco*RI DNA fragments and inserted into the *Eco*RV site of pLAW344. The resulting plasmids, pAB100 and pAB104, respectively, were introduced into *L. pneumophila* JR32 by electroporation, grown in ABYE for 5 h, and plated on ABCYE containing kanamycin. Km^r transformants were streaked on plates containing ABCYE plus kanamycin plus 2% (wt/vol) sucrose (Suc) to select for cells which no longer contained pLAW344 sequences. Km^r Suc^r colonies were streaked on nonselective ABCYE medium and, after 3 days, single isolates were patched on plates containing ABCYE plus kanamycin, ABCYE plus chloramphenicol, and ABCYE plus 2% Suc. One set of ABCYE plates was overlaid with 0.8% agarose containing 0.7 mg of X-Gal per ml, and the Lac phenotype of the patches was determined. The total number of Km^r Suc^r Cm^s and Km^r Suc^r Cm^r isolates for each exchange was counted, and the presence of the transposon (Km^r) and pLAW344 vector sequences (Cm^r) within their genomes was verified by Southern blot analysis (data not shown). The Suc^r phenotype associated with bacteria containing an integrated copy of pLAW344 sequences most likely resulted from *sacB*'s inability to confer sensitivity to Suc in single copy. Alternatively, the *sacB* gene may have been mutated during integration of pLAW344. Considering the frequency of random mutation, we believe this scenario unlikely since all Suc^r isolates obtained by this procedure contain an integrated copy of pLAW344.

DNA sequencing. DNA sequencing was performed by the DNA Synthesis and Sequencing Facility of the Comprehensive Cancer Center, College of Physicians and Surgeons of Columbia University. The primer used to determine the nucleotide sequence across the *lacZ* fusion joint was prepared by the DNA Synthesis and Sequencing Facility, as mentioned above. The DNA sequence of the primer, 5′-CCCAGTCAC GACGTTG-3′, corresponds to nucleotides 31 through 46 of the *lacZ* coding region. DNA sequence analysis was done by using the MacDNASIS version 1.0 program. Nucleic acid and amino acid similarity searches of the GenBank/EMBL and SwissProt data bases were performed by using the Sequence Analysis Software Package obtained from Genetics Computer Group, Inc.

HL-60 cell culture. The human leukemia cell line HL-60 (9) was maintained in RPMI 1640 medium supplemented with 2 mM Gln and 10% fetal calf serum. HL-60 cells were differentiated into macrophages by incubating them for 2 days with 10 ng of phorbol 12-myristate 13-acetate per ml in RPMI containing 2 mM Gln plus 10% normal human serum. Adherent cells were washed three times with RPMI containing 2 mM Gln and incubated with RPMI containing 2 mM Gln plus 10% normal human serum prior to infection.

Measurement of β-galactosidase activity. β-Galactosidase activity was measured by quantitating the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as described by Miller (30). During growth of *L. pneumophila* in HL-60 cells, β-galactosidase activity was determined as described by Wiater et al. (57).

Southern blotting. Genomic DNA was prepared according to the method of Silhavy et al. (45), digested with *Eco*RI, separated by electrophoresis in 0.7% agarose gels, and transferred to Hybond-N nylon membranes (Amersham) by the method of Southern (47). DNA probes were prepared by random-primed labelling with [^{32}P]dATP and the large Klenow fragment of DNA polymerase I (13).

Determination of FeSOD and aconitase activities. SOD activity was visualized after nondenaturing electrophoresis in polyacrylamide gels by the riboflavin-nitroblue tetrazolium method (50). SOD activity was measured in solution by the pyrogallol method (40). Aconitase activity was quantitated, with citrate as a substrate, by coupling with isocitrate dehydrogenase and measuring reduction of $NADP^+$ (18). One unit of aconitase activity is defined as 1 nmol of NADPH formed per min.

RESULTS

Subcloning and transposon mutagenesis of *sodB* with Tn*903*dII*lacZ*. Because repeated attempts to mutagenize *sodB* with Tn*903*dII*lacZ* on pCAR1, a 29-kb cosmid containing the *L. pneumophila sodB* gene (47a, 51), were unsuccessful (data not shown), the *sodB* gene was isolated on a smaller DNA fragment. Plasmid pCAR1 was digested with *Eco*RI, and five DNA fragments (21.6, 20, 4.5, 3, and 1.5 kb) were identified. Four of the 5 *Eco*RI fragments (the 21.6-kb fragment was not isolated because it corresponded to pLAFR1 vector DNA)

were cloned into the RSF1010 derivative pMMB207 and tested for their Sod phenotype in the *E. coli* Δ*sodA* Δ*sodB* strain OX326A (see Materials and Methods). The Sod$^+$ phenotype was encoded by the 4.5-kb *Eco*RI DNA fragment; hence, the corresponding plasmid pJW2 was used for Tn*903*dII*lacZ* mutagenesis.

Following the mutagenesis of pJW2, the location of Tn*903*dII*lacZ* within the plasmid was determined for 51 Lac$^+$ and 5 Lac$^-$ mutants. Of 28 insertions within the 4.5-kb insert DNA, only 1 (pJW26) exhibited a Sod$^-$ Lac$^+$ phenotype. The Lac$^+$ phenotype suggested that the *lacZ* gene from Tn*903*dII*lacZ* had fused with *L. pneumophila sodB* sequences. The low frequency of Sod$^-$ mutants (1 of 28, 3.6%) may reflect a small target size for transposition or a cold spot for Tn*903*dII*lacZ* transposition.

DNA sequencing of the *sodB*::*lacZ* fusion joint. To determine if the Sod$^-$ phenotype associated with pJW26 was due to Tn*903*dII*lacZ* insertion within the *sodB* structural gene, the DNA sequence across the *lacZ* fusion joint was determined. The nucleotide sequence showed 100% identity with that of the *L. pneumophila sodB* gene submitted by J. Amemura-Maekawa and H. Watanabe in Tokyo, Japan (GenBank accession no. D12922). With the complete *sodB* sequence as a reference, the Tn*903*dII*lacZ* insertion in pJW26 (*sodB-1*) was mapped to nucleotide 346 (codon 116) in the *sodB* coding region, as shown in Fig. 1. Examination of the *L. pneumophila sodB*::*lacZ* fusion joint shown in Fig. 2A revealed that *sodB* was fused out of frame with respect to the *lacZ* gene in Tn*903*dII*lacZ*, resulting in a transcriptional fusion.

Identification of an expressed ORF. To consider the possibility that the Sod$^-$ phenotype associated with pJW26 was not due to an insertion within the *sodB* structural gene itself, but rather was due to failure to express an open reading frame (ORF) downstream from *sodB*, a number of Lac$^+$ and Lac$^-$ Tn*903*dII*lacZ* insertions within pJW2 were examined. One Lac$^+$ Sod$^+$ Tn*903*dII*lacZ* insertion (pJW28) mapped very close to the Lac$^+$ Sod$^-$ insertion in pJW26, suggesting a Tn*903*dII*lacZ* insertion in *sodB* that had not lost SOD activity. The sequence across the *lacZ* fusion joint showed identity with the 3′ end of the *sodB* coding region. Rather than being inserted within *sodB*, however, Tn*903*dII*lacZ* had transposed into an ORF that began 60 nucleotides downstream from the stop codon of *sodB*, as illustrated in Fig. 1. The transposon was situated 419 nucleotides downstream from the putative start site of *orf1*, and examination of the fusion joint revealed that in contrast to *sodB*, a translational fusion had been generated between *orf1* and *lacZ* in Tn*903*dII*lacZ*, as shown in Fig. 2B. Because the *orf1*::*lacZ* insertion was able to complement OX326A to Sod$^+$, we believe that sequences downstream from the *sodB* gene are not required for expression of the Sod$^+$ phenotype in *E. coli*. We cannot exclude the possibility that the *orf1*::*lacZ* fusion retains the *orf1* and downstream gene activity required for the Sod$^+$ phenotype, since the 3′ end of the *orf1* coding region has not been obtained.

The *L. pneumophila sodB* gene is essential for viability. We attempted to construct a *sodB* mutant of *L. pneumophila* to study the role of the FeSOD during oxidative damage. The *sodB*::*lacZ* fusion on pJW26 (conferring a Sod$^-$ phenotype to OX326A) was transferred on a 9-kb *Eco*RI DNA fragment into the *Eco*RV site of pLAW344 after the 5′ cohesive ends had been filled in with DNA polymerase I, Klenow fragment. The resulting plasmid pAB100, was introduced into wild-type *L. pneumophila* JR32, with selection for Cm^r (from the vector) and Km^r (from the transposon). Kanamycin-resistant colonies were isolated and streaked on ABCYE medium containing kanamycin plus 2% Suc (see Materials and Methods for details), and then Km^r Suc^r colonies were screened for Cm^r and β-galactosidase expression. *L. pneumophila* is naturally Lac$^-$; therefore, *lacZ* expression is entirely due to the *sodB*::*lacZ* fusion. Of 22 Km^r Suc^r isolates tested, all 22 were Lac$^+$ and Cm^r, indicating that pLAW344 vector DNA was present in the genomes of the putative exchange mutants. Southern blot analysis of genomic DNA prepared from 6 of the 22 Km^r Suc^r Cm^r isolates verified the presence of both the wild-type *sodB* and mutant *sodB*::*lacZ* alleles (data not shown).

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1                                                                    66
ATGACTTTTACCCTGCCGCAATTACCTTATGCATTGGATGCACTGGCTCCTCATGTTTCCAAAGAA sodB
 M  T  F  T  L  P  Q  L  P  Y  A  L  D  A  L  A  P  H  V  S  K  E

67                                                                  132
ACCTTGGAATACCATTATGGTAAACACCATAACACTTACGTCACCAACTTAAATAAATTAATTCCC sodB
 T  L  E  Y  H  Y  G  K  H  H  N  T  Y  V  T  N  L  N  K  L  I  P

133                                                                 198
GGAACAGAATTTGAATCCATGACTCTGGAAGAGATCATCATGAAAGCTAAAGGCGGTATTTTTAAC sodB
 G  T  E  F  E  S  M  T  L  E  E  I  I  M  K  A  K  G  G  I  F  N

199                                                                 264
AACGCCGCTCAGGTTTGGAATCACACCTTTTATTGGCACAGCATGAGCCCTAATGGTGGAGGTGAG sodB
 N  A  A  Q  V  W  N  H  T  F  Y  W  H  S  M  S  P  N  G  G  G  E

265                                                                 330
CCAAAAGGCAGGCTGGCGGAAGCCATTAATAAAAGCTTTGGTTCTTTTGCAGCATTCAAAGAACAA sodB
 P  K  G  R  L  A  E  A  I  N  K  S  F  G  S  F  A  A  F  K  E  Q

                 26
331              ▽                                                  396
TTTTCACAAACAGCTGCTACAACATTCGGTTCCGGGTGGGCATGGCTAGTCCAGGATCAGTCAGGG sodB
 F  S  Q  T  A  A  T  T  F  G  S  G  W  A  W  L  V  Q  D  Q  S  G

397                                                                 462
GCTTTAAAAATTATCAACACAAGCAATGCTGGAACACCAATGACTGAAGGATTAAATGCCTTATTA sodB
 A  L  K  I  I  N  T  S  N  A  G  T  P  M  T  E  G  L  N  A  L  L

463                                                                 528
ACTTGTGACGTATGGGAGCACGCTTATTACATCGATTATAGAAATAGGCGCCCGGATTATATTGAA sodB
 T  C  D  V  W  E  H  A  Y  Y  I  D  Y  R  N  R  R  P  D  Y  I  E

529                                                                 594
GCCTTTTGGTCATTAGTTAATTGGGATTTTGCGAGCAGTAATCTGAAATAAAATAACAAGCTGGAT sodB
 A  F  W  S  L  V  N  W  D  F  A  S  S  N  L  K  *

595                                                                 660
TTGTTGCAATCCCTACCGGATAATTTTATGTTTATGGAGACATCNATGAGTTTAATTACAAGTTAC orf1
                                                 M  S  L  I  T  S  Y

661                                                                 726
AGCCCAATGCCTGTAACCTTCACGCACGGAGAAGGCGTGTGGTTGTATGATGAGCAGGGAAAAAA orf1
 S  P  M  P  V  T  F  T  H  G  E  G  V  W  L  Y  D  E  Q  G  K  K

727                                                                 792
TACCTCGATGGCTTAAGCGGGATAGCAGTTTGTGGATTAGGTCATGCACACCCTGATGTCACCCGT orf1
 Y  L  D  G  L  S  G  I  A  V  C  G  L  G  H  A  H  P  D  V  T  R

793                                                                 858
ACCATACAGGAGCAGGCAGCCAGGCTAATACATACTTCTAATGTCTTTCATATTAAAGAACAAGAA orf1
 T  I  Q  E  Q  A  A  R  L  I  H  T  S  N  V  F  H  I  K  E  Q  E

859                                                                 924
TTATTAGCCGCCAAACTCACTTCAATGGCACGAATGGAGCAGGTTTTTTTTGCTAATTCTGGAGCA orf1
 L  L  A  A  K  L  T  S  M  A  R  M  E  Q  V  F  F  A  N  S  G  A

925                                                                 990
GAAGCTAATGAAGCAGCCATTAAACTTACTCGTCTGTTTGGTCATAAAAAGGGAATTGAAACGCCT orf1
 E  A  N  E  A  A  I  K  L  T  R  L  F  G  H  K  K  G  I  E  T  P

991                                                                1056
TCTATTATTGTAATGGAAAAAGCATTTCATGGTCGCACTATGGCCACCTTAACTGCATCAGGCAGT orf1
 S  I  I  V  M  E  K  A  F  H  G  R  T  M  A  T  L  T  A  S  G  S

 28
 ▽
CGTAAAGTT orf1
 R  K  V
```

FIG. 1. Location of Tn*903*dII*lacZ* within *sodB* and *orf1*. The DNA sequence is shown above the predicted amino acid sequence (shown in single-letter abbreviation) encoded by *sodB* and *orf1*. Methionines represent potential start sites for *sodB* and *orf1* translation, and the asterisk marks the putative stop codon for *sodB*. The sites of Tn*903*dII*lacZ* insertion into *sodB* and *orf1* are shown as open triangles, and the number above this symbol identifies the plasmid from which the nucleotide sequence was derived: pJW26 for *sodB* and pJW28 for *orf1*.

A *sodB::lacZ* fusion joint:

```
                sodB                    lacZ
    K   E   Q   F   S   Q   T   A       A   L   L   N   K
... AAA GAA CAA TTT TCA CAA ACA GCT G*C GCT TTG TTG AAT AAA
...
```

B *orf1::lacZ* fusion joint:

```
                orf1                    lacZ
    T   L   T   A   S   G   S   R   A   L   L   N   K
... ACC TTA ACT GCA TCA GGC AGT CG*C GCT TTG TTG AAT AAA ...
```

FIG. 2. Nucleotide sequence of the Tn*903*dII*lacZ* fusion joint for *sodB* (A) and *orf1* (B). The DNA sequence was determined by using a primer derived from the noncoding strand of the *lacZ* gene (see Materials and Methods). The fusion joints for both *sodB* and *orf1* with *lacZ* are marked by an asterisk.

These results contrasted with those obtained for *orf1*, for which all six allelic exchange mutants tested contained only the mutant *orf1::lacZ* allele within their genomes (data not shown).

The inability to generate a *sodB* null allele suggested either that the FeSOD is essential for viability or that there may be a recombination block on one side of the *sodB* gene, making it impossible to obtain a double crossover between the wild-type and mutant *sodB* alleles. The latter scenario does not appear to be due to a limitation in the amount of homologous DNA flanking the transposon, since Tn*903*dII*lacZ* is situated near the middle of the 4.5-kb *Eco*RI DNA fragment (data not shown). In addition, because there was no problem with the exchange of *orf1*, located immediately downstream from *sodB*, there does not appear to be a block in the ability to recombine within flanking DNA sequences. Similarly, it is unlikely that the insertion of Tn*903*dII*lacZ* in *sodB* is polar to the expression of a downstream gene that is essential for cell viability, because the chromosomal *orf1*::Tn*903*dII*lacZ* insertion mutant of *L. pneumophila* is viable.

Loss of *sodB*$^+$ from a *sodB*$^+$/*sodB::lacZ* merodiploid. All of the *sodB* exchange mutants would contain both a wild-type and a mutant copy of *sodB* within their genomes, because pAB100 had integrated by homologous recombination into the *L. pneumophila* chromosome at the *sodB* locus. Possible cointegrate structures that could result from recombination between the wild-type chromosomal copy of *sodB* and the mutant (*sodB::lacZ*) copy on pAB100 are shown in Fig. 3A.

The loss of pLAW344 vector sequences and either the wild-type *sodB* or mutant *sodB::lacZ* gene should occur by homologous recombination at some frequency. Possible outcomes resulting from recombination between the wild-type and mutant *sodB* alleles within the cointegrate and subsequent loss of plasmid sequences from these strains are illustrated in Fig. 3B. Loss of the *sodB::lacZ* mutant allele would result in a Km^s Cm^s Lac$^-$ phenotype, whereas loss of the wild-type *sodB* allele would confer a Km^r Cm^s Lac$^+$ phenotype. If *sodB* were essential, only isolates that had lost the mutant allele (Km^s Cm^s Lac$^-$) would be anticipated.

To test the hypothesis that *sodB* is essential, a Km^r Suc^r Cm^r isolate that contained a wild-type and a mutant copy of *sodB*, *L. pneumophila* AB1688, was tested for the loss of either the wild-type or mutant *sodB* allele. Strain AB1688 was grown overnight in ABYE without selection, diluted 1:100 into fresh culture medium, and grown again to saturation. The bacteria

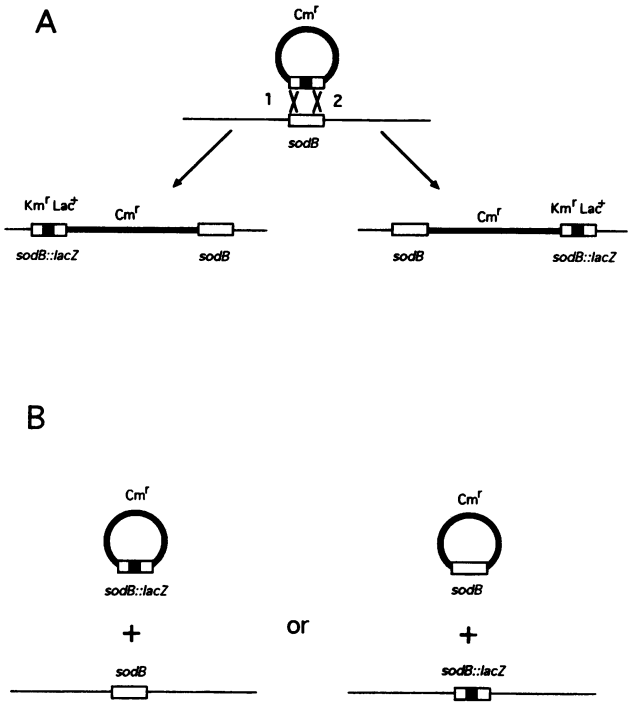

FIG. 3. (A) Integration of the *sodB*-containing plasmid pAB100 into the chromosome of strain JR32 by homologous recombination. The two possible outcomes, shown as 1 and 2, for integration of pAB100 (circle) into wild-type strain JR32 depend upon the site at which the crossover between the wild-type *sodB* (open rectangles) and mutant *sodB::lacZ* (black-centered rectangles) alleles occurs. (B) Excision of pLAW344 DNA sequences by homologous recombination between *sodB* and *sodB::lacZ*. Illustrated are two possible scenarios for excision, dependent on the site of recombination between the integrated *sodB* and *sodB::lacZ* genes. Plasmid sequences (encoding Cm^r) are represented by thick lines, and chromosomal DNA sequences are represented by thin lines.

were plated on ABCYE medium, and after 3 days at 37°C, the bacteria were replica plated on ABCYE plates containing chloramphenicol or kanamycin. For four independent experiments, the *sodB::lacZ* mutant allele was lost 1.4 to 1.8% of the time, as shown in Table 2. No Km^r Cm^s Lac$^+$ colonies were

TABLE 2. Loss of the mutant and wild-type *sodB* alleles from the cointegrate AB1688

Expt no.	No. of colonies[a]	No. of Km^r Cm^s Lac$^+$ colonies[b]	No. of Km^s Cm^s Lac$^-$ colonies[c]	Frequency of *sodB::lacZ* loss
1	212	0	3	0.014
2	222	0	3	0.014
3	899	0	15	0.017
4	965	0	17	0.018

[a] *L. pneumophila* AB1688 was grown in the absence of antibiotic selection as described in the text, and the total number of colonies for each experiment was counted.

[b] The number of bacterial colonies that have lost pLAW344 (encoding Cm^r) and the wild-type *sodB*$^+$ allele from strain AB1688, having a Km^r Cm^s Lac$^+$ phenotype.

[c] The number of bacterial colonies that have lost pLAW344 (encoding Cm^r) and the mutant *sodB::lacZ* allele from strain AB1688, having a Km^s Cm^s Lac$^-$ phenotype.

TABLE 3. Loss of the chromosomal $sodB^+$ gene in the presence of an additional copy of $sodB^+$

Strain	Plasmid	No. of colonies[a]	No. of Cm^s colonies[b]	No. of colonies with allele present in bacterial chromosome	
				$sodB^+$[c]	*sodB*::*lacZ*[d]
AB1703	pAB101	487	3	1	2
AB1704	pAM6	397	0	0	0

[a] *L. pneumophila* strains were grown as described in the text, and the total number of Gm^r colonies containing either pAB101 ($sodB^+$ in pAM6) or pAM6 were counted.

[b] The number of Cm^s isolates that have presumably lost pLAW344 DNA sequences (encoding Cm^r) from the chromosome of strain AB1688.

[c] The number of Cm^s colonies that have lost pLAW344 DNA sequences and the *sodB*::*lacZ* mutant allele from the chromosome of parent strain AB1688, having a Km^s Cm^s Lac^- phenotype.

[d] The number of Cm^s colonies that have lost pLAW344 DNA sequences and the wild-type $sodB^+$ allele from the parent strain AB1688, having a Km^r Cm^s Lac^+ phenotype.

observed, indicating that the wild-type *sodB* gene was lost from *L. pneumophila* AB1688 at a frequency of <0.04%.

If the wild-type *sodB* gene was not lost from strain AB1688 because it is essential, then supplying a second copy of $sodB^+$ should permit recovery of recombinants in which the chromosomal *sodB* gene is lost. This was tested by transforming strain AB1688 with either pAM6, a gentamicin-resistant (Gm^r) pBluescript II derivative, or pAB101 ($sodB^+$ on pAM6) and selecting for growth on ABCYE medium containing gentamicin. Gm^r transformants AB1703 (AB1688 with pAB101) and AB1704 (AB1688 with pAM6) were grown without selection as previously described and plated on ABCYE medium for 3 days at 37°C, and bacterial colonies were replica plated on ABCYE plates containing gentamicin, gentamicin plus chloramphenicol, and gentamicin plus kanamycin. For both strains AB1703 and AB1704, approximately 450 colonies were tested for loss of either the mutant or wild-type *sodB* allele.

For isolates of strain AB1688 containing pAB101, loss of the chromosomal *sodB*::*lacZ* allele occurred 0.2% of the time, as identified by Gm^r Km^s Cm^s Lac^- bacterial colonies. Loss of the wild-type $sodB^+$ allele from the chromosome, indicated by a Gm^r Km^r Cm^s Lac^+ phenotype, was observed for 2 of the 487 isolates (0.4%) (Table 3). No loss of the wild-type or mutant *sodB* allele was observed for isolates of AB1688 containing only the vector pAM6 (Table 3). The two Gm^r Km^r Cm^s Lac^+ isolates of AB1688 containing pAB101 were saved as strains AB1716 and AB1717. As determined by plasmid DNA and Southern blot analyses, both strains contained a wild-type copy of $sodB^+$ on pAB101, with only the mutant *sodB*::*lacZ* allele within their chromosomes (data not shown). These results demonstrate that loss of the wild-type chromosomal locus could occur if a second copy of $sodB^+$ was provided in *trans*.

Failure to observe loss of the plasmid copy of $sodB^+$ from two *sodB*::*lacZ* mutants. If *sodB* is essential, bacteria must maintain a functional copy of the gene. For wild-type *L. pneumophila* JR32 and the merodiploid AB1688, this copy is located within the bacterial chromosome, whereas for strains AB1716 and AB1717, this copy is maintained episomally on pAB101. It should be possible, therefore, to lose pAB101 from strain AB1703 (pAB101 in AB1688), yet impossible to lose pAB101 from strains AB1716 and AB1717. Because the presence of pAB101 confers Gm^r to strains of *L. pneumophila*, the loss of pAB101 can be easily monitored.

Strains AB1703, AB1704 (pAM6 in AB1688), AB1716, and AB1717 were grown overnight at 37°C in ABYE medium without antibiotic. After 24 h, the cultures were diluted 1:100 into fresh ABYE and grown again to saturation to allow for loss of plasmid DNA. The procedure was repeated, and dilutions of bacteria were plated on ABCYE medium and incubated at 37°C for 4 days. To look for cells that had lost pAB101 or pAM6, the bacteria were replica plated on ABCYE medium and ABCYE containing gentamicin and incubated overnight at 37°C, and the total numbers of Gm^r and Gm^s CFU were determined for each strain. In all cases, the Gm^s isolates were further analyzed by patching on plates containing ABCYE and ABCYE plus kanamycin to check for the presence of Tn*903*dII*lacZ*. After 2 days at 37°C, the ABCYE plates were overlaid with X-Gal to determine the Lac phenotype of the patched colonies.

TABLE 4. Loss of pAB101 ($sodB^+$) from *sodB*::*lacZ* mutants of *L. pneumophila*

Strain	No. of colonies[a]	No. of Gm^s colonies[b]	No. of Gm^s colonies that contain chromosomal *sodB*::*lacZ*[c]
AB1703	2,166	83	4 of 20
AB1704	2,445	72	30 of 32
AB1716	2,288	6	0 of 6
AB1717	1,594	8	0 of 8

[a] *L. pneumophila* strains were grown as described in the text, and the number of bacterial colonies was counted.

[b] The number of isolates that have lost either pAB101 ($sodB^+$ on pAM6) or pAM6 DNA sequences and are therefore Gm^s.

[c] The number of Gm^s isolates tested that contain the *sodB*::*lacZ* mutant allele within their genomes, as denoted by their Km^r Lac^+ phenotype.

Approximately 2,000 colonies were screened for each of strains AB1703, AB1704, AB1716, and AB1717, and the frequency of obtaining Gm^s isolates was determined to be 3.8% for strain AB1703 and 2.9% for strain AB1704 (Table 4). Gm^s isolates of the *sodB*::*lacZ* mutant strains AB1716 and AB1717 were observed at lower frequencies, 0.3 and 0.5%, respectively (Table 4). Plasmid loss occurred more frequently, therefore, in those strains that contained a chromosomal copy of the wild-type *sodB* gene. Interestingly, all Gm^s derivatives of strains AB1716 and AB1717 were Km^s and Lac^-, suggesting that the mutant *sodB*::*lacZ* allele had been lost from the bacterial chromosome and replaced with a wild-type copy of *sodB* prior to loss of the plasmid. Exchange of the mutant *sodB*::*lacZ* allele for the wild-type *sodB* allele was verified, in all cases, by Southern blot analysis of genomic DNA prepared from a representative number of isolates (data not shown). From these results, we conclude that the *sodB* gene is essential during growth of *L. pneumophila* on standard bacteriological medium.

The effect of *sodB* copy number on FeSOD activity, paraquat resistance, and aconitase activity. Because the *L. pneumophila* *sodB* gene is essential, it is not possible to generate a null mutant to study the effects of oxidative damage on a strain lacking FeSOD activity. In *E. coli*, resistance to paraquat is strongly dependent on SOD. Strains with elevated SOD levels show increased resistance to paraquat. Therefore, the level of paraquat resistance should depend on the number of copies of *sodB* within *L. pneumophila*. To test if increased copy number of *sodB* results in increased FeSOD production and, correspondingly, increased paraquat resistance, pBC SK(+) and pAB102 ($sodB^+$) were introduced into wild-type *L. pneumophila* JR32 and the strains were tested for SOD activity and paraquat resistance.

Bacterial strains were grown to saturation in ABYE containing chloramphenicol and measured for SOD activity and

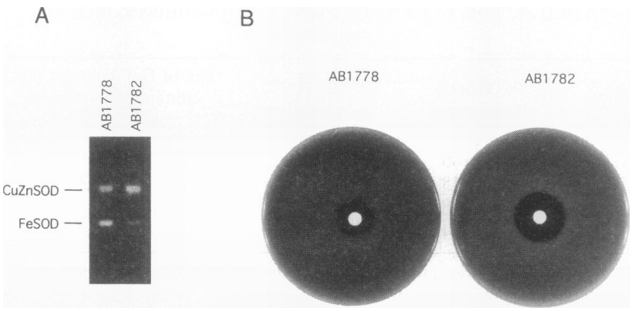

FIG. 4. (A) Effect of multiple copies of *sodB* on FeSOD activity. Extracts from strains AB1778 (pAB102 in JR32) and AB1782 [pBC SK(+) in JR32] were prepared, protein concentrations were determined by the bicinchoninic acid method (Pierce Chemical), and approximately equal amounts of protein were electrophoresed in a nondenaturing polyacrylamide gel as described in the text. Bands migrating as the *L. pneumophila* CuZnSOD and FeSOD are shown. (B) Effect of multiple copies of *sodB* on paraquat resistance. The left and right panels represent strains AB1778 and AB1782 as described above. Briefly, *L. pneumophila* strains were grown overnight to saturation at 37°C on a roller drum. Approximately 10^8 bacteria were added to 3 ml of 0.8% top agar, and the mixture was overlaid on plates containing ABCYE plus chloramphenicol. After the agar solidified, sterile discs were placed in the center of each plate and 5 μl of a freshly prepared 10-mg/ml stock solution of paraquat dichloride was added to each disc. The plates were incubated for 48 h at 37°C, and the zone of clearing, i.e., zone of growth inhibition, was observed.

sensitivity to paraquat as described above. The two SODs of *L. pneumophila* are easily resolved by nondenaturing polyacrylamide gel electrophoresis, upon which the FeSOD migrates as the more anodic SOD (51). Figure 4A illustrates that wild-type strain JR32 with multiple copies of $sodB^+$ (strain AB1778) demonstrated an increase in FeSOD activity in comparison with JR32 containing pBC SK(+) (strain AB1782). This contrasted with the slight decrease in CuZnSOD activity observed for wild-type JR32 containing multiple copies of *sodB* rather than *sodB* in single copy (Fig. 4A, compare strain AB1778 with AB1782). Quantitative assays of SOD activity in extracts of strains AB1778 and AB1782 indicated that FeSOD activity increased by a factor of about 1.4, from 35 to 50% of the total SOD activity (data not shown).

This increased FeSOD activity correlated with increased resistance to paraquat (Fig. 4B). Similar results were obtained with cells grown to saturation on plates and in liquid culture. The zone of growth inhibition for strain JR32 with pAB102 (AB1778) was 15.8 mm in diameter, much smaller than the 23.8-mm-diameter zone observed for strain JR32 containing pBC SK(+) (AB1782). Similar but less pronounced effects were observed with other redox cycling compounds like plumbagin and menadione (data not shown), demonstrating that additional copies of *sodB* increase FeSOD activity and confer greater resistance to paraquat and other redox cycling agents. No difference was observed for bacteria exposed to H_2O_2 (data not shown).

Aconitase is one of several *E. coli* 4Fe-4S enzymes whose activities are inversely related to SOD activity because they are rapidly inactivated by O_2^- (19). The gene encoding the *L. pneumophila* aconitase (*acn*) is highly homologous to the gene encoding *E. coli* aconitase (29), and it is likely that the *L. pneumophila* aconitase is sensitive to O_2^-. To determine if higher levels of FeSOD resulted in increased aconitase activity, measurements were made for strains containing a single copy (AB1782) or multiple copies (AB1778) of the *sodB* gene. No difference in aconitase activity was observed, 0.024 and 0.027 units/mg of protein for each strain, respectively. This result suggests that wild-type (single-copy) levels of FeSOD are adequate to protect aconitase from inactivation and that multiple copies of *sodB* provide no further protection. In addition, observed differences in paraquat resistance between these two strains are therefore not attributable to differences in aconitase, the major iron-containing protein of *L. pneumophila*.

Effect of growth conditions on *sodB* expression. β-Galactosidase activity was measured for strains AB1688 (chromosomal *sodB*::*lacZ*) and JW1028 (plasmid *sodB*::*lacZ*) to determine the effect of different parameters on FeSOD gene expression. Hydrogen peroxide or paraquat at concentrations that produced slight growth inhibition (5 and 0.75 mM, respectively) produced no change in *sodB* expression (data not shown). β-Galactosidase activity was measured following growth on plates and in stationary-phase and log-phase (1- to 2-h) cultures in liquid medium.

The effect of iron availability was tested, because *E. coli sodB* is under control of *fur* (ferric uptake regulation) (35) and because *L. pneumophila* requires high concentrations of iron for optimal growth (7). To determine if *sodB* expression is dependent on the levels of iron in the growth medium, β-galactosidase activity was measured in cultures grown for 2 h in liquid medium with or without 250 μg of ferric nitrate per ml. For both strains AB1688 and JW1028, β-galactosidase activity was not significantly different in control versus treated cultures (data not shown). Moreover, no change was seen for either strain upon addition of 1,10-phenanthroline, an iron chelator, at a concentration (50 μM) that reduces growth of an overnight culture by about 50%.

Expression of *sodB* was not dependent on the stage of growth in liquid medium (Fig. 5) for both strains AB1688 and JW1028. The plasmid-borne fusion generated more β-galactosidase activity (an average of 95 units for strain JW1028 compared with 15 units for AB1688), most likely from the increased copy number of the fusion. The increased level of β-galactosidase activity in strain JW1028 permitted us to measure *sodB* expression during growth in human macrophages, where bacterial titers are very low. Similar to expression during growth in culture, the *sodB* gene was expressed constitutively during growth of *L. pneumophila* in macrophage-like HL-60 cells (Fig. 6). The levels of β-galactosidase per CFU were comparable to those measured for strain JW1028 grown in culture (data not shown).

In contrast to expression of *sodB* during growth of *L. pneumophila* in culture and within human macrophages, the expression of *sodB* was influenced by charcoal during growth on ABCYE agar plates. Charcoal is frequently added to agar media for *L. pneumophila*, presumably to diminish the concentration of toxic substances or to scavenge free radicals. A two- to threefold decrease in *sodB* expression was observed for bacteria grown in the presence of charcoal (Table 5). A fusion of *lacZ* with *pig*, an *L. pneumophila* gene required for pigmentation, resulted in no change in gene expression under these conditions. Expression of the *E. coli lacZ* gene from the *tac* promoter was slightly increased in the presence of charcoal.

DISCUSSION

To examine the role of the FeSOD during oxidative stress, the *L. pneumophila sodB* gene was isolated and mutagenized with the intention of generating a *sodB* mutant. To generate a *sodB* mutant of *L. pneumophila*, the *sodB*::*lacZ* gene fusion on pJW26 was transferred to the allelic exchange vector

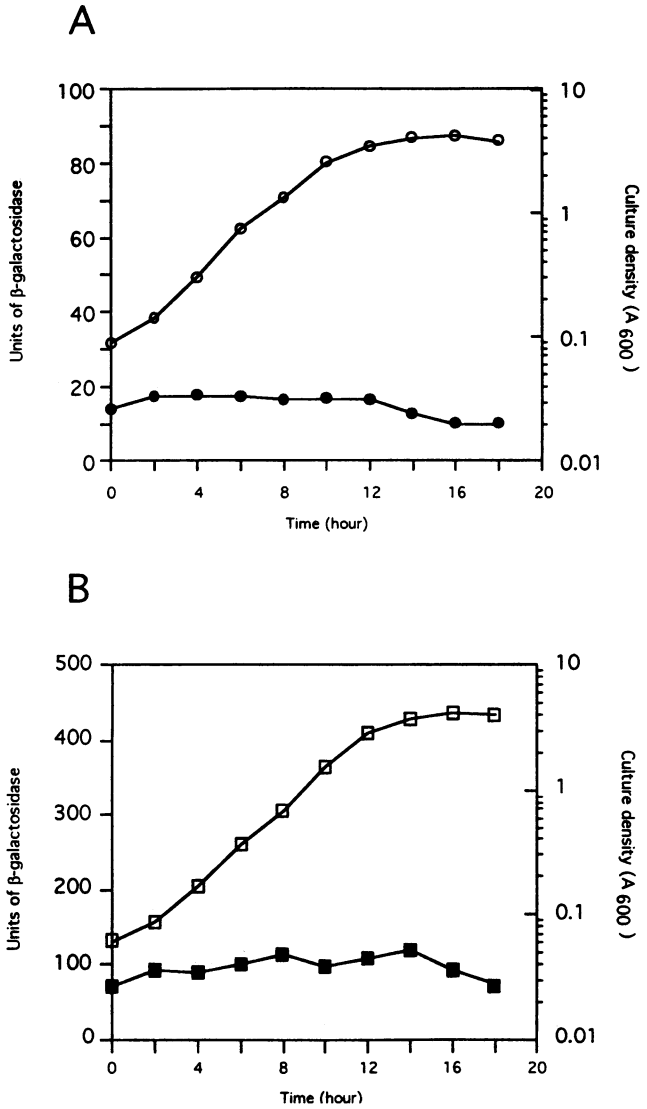

FIG. 5. Expression of the *L. pneumophila sodB* gene during growth in ABYE. (A) β-Galactosidase activity measured from the chromosomal copy of *sodB*::*lacZ* in strain AB1688. Every 2 h, bacterial growth was measured by reading A_{600} (open circles) and β-galactosidase activity (closed circles) was measured from *sodB*::*lacZ* as described in the text. (B) β-Galactosidase activity measured from the plasmid-borne (pJW26) copy of *sodB*::*lacZ* in strain LW1028. Open squares denote bacterial growth measured at A_{600}, and closed squares represent β-galactosidase activity measured from *sodB*::*lacZ*.

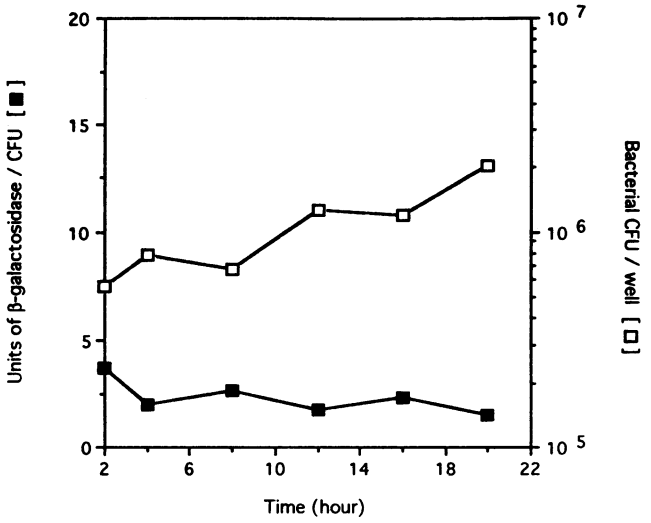

FIG. 6. *L. pneumophila sodB* expression during macrophage infection. Monolayers containing 1.5×10^6 differentiated HL-60 cells were infected with a one-to-one ratio of *L. pneumophila* JW1028 (pJW26 in JR32). Units of β-galactosidase activity per CFU and bacterial CFU/well were determined over a period of 20 h as described in the text. Data are plotted as the mean for two independent experiments. One unit of β-galactosidase activity is defined as 1×10^{-7} mol of ONPG hydrolyzed per min.

pLAW344 and introduced into wild-type strain JR32. Repeated attempts to generate a *sodB* mutant failed, suggesting that the *sodB* gene was essential for viability.

If *sodB* is essential, our inability to mutate the chromosomal copy of *sodB* suggested that a single functional copy of *sodB* exists within the *L. pneumophila* genome. This was verified by Southern blot analysis of genomic DNA prepared from wild-type strain JR32, when a single DNA fragment hybridized with *sodB* sequences after digestion with a variety of different restriction enzymes (data not shown).

It was possible to replace the chromosomal copy of *sodB* by mutant *sodB* only when bacteria were provided with a second wild-type copy of *sodB*$^+$. Two mutant strains, AB1716 and AB1717, were isolated by this means, and both were shown to contain the mutant *sodB*::*lacZ* gene within their chromosomes and a plasmid-borne copy of the wild-type *sodB*$^+$ gene. In no instance was it possible to lose the plasmid copy of *sodB*$^+$ from strains AB1716 and AB1717 and for the strains to maintain the mutated copy within their genomes. In all 14 cases in which the plasmid copy of *sodB*$^+$ was lost from these strains (Table 4), a wild-type copy of the *sodB* gene had replaced the mutant chromosomal copy, presumably by homologous recombination. These results further support the idea that *sodB* is an essential gene for *L. pneumophila*.

Mutants of *E. coli* (8) and *Streptococcus mutans* (34) lacking SOD are viable. *E. coli* contains two cytoplasmic SODs, an FeSOD (60) and an MnSOD (24). *S. mutans* contains a single cytoplasmic MnSOD (34). There are several possible explanations for the viability of a Sod$^-$ mutant in those species. Alternative antioxidants may compensate for the absence of SOD as an oxyradical scavenger, targets of ROIs may be less sensitive than they are in *L. pneumophila*, or generation of oxyradicals might be attenuated when SOD is lacking. It is

TABLE 5. Growth medium-dependent *sodB* gene expression

L. pneumophila strain	Genotype	Units of β-galactosidase activity[a]		ABYE/ABCYE[b]
		ABYE	ABCYE	
AB1688	*sodB*::*lacZ*	17.8 ± 1.62	6.79 ± 0.43	2.6
AB1156	*pig*::*lacZ*	35.53 ± 0.74	29.35 ± 1.84	1.1
AB1267	*lacZ*$^+$	7.13 ± 0.43	11.78 ± 0.90	0.6

[a] *L. pneumophila* strains were grown for 3 days on ABYE and ABCYE agar media, scraped into 1× M63 salts, and assayed immediately for β-galactosidase activity. Measurements were performed in triplicate, and the average and standard error of the mean are shown.

[b] Ratio of β-galactosidase activity measured for *L. pneumophila* strains grown on ABYE medium versus those grown on ABCYE medium.

interesting that *L. pneumophila* is an obligate aerobe while *E. coli* and *S. mutans* are able to grow anaerobically and thus avoid formation of oxygen-derived free radicals.

In contrast to *E. coli* and *S. mutans*, *L. pneumophila* contains a periplasmic CuZnSOD in addition to a cytoplasmic SOD. The inability to construct a *sodB* mutant strain of *L. pneumophila* indicates that its periplasmic CuZnSOD is unable to compensate for the absence of cytoplasmic FeSOD. This suggests that superoxide pools generated in the periplasm and cytoplasm are not exchangeable. This is consistent with the known impermeability of the inner membrane to superoxide and implies that the two *L. pneumophila* SODs serve functionally discrete roles.

Although it was not possible to construct a *sodB* mutant, we were able to demonstrate a role for FeSOD in protection against ROIs by comparing the wild-type strain with a strain harboring multiple copies of plasmid-encoded *sodB*. The strain with multiple copies of *sodB* showed increased FeSOD activity and increased resistance to the redox cycling compounds paraquat, plumbagin, and menadione.

Using the *sodB*::*lacZ* fusion, we demonstrated that the expression of *L. pneumophila sodB*, like that of *E. coli sodB* (11, 52), is largely constitutive. The *E. coli* cytoplasmic Mn-SOD is induced by a variety of oxidative and other stresses (11, 31, 52–54). Although the FeSOD of *L. pneumophila* is the only cytoplasmic SOD present, it is not induced like *E. coli* Mn-SOD. No change in expression was seen with different degrees of aeration or in the presence of various oxidants or redox cycling compounds. In addition, manipulation of iron concentration in the medium failed to produce a significant change in *L. pneumophila sodB* expression. Finally, expression did not change during growth from exponential to stationary phase.

The constitutivity of *sodB* expression suggests that the basal level of gene expression is sufficient to provide protection against superoxide generated during normal aerobic metabolism. It could be argued that the basal level of FeSOD is so high that no induction is necessary during oxidative stress. However, this seems unlikely, because the total SOD activity of *L. pneumophila* (about 150 units per mg of protein) is comparable to that in aerobically grown *E. coli*.

A decrease in *sodB* expression was found in agar medium containing charcoal. Charcoal has been proposed as an adsorbent of toxic substances, including free radicals (22, 37) and fatty acids (5, 33, 39). It might be that charcoal decreases the superoxide concentration in the *L. pneumophila* cytoplasm, diminishing the requirement for FeSOD and decreasing *sodB* expression. The mechanism by which this might occur is unclear.

Similar to *L. pneumophila* growth in culture, growth in macrophage-like HL-60 cells occurred without significant changes in *sodB* expression. Although *L. pneumophila* enters host cells without triggering the respiratory-burst production of oxyradicals, the bacterium is likely to encounter ROIs during growth within human macrophages. It is possible that these extracellular ROIs are scavenged by the periplasmic CuZnSOD. In contrast, the FeSOD probably serves to decompose intracellular, cytoplasmic ROIs generated during aerobic metabolism. The lack of induction of *sodB* during macrophage infection is consistent with this proposal.

Finally, it should be noted that although our results demonstrate a constitutivity of *sodB* expression, there may be control of FeSOD activity posttranslationally. Posttranslational control of enzyme function is particularly feasible for a metalloenzyme, for which conversion of apo- to holoenzyme may be the rate-limiting or regulatory step. The MnSOD of *E. coli*, for example, is subject to posttranslational control on activity relating to availability of manganese and iron (55).

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