CLONING AND NUCLEOTIDE SEQUENCES OF IRON AND COPPER-ZINC SUPEROXIDE DISMUTASE GENES OF LEGIONELLA PNEUMOPHILA AND THEIR DISTRIBUTION AMONG LEGIONELLA SPECIES

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SUMMARY: A facultative intracellular parasite Legionella pneumophila has two kinds of superoxide dismutase (SOD), iron-containing superoxide dismutase (Fe-SOD) and copper,zinc-containing one (Cu,Zn-SOD). We cloned both SOD genes of L. pneumophila and determined their DNA sequences. The Fe-SOD gene (sodB), isolated by functional complementation of a SOD-deficient Escherichia coli strain, encoded a protein of 192 amino acids conserving the Fe-SOD-specific amino acid residues. A clone containing entire Cu,Zn-SOD gene (sodC) was constructed by connecting two contiguous DNA fragments; one with a lower part of the gene was obtained by colony hybridization with a probe acquired by polymerase chain reaction (PCR) with degenerate oligonucleotide primers corresponding to conserved regions of known Cu,Zn-SOD genes and the other with an upper part of the gene was by IPCR (inverted PCR). The sodC gene encoded a protein of 162 amino acids, of which the first 20 amino acids inferred a signal peptide similar to other bacterial Cu,Zn-SODs reported previously. Both clones expressed their SOD activities in E. coli K-12 through their own plausible promoters. We examined for SOD genes on chromosomes of several Legionella species. All chromosomes were hybridized with Fe-SOD gene of L. pneumophila, but Cu,Zn-SOD gene did not hybridize to the chromosomes of other than L. pneumophila strains.
INTRODUCTION

*Legionella pneumophila*, the causative agent of Legionnaires' disease, is an intracellular pathogen of a gram-negative facultative bacterium. It multiplies in human monocytes and alveolar macrophages, since it is able to escape the bacteriocidal oxidative burst and to inhibit phagosome-lysosome fusion. In the natural environment, *L. pneumophila* is considered to flourish in the water as an intracellular parasite of protozoa, although it is capable of extracellular growth (reviewed in ref. 1,2).

Superoxide dismutase (SOD), which converts superoxide to hydrogen peroxide, exists commonly in most organisms and works as a part of the protection against oxidative stress. Three types of SOD are known; Mn-SOD, Fe-SOD and Cu,Zn-SOD (3). Mn-SOD exists in prokaryotes and the matrix of mitochondria (4), and Fe-SOD in prokaryotes and the chloroplast of some plants (5), and they are very similar each other in the amino acid sequence and tertiary structure (6). Cu,Zn-SOD is found in cytosol of eukaryotic cells and the chloroplast of higher plants (7), while extracellular Cu,Zn-SOD is also found in eukaryotes (8).

A few species of bacteria were considered to contain Cu,Zn-SOD (9-13). Recently, however, it was detected in *E. coli* (14) and among wide-spread gram-negative bacterial species (15,16). All bacterial Cu,Zn-SOD examined possessed signal peptides.

For intracellular pathogenic bacteria, SOD is considered to play an important role in the resistance against host-cellular oxidative burst. An Fe-SOD defective mutant of *Shigella flexneri* was found to be less virulent (17). The loss of Cu,Zn-SOD reduced the survivals of *Brucella abortus* in mice (18).

To begin studies on the role of SOD in the pathogenicity of *Legionella*, we report here the cloning and nucleotide sequences of *L. pneumophila* SOD genes, characterization of their products and distribution of SOD genes among *Legionella* species.

MATERIALS AND METHODS

*Bacterial strains and plasmids*: Bacterial strains and plasmids used in this study are listed in Table I.
<table>
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<th>E. coli K-12 strains</th>
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<tr>
<td>DH5α</td>
<td>F− endA1 hadR17 supE44 thi-1 λ− recA1 gyrA (Nal') relA1 Δ lacZΔM15K12 (φ80dlacZΔM15)</td>
<td>Bethesda Research Labs</td>
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<td>GC4468</td>
<td>F− ΔlacI169 rpsL</td>
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<td>QC774</td>
<td>GC4468 φ(sodA-lacZ49 φ(sodB-km)1-Δ2 Cm' Km'</td>
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<td>Philadelphia 1</td>
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<td>AM511</td>
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<td>Chicago 2</td>
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<td>pUC19</td>
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<td>pJAM110</td>
<td>1.5-kb Sau3AI fragment of chromosomal DNA from L. pneumophila AM511 in BamHI site of pUC18; sodB</td>
<td>This study</td>
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<td>pJAMP1</td>
<td>541-bp Psfl fragment of chromosomal DNA from L. pneumophila AM511 including C-terminal region of the Cu,Zn-SOD in Psfl site of pUC18</td>
<td>This study</td>
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<td>pGEM-T</td>
<td>Cloning vector for PCR product; Ap²</td>
<td>Promega, Madison, Wisconsin</td>
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<td>pJAMT1</td>
<td>617-bp IPCR product provided using TaqI sites cloned into pGEM-T</td>
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<td>pJAM19C</td>
<td>826-bp fragment constructed in this study in SmaI-Psfl site of pUC19; sodC²</td>
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* Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; Ap, ampicillin; sodB, L. pneumophila Fe-superoxide dismutase gene; sodC, L. pneumophila Cu,Zn-superoxide dismutase gene.
**Bacterial cultures and enzymes:** For SOD assay, *L. pneumophila* was cultivated on buffered charcoal-yeast extract (BCYE) agar (Difco Laboratories, Detroit, MI) plates at 35°C for 72 hr before harvest. For chromosomal DNA preparation from *Legionella*, yeast extract broth (YEB) (22) was used. For *E. coli*, L broth and M63 (23) were used as rich and minimum media, respectively, and SOC medium (24) for incubation of cells after electroporation. In solid media, 1.5% agar (Difco Laboratories) was included. When needed, used was 50 μg of ampicillin per ml. All restriction endonucleases except *TaqI* purchased from Toyobo (Osaka), calf intestine alkaline phosphatase and T4 DNA ligase were purchased from Takara (Kyoto).

**DNA manipulations:** *Legionella* chromosomal DNA was extracted as described by Miura (25). Plasmid DNA was isolated by the alkaline lysis method (24). DNA sequencing was performed with a Deletion Kit (Takara) and Sequenase (United States Biochemical, Cleveland, OH). Southern hybridization and colony hybridization were performed with a DIG (digoxigenin) Labeling and Detection Kit (Boehringer Mannheim, Mannheim, Germany). These kits were used as recommended by the suppliers. DNA cloning and other DNA manipulations were carried out essentially as described by Sambrook et al (24).

**PCRs:** A Cetus/Perkin-Elmer Thermocycler (Cetus, Emeryville, CA) was used for PCR. For degenerate primers, a reaction mixture (100 μl) contained 100 pmol of the primers, 100 ng of *Legionella* chromosomal DNA, all four deoxynucleoside triphosphates at 250 μM each, and 1 U of recombinant *Taq* DNA polymerase (Takara) with the supplied buffer. The sequences of the degenerate primers were as follows: Primer N (17 bases), 5’-CA(TC)GG(TCAG)TT(TC)-CA(TC)(TC)T(TCAG)CA-3’; Primer C (16 bases), 5’-AT(TCAG)AC(TCAG)-CC(AG)CA(TCAG)GC(TCAG)A-3’. The mixture was subjected to 30 cycles consisting of 1-min denaturation at 94°C, 2-min annealing at 40°C, and 2-min extension at 72°C.

IPCR (inverted PCR) (26) was performed as follows: *Legionella* chromosomal DNA was completely cut with endonuclease *TaqI*, and then self-ligated at a low DNA concentration (0.1 μg/ml). The products were purified by phenol-chloroform extraction and ethanol precipitation. Then, PCR was performed with a reaction mixtures (100 μl) containing 50 pmol of the primers which were designed at opposite orientation to each other (see Fig. 4), 5 ng of the self-ligated product, all four deoxynucleoside triphosphates at 1 mM each, and 2.5 U of recombinant *Taq* DNA polymerase (Takara) with the supplied buffer. The mixture was subjected to 30 cycles consisting of 1-min denaturation at 94°C, 2-min annealing at 55°C, and 3-min extension at 72°C.

PCR products were analyzed on 2% agarose gel and purified from the gel by with GeneClean (Bio 101, La Jolla, CA) when required.

**Preparation of cell lysates and SOD assays in polyacrylamide gel:** Overnight *E. coli* cultures or *Legionella* colonies grown for 72 hr were harvested, suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and sonicated for 30 s
five times at 200 W with a Bioruptor UCD-200T (CosmoBio, Tokyo). The debris was removed by centrifugation. SOD activities of the cell lysates obtained were assayed by nondenaturing polyacrylamide gel electrophoresis and staining as described by Beauchamp and Fridovich (27).

RESULTS

Fe- and Cu,Zn-SOD Possessed by Legionella pneumophila

To identify SOD(s) of Legionella pneumophila, we performed native polyacrylamide gel electrophoresis followed by staining for the SOD activity. The lysate of L. pneumophila AM511 cells contained two SOD activities (Fig. 1A, lane 1). These SODs were tested for the cyanide and hydrogen peroxide sensitivities to identify them with Mn-SOD, Fe-SOD or Cu,Zn-SOD. It is well known that treatment with cyanide inhibits the activity of Cu,Zn-SOD but neither of Fe-SOD nor

Fig. 1. Native polyacrylamide gel electrophoresis for the detection of SOD activity. Cell extracts were prepared as described in Materials and Methods, electrophoresed on a 10% polyacrylamide gel, and stained for SOD activities without any inhibitors (A), with 5 mM cyanide (B), or with 20 mM hydrogen peroxide (C). Lanes: 1, L. pneumophila AM511 lysate; 2, E. coli GC4468 lysate*; 3, purified Cu,Zn-SOD derived from beef erythrocytes (ICN Biochemicals, Costa Mesa, CA). *Cu,Zn-SOD of E. coli was not detected under these conditions.
of Mn-SOD, and that hydrogen peroxide inhibits both Cu,Zn-SOD and Fe-SOD, but not Mn-SOD (see Fig. 1B and C, lanes 2 and 3). The upper band of the *L. pneumophila* lysate disappeared by the treatment with cyanide or hydrogen peroxide, and so did the lower band with hydrogen peroxide but not with cyanide (Fig. 1B and C, lane 1). Therefore, we concluded that the upper and lower bands were Cu,Zn-SOD and Fe-SOD, respectively, being consistent with the previous result by Steinman (28).

**Cloning sodB Gene of Legionella pneumophila by Complementation of the E. coli SOD-defective Mutant**

An *E. coli* sodA and sodB mutant, QC774, cannot survive on minimal medium agar plates when cultivated in the presence of oxygen (19). If the SOD gene of *Legionella* can functionally complement the defect, the mutant harboring the recombinant *Legionella* SOD gene will be able to grow on the minimal medium. The cloning of other bacterial SODs also succeeded by using this method (29,30). Therefore, we performed screening a plasmid library of *L. pneumophila* chromosomal DNA for SOD genes by functional complementation. Sau3AI-partially digested chromosomal DNA of *L. pneumophila* AM511 was ligated with BamHI-linearized and calf intestine alkaline phosphatase-treated pUC18, and the resultant plasmid library was introduced into *E. coli* QC774 by electroporation. The transformants were selected on minimal medium (M63) plates containing ampicillin. Eight independent clones were obtained by screening about 17,000 recombinant plasmids. The eight plasmids contained inserts of 1.5 to 6.5 kb. According to the restriction enzyme analysis, all the inserts included a common 1.3 kb region (Fig. 2A). Moreover, all clones produced SOD with identical mobilities on nondenaturing polyacrylamide gel. The mobility was identical to that of Fe-SOD of *L. pneumophila* AM511 (Fig. 5, lanes 1 and 3), and the activity was inhibited with hydrogen peroxide (data not shown). Therefore, we concluded that these cloned SOD genes were the Fe-SOD ones (*sodB*).

We determined the nucleotide sequence of the insert of pJAM110 that contained the shortest fragment of the eight clones (31). An open reading frame with a coding capacity for 192-amino-acid protein was identified (Fig. 2B). Sadosky et al. (32) cloned sodB from *L. pneumophila* JR32 that was an AM511 derivative. They mentioned that their sodB sequence showed 100% identity with that of ours. The predicted amino acid sequence showed a strong homology to bacterial Mn- and Fe-SODs reported previously. The nucleotide sequence of *Legionella* Fe-
SOD gene (sodB) showed a potential Shine-Dalgarno ribosome-binding site, putative -35 and -10 RNA polymerase-binding sites and no obvious rho-independent termination sequence (Fig. 2B). The sodB gene was considered to express SOD by its own promoter in E. coli cells (Fig. 5, lane 3), since the orientation of sodB gene of pJAM110 was opposite to that of lacZ promoter of pUC18. However, we did not rule out a reading-through from cryptic promoters belonging to the vector when the insert is present in opposite orientation in respect to the lac promoter.

Fig. 2. [A]. The restriction map around L. pneumophila Fe-SOD gene (sodB). The region common to all eight clones is marked with the striped box on the restriction map. The region of the insert of pJAM110 and the position of the sodB gene are also indicated under the restriction map. The arrow on the sodB gene shows the direction of transcription. Restriction enzymes: H, HindIII; P, PvuII; P, PstI; S, ScaI. No site of EcoRI, SalI or KpnI exists on this map. The 1.5-kb EcoRI-SalI DNA fragment used for Southern hybridization probe was isolated after digestion of pJAM110 by using multiple cloning restriction sites of pUC18. [B]. Nucleotide sequence of the sodB gene and deduced amino acid sequence of L. pneumophila Fe-SOD. The putative Shine-Dalgarno (SD) sequence and possible -35 and -10 regions are indicated by solid lines under the nucleotides.

Fig. 3. Amino acid sequence comparison of L. pneumophila Fe-SOD with Fe- and Mn-SODs of other bacteria. The derived amino acid sequence of L. pn. (L. pneumophila) Fe-SOD is aligned with those of E. co. (Escherichia coli) Fe-SOD (6), C. bu. (Coxiella burnetii) Fe-SOD (33), P. ov. (Pseudomonas ovalis) Fe-SOD (6), P. le. (Photobacterium leiognathi) Fe-SOD (6), S. sp. (Synechococcus species) Fe-SOD (34), B. gi. (Bacteriodes gingivalis) Fe/Mn-SOD (35), M. sp. (Methylomonas species) Fe/Mn-SOD (36), E. co. (Escherichia coli) Mn-SOD (6), B. st. (Bacillus stearothermophilus) Mn-SOD (6), L. iv. (Listeria ivanovii) Mn-SOD (29), M. th. (Methanobacterium thermoautotrophicum) Mn-SOD (37), T. aq. (Thermus aquaticus) Mn-SOD (6), M. tu. (Mycobacterium tuberculosis) Mn-SOD (38) and H. ha. (Halobacterium halobium) Mn-SOD (39). Amino acid residues that are common to all the listed SODs are enclosed in dotted boxes. Fe-SOD-specific conserved residues are boxed. Asterisks indicate the metal ligand residues.
The amino acid sequence of *L. pneumophila* Fe-SOD was aligned with those of other bacterial Fe- and Mn-SODs reported previously (Fig. 3). We identified several Fe-SOD-specific and Mn-SOD-nonconserved amino acid residues by this alignment (Fig. 3). In *L. pneumophila* Fe-SOD, all of the Fe-SOD-specific residues were also conserved.

**PCR Amplification of *L. pneumophila* Cu,Zn-SOD Gene**

We tried cloning *L. pneumophila* Cu,Zn-SOD gene in *E. coli* using the sequence conserved among Cu,Zn-SODs derived from other organisms. With DNA primers (Fig. 4, primer N and primer C, and Materials and Methods), a DNA fragment about 300-bp long was amplified, ligated with the pGEM-T vector and transformed into DH5α. Nucleotide sequence analysis of six independent transformants revealed that the about 300-bp DNA fragment generated by PCR was a mixture of at least three products, 305 bp, 301 bp and 296 bp. The 296-bp product alone had the potential sequence to code for a peptide that resembled a part of Cu,Zn-SODs from several organisms reported previously (Fig. 4).
Acquisition, DNA Sequencing and Analysis of the Full Length of L. pneumophila Cu,Zn-SOD Gene

We tried to obtain pUC18 clones containing either a 3.5-kb EcoRI (data not shown) or a 5.0-kb HindIII fragment (see Fig. 6b) of L. pneumophila AM511 genomic DNA, which seems to contain the entire Cu,Zn-SOD gene, by colony hybridization with the 296-bp DNA fragment but failed. Then, we tried to obtain a PstI DNA clone containing a part of Cu,Zn-SOD gene by colony hybridization with the probe. The resultant pUC18 clone, named pJAMP1, contained a 541-bp PstI fragment that included the C-terminal region of the Cu,Zn-SOD (Fig. 4).

To acquire the remaining N-terminal region, we performed IPCR (26) using primers shown in Fig. 4 and a template of L. pneumophila AM511 genomic DNA which was digested with TaqI and self-ligated. Consequently, a 617-bp DNA fragment was amplified, which was cloned into pGEM-T vector. The resultant three clones obtained independently were sequenced and found to be identical one another, one of which was designated as pJAMT1.
Fig. 6. DNA-DNA hybridization experiments. Chromosomal DNAs were derived from several *Legionella pneumophila* strains and other *Legionella* species. Used probes: the *sodB* DNA probe, which was the 1.5-kb-*EcoRI*-SalI fragment from pJAM110 (A); the *sodC* DNA probe, which was the IPCR product with *SspI*, containing about 95% region of *sodC* structure gene (B). Chromosomal DNAs (1.5 µg) were digested completely with *HindIII*, and electrophoresed on 1% agarose. Southern blotted DNAs were hybridized with the DIG-labeled probes under a less stringent condition. Lane: 1, *L. pneumophila* Philadelphia 1 AM511; 2, *L. pneumophila* Philadelphia 1 (serogroup 1); 3, *L. pneumophila* Chicago 2 (serogroup 6); 4, *L. feeleii*; 5, *L. jordanis*; 6, *L. micdadei*; 7, *L. parisiensis*. Numbers at left show kilobase pairs of markers.

The nucleotide sequence of the Cu,Zn-SOD gene (*sodC*) and the deduced sequence of the protein are shown in Fig. 4 (40). A ribosome-binding domain was located 7 bp upstream and putative -10 and -35 RNA polymerase-binding sites were located 40 and 63 bp upstream, respectively, from the initiation codon. A peculiar sequence, the 17 bp perfectly matched inverted repeats, was observed downstream from the stop codon instead of the normal termination structure (Fig. 4). The predicted 162 amino acid residues, the first 20 residues of which implied a signal peptide, were similar to other bacterial Cu,Zn-SODs previously reported.
All of the catalytically important metal-ligand sites (42) were conserved in *L. pneumophila* Cu,Zn-SOD (Fig. 4).

To examine the expression of the *L. pneumophila* Cu,Zn-SOD gene (*sodC*) in *E. coli* cells, we constructed plasmids containing the entire *sodC* gene; a 285-bp *DraI*-*PstI* fragment of pJAMT1 containing the N-terminal region of SodC and a 541-bp *PstI* fragment of pJAMP1 containing the C-terminal region of SodC were extracted from the gel and ligated (Fig. 4). A resultant 826-bp fragment was cloned into *SmaI*-*PstI*-digested pUC18 and pUC19 vectors, which were transformed into *E. coli* QC774. The transformants carrying pUC18*sodC* and pUC19*sodC* clones designated as pJAM18C and pJAM19C, respectively, showed a single band of SOD on native polyacrylamide gel, of which the mobility was the same as that of *L. pneumophila* Cu,Zn-SOD (Fig. 5, lanes 1 and 2; data of pJAM18C not shown), and of which the activity was inhibited with both cyanide and hydrogen peroxide (data not shown). The restriction enzyme analysis indicated that the inserts of pJAM18C and JAM19C were the same in size and structure, and that the orientation of the *sodC* gene against the *lacZ* promoter of the vector was the same in pJAM18C but opposite in pJAM19C. This finding strongly suggested that the *sodC* gene expressed its product through its own promoter.

Furthermore, QC774 harboring pJAM18C or pJAM19C grew more slowly on minimal medium agar than did QC774 carrying pJAM110 (*sodB*), indicating that the *sodC* gene of *L. pneumophila* could partially complement the defect of QC774.

*Identification of SOD Genes in Legionella Species*

To examine for the existence of SOD genes on chromosomal DNAs from other *Legionella* species, we performed Southern hybridization analysis. First, to detect Fe-SOD genes, we used 1.5-kb *EcoRI*-SalI fragment from pJAM110 (see Fig. 2A), which contained *sodB* of *L. pneumophila*, as a probe. When *L. pneumophila* Philadelphia 1 (serogroup 1) AM511 chromosomal DNA was digested with *PstI* or *EcoRI* and subjected to Southern hybridization, a single hybridized band was detected (data not shown). We concluded that single Fe-SOD gene is present on the *L. pneumophila* chromosome. When the same chromosomal DNA was digested with *HindIII*, the probe hybridized to two DNA fragments, as a *HindIII* site existed in the *sodB* gene (Fig. 6A, see also Fig. 2A). *L. pneumophila* Philadelphia 1 (serogroup 1) and *L. pneumophila* Chicago 2 (serogroup 6) also showed the same results with that of AM511. When chromosomal DNAs of other *Legionella* species, *L. feeleii, L. jordanis, L. micdadei* and *L. parisiensis*, were digested with
HindIII and subjected to Southern hybridization, all chromosomal DNAs exhibited one positive band, but their sizes were all different and their signals were much weaker than those of *L. pneumophila* strains (Fig. 6A). Therefore, these *Legionella* species seem to possess one *sodB*-like gene with no HindIII sites and sequence diversity exists in and around it.

Second, we examined for the existence of Cu,Zn-SOD genes among the *Legionella* species described above. We used the purified IPCR product provided with *SspI*, which contained about 95% of the *sodC* gene (see Fig. 4), as a probe. Three *L. pneumophila* strains indicated the single hybridized band of the identical mobility. Other *Legionella* species showed no signals (Fig. 6B). It suggested that the chromosomes of other *Legionella* species contained no Cu,Zn-SOD gene or less conserved one that was not detectable under this condition.

**DISCUSSION**

We cloned *sodB* and *sodC* genes encoding Fe-SOD and Cu,Zn-SOD, respectively, of *L. pneumophila*. Southern blotting analysis suggested that a *sodB*-like gene was conserved among *Legionella* species, while no other *Legionella* species strains except *L. pneumophila* hybridized with the *sodC* gene of *L. pneumophila* as far as we examined, even under less stringent conditions. Strains of other species may lack *sodC* gene or possess diverse *sodC* gene. We examined the SOD activity of the *Legionella* strains that were the same as those used in Southern hybridization and found that *L. jordanis* expressed Cu,Zn-SOD activity (data not shown). PCR of the *Legionella* strains was also performed with the degenerate primers (Primer N and Primer C, see Fig. 4) by which the 296-bp DNA fragment was amplified from the chromosome of *L. pneumophila*. Only when the chromosome of *L. jordanis* was used as a template, an amplified fragment about 350 bp was detected (data not shown). These results suggest that *L. jordanis* has a Cu,Zn-SOD gene that is not strongly homologous to *L. pneumophila* Cu,Zn-SOD gene. Therefore, *sodC* gene might be a useful marker in identification of *L. pneumophila*.

*L. pneumophila* requires iron for the growth. Mengaud and Horwitz (43) showed that *L. pneumophila* has seven iron-containing proteins; Fe-SOD and so on. The reduction of intracellular iron inhibits *L. pneumophila*'s intracellular
multiplication (44,45). Fe-SOD may contribute to the intracellular multiplication of *L. pneumophila*.

Sodosky et al. (32) indicated that a sodB mutant could not be isolated by gene replacement, suggesting that Fe-SOD is essential for viability and provides a protection against superoxide generated during normal bacterial aerobic metabolism. The result may support the hypothesis that *L. pneumophila* SODs play independent roles in the protection against superoxide; *L. pneumophila* Fe-SOD and Cu,Zn-SOD inactivate superoxide produced by bacterial metabolite and by host-cellular oxidative burst, respectively.

It has been suggested that secretory SODs of intracellular pathogens could play a role in defence against oxidative burst in host phagosomes. SOD of *Nocardia asteroides*, which is cell surface-associated and secreted into the growth medium, protects the bacterium from oxidative killing by host phagocytes (46,47). Mn-SOD of *Mycobacterium tuberculosis*, a major human pathogen multiplying intracellularly, is also secreted, but SOD of nonpathogenic *M. smegmatis* is not (38). Their SODs do not have any signal peptide. They are, however, considered to be secreted by unknown transport machinery. All bacterial Cu,Zn-SODs sequenced previously have putative signal peptides, some of which were revealed to locate in the periplasm (13,48), and the importance of Cu,Zn-SOD for virulence has been shown in *Brucella abortus* (18). St. John and Steinman (41) indicated that the sodC mutant of *L. pneumophila* could grow in cultured macrophages. They mentioned, however, that the respiratory burst was not induced in macrophages under their experimental conditions. Using the constructed sodC mutant of *L. pneumophila*, we are examining the function of sodC for its survival and multiplication in activated macrophages.

**ACKNOWLEDGMENTS**

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