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Identification and Characterization of the *sodA* Genes Encoding Manganese Superoxide Dismutases in *Vibrio parahaemolyticus*, *Vibrio mimicus*, and *Vibrio vulnificus*

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***Abstract*: Sequencing of Fur titration assay-positive clones obtained from genomic DNA libraries of *Vibrio parahaemolyticus*, *V. mimicus* and *V. vulnificus* revealed open reading frames encoding proteins of 202, 205 and 202 amino acid residues, respectively. Each open reading frame was preceded by a predicted Fur box which overlaps a likely promoter with similarity to the −10 and −35 consensus sequence of *Escherichia coli*. The deduced amino acid sequences shared considerable homology with bacterial Mn-containing superoxide dismutases (MnSODs). Consistent with this, these *Vibrio* strains produced proteins with SOD activity resistant to inhibition by H_2O_2 and KCN only when grown under iron-limiting conditions. Primer extension analysis of the total RNA from these vibrios revealed iron-repressible expression of the genes. Furthermore, when grown under iron-limiting conditions, *E. coli* carrying a plasmid with each cloned gene overexpressed protein with the same electrophoretic mobility and insensitivity of SOD activity to H_2O_2 and KCN. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by N-terminal amino acid sequencing revealed that proteins (MnSODs) having N-terminal amino acid sequences consistent with those deduced from the corresponding genes were present in cell lysates of the vibrios grown under these iron-limited conditions. These results demonstrate that the genes cloned in this study are *sodA* homologs encoding MnSODs, whose expression is regulated by the iron status of the growth medium. PCR using a primer set based on the *V. parahaemolyticus sodA* sequence revealed the presence of homologous genes in certain other *Vibrio* species.**

Key words*: *sodA*, Manganese superoxide dismutase, *Vibrio

Metalloenzyme superoxide dismutase (SOD) catalyzes the dismutation of the superoxide radical O_2^- according to the reaction $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$, and plays a crucial role in prevention of oxidative cell damage (9). There are three kinds of SODs, in which the metal cofactors are different, iron- and manganese-containing SODs (FeSOD and MnSOD), and a copper, zinc-containing SOD (CuZnSOD). MnSOD and FeSOD are quite homologous in amino acid sequences, and are present in the cytoplasm. While *Escherichia coli* FeSOD encoded by the *sodB* gene is a constitutive enzyme, expression of the gene (*sodA*) encoding MnSOD involves intricate mechanisms (5 and references therein), including negative regulation by iron via the ferric uptake regulation (Fur) system (15). Iron-regulated expression of the *sodA* genes has also been reported for other bacterial species (2, 11, 17, 18). In these bacteria, transcription of the *sodA* genes is also negatively regulated by a Fur-like protein acting as a co-repressor in the presence of sufficient iron, however, negative regulation of transcription does not occur under low-iron conditions and transcription can proceed. CuZnSOD has been generally considered almost exclusively a eukaryotic enzyme, but it has become apparent that a wide range of Gram-negative pathogens export CuZnSOD to their periplasmic spaces to protect cells from exogenous superoxide such as produced by an oxidative burst by phagocytes (10, 13). Interestingly, CuZnSOD shows no sequence similarity to

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Abbreviations: FURTA, Fur titration assay; LB, Luria-Bertani; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TB, Tris-buffered.

MnSOD or FeSOD.

In *Vibrio* species, a single SOD enzyme has been detected (1, 7), which was identified as FeSOD due to its inactivation by H_2O_2 (1). More recently, Shyu et al (20) reported cloning of the *V. alginolyticus sodA* gene, but it remains to be clarified whether the gene is indeed expressed in this bacterium and is regulated by iron.

During the course of our study to clone iron-regulated genes of *Vibrio parahaemolyticus*, *V. mimicus* and *V. vulnificus*, results of the Fur titration assay (FURTA) (22) showed that some FURTA-positive clones contained open reading frames (ORFs) with significant amino acid sequence similarity to bacterial MnSODs. We describe here the identification and characterization of iron-regulated genes encoding MnSODs in these three *Vibrio* species.

Materials and Methods

Bacterial strains, media and growth conditions. To assess the expression of MnSOD, *V. parahaemolyticus* WP1, *V. mimicus* PT7 and *V. vulnificus* M2799 were cultured aerobically at 37 C in Tris-buffered (TB) medium (25), which was supplemented with 100 μM $MnCl_2$ and either 0.2 μM (iron-limiting) or 20 μM (iron-sufficient) $FeCl_3$. The TB medium used contained 0.5% NaCl for *V. mimicus* PT7 and 2% NaCl for the others. For preparation of genomic DNAs, *Vibrio* strains were grown in Luria-Bertani (LB) medium containing either 2% or 0.5% NaCl. *E. coli* strains H1717 (*aroB fhuF*::λ*plac*Mu) (22) and DH5α were grown in LB medium, which included ampicillin at 100 μg/ml when required. To generate iron-limiting growth conditions, LB medium was supplemented with ethylenediamine-di-(*o*-hydroxyphenyl acetic acid) at a final concentration of 140 μM.

Fur titration assay. FURTA was performed as described by Stojiljkovic et al (22). Briefly, genomic libraries of *V. parahaemolyticus* WP1, *V. mimicus* PT7 and *V. vulnificus* M2799, obtained by partial digestion of chromosomal DNAs with *Sau*3AI followed by ligation with the *Bam*HI sites of pUC19, were transformed into *E. coli* H1717. The transformants were plated on MacConkey agar plates containing 1% lactose, 20 μM $Fe(NH_4)_2(SO_4)_2$, and 100 μg ampicillin per ml for selecting red colonies. After several rounds of selection, some 20 red colonies harboring a plasmid with a different insert of about 0.8–3.5 kbp in length were isolated from the genomic DNA library of each *Vibrio* strain, and the plasmid inserts were individually sequenced.

Standard DNA techniques. Isolation of chromosomal DNA and plasmid DNA, DNA cleavage with restriction enzymes, recovery of DNA fragments from agarose gels, DNA ligation, electroporation and PCR were performed using standard techniques (19) or as recommended by the commercial suppliers of the materials. Nucleotide sequences were determined by the dideoxy chain termination method with a Thermo Sequenase fluorescent labeled primer cycle sequencing kit equipped with a 7-deaza-dGTP and an ALF DNA sequencer (Pharmacia Biotech).

Primer extension analysis. Vibrio strains were each grown in either iron-sufficient or iron-limiting LB medium until the optical density at 660 nm reached 0.5 and the total RNA was extracted with Isogene (Wako Pure Chemical Ind., Osaka, Japan) according to the manufacturer's protocol. Primers 5′-CGGCGGTGACGAATT TATCG-3′ for *V. parahaemolyticus*, 5′-CGTAATAAGT-GCGATGGTG-3′ for *V. mimicus* and 5′-CAAGCT-CACTGCCTTGAATC-3′ for *V. vulnificus* complementary to the corresponding *sodA* genes were labeled with a 5′ oligonucleotide Texas Red Labeling kit (Hitachi Co., Tokyo) and extended with avian myeloblastosis virus reverse transcriptase XL supplied with an RNA PCR kit ver. 2.1 (Takara Biomedicals). The resultant cDNAs obtained after primer extension were analyzed on the sequencing gel alongside the DNA sequence reaction products obtained by the dideoxy chain-termination method with the same primer using Hitachi DNA Sequencer SQ5500E.

Gel electrophoresis and detection of SOD activity. Crude cell lysates were prepared by sonication of cells from stationary-phase cultures as described by Steinman (21). One hundred micrograms of soluble proteins in non-denaturing loading buffer (100 mM Tris-HCl, pH 8.9, and 14% glycerol) were loaded onto a 10% polyacrylamide separating gel prepared with 1.5 M Tris-HCl, pH 8.9, and a 4% polyacrylamide stacking gel prepared with 0.5 M Tris-HCl, pH 8.3, and then electrophoresed in the non-denaturing discontinuous Tris-glycine buffer system (8) at 180 V for 1 hr. SOD activity was visualized in gels by the activity-staining method of Beauchamp and Fridovich (4). To ascertain the class of SOD present, H_2O_2 and KCN were added to the riboflavin-tetramethylenediamine solution at 2.5 mM and 1 mM, respectively (6). Purified MnSOD (Sigma) served as a control.

N-Terminal amino acid sequencing. Crude cell lysates were prepared as described above from cells of *Vibrio* strains and *E. coli* DH5α bearing each of the recombinant *sodA* plasmids under iron-limiting conditions, and the proteins were resolved by sodium dodecyl sulfate (SDS)-PAGE on 12% polyacrylamide gels (14) and were visualized by Coomassie blue staining. In parallel, the gel was electroblotted with a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) to a prewetted

polyvinylidene difluoride membrane (ProBlott, Applied Biosystems) in Towbin buffer (23), at 25 V for 2 hr according to the manufacturer's protocol. Proteins on the membrane were stained with 0.025% Coomassie blue in 40% methanol for 5 min, then destained in 50% methanol for 15 min, rinsed four times with distilled water and air-dried. The approximately 23-kDa bands, whose electrophoretic mobilites were same as those of the corresponding MnSOD proteins expressed in *E. coli* DH5α, were excised from the membrane, and the N-terminal amino acid sequences were determined by automated Edman degradation with an Applied Biosystems Mode 491 protein sequencer equipped with an online Model 120A PTH-amino acid analyzer.

Amplification of genomic DNA by PCR. The genomic DNAs were amplified by PCR with Ready-To-Go PCR beads (Amersham Pharmacia Biotech) and a set of primers (5′-GATGCGTTAGAGCCATACATCGACG-3′ and 5′-GATGTGTGGGAGCACGCGTACTACA-3′) spanning bases 253 to 277 and 703 to 727, respectively, of the sequence of the *V. parahaemolyticus sodA* gene. After a 5-min denaturation at 95 C, the reactions were performed for 35 cycles with a 1-min denaturation at 95 C, and a 1-min annealing at 60 C, and a 2-min extension at 72 C. The PCR products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Other methods. Protein concentrations were determined by the Lowry method with bovine serum albumin as the standard. Sequence analysis, alignment and construction of an evolutionary tree were performed with GENETYX-Mac software (Software Development, Tokyo).

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper will appear in DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB041845, AB041846 and AB041847.

Results and Discussion

Identification and Sequencing of the Genes Expected to Encode MnSODs

Application of the FURTA system to *V. parahaemolyticus*, *V. mimicus* and *V. vulnificus* allowed us to isolate many positive clones which might have Fur-regulated gene fragments. Nucleotide sequence determinations of these clones revealed the presence of full-length ORFs in the plasmids termed pVP236 from *V. parahaemolyticus*, pVM11 from *V. mimicus* and pVV30 from *V. vulnificus*, whose deduced amino acid sequences exhibited considerable homology with the bacterial SOD family. The regulatory regions of these genes are shown in Fig. 1. A predicted Fur box highly homologous to the *E. coli* consensus Fur box sequence (5′-GATAAT-GATAATCATTATC-3′) (3) was found in each gene, which overlaps a putative promoter with similarity to the *E. coli* −10 and −35 consensus sequences. The base-pair matches to the *E. coli* Fur box ranged from 15/19 for *V. vulnificus* to 16/19 for *V. parahaemolyticus* and *V. mimicus*. Each ORF preceded by the predicted Fur box initiates at an ATG with a putative ribosome-binding sequence, GGAGG, detected 8 bp upstream of each ATG codon. Moreover, an inverted repeat is located beyond each transcriptional stop codon (TAA) (data not shown), and may serve as a transcriptional terminator. Our inspection of the promoter region of the *V. alginolyticus sodA* gene (20) revealed a predicted Fur box sequence with a moderate match (sharing 12 of 19 nucleotides) to the consensus sequence of the *E. coli* Fur box, but whether expression of this gene is indeed iron-regulated is at present unclear.

After translation of the ORFs, protein sequences composed of 202, 205 and 202 amino acids were deduced for *V. parahaemolyticus*, *V. mimicus* and *V. vulnificus*, respectively (Fig. 2). The deduced amino acid sequences of *V. mimicus* and *V. vulnificus* showed 74% identity (94% similarity) and 82% identity (98% similarity), respectively, to that of *V. parahaemolyticus*. A homology search using the FASTA program revealed that the deduced amino acid sequences of these *Vibrio* species had the highest degree of homology with the *Bacillus stearother-*

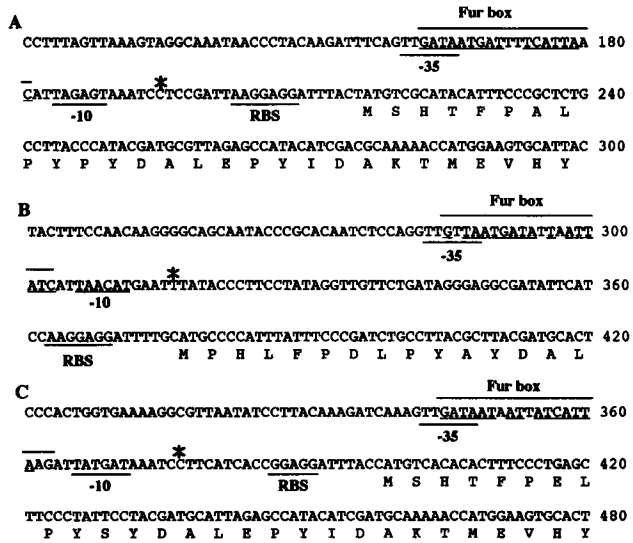

Fig. 1. Regulatory regions of the *sodA* genes of *V. parahaemolyticus* (A), *V. mimicus* (B) and *V. vulnificus* (C). The deduced amino acid sequence is shown below the nucleotide sequence. The −35 and −10 regions, and the ribosome-binding site (RBS) are underlined and labeled. The predicted Fur box is indicated by a line above the sequence, and the bases shared with the *E. coli* Fur box consensus sequence are underlined.

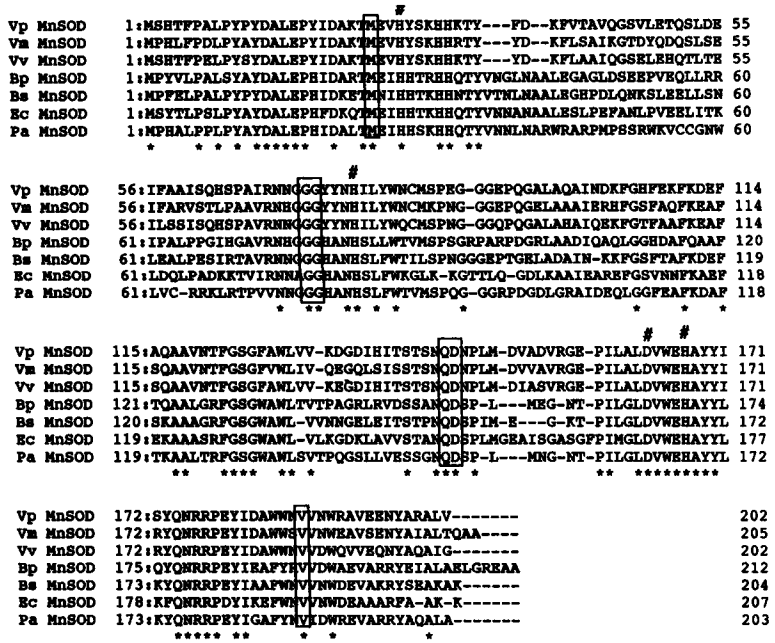

Fig. 2. Alignment of the amino acid sequences of *V. parahaemolyticus* (Vp), *V. mimicus* (Vm) and *V. vulnificus* (Vv) MnSODs with those of other bacterial MnSODs. Amino acid sequences of *Bordetella pertussis* (Bp) (accession no. X84800), *Bacillus stearothermophilus* (Bs) (accession no. M81914), *E. coli* (Ec) (accession no. X03951), and *Pseudomonas aeruginosa* (Pa) (accession no. L25672A) were retrieved from DDBJ/EMBL/GenBank nucleotide sequence databases. Asterisks indicate identical amino acid residues. Four metal-binding sites deduced from the three-dimensional structural analysis of *B. stearothermophilus* MnSOD (16) are each marked with a sharp. Amino acids characteristic of MnSODs (11, 16) are boxed.

mophilus SodA (more than 54% identity), and that most amino acid residues characteristic of MnSOD (11, 16, 18) were conserved (Fig. 2). The evolutionary tree of many bacterial SODs placed these three vibrio proteins within the MnSOD family (Fig. 3). From these comparisons, we concluded that the genes we isolated were *sodA* homologues encoding MnSODs. Surprisingly, the deduced amino acid sequence of the *V. parahaemolyticus sodA* gene more recently deposited by Shyu and Lin (EMBL/GenBank/ DDBJ accession number AF085191) was grouped into the FeSOD family with only 46% identity to that of the *V. parahaemolyticus* gene in this study. In addition, a Fur box-like sequence was absent from the promoter region of the gene of Shyu and Lin, implying that it is *sodB* gene. These results strongly suggested that the genes we isolated might be *sodA* homologues encoding MnSODs.

Iron-Regulated Transcription and Transcriptional Start Sites of sodA *Genes*

The primer extension products were detected only when the total cellular RNAs from iron-deficient cells were used (Fig. 4), indicating that transcription of the *sodA* genes in these *Vibrio* strains are strictly controlled by the iron status of the medium. Moreover, primer extension identified the likely transcriptional start sites: cytosine (position 195) for *V. parahaemolyticus sodA*, thymine (position 316) for *V. mimicus sodA* and cytosine (position 377) for *V. vulnificus sodA*, as indicated by asterisks in Fig. 1.

Expression of MnSOD in Vibrio *Species*

To verify the presence of MnSOD protein in these *Vibrio* species, lysates of cells grown under either iron-sufficient or iron-deficient conditions were examined for SOD activity after separation by non-denaturing PAGE followed by activity staining (Fig. 5). Under iron-sufficient conditions, each strain showed a single band with SOD activity, which disappeared upon treatment with H_2O_2 (data not shown), indicative of an FeSOD enzyme (4). No additional band was found even if the medium was fortified with $MnCl_2$ (100 μM). In contrast, under iron-deficient conditions, distinct proteins with SOD activity were detected. These SODs were resistant to inhibition by either H_2O_2 or KCN, a characteristic suggestive of an MnSOD (4). Moreover, the addition of $MnCl_2$ (100 μM) to the growth medium resulted in a slight increase in band intensity reflecting MnSODs (data not shown), suggesting a requirement for adequate amounts of manganese to ensure the full catalytic activity of the enzyme (11). The appearance of MnSOD activity in response to iron limitation is consistent with the above genetic evidence that these *Vibrio* strains have *sodA* homologs whose expression is mediated by Fur. A

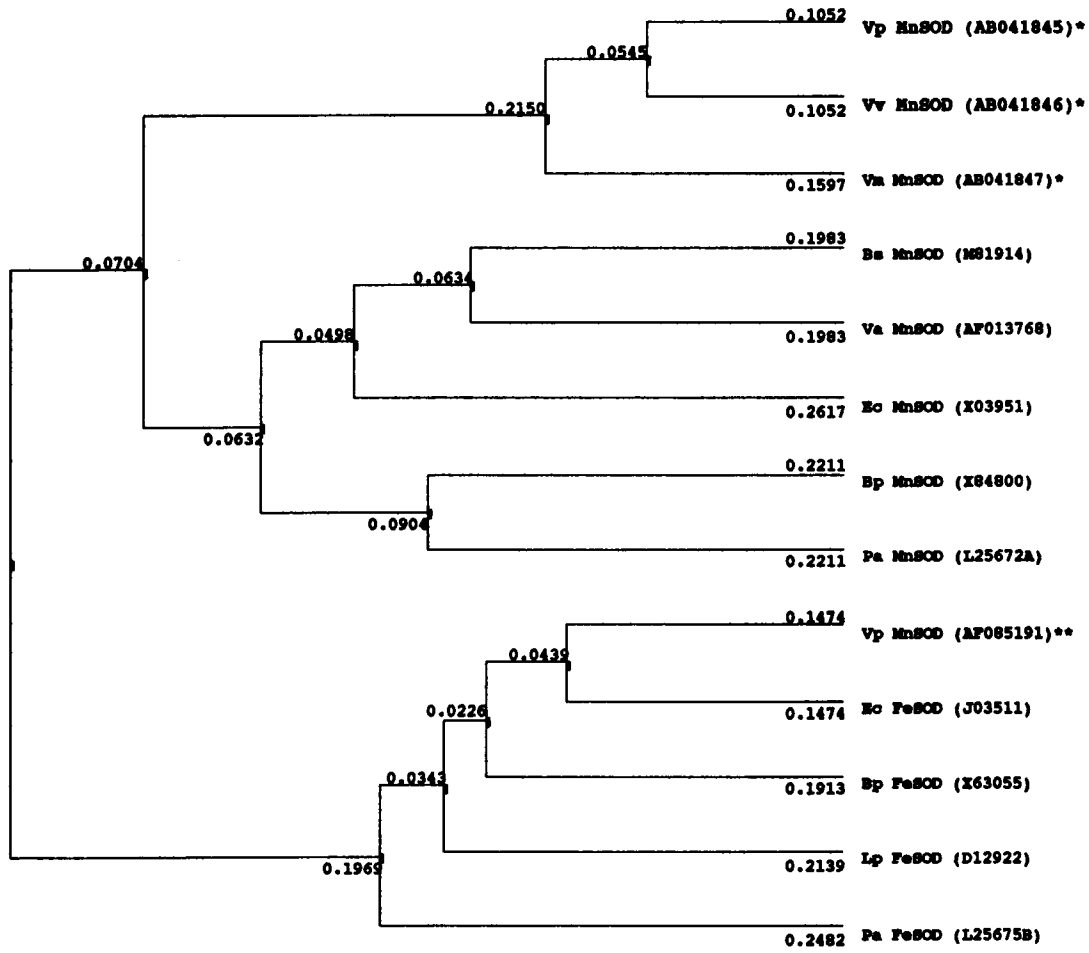

Fig. 3. Evolutionary tree (dendrogram) of bacterial MnSODs and FeSODs. The amino acid sequences were aligned and a dendrogram was generated using the UPGMA method. Lp, *Legionella pneumophila*; Va, *V. alginolyticus*. Abbreviations for other species are the same as in Fig. 2. Accession numbers of DDBJ/EMBL/GenBank nucleotide sequence databases are shown in parentheses. *, this study; **, Shyu and Lin.

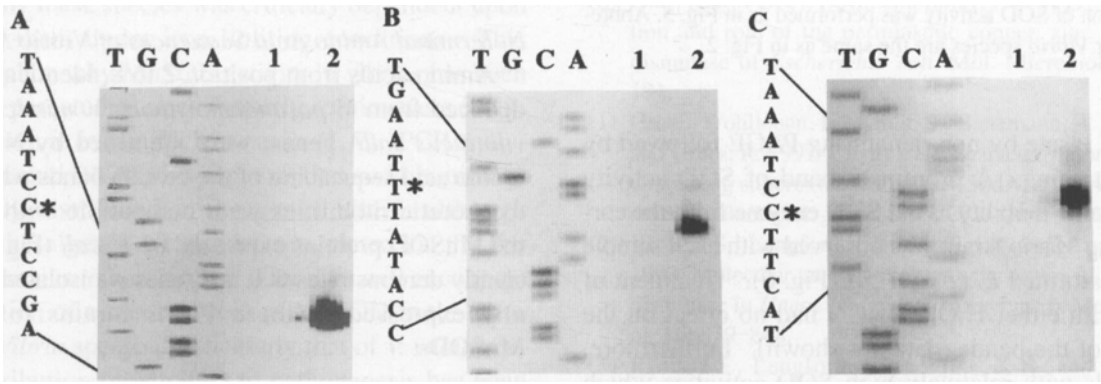

Fig. 4. Primer extension analyses of the total cellular RNAs from *V. parahaemolyticus* WP1 (A), *V. mimicus* PT7 (B) and *V. vulnificus* M2799 (C) grown under iron-sufficient (lane 1) and iron-limiting (lane 2) conditions. Lanes A, C, G, T are lanes corresponding to the DNA sequencing ladder. The identified transcriptional start sites are shown with asterisks in Fig. 1.

great reduction in FeSOD under iron-deficient conditions is probably due to the lack of iron essential for its maturation (11).

Expression of Cloned sodA *Genes in* E. coli

E. coli DH5α was transformed with either pVP236, pVM11 or pVV30, and each clone was grown either in iron-limiting or iron-sufficient medium prior to analysis

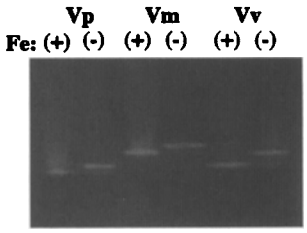

Fig. 5. Detection of SOD on a non-denaturing polyacrylamide gel stained for SOD activity. The *Vibrio* strains were grown under iron-sufficient (+) and iron-limiting (−) conditions, and 100 μg of protein in the lysate were loaded in each lane. Vp, *V. parahaemolyticus* WP1; Vm, *V. mimicus* PT7; Vv, *V. vulnificus* M2799. Only the activity of the upper band of each strain was resistant to inhibition by either 2.5 mM H_2O_2 or 1 mM KCN, suggesting activity due to an MnSOD.

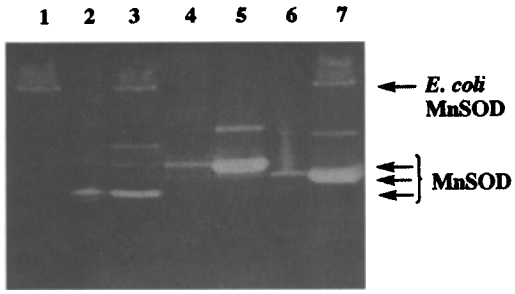

Fig. 6. Expression of the cloned *sodA* genes in the *E. coli* DH5α background. *E. coli* DH5α/pUC19 (lane 1) and that bearing a *sodA* recombinant plasmid, pVP236 (lane 3), pVM11 (lane 5) or pVV30 (lane 7), were grown under iron-limiting conditions. Vp MnSOD (lane 2), Vm MnSOD (lane 4) and Vv MnSOD (lane 6) were used as controls for detection of SOD activities derived from the cloned genes (see Fig. 5). *E. coli* MnSOD was identified by comparison with the corresponding enzyme purified from *E. coli* (Sigma). Each lane received 100 μg of the cell lysate protein, and detection of SOD activity was performed as in Fig. 5. Abbreviations for *Vibrio* species are the same as in Fig. 2.

of its cell lysate by non-denaturing PAGE followed by activity staining. A prominent band of SOD activity with the same mobility as the SOD enzyme from the corresponding *Vibrio* strain was observed with each sample from transformed *E. coli* DH5α (Fig. 6). Treatment of the gel with either H_2O_2 or KCN had no effect on the intensity of the bands (data not shown). Furthermore, new bands with relatively high SOD activities which had migrated to intermediate positions were observed. These were also insensitive to H_2O_2 and cyanide and coincided with neither of the *Vibrio* nor *E. coli* MnSOD. These activities were presumed to indicate hybrid MnSOD proteins formed between the MnSOD monomer of the *Vibrio* species and the counter monomer of *E. coli*, since bacterial MnSODs are in general homodimeric (9) and formation of such hybrids has been reported for *Haemophilus influenzae* MnSOD expressed in *E. coli* (12). It is noteworthy that when the *V. mimicus sodA* gene was coexpressed, the MnSOD band of *E. coli* disappeared with the formation of a more intense hybrid SOD band as compared with the others (lane 5 of Fig. 6). However, in the iron-sufficient cells, such prominent MnSOD bands together with the hybrid bands were not found (data not shown), indicating that the expression of the *Vibrio sodA* genes in the *E. coli* background is also regulated by iron.

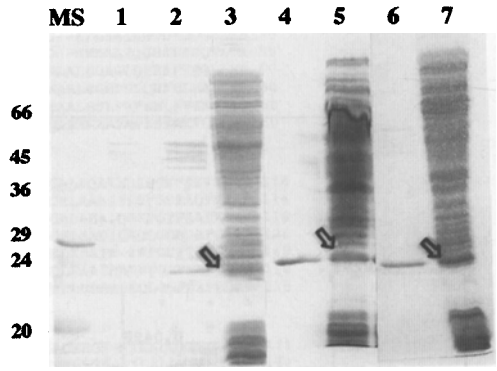

Fig. 7. SDS-PAGE analysis of crude cell lysates prepared from *Vibrio* strains and *E. coli* DH5α which had been grown under iron-limiting conditions. MS, molecular mass standards shown in kilodaltons on the left; lanes 1, *E. coli* DH5α; 3, *V. parahaemolyticus* WP1; 5, *V. mimicus* PT7; 7, *V. vulnificus* M2799. *E. coli* DH5α bearing a *sodA* recombinant plasmid, pVP236 (lane 2), pVM11 (lane 4) or pVV30 (lane 6) was used for comparison. The amounts of protein loaded per well were as follows: lane 1, 30 μg; lanes 3, 5 and 7, 200 μg; and lanes 2, 4 and 6, 30 μg, 5 μg and 6 μg, respectively. The bands excised for the N-terminal amino acid sequence determination are indicated by an arrow.

N-Terminal Amino Acid Sequences of Vibrio *MnSODs*

Amino acids from position 2 to 8, identical to those deduced from *V. parahaemolyticus*, *V. mimicus* and *V. vulnificus sodA* genes, were identified by N-terminal amino acid sequencing of the protein bands whose electrophoretic mobilities were compatible with those of the MnSOD proteins expressed by *E. coli* (Fig. 7). This clearly demonstrates that the genes we isolated are actually expressed in these *Vibrio* strains to produce MnSODs.

Presence of sodA *Homologs in Other* Vibrio *Strains and Species*

The genomic DNAs were amplified by PCR with a set of oligonucleotide primers, whose preparation was based on the *V. parahaemolyticus sodA* gene sequence, to examine whether *sodA* homologs occur in other *Vibrio* strains and species. A PCR product of the expected length was detected for randomly selected strains of *V.*

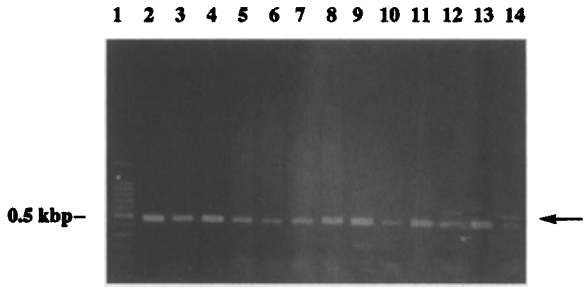

Fig. 8. Detection of *sodA* homologs in other *Vibrio* species by PCR. A primer set prepared according to the nucleotide sequence of the *V. parahaemolyticus sodA* gene was used for PCR amplification of the genomic DNAs from the strains indicated. An arrow indicates an expected amplicon band (ca. 0.5 kbp) in *V. parahaemolyticus* WP1. Lanes 1 to 14; 100 base pair ladder, *V. parahaemolyticus* WP1, *V. mimicus* PT7, *V. vulnificus* M2799, *V. furnissii* ATCC35016, *V. fluvialis* ATCC33809, *V. cholerae* Non-O1 NCTC8042, *V. alginolyticus* ATCC17749, *V. harveyi* ATCC14126, *V. metschnikovii* NCTC11171,*V. proteolyticus* ATCC15338, *V. anguillarum* NCBM828, *V. piscium* NCBM571 and *V. hollisae* ATCC33564, respectively.

parahaemolyticus, *V. mimicus* and *V. vulnificus* (data not shown), as well as for some other vibrios (Fig. 8). However, *V. metschnikovii*, *V. anguillarum* and *V. hollisae* provided only a faint band of ca. 500 bp, at least suggesting that their *sodA* nucleotide sequences may not be particularly homologous to that of *V. parahaemolyticus*.

In summary, the present study demonstrates that the *sodA* gene of *V. parahaemolyticus*, *V. mimicus* or *V. vulnificus* contains a potential Fur box, thus contributing to the control of gene expression in response to the iron concentration of the culture medium. Indeed, production of MnSOD by these species was critically dependent upon culturing them under iron-limiting conditions. This would explain why MnSOD activity in vibrios has been elusive in past studies since formerly, vibrios were exclusively grown in iron-sufficient media (1, 7). PCR analysis of genomic DNAs also suggests that *sodA* homologs are widely distributed in the genus *Vibrio*, although whether these genes are also iron-regulated via the Fur system remains to be clarified. Our data will encourage investigation of a possible role of MnSOD in the virulence of *Vibrio* species, particularly that of *V. vulnificus*. The contribution of MnSOD to pathogenesis has been demonstrated in some bacterial models including *Aeromonas salmonicida* (2), *Salmonella typhimurium* (24) and *Yersinia enterocolitica* (18).

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References

1) Bang, S.S., Baumann, L., Woolkalis, M.J., and Baumann, P. 1978. Electrophoretic mobilities of superoxide dismutases from species of *Photobacterium, Beneckea, Vibrio*, and selected terrestrial enterobacteria. Curr. Microbiol. **1**: 371–376.
2) Barnes, A.C., Horne, M.T., and Ellis, A.E. 1996. Effect of iron on expression of superoxide dismutase by *Aeromonas salmonicida* and associated resistance to superoxide anion. FEMS Microbiol. Lett. **142**: 19–26.
3) Baun, V., and Hantke, K. 1991. Genetics of bacterial iron transport, p. 107–138. *In* Winkelmann, G. (ed), Handbook of microbial iron chelates, CRC Press, Boca Raton, Fla.
4) Beauchamp, C.O., and Fridovich, I. 1971. Superoxide dismutase: improved assays and an applicable to acrylamide gels. Anal. Biochem. **72**: 276–287.
5) Compan, I., and Touati, D. 1993. Interaction of six global transcriptional regulators in expression of manganese superoxide dismutase in *Escherichia coli* K-12. J. Bacteriol. **175**: 1687–1696.
6) Crapo, J.D., McCord, J.M., and Fridovich, I. 1978. Preparation and assay of superoxide dismutase. Methods Enzymol. **53**: 382–393.
7) Daily, O.P., Debell, R.M., and Joseph, S.W. 1978. Superoxide dismutase and catalase levels in halophilic vibrios. J. Bacteriol. **134**: 375–380.
8) Davis, B.J. 1964. Disc electrophoresis. II. Method and applications to human serum proteins. Ann. NY Acad. Sci. U.S.A. **121**: 404–427.
9) Fridovich, I. 1995. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem. **64**: 97–112.
10) Gort, A.S., Ferber, D.M., and Imlay, J.A. 1999. The regulation and role of the periplasmic copper, zinc superoxide dismutase of *Escherichia coli*. Mol. Microbiol. **32**: 179–191.
11) Graeff-Wohlleben, H., Killat, S., Banemann, A., Guiso, N., and Gross, R. 1997. Cloning and characterization of an Mn-containing superoxide dismutase (SodA) of *Bordetella pertussis*. J. Bacteriol. **179**: 2194–2201.
12) Kroll, J.S., Langford, P.R., Saah, J.R., and Loynds, B.M. 1993. Molecular and genetic characterization of superoxide dismutase in *Haemophilus influenzae* type b. Mol. Microbiol. **10**: 839–848.
13) Kroll, J.S., Langford, P.R., Wilks, K.E., and Keil, A.D. 1995. Bacterial [Cu,Zn]-superoxide dismutase: phylogenetically distinct from the eukaryotic enzyme, and not so rare after all! Microbiology **141**: 2271–2279.
14) Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature **227**: 680–685.
15) Niederhoffer, E.C., Naranjo, C.M., Bradley, K.L., and Fee, J.A. 1990. Control of *Escherichia coli* superoxide dismutase (*sodA* and *sodB*) genes by the ferric iron uptake regulation

(*fur*) locus. J. Bacteriol. **172**: 1930–1938.
16) Parker, M.W., and Blake, C.C.F. 1988. Iron- and manganese-containing superoxide dismutase can be distinguished by analysis of their primary structures. FEBS Lett. **2**: 377–382.
17) Polack, B., Dacheux, D., Delic-Attree, I., Toussaint, B., and Vignais, P.M. 1996. The *Pseudomonas aeruginosa fumc* and *soda* genes belong to an iron-responsive operon. Biochem. Biophys. Res. Commun. **226**: 555–560.
18) Roggenkamp, A., Bittner, T., Leitritz, L., Sing, A., and Heesemann, J. 1997. Contribution of the Mn-cofactored superoxide dismutase (SodA) to the virulence of *Yersinia enterocolitica* serotype O8. Infect. Immun. **65**: 4705–4710.
19) Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual, 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
20) Shyu, Y.-C., Chiu, C.-C., and Lin, F.-P. 1999. Cloning, sequencing, expression and characterization of the manganese superoxide dismutase gene from *Vibrio alginolyticus*. Biochem. Mol. Biol. Intern. **47**: 803–814.
21) Steinman, H.M. 1985. Bacteriocuprein superoxide dismutases in Pseudomonads. J. Bacteriol. **162**: 1255–1260.
22) Stojiljkovic, I., Bäumler, A.J., and Hantke, K. 1994. Fur regulon in gram-negative bacteria: identification and characterization of new iron-regulated *Escherichia coli* genes by a *fur* titration assay. J. Mol. Biol. **236**: 531–545.
23) Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**: 4350–4354.
24) Tsolis, R.M., Bäumler, A.J., and Heffron, R. 1995. Role of *Salmonella typhimurium* Mn-superoxide dismutase (SodA) in protection against early killing by J774 macrophages. Infect. Immun. **63**: 1739–1744.
25) Yamamoto, S., Fujita, Y., Okujo, N., Minami, C., Matsuura, S., and Shinoda, S. 1992. Isolation and partial characterization of a compound with siderophore activity from *Vibrio parahaemolyticus*. FEMS Microbiol. Lett. **94**: 181–186.