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Molecular Characterization and Quantitative Analysis of Superoxide Dismutases in Virulent and Avirulent Strains of *Aeromonas salmonicida* subsp. *salmonicida*

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***Aeromonas salmonicida* subsp. *salmonicida* is a facultatively intracellular gram-negative bacterium that is the etiological agent of furunculosis, a bacterial septicemia of salmonids that causes significant economic loss to the salmon farming industry. The mechanisms by which *A. salmonicida* evades intracellular killing may be relevant in understanding virulence and the eventual design of appropriate treatment strategies for furunculosis. We have identified two open reading frames (ORFs) and related upstream sequences that code for two putative superoxide dismutases (SODs), *sodA* and *sodB*. The *sodA* gene encoded a protein of 204 amino acids with a molecular mass of approximately 23.0 kDa (SodA) that had high similarity to other prokaryotic Mn-SODs. The *sodB* gene encoded a protein of 194 amino acids with a molecular mass of approximately 22.3 kDa that had high similarity to other prokaryotic Fe-SODs. Two enzymes with activities consistent with both these ORFs were identified by inhibition of O_2^--catalyzed tetrazolium salt reduction in both gels and microtiter plate assays. The two enzymes differed in their expression patterns in in vivo- and in vitro-cultured bacteria. The regulatory sequences upstream of putative *sodA* were consistent with these differences. We could not identify other SOD isozymes such as *sodC* either functionally or through data mining. Levels of SOD were significantly higher in virulent than in avirulent strains of *A. salmonicida* subsp. *salmonicida* strain A449 when cultured in vitro and in vivo.**

Microorganisms have several highly specific and effective enzymatic pathways that confer protection from reactive oxygen species (ROS). Oxidant inactivation relies upon a variety of enzymes including glutathione peroxidase, glutathione reductase, catalase-peroxidase, and the metalloenzymes superoxide dismutases (SODs). The pathway involving SOD is one of the most extensively studied. SOD is responsible for the first step in the detoxification of the superoxide anion (O_2^-) to H_2O and O_2, via hydrogen peroxide (H_2O_2) (4). In addition to protecting cells against O_2^--mediated toxicity the removal of O_2^- prevents the O_2^--mediated reduction of Fe and the subsequent production of $^{\cdot}OH$ via the Haber-Weiss reaction (26).

SOD isozymes are classified into groups depending on their required prosthetic metal. These groups include manganese-cofactored SodA encoded by *sodA*, iron-cofactored SodB encoded by *sodB*, and copper-zinc-cofactored SodC encoded by *sodC*. In gram-negative bacteria both SodA and SodB are usually cytoplasmic (7). SOD functions to remove endogenously oxidants produced during normal oxidative metabolism. It has also been considered to protect cells from exogenously generated oxidants. The SodB of *Mycobacterium tuberculosis* is located in the glycocalyx or capsule secreted by this organism. *M. tuberculosis* also secretes SodB (20). Recently a variety of other forms of SOD have been identified in bacteria. These include a nickel-containing isozyme (24) and hybrid isoforms containing iron and zinc (23).

SOD expression is under the control of environmental stimuli. In all bacterial species, SodB, the iron-cofactored isozyme, is constitutively expressed under iron-replete conditions. The manganese-cofactored SodA is produced when bacteria are exposed to oxidative stress or under iron-limited conditions (2, 6, 26). Host phagocytic cells exploit the deleterious biological effects of ROS in their nonspecific host defense against pathogens (9, 26). It is unsurprising given the role of SOD that it has been implicated in intracellular pathogen survival and as a virulence factor in numerous species of bacteria including *M. tuberculosis*, (20, 30), *Salmonella enterica* serovar Typhimurium (14), *Shigella flexneri* (15), *Streptococcus agalactiae* (32), and *Escherichia coli* (3).

Aeromonas salmonicida is a gram-negative bacterium from the gamma class of the *Proteobacteria* phylum. There are four subspecies of *A. salmonicida*; the subspecies *salmonicida* is the etiological agent of a systemic infectious disease of fish called furunculosis. It is a facultative intracellular pathogen with the intracellular stage thought to allow this species to avoid temporarily the host immune system (18). Although the mechanisms by which this species survives within macrophages are not fully understood, the presence of the S-layer protein and SodA expression are thought to be important (2, 16). Both the role of ROS in the anti-*Aeromonas* immune response and the role of SOD in the response of *A. salmonicida* subsp. *salmonicida* to the host are unclear (33). The importance of these enzymes in the avoidance of host responses in this species has

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not been fully elucidated, although the suggestion has been made that it is not important, as both virulent and avirulent strains of *A. salmonicida* subsp. *salmonicida* possess the enzyme (2, 12, 16).

In this paper, we describe the genes encoding SodA and SodB in *A. salmonicida* subsp. *salmonicida* strain A449. We also quantitatively compared SOD activities in virulent and avirulent strains grown in vitro as well as in vivo to demonstrate that the role of SOD in this bacterium's virulence may depend upon the level of gene expression rather than a gene deficiency.

MATERIALS AND METHODS

Identification of the *A. salmonicida* subsp. *salmonicida sodA* and *sodB* genes. The complete sequences of *sodA* and *sodB* were identified from a genomic DNA sequence database for *A. salmonicida* subsp. *salmonicida* strain A449 (R. K. Singh et al., unpublished data) by using BLAST searches (1).

Two-dimensional electrophoresis. Outer membrane proteins were isolated by glycine extraction (29) from *A. salmonicida* subsp. *salmonicida* at stationary phase following growth in tryptic soy broth (Difco, Detroit, Mich.) at 17°C. The extracted outer membrane proteins were diluted with 7 M urea, 2 M thiourea, 4% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 40 mM Tris, 2 mM tributylphosphine, and 0.05% carrier ampholytes. Isoelectric focusing was performed on an 18-cm Immobiline dry strip, pH 3 to 10, controlled with an IPGphor isoelectric focusing unit (AP-Biotech, Montreal, Quebec, Canada) with a voltage profile of 1 h at 500 V, 1 h at 2,000 V, and 8 h at 8,000 V. After electrophoresis the strip was equilibrated with 50 mM Tris-HCl (pH 8.8)–6 M urea–2% sodium dodecyl sulfate (SDS)–65 mM dithiothreitol–0.05% bromophenol blue–135 mM iodoacetamide for 30 min and then loaded onto an SDS–14% polyacrylamide gel and electrophoresed at 100 V until the dye front was within 5 mm of the bottom of the gel. Proteins were silver stained (34) and excised from the gel.

Mass spectrometry (MS) analysis. Protein spots were processed using the Investigator ProGest automated digestion unit (Genomic Solutions, Ann Arbor, Mich.). Briefly, the procedure involved spot destaining with potassium ferricyanide solution (15 mM potassium ferricyanide, 50 mM sodium thiosulfate). Gel pieces were then rinsed three times with Milli-Q water and were shrunk with high-pressure liquid chromatography-grade acetonitrile. They were reswelled with 20 μl of sequencing-grade modified trypsin solution (Promega, Madison, Wis.) prepared as 0.01 μg/μl in 50 mM ammonium bicarbonate. An additional 50 μl of 50 mM ammonium bicarbonate was added to cover the gel pieces prior to overnight incubation at 37°C. To recover the peptides, an organic solution (50 μl of 50% methanol–5% acetic acid) was added to the digestion solution and the mixture was separated from the gel pieces onto a clean 96-well plate. Peptide extracts were evaporated to dryness on a SpeedVac concentrator (Thermo Savant, Holbrook, N.Y.), and they were then reconstituted uniformly with 20 μl of 5% acetonitrile–1% acetic acid solution.

Partial peptide sequencing was achieved using liquid chromatography coupled to electrospray MS. The online liquid chromatography-electrospray MS experiments were carried out using a CapLC high-pressure liquid chromatography system coupled to a Q-ToF 2 hybrid quadrupole–time-of-flight instrument (Micromass, Manchester, United Kingdom). Peptide extract solution was loaded onto a 5-mm by 300-μm μ-Precolumn PepMap C_{18} cartridge (LC Packings, Amsterdam, The Netherlands) at 30 μl/min and eluted using a fast gradient of 5 to 70% acetonitrile–0.2% formic acid in 4 min at 1 μl/min. Mass spectral data were recorded using the automated data-dependent switching function. The MS survey scan was acquired over the mass range *m/z* 400 to 1,500 with a scan duration of 1 s. When the survey scan detected doubly or triply charged precursor ions with an intensity higher than 10 counts/s, the automated MS to MS-MS switching function was activated. A total of two channels for recording MS-MS spectra were selected, and the mass range was acquired from *m/z* 50 to 2,000 with a scan duration of 2 s. Data were acquired and processed in the MassLynx Windows NT-based data system version 3.4.

Bacterial strains and growth conditions. Five strains of *A. salmonicida* subsp. *salmonicida* were used (Table 1): two virulent strains, A449 and 80204, and three avirulent strains, 80204-1, 84222-5, and SS70-1. Strain 80204-1 is an S-layer-negative mutant of 80204. Each strain was cultured to mid-exponential phase of growth from a glycerol stock in tryptic soy broth with agitation at 17°C. Strain A449 was also cultured in an iron-free minimal salts medium, a modification of simplified Griffin's medium (27) with iron sulfate added to give final iron concentrations between 0 and 40 mM. Bacterial number was estimated by A_{600} with a spectrophotometer (Ultrospec 2000; Pharmacia). A more accurate estimate of bacterial number was achieved by direct colony counts on tryptic soy agar (Difco) after incubation at room temperature for 24 h.

TABLE 1. *A. salmonicida* subsp. *salmonicida* strains used in this study[a]

Strain	S-layer	Virulence	Origin
A449	+	+	Natural epizootic
80204	+	+	Natural epizootic
80204-1	−	−	Laboratory-derived mutant of 80204
84222-5	−	−	Laboratory-derived mutant
SS70-1	+	−	Laboratory-derived mutant

[a] A449 and 80204 were both derived from naturally occurring salmonid epizootics. 80204-1, 84222-5, and SS70-1 are all laboratory-derived avirulent mutants. The phenotype for A449 came from the late Julian Thornton (Microtek International, Saanichton, British Columbia, Canada, personal communication), and the phenotypes for 80204, 80204-1, 84222-5, and SS70-1 came from Gilles Olivier (Department of Fisheries and Oceans, Moncton, New Brunswick, Canada, personal communication).

In vivo culture of *A. salmonicida*. The in vivo culture procedure was approved by the Dalhousie University Committee for Laboratory Animals and the National Research Council—Halifax Local Animal Care Committee. In vivo culture was performed as per the work of Garduño et al. (17) with some modifications. The in vivo growth chambers were lengths of autoclaved 12- to 14-kDa-molecular-mass-cutoff dialysis tubing (Spectrapor; Spectrum Laboratories, Rancho Dominguez, Calif.) ligated at both ends (10). Chambers were filled with a bacterial suspension at an A_{600} of 0.5 in 1× Hanks' balanced salt solution and implanted in the abdominal cavities of buffered tricaine methanosulfonate (Syndel Laboratories, Vancouver, British Columbia, Canada)-anesthetized juvenile Atlantic salmon (*Salmo salar* Linnaeus 1758; mean weight, 50.3 ± 12.0 g) via a ventral midline incision. The incision was closed with 4-0 polypropylene sutures on a three-eighths reverse cutting needle placed in a simple interrupted pattern. After recovery the fish were maintained in flowthrough, dechlorinated fresh water at 12°C. Fish were euthanized 24 h postsurgery with a lethal overdose of tricaine methylsulfonate. The implants were retrieved by dissection, the implant contents were removed, and their volume was noted. Bacterial numbers within the implants were determined by direct colony counts as described above. We have previously demonstrated that when cultured in this manner the bacterial cultures were in the exponential phase of growth at the time of harvest (A. Dacanay, unpublished observations).

Bacterial preparation. Suspensions of both broth- and in vivo-cultured bacteria were pelleted at 4°C for 10 min at 3,000 × *g*. The culture supernatant was carefully removed, and the pellet was stored at −20°C prior to extraction. Cell-free lysates of the bacteria were obtained by resuspending the bacterial pellets in a small volume of ice-cold double-distilled water and disrupting the cells by passage through a French press (American Instrument Company, Silver Spring, Md.) with an internal cell pressure of >18,000 lb/in^2. The resulting lysate was centrifuged for 10 min at 10,000 × *g* at 4°C to pellet any cell debris, frozen at −20°C, and then freeze-dried to a powder that was stored at −20°C until use.

SOD: zymography. Zymography was conducted using 100- by 105- by 1-mm 12% nondenaturing, nonreducing (native) polyacrylamide gels (polyacrylamide gel electrophoresis). Loading volumes were adjusted to the equivalent of 10^8 CFU per well. Samples were mixed with nondenaturing, nonreducing loading buffer (125 mM Tris [pH 6.8], 20% glycerol, 0.4% bromophenol blue) and were not heated before loading. The running buffer was a 190 mM Tris–25 mM glycine solution that was unadjusted for pH. Electrophoresis was carried out at 200-V constant voltage in a gel apparatus maintained at 4°C. SOD activity was visualized as inhibition of the reduction of the tetrazolium salt 2,2′-bis(4-nitrophenyl)-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)ditetrazolium chloride (NBT) according to the method of Beauchamp and Fridovich (4). Commercially available *E. coli* SOD (Fe-SOD or Mn-SOD, EC 1.15.1.1; Sigma-Aldrich, Oakville, Ontario, Canada) was used as a positive control for activity only. Conventional molecular weight standards were not run. An assessment of migration was made by determining the migration of any zones of clearance observed relative to the migration of the dye front (R_f = distance migrated by band/distance migrated by dye front).

SOD: quantitative analysis. SOD activity was quantitatively determined using a commercially available colorimetric microtiter plate method (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. SOD

activity was assayed colorimetrically at 450 nm as the inhibition of the reduction of the tetrazolium salt 2-(4-iodophenyl) 3-4-(nitrophenyl)-5(2,4-disulfophenyl)-2H tetrazolium (WST-1) by O_2^-. Sample volumes were adjusted so that a protein amount equivalent to 10^7 CFU of *A. salmonicida* was loaded per well. Total SOD activities were determined kinetically by the initial rate of the reaction, v_o. The reaction rate was determined using a Thermomax microplate reader and the Softmax Pro suite software (Molecular Devices Corp., Sunnyvale, Calif.). Commercially available *E. coli* Fe-SOD was used to construct standard curves. The identity of the SOD isozyme was determined by the ability of either 10 mM KCN or 10 mM H_2O_2 to inhibit the colorimetric change. Inhibitors were added to the reaction mixture and incubated for 20 min at room temperature before assay. Enzyme activity, defined as percent inhibition of WST-1 reduction, was determined as {[(reagent control v_o − buffer blank v_o) − (sample v_o − sample blank v_o)]/(reagent control v_o − buffer blank v_o)} × 100 for both samples and standards. SOD activity per well was determined from the standard curve, and the amount of SOD per cell was calculated. Data were transformed, and any difference in SOD activity was determined using one-way analyses of variance (ANOVAs) and Tukey's multiple comparisons with Prism 3.0 (Graphpad Software Inc., San Diego, Calif.).

Nucleotide sequence accession numbers. The *sodA* and *sodB* sequences have been deposited in GenBank under accession no. AY321354 and AY321353, respectively.

RESULTS

Sequence analysis of *A. salmonicida* subsp. *salmonicida sodA* gene. The deduced amino acid sequence of the putative manganese-cofactored SOD (SodA) is shown in Fig. 1. This protein consisted of 204 amino acids and had a predicted molecular mass of 22.9 kDa and a predicted pI of 5.47. The first methionine is removed in the active enzyme, and the amino acid numbering system used reflects this. The four conserved metal-binding residues His_{23}, His_{81}, Asp_{164}, and His_{168} (*A. salmonicida* SodA numbering) are present along with residues that predominate in Mn-cofactored SODs: Met_{23}, Ile_{25}, Asn_{73}, His_{74}, Gly_{75}, Gly_{76}, Gly_{77}, His_{78}, Ala_{79}, Gln_{149}, Asp_{150}, Gly_{158}, Gln_{175}, and Glu_{193} (22). Immediately upstream of *sodA* there was a 477-bp open reading frame (ORF) that encoded a protein of 159 amino acids with a predicted molecular mass of 17.8 kDa and a predicted pI of 6.6 (Fig. 2). Comparison of the deduced amino acid sequence with other sequences revealed a 33% identity (49% similarity) to a hypothetical 17-kDa protein (OrfX) from *Pseudomonas syringae* (accession number AF121078) and a slightly lower similarity to OrfX region deduced amino acid sequences from *Pseudomonas putida* (33% identity, 45% similarity) and *Pseudomonas aeruginosa* (28% identity, 40% similarity) (accession numbers AF1022780 and JC4983, respectively). A putative iron box region of 18 nucleotides (positions 358 to 375) upstream from OrfX was identified using the consensus GATATTGATAATCA TTATC iron box sequence of *E. coli* given in the work of Calderwood and Mekalanos (8). This region has a 15-of-19-base match with the *E. coli* consensus sequence.

Sequence analysis of *A. salmonicida* subsp. *salmonicida sodB* gene. The deduced amino acid sequence for a putative iron-cofactored SOD (SodB) is shown in Fig. 3. This protein consisted of 194 amino acids and had a predicted molecular mass of 21.6 kDa and a calculated pI of 5.55. The four conserved metal-binding residues His_{27}, His_{74}, Asp_{158}, and His_{162} (*A. salmonicida* subsp. *salmonicida sodB* numbering) characteristic of SodB are present. This sequence was 100% similar to the partial sequence for *A. salmonicida* SodB (accession number BAA94568, subspecies not given). Comparison of the deduced amino acid sequence with those of other bacterial SodB proteins from different genera revealed the highest similarity (82% identity) to a manganese SOD gene product from *Vibrio parahaemolyticus* (accession number AAD54651). Comparisons to other SOD sequences revealed high similarities (>73% identity) only to SodB from a variety of bacterial species outside the genus *Aeromonas*.

Identification of SOD peptides. Two tryptic peptides were identified during proteomic screening of A449 cultured under iron-replete and iron-limiting conditions.

A tryptic peptide with an amino acid sequence of RFGSG WAWLVVDK was obtained from a cytosolic protein with a molecular mass of 22.3 kDa and a pI of 5.74 from strain A449 cultured in low-iron medium. This partial sequence was 100% identical to the putative SodA amino acid sequence (positions 126 to 138, Fig. 1).

A second tryptic nonapeptide with an amino acid sequence of AFGSFAEFK was obtained from an outer membrane protein with a molecular mass of 22.3 kDa and a pI of 5.0 from strain A449 cultured under iron-replete conditions. This partial sequence was 100% identical to the putative SodB amino acid sequence (positions 100 to 108, Fig. 3) and also to the existing database sequence.

Bacterial strain and growth condition expression of SOD. (i) Zymography (NBT reduction). Cell-free lysates produced from the virulent strain (80204) and two avirulent strains (80204-1 and 84222-5) grown in vitro under low aeration to log phase at 17°C produced a single zone of clearance on native PAGE zymography with an R_f value of 0.31 (Fig. 4). This band was sensitive to 10 mM H_2O_2 (Fig. 5). Cell-free lysates from the same strains cultured in vivo produced a zone of clearance on native PAGE zymography with an R_f value of 0.36 (Fig. 4). This band was insensitive to both 10 mM H_2O_2 and 10 mM KCN (Fig. 5). Lysates from strain A449 cultured in modified Griffin's medium with differing amounts of iron sulfate were also analyzed by zymography. The R_f 0.31 zone of clearance was seen at all concentrations of iron sulfate whereas the R_f 0.36 zone of clearance was seen only at a medium concentration of 0 mM iron sulfate (lane A, Fig. 5).

(ii) Quantitative analysis (WST-1 reduction). SOD activity was quantified in two virulent (A449 and 80204) and three avirulent (80204-1, 84222-5, and SS70-1) strains cultured to mid-exponential phase in vitro in tryptic soy broth or cultured for 24 h in vivo. Under both growth conditions SOD activity was present in the cell-free lysates of all strains (Fig. 6). Following in vitro culture there were significant differences in SOD activity in the lysates of the different strains (one-way ANOVA; $P < 0.001$): SOD activity was significantly higher in the virulent strains than in the avirulent strains (Tukey's test; $P < 0.01$). There was no significant difference between the levels of SOD activity of the virulent strains in vitro. With in vivo-cultured bacteria there were significant differences in lysate SOD activity between the different strains (one-way ANOVA; $P < 0.001$) (Fig. 6). There was no significant difference in the levels of SOD activity between the two virulent strains. However, there was significantly higher SOD activity in the virulent strains than in avirulent strains 80204-1 and SS70-1 (Tukey's test; $P < 0.01$) The level of SOD activity in 84222-5 was not significantly different from that of 80204, but it was significantly lower than the levels of SOD activity in A449 (Tukey's test; $P < 0.01$). Inhibitor analyses were performed on A449 lysates from in vitro- and in vivo-cultured cells. In the in

Aeromonas salmonicida SodA	(AY321354)	
Yersinia pestis SodA	(NP_407488)	55% AA identity
Yersinia enterocolitica SodA	(CAA65596)	55% AA identity
Pectobacterium chrysanthemi SodA	(CAC69393)	55% AA identity
Salmonella typhimurium SodA	(AAC43331)	55% AA identity
Escherichia coli K12 SodA	(NP_418344)	55% AA identity
Vibrio alginolyticus SodA	(AAC26483)	58% AA identity
Vibrio cholerae SodA	(NP_2323220)	<55% AA identity

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A. salmonicida       1   ---------- -------MSH TLPDLSYAYD ALEPHIDALT MEIHHSRHHQ
Y. pestis            1   ---------- -------MSY SLPSLPYAYD ALEPHFDKQT MEIHHTKHHQ
Y. enterocolitica    1   ---------- -------MSY SLPSLPYAYD ALEPHFDKQT MEIHHTKHHQ
P. chrysanthemi      1   memln----- -------MSY SLPSLPYAYD ALEPHFDKQT MEIHHSKHHQ
S. typhimurium       1   ---------- -------MSY TLPSLPYAYD ALEPHFDKQT MEIHHTKHHQ
E. coli              1   ---------- -------MSY TLPSLPYAYD ALEPHFDKQT MEIHHTKHHQ
V. alginolyticus     1   ---------- -------MTY TLPDLPYAYD ALEPYIDEET MHLHHDKHHN
V. cholerae          1   mlsalliakr ysskedtMPH LFPDLPYAYD ALEPYIDTKT MEVHYSKHHR

A. salmonicida      34   TYITNLNVAL EAFPELAALP VDELLARFDS LPVKVQGAVR NHGGGHANHS
Y. pestis           34   TYVNNANTVL ESFPELADLS VEDLIKDLDK VPAEKRTFMR NNAGGHANHS
Y. enterocolitica   34   TYVNNANTVL ESFPELAKFS VEDLIKDLDK VPAEKRTFMR NNAGGHANHS
P. chrysanthemi     39   AYVNNANAAL ESLPEFAGLS AEELITKLDQ LPADKKGPLR NNAGGHANHS
S. typhimurium      34   TYVNNANAAL ENLPEFASLP VEELITKLDQ VPADKKTVLR NNAGGHANHS
E. coli             34   TYVNNANAAL ESLPEFANLP VEELITKLDQ LPADKKTVLR NNAGGHANHS
V. alginolyticus    34   TYVTNLNAAI EKHPELGEKT VEELLADFSS VPEDIQTAVR NNGGGHANHT
V. cholerae         51   TYydkflsai -KGTEHEDRP LSEIFARVST LPA----AVR NHGGGYYNHI

A. salmonicida      84   LFWQVMSPQG GSEPTGELAE AIRRDLGGLD AFKQAFTQAA LSRFGSGWAW
Y. pestis           84   LFWKGLKL-- GTTLAGDLKA AIERDFGSVD SFKEKFEQAA ATRFGSGWAW
Y. enterocolitica   84   LFWKGLKL-- GTTLTGDLKA AIERDFGSVD SFKEKFEAAA ATRFGSGWAW
P. chrysanthemi     89   LFWKGLKL-- GTTLTGELKA AIERDFGSVD AFKEKFEQAA ATRFGSGWAW
S. typhimurium      84   LFWKGLKT-- GTTLQGDLKA AIERDFGSVD NFKAEFEKAA ATRFGSGWAW
E. coli             84   LFWKGLKK-- GTTLQGDLKA AIERDFGSVD NFKAEFEKAA ASRFGSGWAW
V. alginolyticus    84   FFWEILGPNA GGEPTGAIKE AIEETFGSFE DFKEEFKTAA TGRFGSGWAW
V. cholerae         96   VYWNCMKPNA GGEPQGELAA EIERQFGSFA QFKEAFSQAA VNTFGSGFVW

A. salmonicida     134   LVVdKEG-KL QVESSANQDS PLMQGHA--- ---PILGLDV WEHAYYLKYQ
Y. pestis          132   LVL-KDDGKL AVVSTANQDS PLMGEAVSGV SGFPIVGLDV WEHAYYLKFQ
Y. enterocolitica  132   LVL-KDDGKL AVVSTANQDS PLMGEAVSGA SGFPIVGLDV WEHAYYLKFQ
P. chrysanthemi    137   LVL-KDDGKL AVVSTPNQDS PLMGEAISGA SGYPIVALDV WEHAYYLQYQ
S. typhimurium     132   LVL-KGD-KL AVVSTANQDS PLMGEAISGA SGFPILGLDV WEHAYYLKFQ
E. coli            132   LVL-KGD-KL AVVSTANQDS PLMGEAISGA SGFPIMGLDV WEHAYYLKFQ
V. alginolyticus   134   LVV-KDG-KL AITSTANQDS PLMDGQT--- ---PVLGLDV WEHAYYLKYK
V. cholerae        146   LIV-QQG-QL SITSTSNQDN PLMdvva--V RGEPILALDV WEHAYYIRYQ

A. salmonicida     177   NKRPDYIAAF YNVIDWDEVE RRYRQAla-- -
Y. pestis          181   NRRPDYIKAF WNVVNWDEAA ARFAQAK--- -
Y. enterocolitica  181   NRRPDYIKAF WNVVNWDEAA ARFAQAK--- -
P. chrysanthemi    186   NRRPDYIKAF WFVVNWDEAA KRFAEAKk-- -
S. typhimurium     180   NRRPDYIKEF WNVVNWDEAA ARFAlk---- -
E. coli            180   NRRPDYIKEF WNVVNWDEAA ARFAakk--- -
V. alginolyticus   176   NVRPDYINAF WSVVNWDKVN EYFAKA---- -
V. cholerae        192   NRRPEYIDAW WNVVNWEAVS ENYaialtqa a
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FIG. 1. Comparison of derived amino acid sequence of *A. salmonicida* subsp. *salmonicida* strain A449 SodA with other similar sequences of proteobacteria. Amino acids involved in metal binding are indicated by reversed type. Amino acids characteristic for Mn-containing enzymes are shaded (see the work of Jackson and Cooper [22]). Sequences were aligned using the Genomatix DiAlign progam (http://genomatix.gsf.de/). Only uppercase letters are considered to be aligned. The tryptic peptide is underlined.

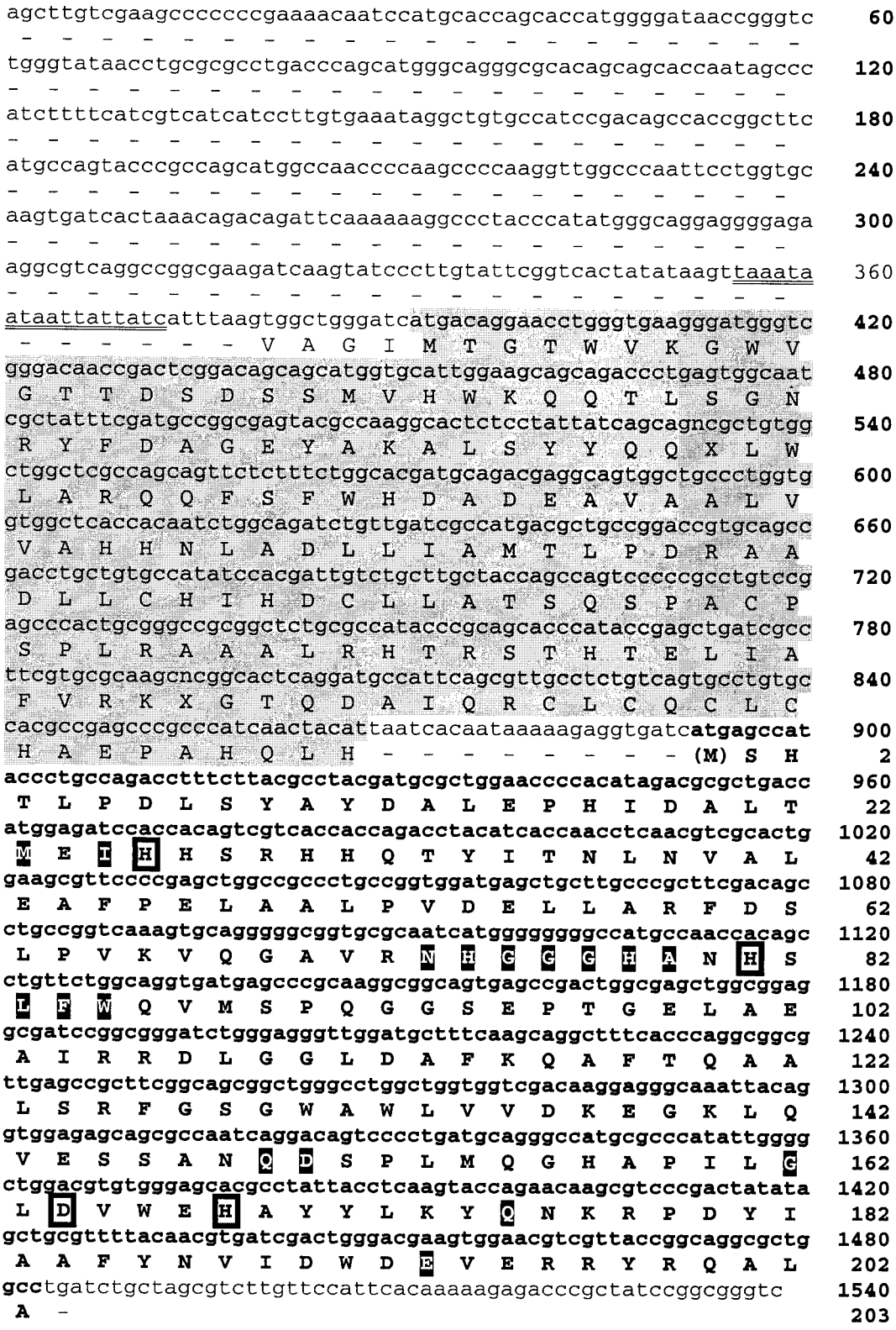

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the manganese-cofactored SOD (*sodA*, SodA) region of *A. salmonicida* subsp. *salmonicida* strain A449 (nucleotides and amino acids in boldface) and its upstream flanking region. The shaded region upstream of *sodA* is an unidentified gene similar to *orfX* of *Pseudomonas syringae* (AF121078). Residues in filled boxes are conserved residues of *sodA* (see the work of Jackson and Cooper [22]). Residues in open boxes are conserved ligands for the metal cofactor. The putative iron box is double underlined.

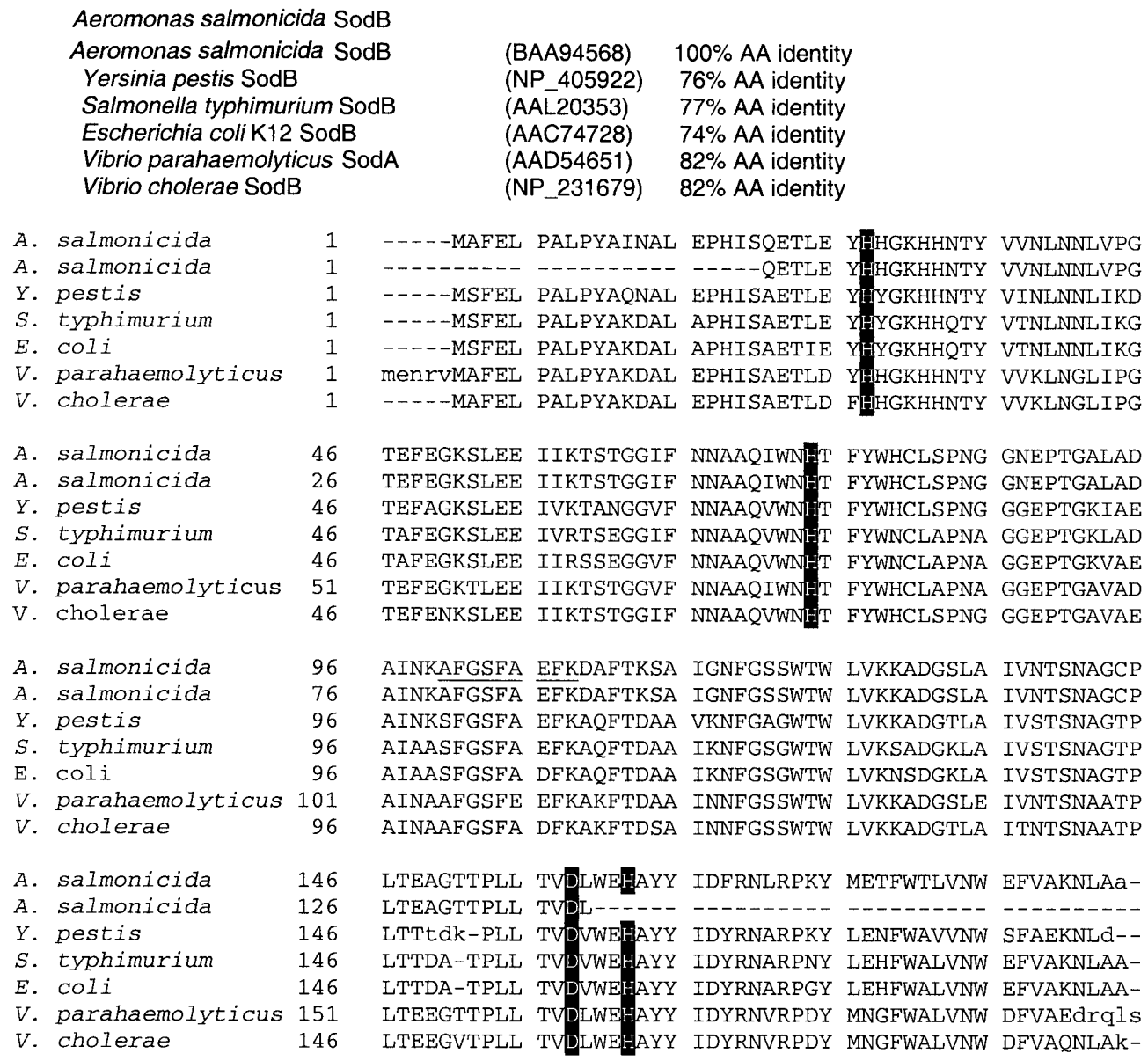

FIG. 3. Comparison of derived amino acid sequence of *A. salmonicida* subsp. *salmonicida* strain A449 iron-cofactored SOD (*sodB*, SodB) with other similar sequences of gamma-proteobacteria. Amino acids involved in metal binding are indicated by reversed type. Sequences were aligned using the Genomatix DiAlign program. Only uppercase letters are considered to be aligned. The tryptic peptide is underlined

vitro lysates, 10 mM hydrogen peroxide significantly reduced v_o (Tukey's test; $P = 0.01$) but 10 mM KCN did not. In the in vivo lysates there were no significant differences in v_o with the addition of either 10 mM H_2O_2 or 10 mM KCN (data not shown).

DISCUSSION

Sequence analysis. As the result of genomic sequencing of *A. salmonicida* subsp. *salmonicida* strain A449 we have identified two ORFs with high identities to prokaryotic SodA and SodB. The putative SodB sequence was identical to the partial *sodB* product sequence for *A. salmonicida* (accession number BAA94568). Our sequence also has high levels of amino acid identity to SodB from other *Aeromonas* species. We report a high similarity of the putative SodB sequence to the SodA gene sequence of *V. parahaemolyticus* (accession number AAD54651); however, this similarity appears to be due to a misannotation of that gene as *sodA*. We feel that this is the case as the reported sequence for *V. parahaemolyticus sodA* shares high identities with numerous SodB sequences but not with other SodA sequences. No other SOD or SOD-like genes were identified.

Iron metabolism genes in bacteria such as *E. coli* are under the control of the ferric uptake regulation protein (Fur), which functions as a transcriptional regulator. Fur both represses and activates the expression of certain target genes (11, 13). Its regulatory role in the control of *E. coli* SodA has been well

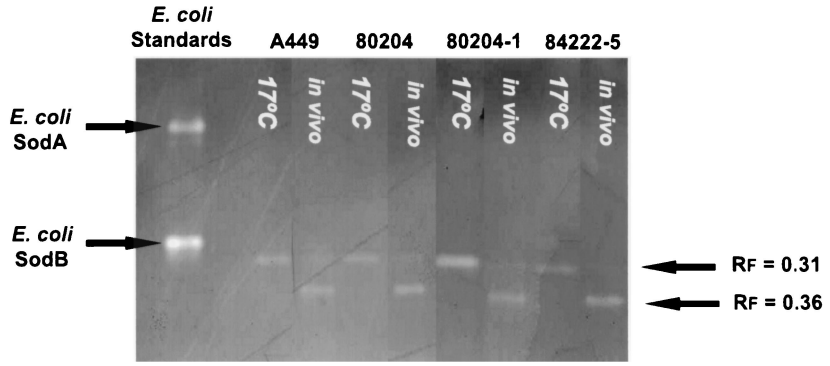

FIG. 4. Zymograms of cell-free lysates of a virulent strain (80204) and two avirulent strains (80204-1 and 84222-5) of *A. salmonicida* subsp. *salmonicida* cultured in vitro at 17°C in vitro and in vivo. Note the presence of SOD activity with an R_f value of 0.31 in all strains cultured in vitro. Strains cultured in vivo show SOD activities with R_f values of 0.36.

studied (21). In *P. aeruginosa*, iron-limiting conditions appear to increase SodA activity (6). An 891-bp sequence upstream from *sodA* was examined to identify putative regulatory elements. Immediately upstream of *sodA* there was a 477-bp ORF that encoded a protein similar to the hypothetical 17-kDa protein (*orfX*) of *Pseudomonas* spp. that is also upstream of the *P. aeruginosa sodA* gene (31). Using the consensus iron box sequence for *E. coli*, we identified a putative iron box region of 18 nucleotides upstream from *orfX* that has a 15-of-19-base match with the *E. coli* consensus sequence (8).

Expression studies. Zymography demonstrated a SOD activity with an R_f of 0.36 in cell-free lysates of A449 cultured in vitro in iron-deficient medium and in all five strains cultured in vivo (Fig. 4). Both zymographic (Fig. 5) and colorimetric (data not shown) inhibitor studies demonstrated that this activity was H_2O_2 and KCN insensitive, which suggested that this activity was due to SodA. A protein identified as SodA by MS was identified in the A449 proteome when the strain was cultured in iron-deficient medium. SodA levels were significantly higher in virulent than in avirulent strains in in vivo-cultured bacteria with the exception of 84222-5 (Fig. 6). The molecular mass as determined by reducing-denaturing SDS-PAGE for this isozyme was estimated to be 22.3 kDa, which is close to the predicted molecular mass of 22.9 kDa based on the sequence data. Barnes et al. (2) have previously reported a SodA produced under iron-limited conditions with a molecular mass of 45.6 kDa in another virulent strain of *A. salmonicida* subsp. *salmonicida*. SodA, therefore, likely exists as a homodimer.

Zymography identified a SOD activity with an R_f of 0.31 in virulent and avirulent strains of *A. salmonicida* subsp. *salmonicida* cultured in vitro in iron-replete medium (Fig. 4). The same activity was obtained when the virulent strain A449 was grown in modified Griffin's medium with high or low iron levels. Both zymographic (Fig. 5) and colorimetric (data not shown) inhibitor studies demonstrated that this activity was H_2O_2 sensitive and KCN insensitive, which suggested that this activity was due to SodB. A protein identified as SodB by MS was identified in the A449 proteome when the strain was cultured in iron-replete medium. SodB levels were significantly higher in virulent than in avirulent strains in broth-cultured bacteria (Fig. 6). The estimated size of SodB under native conditions is 50.9 kDa (2). The molecular mass of *A. salmonicida* subsp. *salmonicida* SodB was estimated to be 22.3 kDa under reducing and denaturing conditions. This figure is close to the deduced molecular mass for the *sodB* ORF product of 21.6 kDa. As with SodA, SodB would appear to exist as a homodimer.

These data suggested that the virulent strains had an enhanced antioxidative capacity over that of the avirulent strains with respect to both SodA and SodB. There are two possible interpretations as to the biological relevance of this finding. SOD has been implicated in the detoxification of O_2^- produced by phagocytic cells as an antimicrobial, the so-called respiratory burst (3, 14, 15, 20, 30, 32). In contrast to these data a recent study showed no significant difference in SOD levels between virulent and avirulent *P. aeruginosa* strains (6). This was linked to the unimportance of ROS-mediated killing in neutrophils, the primary leukocyte subpopulation involved in the immune response against *P. aeruginosa*. It is also clear that salmonid mononuclear phagocytic cells capable of a robust respiratory burst play an important role in the immune response to *A. salmonicida* subsp. *salmonicida* (33); therefore, up-regulation of SOD might be more relevant in *A. salmonicida* subsp. *salmonicida* pathogenesis. However, the antimicrobial action of exogenous O_2^- is likely through reactions with nitric oxide to produce peroxynitrous acid, which unlike O_2^- can penetrate cell membranes. It has been suggested that the cytosolic subcellular localization of both SodA and SodB in many species of bacteria (7) posits a role in detoxifying endogenous, rather than exogenous, O_2^- (5, 19). This latter interpretation offers a new view of the *A. salmonicida*-*S. salar* relation-

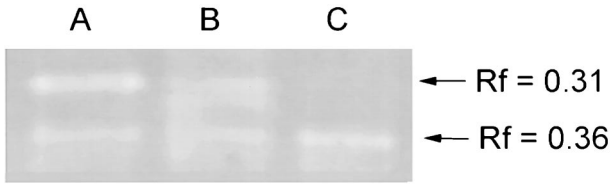

FIG. 5. Zymograms of cell-free lysates of *A. salmonicida* subsp. *salmonicida* strain A449, a virulent strain, cultured in vitro in iron-free simplified Griffin's medium. Lysates were incubated for 30 min prior to zymography with either buffer (A), 10 mM KCN (B), or 10 mM H_2O_2 (C).

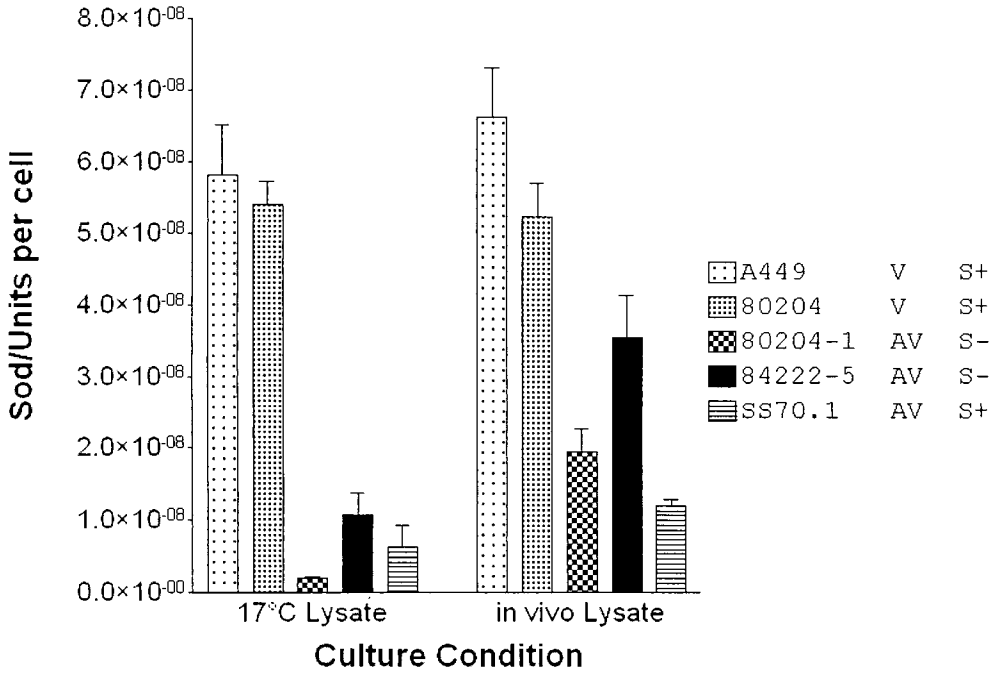

FIG. 6. Quantitative analysis of SOD in cell-free lysates for two virulent strains (A449 and 80204) and three avirulent strains (80204-1, 84222-5, and SS70-1) of *A. salmonicida* subsp. *salmonicida* cultured in vitro and in vivo. V, virulent strain; AV, avirulent strain; S+, S-layer-positive strain; S−, S-layer-deficient strain. Values are the mean (± standard deviation) SOD units per cell ($n = 3$). One unit of SOD (EC 1.15.1.1) is defined as that required to inhibit reduction of cytochrome *c* by 50% in a coupled system with xanthine oxidase at pH 7.8 at 25°C in a 3.0-ml reaction volume.

ship. The literature (reviewed in reference 33) has assumed that SOD acts on exogenous O_2^-. Certainly the periplasmic localization of SodB (2) supports this. However, under in vivo conditions, when it might be more likely to encounter O_2^-, especially if it is facultatively intracellular (18), the predominant form of SOD is cytoplasmically located SodA (2). Given the inability of O_2^- to cross lipid membranes, the significant increase in SodA in vivo reported here might be seen as irrelevant. Indeed this may be the case if one considers only oxidative stress as O_2^- applied as an antimicrobial. However, we argue that the avirulent strains may have been attenuated in their ability to cope with endogenously generated aerobic stress.

Lysates from *A. salmonicida* subsp. *salmonicida* cultured in iron-limited medium or in vivo had SodA activity, but its expression was abolished by the addition of 5 mM iron sulfate. Upstream of *sodA* we identified a putative iron box consistent with this and also an ORF of unknown function that is similarly located with respect to *sodA* of *P. aeruginosa*. Barnes et al. (2) reported the expression of only SodA when *A. salmonicida* was cultured in iron-deficient medium. The presence of SodA activity in cells cultured in vivo suggested either that the in vivo culture condition is limited in the amount of available iron or that other mechanisms play a role in stimulating SodA activity, as SodA is also known to be produced under conditions of oxidative stress (26). The presence of SodA due to the bacteria being in stationary phase growth as in *Aeromonas hydrophila* (25) is unlikely as in vivo-cultured bacteria at 24 h in the system used in this study are in exponential growth (data not shown). The molecular mass cutoff of the in vivo culture chamber was chosen to both retain bacteria within the chamber and exclude host cells from it. It is likely, therefore, that SodA expression was a response to low-iron conditions in the peritoneal cavity. Garduño et al. (17) demonstrated that virulent *A. salmonicida* subsp. *salmonicida* strain A450 when grown in vivo with a similar system acquired resistance to bacteriolysis, phagocytosis, and oxidative killing. The differential expression of SodA in vivo, in an arguably more authentic culture condition than in vitro broth culture, suggests that further studies of *A. salmonicida* subsp. *salmonicida*, especially knockout mutants of SodA, would be instructive in the study of the *A. salmonicida-S. salar* host-pathogen relationship. When cultured in vivo, SOD activity was partially restored to the three avirulent strains. This was especially noticeable in the S-layer-deficient strains 80204-1 and 84222-5 (Fig. 5). This suggests that there may be some regulatory hurdle to SOD expression that is overcome in vivo in these strains. It also suggests that, in 80204-1 and 84222-5, S-layer and SOD expression may be linked. This is noteworthy, as the S-layer has often been implicated as a virulence factor in *A. salmonicida* subsp. *salmonicida* (28). The nature of this link may seem intuitive given that Barnes et al. (2) have clearly demonstrated that SodB, the enzyme predominantly expressed in vitro, has a periplasmic localization. However, as it was SodA activity, with a demonstrated cytosolic localization (2), that was restored in vivo, the nature of any possible link between SOD and the S-layer is obscure. Given that 80204-1 and 84222-5 are considered avirulent, the partial restoration of SOD activity in vivo would not seem to be concomitant with a restoration of virulence.

In summary we have demonstrated quantitative differences in SOD levels between virulent and avirulent strains of *A. salmonicida* subsp. *salmonicida* by the inhibition of O_2^--cata-

lyzed tetrazolium salt reduction. We have identified two ORFs that code for two SOD isozymes, SodA and SodB, that are consistent with the observed SOD activities. The *sodA* of *A. salmonicida* subsp. *salmonicida* was a cytoplasmic dimer of a predicted 22.9-kDa protein, with an R_f of 0.36 by zymography, and was insensitive to inhibition by 10 mM H_2O_2 or KCN. Its activity appeared to be at least partly restored in vivo in two S-layer-deficient strains. The *sodB* of *A. salmonicida* subsp. *salmonicida* was a periplasmic dimer of a predicted 21.6-kDa protein, with an R_f of 0.31 by zymography, and was insensitive to inhibition by 10 mM H_2O_2 but not KCN. These two isozymes differ in their expression patterns under in vivo and in vitro culture conditions. No other SOD or SOD-like molecules were identified by data mining or functionally. This does not, however, preclude their existence. The role of SOD in the pathogenesis of *A. salmonicida* subsp. *salmonicida* is unclear. These data do suggest a role in virulence for SOD, although the role has not been fully elucidated. These data together with the subcellular localization of SOD by Barnes et al. (2) suggest that avirulence might be homeostasis rather than defense related. A more rigorous assessment of the role of SOD in virulence would require both *sodA*- and *sodB*-knockout strains. Much has been made of the inability to correlate virulence in *A. salmonicida* subsp. *salmonicida* with putative virulence factors such as the S-layer or a number of secreted toxins (12, 28, 35). However, the view that virulence will be linked to a single gene is overly simplistic and the data presented here suggest that other putative virulence factors should be reassessed quantitatively.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Barnes, A. C., M. T. Horne, and A. E. Ellis.** 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. **Battistoni, A., F. Pacello, S. Folcarelli, M. Ajello, G. Donnarumma, R. Greco, M. G. Ammendolia, D. Touati, G. Rotilio, and P. Valenti.** 2000. Increased expression of periplasmic Cu,Zn superoxide dismutase enhances survival of *Escherichia coli* invasive strains within nonphagocytic cells. Infect. Immun. **68:**30–37.
4. **Beauchamp, C., and I. Fridovich.** 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. **44:**276–287.
5. **Benov, L., and I. Fridovich.** 1995. Superoxide dismutase protects against aerobic heat shock in *Escherichia coli*. J. Bacteriol. **177:**3344–3346.
6. **Britigan, B. E., R. A. Miller, D. J. Hasset, M. A. Pfaller, M. L. McCormick, and G. T. Rasmussen.** 2001. Antioxidant enzyme expression in clinical strains of *Pseudomonas aeruginosa*: identification of an atypical form of manganese superoxide dismutase. Infect. Immun. **69:**7396–7401.
7. **Britton, L., and I. Fridovich.** 1977. Intracellular localization of the superoxide dismutases of *Escherichia coli*: a re-evaluation. J. Bacteriol. **131:**815–820.
8. **Calderwood, S. B., and J. J. Mekalanos.** 1987. Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. J. Bacteriol. **169:**4759–4764.
9. **Chung, S., and C. J. Secombes.** 1988. Analysis of events occurring within teleost macrophages during the respiratory burst. Comp. Biochem. Physiol. B **89:**39–54.
10. **Colquhoun, D., and H. Sørum.** 1998. Outer membrane protein expression during in vivo cultivation of *Vibrio salmonicida*. Fish Shellfish Immunol. **8:**367–377.
11. **Dubrac, S., and D. Touati.** 2000. Fur positive regulation of iron superoxide dismutase in *Escherichia coli*: functional analysis of the *sodB* promoter. J. Bacteriol. **182:**3802–3808.
12. **Ellis, A. E., A. S. Burrows, and K. J. Stapleton.** 1988. Lack of relationship between virulence of *Aeromonas salmonicida* and the putative virulence factors: A-layer, extracellular proteases and extracellular haemolysins. J. Fish Dis. **11:**309–323.
13. **Escolar, L., J. Perez-Martin, and V. de Lorenzo.** 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. J. Bacteriol. **181:**6223–6229.
14. **Fang, F. C., M. A. DeGroote, J. W. Foster, A. J. Baumler, U. Ochsner, T. Testerman, S. Bearson, J. C. Giard, Y. Xu, G. Campbell, and T. Laessig.** 1999. Virulent *Salmonella typhimurium* has two periplasmic Cu, Zn-superoxide dismutases. Proc. Natl. Acad. Sci. USA **96:**7502–7507.
15. **Franzon, V. L., J. Arondel, and P. J. Sansonetti.** 1990. Contribution of superoxide dismutase and catalase activities to *Shigella flexneri* pathogenesis. Infect. Immun. **58:**529–535.
16. **Garduño, R. A., M. A. Kuzyk, and W. W. Kay.** 1997. Structural and physiological determinants of resistance of *Aeromonas salmonicida* to reactive radicals. Can. J. Microbiol. **43:**1044–1053.
17. **Garduño, R. A., J. C. Thornton, and W. W. Kay.** 1993. *Aeromonas salmonicida* grown in vivo. Infect. Immun. **61:**3854–3862.
18. **Garduno, R. A., A. R. Moore, G. Olivier, A. L. Lizama, E. Garduno, and W. W. Kay.** 2000. Host cell invasion and intracellular residence by *Aeromonas salmonicida*: role of the S-layer. Can. J. Microbiol. **46:**660–668.
19. **Gort, A. S., and J. A. Imlay.** 1998. Balance between endogenous superoxide stress and antioxidant defenses. J. Bacteriol. **180:**1402–1410.
20. **Harth, G., and M. A. Horwitz.** 1999. Export of recombinant *Mycobacterium tuberculosis* superoxide dismutase is dependant upon both information in the protein and mycobacterial export machinery. J. Biol. Chem. **274:**4281–4292.
21. **Hassan, H. M., and L. W. Schrum.** 1994. Roles of manganese and iron in the regulation of the biosynthesis of manganese-superoxide dismutase in *Escherichia coli*. FEMS Microbiol. Rev. **14:**315–324.
22. **Jackson, S. M. J., and J. B. Cooper.** 1998. An analysis of structural similarity in the iron and manganese superoxide dismutases based on known structure and sequences. Biometals **11:**159–173.
23. **Kim, E.-J., H.-J. Chung, B. Suh, Y. C. Hah, and J.-H. Roe.** 1998. Expression and regulation of the *sodF* gene encoding iron and zinc-containing superoxide dismutase in *Streptomyces coelicolor* Müller. J. Bacteriol. **180:**2014–2020.
24. **Kim, E.-J., H.-J. Chung, B. Suh, Y. C. Hah, and J.-H. Roe.** 1998. Transcriptional and post-transcriptional regulation by nickel of *sodN* gene encoding nickel-containing superoxide dismutase from *Streptomyces coelicolor* Müller. Mol. Microbiol. **27:**187–195.
25. **Leclère, V., A. Chotteau-Lelièvre, F. Gancel, M. Imbert, and R. Blondeau.** 2001. Occurrence of two superoxide dismutases in *Aeromonas hydrophila*: molecular cloning and differential expression of the *sodA* and *sodB* genes. Microbiology **147:**3105–3111.
26. **Miller, R. A., and B. E. Britigan.** 1997. Role of oxidants in microbial pathophysiology. Clin. Microbiol. Rev. **10:**1–18.
27. **O'Leary, M. O., C. Panos, and G. E. Helz.** 1956. Studies on the nutrition of *Bacterium salmonicida*. J. Bacteriol. **72:**673–676.
28. **Olivier, G.** 1990. Virulence of *Aeromonas salmonicida*: lack of correlation with phenotypic characteristics. J. Aquat. Anim. Health **2:**119–127.
29. **Phipps, B. M., T. J. Trust, E. E. Ishiguro, and W. W. Kay.** 1983. Purification and characterization of the cell surface virulent A protein from *Aeromonas salmonicida*. Biochemistry **22:**2934–2939.
30. **Piddlington, D. D., F. C. Fang, T. Laessig, A. M. Cooper, I. M. Orme, and N. A. Buchmeier.** 2001. Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis* contributes to survival in activated macrophages that are generating an oxidative burst. Infect. Immun. **69:**4980–4987.
31. **Polack, B., D. Dacheux, I. Delic-Attree, B. Toussaint, and P. M. Vignais.** 1996. The *Pseudomonas aeruginosa fumc* and *soda* genes belong to an iron-responsive operon. Biochem. Biophys. Res. Commun. **226:**555–560.
32. **Poyart, C., E. Pellegrini, O. Gaillot, C. Boumaila, M. Baptista, and P. Trieu-Cuot.** 2001. Contribution of Mn-cofactored superoxide dismutase (SodA) to the virulence of *Streptococcus agalactiae*. Infect. Immun. **69:**5098–5106.
33. **Secombes, C. J., and G. Olivier.** 1997. Host-pathogen interactions in salmonids, p. 269–296. *In* E.-M. Bernoth, A. E. Ellis, P. J. Midtlyng, G. Olivier, and P. Smith (ed.), Furunculosis: multidisciplinary fish disease research. Academic Press, London, United Kingdom.
34. **Shevchenko, A., M. Wilm, O. Vorm, and M. Mann.** 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. **68:**850–858.
35. **Vipond, R., I. R. Bricknell, E. Durant, T. J. Bowden, A. E. Ellis, M. Smith, and S. McIntyre.** 1998. Defined deletion mutants demonstrate that the major secreted toxins are not essential for the virulence of *Aeromonas salmonicida*. Infect. Immun. **66:**1990–1998.