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NOTE

Molecular Cloning of the Superoxide Dismutase Gene from *Orientia tsutsugamushi*, the Causative Agent of Scrub Typhus

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A superoxide dismutase (SOD) gene from the obligate intracellular bacterium *Orientia tsutsugamushi* has been cloned by using the polymerase chain reaction with degenerate oligonucleotide primers corresponding to conserved regions of known SODs. Nucleotide sequencing revealed that the predicted amino acid sequence was significantly more homologous to known iron-containing SODs (FeSOD) than to manganese-containing SODs (MnSOD). Conserved regions in bacterial FeSOD could also be seen. Isolation of the oriential SOD gene may provide an opportunity to examine its role in the intracellular survival of this bacterium.

Key words: *Orientia tsutsugamushi,* superoxide dismutase, gene cloning, PCR, intracellular survival

Orientia tsutsugamushi, an obligate intracellular bacterium, is the causative agent of scrub typhus (tsutsugamushi disease), which is one of the most prevalent febrile illnesses in South Korea (Chang *et al.*, 1990; Kawamura *et al.*, 1995). This bacterium infects a variety of host cells *in vitro* and *in vivo*, including macrophages, polymorphonuclear leukocytes (PMN), lymphocytes, and endothelial cells, where it replicates in the cytoplasm without being surrounded by a phagolysosomal membrane (Rikihisa and Ito, 1979; Murata *et al.*, 1985; Ng *et al.*, 1985; Kawamura *et al.*, 1995). This intracellular bacterium enters the cell by a process of induced phagocytosis and escapes from the phagosome into the host cytoplasm by unknown mechanisms (Urakami *et al.*, 1983; Kawamura *et al.*, 1995). *O. tsutsugamushi* infects professional and nonprofessional phagocytes, where it resists oxygen-dependent and -independent microbicidal activities induced upon phagocytosis. The oxygen-dependent arm corresponds with a phagocyte respiratory burst and produces toxic oxygen metabolites including superoxide radical, hydrogen peroxide, and hydroxyl radical.

The metalloenzyme superoxide dismutases (SODs) detoxify superoxide anion by converting it to hydrogen peroxide and oxygen (McCord and Fridovich, 1969). They have been found in nearly all organisms examined to date and play a major role in the defense against oxidative stress (Fridovich, 1995). There are three general classes of SODs in bacteria, which differ in their metal cofactors. The manganese-containing SOD (MnSOD) and iron-containing SOD (FeSOD) are cytoplasmic, while the copper- and zinc-containing SOD (CuZnSOD) is periplasmic. In addition, a new class of nickel-containing SODs has been recently discovered in *Streptomyces griseus* and *S. coelicolor* (Kim *et al.*, 1996; Youn *et al*, 1996). The MnSODs and FeSODs have very similar sequences and structures and are evolutionarily unrelated to CuZnSODs (Beyer *et al.*, 1991). The detoxifying ability of SOD has established it as a virulence factor in some bacterial pathogens. For example, the ability of certain strains of *Nocardia asteroides* to escape killing by neutrophils is related to the production of SOD that is secreted (Beaman *et al.*, 1985; Beaman and Beaman, 1990). In addition, a *Shigella flexneri* mutant that is unable to produce FeSOD is more sensitive than the wild type to killing by both macrophages and PMNs (Frazon *et al.*, 1990).

Since the ability of *O. tsutsugamushi* to invade, grow within, and ultimately damage a host cell is the basis of its pathogenicity, an understanding of oriential intracytoplasmic growth and intracellular survival mechanisms is crucial. Unfortunately, the lack of oriential mutants and a genetic manipulation system for this organism has frustrated attempts to elucidate molecular mechanisms of

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pathogenesis. The *O. tsutsugamushi* gene encoding SOD has not been characterized and its roles in the intracellular survival of orientiae have not been elucidated so far. To understand the role of *O. tsutsugamushi* SOD as a virulence factor at the molecular level, we have isolated the gene encoding SOD from *O. tsutsugamushi*.

O. tsutsugamushi Karp (American Type Culture Collection, Manassas, VA) was propagated in monolayers of L929 cells (Cho *et al.*, 2000). When more than 90% of the cells were infected, as determined by an indirect immunofluorescent-antibody technique (Chang *et al.*, 1990), the cells were collected, homogenized with a glass Dounce homogenizer (Wheaton Inc., Millville, N.J.), and centrifuged at 430 × *g* for 5 min at 4°C. The supernatant was mixed with percoll at the final concentration of 40% and ultracentrifuged (SW41 Ti rotor, Beckman) at 25,000 × *g* for 1 h at 4°C (Tamura *et al.*, 1982). The oriential bands were harvested and diluted with phosphate-buffered saline (PBS) and centrifuged at 22,000 × *g* for 5 min at 4°C. The oriential pellets were harvested, diluted with PBS, and centrifuged again. The pellets were stored in liquid nitrogen until use. Bacterial cell pellets of *E. coli* grown overnight in LB broth and *O. tsutsugamushi* were resuspended in 50 mM potassium phosphate buffer (pH 7.8). Crude extracts were prepared by sonicating the bacterial suspensions by using five 30-s bursts in a model UP 50 H Ultrasonic Processor (Dr. Hielscher GmbH, Germany) at a constant pulse and 80% amplitude setting. The total protein concentration was measured by using the bicinchoninic acid protein assay kit (Sigma, St. Louis, MO). SOD activity in nondenaturing 10% polyacrylamide gels was visualized by nitroblue tetrazolium negative staining as described previously (Beauchamp and Fridovich, 1971). Genomic DNA was extracted from *O. tsutsugamushi* by using an Easy-DNA kit (Invitrogen, Carlsbad, CA). The PCR reaction mixture contained 100 pmol of each primer, 300 ng of genomic DNA, 0.2 mM deoxynucleoside triphosphate (Promega, Madison, WI), 1.25 mM $MgCl_2$, 1 × *Taq* polymerase buffer, and 0.4 U *Taq* DNA polymerase (Promega, Madison, WI). The first reaction step was 94°C at 5 min and the reaction parameters were 4 cycles (2 min at 94°C, 2 min at 40°C, 2 min at 72°C) followed by 30 identical cycles with 45°C as the annealing temperature and a final elongation step of 10 min at 72°C. Degenerate primers were listed in Table 1 and designed according to the conserved amino acid regions 2, 3 and 4 of SOD proteins as described in Fig. 2. The molecular cloning techniques and gel electrophoresis were conducted by standard methods (Sambrook *et al.*, 1989). Cloning of PCR-amplified fragments was carried out by using a pGEM-T vector system (Promega, Madison, WI). Pure plasmid DNA was prepared by using a High pure plasmid isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). DNA fragments were isolated from agarose gels with a QIAEX II gel extraction kit (QIAGEN, Valencia, CA). Plasmid double-stranded DNA was sequenced by the dye terminator method on an ABI Prism 3100 Genetic Analyzer (ABI prism, Foster, CA).

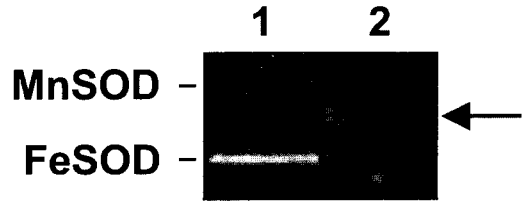

Fig. 1. Detection of SOD in *O. tsutsugamushi* on a nondenaturing polyacrylamide gel stained for SOD activity. Fifty μg of proteins from *E. coli* XL1-Blue (lane 1) and *O. tsutsugamushi* (lane 2) crude lysates were loaded. The *E. coli* MnSOD and FeSOD are indicated. Activity band of *O. tsutsugamushi* SOD is indicated with an arrow.

Table 1. Degenerate primer sequences used in this study

Primer name	Sequences
SOD2A	5'-CAYCAYGGNAARCAYCA-3'
SOD2B	5'-CAYCAYGAYAARCAYCA-3'
SOD2C	5'-AARCAYCAYAAYACNTAYGT-3'
SOD2D	5'-AARCAYCAYCARACNTAYGT-3'
SOD3FA	5'-TTYGGNTCNGGNTGGRCNTGG-3'
SOD3FB	5'-TTYGGNAGYGGNTGGRCNTGG-3'
SOD3RA	5'-CCANCCNGANCCRAA-3'
SOD3RB	5'-CCANCCRCTNCCRAA-3'
SOD4A	5'-TARTANGCRTGYTCCCANACRTC-3'
SOD4B	5'-RTARTANGCRTGYTCCCA-3'

Abbreviations: R(A,G), Y(A,C), N(A,G,C,T)

To test whether *O. tsutsugamushi* has SOD activity, we performed native polyacrylamide gel electrophoresis (PAGE) followed by staining for SOD activity. As shown in Fig. 1, lysates from *O. tsutsugamushi* contained a single activity band that migrated between *E. coli* MnSOD and FeSOD. No additional bands of activity were seen with the *O. tsutsugamushi* lysate, suggesting that the orientia produces a single type of SOD. A multiple-sequence alignment of various bacterial SODs demonstrated many clusters of conserved residues (Fig. 2). With this high degree of sequence conservation, we reasoned that a PCR approach to isolatation *of O. tsutsugamushi* SOD was feasible. The conserved SOD regions chosen for this study are illustrated in Fig. 2. Some of the depicted domains are intimately involved in metal-ligand binding. When SOD2A/2B-SOD4A primer pair and *O. tsutsugamushi* genomic DNA were used as the template, PCR generated a product with a predicted molecular size of 430 bp (Fig. 3A and B). To further confirm that PCR products represented amplified SOD coding sequences, we performed nested-PCR analysis using region 3 primers in both orientations. The expected size fragments were obtained by the pairs SOD2A/2B-SOD3RA/3RB and SOD3FA/3FB-SOD4A (Fig. 3C). The amplified 430 bp SOD2A/2B-SOD4A frag-

	Region 1	Region 2	Region 3	Region 4
	12 17	26 35	117 122	156 163
E. coli (Fe)	DALAPH	HYGKHHQTYV	NFGSGW	DVWEHAYY
E. coli (Mn)	DALEPH	HHTKHHQTYV	RFGSGW	DVWEHAYY
B. stearotherm. (Mn)	DALEPH	HHTKHHNTYV	RFGSGW	DVWEHAYY
P. leiognathi (Fe)	NALEPH	HYGKHHNTYV	QFGSGW	DLWEHAYY
R. prowazekii (Fe)	ESFKPH	HHGKHHNSYV	QFGSGW	DVWEHAYY
L. pneumophila (Fe)	DALAPH	HYGKHHNTYV	TFGSGW	DVWEHAYY
C. burnetii (Fe)	NALEPH	HHGKHHRAYV	HFGSGW	DVWEHAYY
S. cerevisiae (Mn)	GALEPH	HYTKHHQTYV	VQGSGW	DVWEHAYY
Human liver (Mn)	GALEPH	HHSKHHAAYV	VQGSGW	DVWEHAYY
Consensus	dalePH	HhgKHHntYV	rfGSGW	DvWEHAYY
Primers		(SOD2A)	(SOD3FA)	(SOD4A)
		(SOD2B)	(SOD3FB)	(SOD4B)
		(SOD2C)	(SOD3RA)	
		(SOD2D)	(SOD3RB)	

Fig. 2. Conserved regions of SOD from various sources. *Escherichia coli* iron (Fe) and manganese (Mn) SODs are compared with the Mn enzymes produced by *Bacillus stearothermophilus, Saccharomyces cerevisiae*, and human liver and Fe enzyme of *Photobacterium leiognathi, Rickettsia prowazekii, Legionella pneumophila*,and *Coxiella burnetii*. Degenerate PCR primers corresponding to the consensus sequence of each conserved region are synthesized. Regions 2 and 3 are used in the design of the forward primers SOD2A/2B/2C/2D, and SOD3FA/3FB, respectively. Regions 3 and 4 are used in the design of the reverse primers SOD3RA/3RB and SOD4A/4B, respectively. The numbering reflects the amino acid position in *E. coli* FeSOD with the initiator methionine excluded. Capital letters in the derived consensus sequence represent residues conserved in all sequences and lowercase letters represent residues present in the majority of sequences.

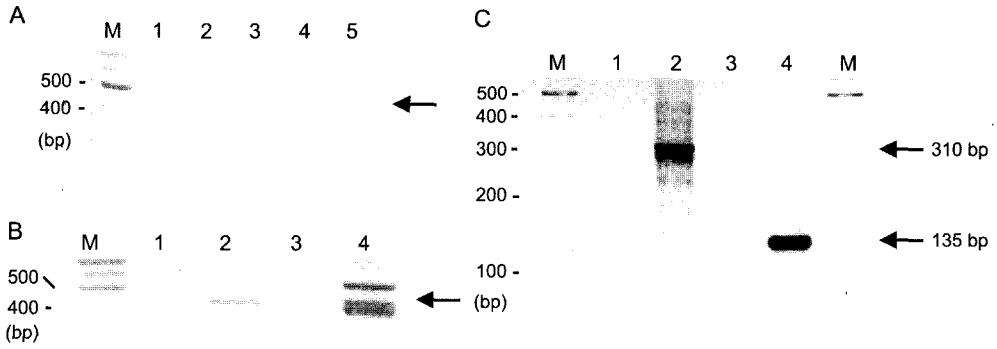

Fig. 3. (A) Analysis of PCR products resulting from different forward and reverse primer pairs. Lanes: M contains 100 bp molecular size markers (Promega); 1 through 4 contain PCR products produced from *O. tsutsugamushi* genomic DNA and the following primer pairs: lane 1, SOD2A/2B-SOD4A; lane 2, SOD2C/2D-SOD4A; lane 3, SOD2A/2B-SOD4B; lane 4, SOD2C/2D-SOD4B; lane 5, no DNA. Arrows indicate the 430 bp PCR-amplified band. (B) Analysis of PCR products from genomic DNA extracted from different organisms. Lanes: 1 contains no DNA; 2 through 4 contain PCR products produced from SOD2A/2B-SOD4A primer pair and the genomic DNA from *O. tsutsugamushi*, L-929 cell, and *E. coli*, respectively. Arrows indicate the 430 bp PCR-amplified band. (C) PCR analysis using region 3 degenerate primers as nested primer pairs. Lanes: M contains 100 bp molecular size markers (Promega); 2 and 4 contain PCR products produced from 430 bp PCR-amplified fragment and the following primer pairs: lane 2, SOD2A/2B-SOD3RA/3RB; lane 4, SOD3FA/3FB-SOD4A; lanes 1 and 3 have no template DNA with the following primer pairs: lane 1, SOD2A/2B-SOD3RA/3RB; lane 3, SOD3FA/3FB-SOD4A. Arrows indicate the nested PCR-amplified band.

ment was cloned into pGEM-T and the sequence was determined. The DNA sequence was deposited in the GenBank database under accession no. AF484107. The deduced *O. tsutsugamushi* SOD amino acid sequence was used to search protein databases on the National Center for Biotechnology Information BLAST server. It was very similar to prokaryotic and eukaryotic SODs, being most like FeSODs (Fig. 4). It exhibited the highest similarity to the *Rickettsia conorii* (58% identity and 69% similarity) and *R. prowazekii* (53% identity and 69% similarity) SODs. It showed about 67% similarity to FeSOD from the cyanobacteria *Nostoc commune*. The majority of residues used to distinguish between the Mn- and Fe-type enzymes matched an FeSOD (Parker and Blake, 1988; Jackson and Cooper, 1998; Santos *et al.*, 1999). These residues were Phe-65, Ala-68, Ala-69, Gln-70, Phe-76, and Ala-142 (Fig. 4). Four residues which are known to be ligands for the metal cofactor (indicated with the plus sign in Fig. 4) were found at conserved positions. However, further investigations are necessary to test whether this oriential SOD gene is expressed functionally in *E. coli* and/or complements functionally SOD-deficient *E. coli* mutant strains.

Isolation of the oriential SOD gene may provide an

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     +          40                          60              +    80
Ot   HRGKHHAAYVNNLNRLLEHKTELQQKSLEDII-LTSFNCEGSKAIFNNAAQIWNHNFFWHS
Rc   HHGKHHNTYVQNLNNLLKDKEELQKKDLEEII-EWSSQ-NSNAAIFNNAAQIWNHTFFWHS
Rp   HHGKHHNSYVQNLNNLIKDREELQKKDLEEIIEWSSQNAE--VAILNNASQIWNHTFFWYS
Nc   HYGKHHKAYVDNLNKLTEG-TELANKSLEEVI-KTSFNDSSKAGIFNNAAQVWNHTFFWNS
St   HYGKHHQTYVTNLNNLIKG-TAFEGKSLEEIV-RTS---EGG--IFNNAAQVWNHTFYWNC
Lp   HYGKHHNTYVTNLNKLIPG-TEFESMTLEEII-M-----KAKGGIFNNAAQVWNHTFYWHS
Ec   HYGKHHQTYVTNLNNLIKG-TAFEGKSLEEII-R-----SSEGGVFNNAAQVWNHTFYWNC
     * **** ** *** *           **●●●           ● ***●*●*** *●*●

                       100                 120                 140
Ot   ICNNNATKPEGNFLQKINQDFGSYENFTNQFQELALAQFGSGWVWLI-SENGKLKIITTSN
Rc   IKPQGGGKPSGKILEQINKDFGSFEEFCEQFKQEAVGQFGSGWAWLI-YHDNRLQIIKTAN
Rp   IKPHGGGKPSGKVFEQISKDFGSFEQFCAQFKQEAVGQFGSGWTWVV-YHDNKLQIIKTSN
Nc   LKPAGGGAPTGDLAARIDKDFGSFDKFKEEFSNAAATQFGSGWSWLI-DDGGTLKVIKTPN
St   LAPNAGGEPTGKLADAIAASFGSFAEFKAQFTDAAIKNFGSGWTWLVKSADGKLAIVSTSN
Lp   MSPNGGGEPKGRLAEAINKSFGSFAAFKEQFSQTAATTFGSGWAWLVQDQSGALKIINTSN
Ec   LAPNAGGEPTGKVAEAIAASFGSFADFKAQFTDAAIKNFGSGWTWLVKNSDGKLAIVSTSN
     ●       * *     *   ***●  *  ●*   *   ***** *●●      * ●● * *

                     +160+
Ot   AELPIIYNQIPLLTCDVWEHAY
Rc   AGTPIAHGMKPLLACDVWEHAY
Rp   AGTPIVNFMKPILACDVWEHAY
Nc   AENPLAHGKKALLTLDVWEHAY
St   AGTPLTTDATPLLTVDVWEHAY
Lp   AGTPMTEGLNALLTCDVWEHAY
Ec   AGTPLTTDATPLLTVDVWEHAY
     *  *●      ●*  *******
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Fig. 4. Comparison of the amino acid sequence of *O. tsutsugamushi* SOD with those of other bacterial SODs. The deduced amino acid sequence of *O. tsutsugamushi* (Ot) was aligned by using the CLUSTAL V program with those of other bacterial SODs. Amino acid sequences of SODs from *R. conorii* (Rc, AAL03316), *R. prowazekii* (Rp, Q9ZD15), *N. commune* (Nc, AAF25009), *Salmonella typhimurium* (St, AAL20353), *Legionella pneumophila* (Lp, P31108), and *E. coli* (Ec, P09157) are from SWISS-PROT and GenBank databases. Identical and similar amino acids are marked with asterisks and dots, respectively. +, amino acid residues proposed to be ligands for metal cofactor. The numbering represents the amino acid position in *E. coli* FeSOD.

opportunity to examine its role in the intracellular survival and virulence of this bacterium. An accurate determination of the contribution of SOD to *O. tsutsugamushi* virulence will be acquired only when *sod* mutant strains are created and tested in tissue culture or a suitable animal model. Gene inactivation studies of orientiae require a system for introduction of DNA into the cell. Such a system was not available until recently, when *R. prowazekii* was transformed by electroporation (Rachek *et al.*, 1998). It is hoped that the same technology will lead to the development of a transformation system in *O. tsutsugamushi.*

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