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Cloning and Molecular Characterization of Cu,Zn Superoxide Dismutase from *Actinobacillus pleuropneumoniae*

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Copper-zinc superoxide dismutases (Cu,Zn SODs), until recently considered very unusual in bacteria, are now being found in a wide range of gram-negative bacterial species. Here we report the cloning and characterization of *sodC*, encoding Cu,Zn SOD in *Actinobacillus pleuropneumoniae*, a major pathogen of pigs and the causative organism of porcine pleuropneumonia. *sodC* was shown to lie on a monocistronic operon, at the chromosomal locus between the genes *asd* (encoding aspartate semialdehyde dehydrogenase) and *recF*. The primary gene product was shown to have an N-terminal peptide extension functioning as a leader peptide, so that the mature *Actinobacillus* enzyme, like other bacterial examples, is directed to the periplasm, where it is appropriately located to dismutate exogenously generated superoxide. While the role of these secreted bacterial SODs is unknown, we speculate that in *A. pleuropneumoniae* the enzyme may confer survival advantage by accelerating dismutation of superoxide derived from neutrophils, a central host defense response in the course of porcine infection.

Actinobacillus pleuropneumoniae, the cause of contagious porcine pneumonia, is a veterinary pathogen of major economic significance. Infected pigs may develop fulminant, rapidly fatal infection or a more indolent illness characterized by protracted poor growth (45). Whole herds may be rapidly affected, leading to financial ruin. There is no established vaccine that is generally effective in protecting animals, and the identification of microbial characteristics critically involved in infection, and thus suitable targets for vaccines, is a generally acknowledged research goal. Attention has been focused on antiphagocytic serotype-specific capsular polysaccharides (25) and on cytotoxins that play an important part in the interaction of organisms with phagocytic cells (19). While *A. pleuropneumoniae* elicits a vigorous respiratory burst from porcine neutrophils (15, 16), infected pulmonary lesions with a substantial inflammatory cell (neutrophilic) component are a hallmark of disease (45), indicating that organisms are at least to some extent able to withstand the bactericidal action of this host response. Bacterial characteristics that permit organisms to withstand the toxic action of activated oxygen species produced by host inflammatory cells—superoxide and hydroxyl radicals, various oxyanions, and hydrogen peroxide—may therefore make a significant contribution to their pathogenicity. High-molecular-weight surface carbohydrate structures incorporated into capsule and lipopolysaccharide can contribute by scavenging for free radicals, (6), but we and others have discovered that a range of pathogens, both prokaryotic and eukaryotic, produce a periplasmic Cu,Zn superoxide dismutase (SOD) that is available to dismute exogenously produced superoxide radicals, which may participate in this process (4, 26, 28, 32, 46, 50). We have recently developed a PCR-based approach which has permitted the cloning of partial *sodC* genes encoding Cu,Zn SOD from many bacteria (9, 30), including representative examples of all members of the *Haemophilus-Actinobacillus-Pasteurella* family of pathogens. To study the role that Cu,Zn SOD plays in the interactive biology of *Haemophilus-Actinobacillus-Pasteurella* group bacteria with their hosts, we have selected examples for further study, and in the present work we describe the cloning and genetic and molecular characterization of *sodC* and its product in *A. pleuropneumoniae.*

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MATERIALS AND METHODS

Bacterial strains and culture. *A. pleuropneumoniae* 1421, a virulent serotype 3 strain kindly provided by I. Smith (Royal Veterinary College, London), was grown in brain heart infusion broth supplemented with 2 μg of NAD per ml. Brain heart infusion plates were prepared with 10% Levinthal's base (1). For anaerobic culture, organisms were grown without shaking in a chamber containing 10% hydrogen, 5% carbon dioxide, and 85% nitrogen. Iron restriction was achieved by growing organisms for 18 h in medium to which the iron-chelating agent ethylenediamine-N,N′-diacetic acid was added to a concentration of 100 μM (13). Where appropriate, iron was restored as iron(III) nitrate to a concentration of 100 μM. *Escherichia coli* DH5α was used to propagate plasmid constructions. The *sodA sodB* mutant QC779 was kindly provided by D. Touati (Institut Jacques Monod, Paris). *E. coli* AA10, a *recA* mutant of the minicell strain P678-54, was kindly provided by S. Normark (Karolinska University, Stockholm, Sweden). Antibiotics were added to growth media (Luria broth and L agar) as appropriate at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 5 μg/ml; and kanamycin, 50 μg/ml.

Recombinant DNA experiments. Standard methods were used for chromosomal and plasmid DNA preparation, manipulation, and analysis (endonuclease and exonuclease digestion, Southern blotting, ligation, and sequencing) (42, 43). Total cellular RNA was prepared from 80-ml cultures of exponentially growing cells by the hot phenol method (36) for use as a template for the synthesis of cDNA products by primer extension with avian myeloblastosis virus reverse transcriptase (40).

In vitro protein synthesis in minicells. Minicells harboring the appropriate plasmid were isolated essentially by the method of Thompson and Achtman (55) and labelled with [^{35}S]methionine. Approximately 300,000 cpm of trichloroacetic acid-acetone-precipitable material was separated electrophoretically in sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels. Gels were fixed, stained with Coomassie blue to visualize proteins, infiltrated with Amplify (Amersham), dried onto filter paper, and examined by autoradiography.

Extraction of bacterial cell proteins, gel electrophoresis, and detection of SOD. The cell pellet from 25 ml of an exponentially growing aerobic culture was frozen at −20°C, thawed, resuspended in 1 ml of 50 mM Tris (pH 7.8) containing 1 mM copper sulfate, sonicated for 1 min on ice, and centrifuged at 13,500 × *g* for 15 min. The supernatant was used directly or stored at −20°C. When material of higher purity was required for the preparation of crystals for future structural determination, an extra stage involving heating to 65°C for 1 h was included. This did not reduce the activity of the SOD as judged by assay in nondenaturing gels (31). The protein concentration was measured by comparison to bovine serum albumin as a standard (51). Polyacrylamide gel electrophoresis (PAGE) was

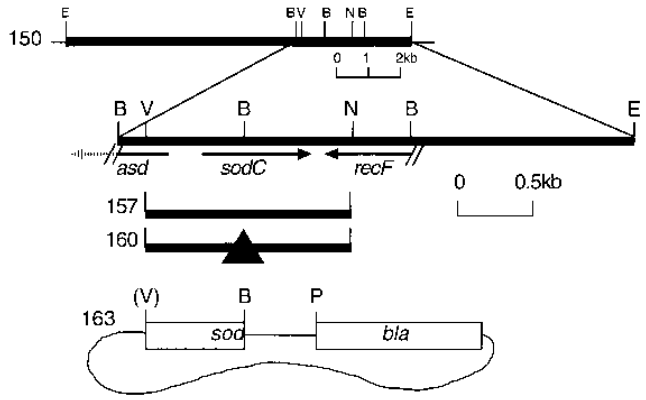

FIG. 1. Plasmid constructions from the *sodC* locus. The numbers on the left identify pJSK plasmids pJSK150 and, in the expansion below, subclones derived from it. Selected restriction sites are indicated as follows: B, *Bam*HI; E, *Eco*RI; N, *Nco*I; P, *Pvu*II; and V, *Eco*RV. The parenthetical V signifies the position of the *Eco*RV site disrupted in the cloning strategy. The location and direction of transcription of genes *asd*, *sodC*, and *recF* are shown with arrows, while the hatching indicates extension beyond the end of the DNA sequenced. The filled triangle indicates the location of the kanamycin resistance cassette in pJSK160. A schematic representation of pJSK163 shows the promoterless, leader-peptide-deficient β-lactamase gene (*bla*) downstream of cloned 5′-*sodC* DNA (*sod*).

performed with 4.5% stacking gels (pH 8.3), 7% separating gels (pH 8.9), and the buffer system of Davis (12), except that the pH of the upper reservoir was raised to pH 8.9 with 10 M NaOH. SOD activity in polyacrylamide gels was visualized by the method of Beauchamp and Fridovich (3) as modified by Steinman (47). When used as an inhibitor of SOD activity, hydrogen peroxide or potassium cyanide was included at a final concentration of 5 mM or 2 mM, respectively. Cu,Zn SODs are inactivated by cyanide and Fe SODs are inactivated by hydrogen peroxide, but Mn SODs are inactivated by neither of these metals (11). Bovine erythrocyte Cu,Zn SOD (Sigma) mixed with a sonicate of *E. coli* DH5α containing Fe SOD and Mn SOD was used as a control preparation of all three forms of the enzyme.

β-Lactamase fusions. Random fusions were made between the 5′ part of *sodC* and upstream DNA and a promoterless gene encoding a leader peptide-deficient version of TEM-1 β-lactamase (Bla) cloned in the plasmid vector pJBS633 with an engineered *Pvu*II site at the start of the *bla* gene (8). Briefly, the *Eco*RV-*Bam*HI fragment from the insert of pJSK157 was cloned into pJBS633 at its *Bam*HI site to give pJSK163 (Fig. 1). This plasmid was subsequently linearized at the unique *Bam*HI site, after which random deletions were made by using *Bal*31 exonuclease and, following *Pvu*II digestion, the products were ligated. *E. coli* transformants were initially selected on medium containing kanamycin and subsequently assessed for their ability to confer resistance to ampicillin when plated out with a large (confluent streak) or small (well-separated single colonies) inoculum. The cellular location of the β-lactamase moiety of the fusion protein can be determined, since only when it is exported to the periplasm can individual cells form single colonies in the presence of ampicillin. Fusion proteins expressed in the cytoplasm do not confer ampicillin resistance on single cells, although growth is seen when organisms are plated with a large inoculum since cell lysis results in the release of β-lactamase, which can protect neighboring organisms. Nucleotide sequence across the *sodC-bla* junction was determined in a series of fusions by sequencing miniprep DNA using the primer MATBLAC (5′-CTCGT GCACCCAACTGA), which is complementary to codons 14 to 18 of the mature β-lactamase.

Western blotting (immunoblotting). Total proteins from minicells harboring pJSK157 were separated by SDS-PAGE followed by transfer to a nitrocellulose membrane (57). Blots were incubated in 10 mM Tris (pH 7.4) containing 0.9% sodium chloride and 0.3% Tween 20 (TN20) for 30 min to prevent nonspecific binding. Subsequently they were incubated in the presence of a 1:200 dilution of pig serum in TN20 for 120 min. Pigs recovering from experimental infection with a serotype 3 strain or gnotobiotic piglets were the source of serum. Following washing, blots were incubated with goat anti-pig immunoglobulin G-alkaline phosphatase conjugate in TN20 for 60 min. After washing, the blots were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Nucleotide sequence accession number. The nucleotide sequence of the *sodC* locus of *A. pleuropneumoniae* 1421 has been deposited in the EMBL database under accession number X99396.

RESULTS

Superoxide dismutase expression in *A. pleuropneumoniae*. *A. pleuropneumoniae* 1421 was harvested from an aerobic liquid culture grown with shaking to mid-exponential phase. Whole-cell sonicates were separated electrophoretically on nondenaturing polyacrylamide gels and stained for SOD activity. As described previously (30), two bands of activity were seen when organisms were grown in rich medium under aerobic conditions. Activity in the well-defined achromatic band at the position of low relative mobility is abolished by KCN, a characteristic of Cu,Zn SOD, while the more diffuse achromatic zone, representing an SOD activity of greater relative mobility (characteristic of Fe or Mn SOD under these gel conditions), is unaffected by KCN and also by hydrogen peroxide (data not shown), characteristics of Mn SOD. The activities of *A. pleuropneumoniae* SOD were determined under aerobic and anaerobic conditions with and without iron (Fig. 2). Active Cu,Zn SOD was present under all growth conditions examined, at levels indistinguishable under the conditions of assay employed, suggesting constitutive expression. In aerobic culture, expression of Mn SOD did not change under conditions of iron restriction (data not shown). In contrast, Mn SOD was not expressed anaerobically unless the conditions included iron restriction.

Cloning and characterization of *sodC*, encoding Cu,Zn SOD of *A. pleuropneumoniae*. The use of degenerate oligonucleotide primers to amplify a 300-bp *sodC* DNA fragment from the chromosome of *A. pleuropneumoniae* 1421 by PCR has been described previously. This fragment, labeled and used as a probe, has revealed a single band of hybridization of around 10 kb in all serotypes of *A. pleuropneumoniae* as well as other *Actinobacillus* species (30). A partial gene bank of 8- to 12-kb fragments was prepared from *A. pleuropneumoniae* 1421 chromosomal DNA in the plasmid vector pACYC184, and a clone containing a 10.5-kb fragment that hybridized strongly to the probe was isolated (pJSK150) (Fig. 1). The region of homology

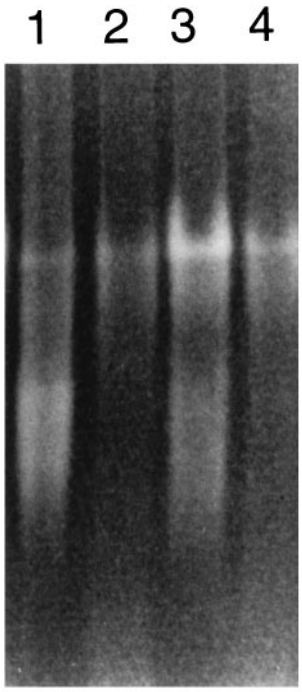

FIG. 2. *A. pleuropneumoniae* 1421 SOD visualized after 7% PAGE. SOD activity is represented by a decolorized zone against the dark background. Lanes contain 20 μg of protein from extracts of whole cells grown under the following conditions: lane 1, aerobic; lane 2, anaerobic; lane 3, anaerobic with iron restriction; and lane 4, like lane 3 but with restoration of iron.

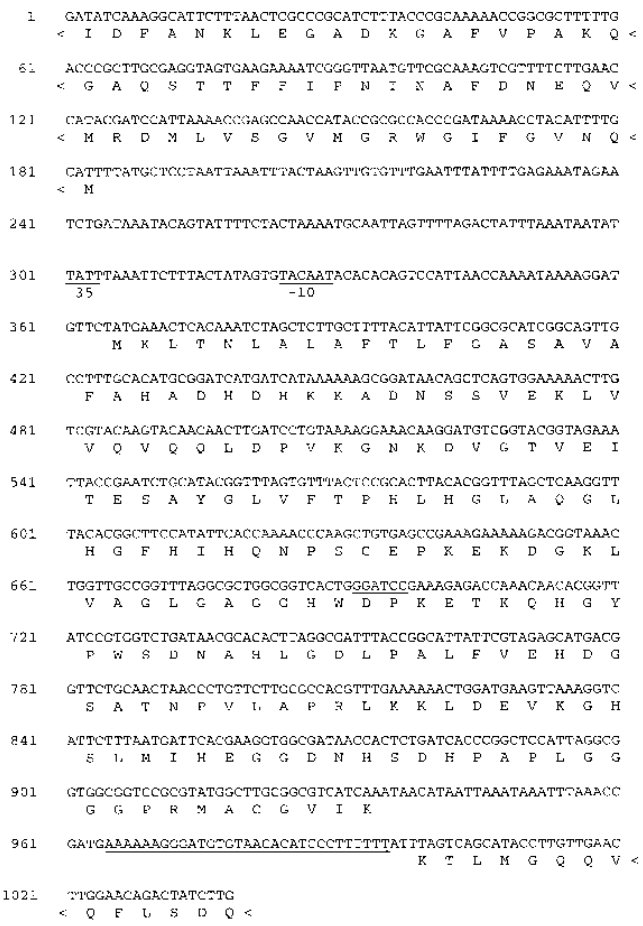

FIG. 3. Nucleotide sequence and its translation at the *sodC* locus of *A. pleuropneumoniae* 1421. Shown are 1,038 nucleotides, from the *Eco*RV site upstream of *sodC* to the 3′ end of *recF* (overlapping with *recF* sequence reported elsewhere [EMBL accession number X63626]), and translated protein sequence of open reading frames (single-letter code). In the DNA sequence, the *Bam*HI site in *sodC*, the stem-loop putative rho-independent terminator shared by *sodC* and *recF*, and −35 and −10 transcriptional signals are underlined. The protein sequence of the lines bordered by the symbol < corresponds to the DNA strand complementary to the one shown.

to the probe was localized to a 1.4-kb *Eco*RV-*Nco*I fragment which was subcloned into the plasmid vector pBluescript to make pJSK157, and the insert was sequenced on both strands (Fig. 3). A 570-nucleotide open reading frame was identified as *sodC* by its similarity, and the similarity of its translated product, to known examples of the bacterial gene and protein. It was followed by a run of adenylate residues; a stem-loop structure, characteristic of a rho-independent terminator ($\Delta G = -90.7$ kJ [56]), located 27 nucleotides downstream of the TAA stop codon; and a run of thymidylate residues. This structure has the potential to act as a terminator for two converging genes, that on the opposite strand being identified by the 56% identity of its translated product to the gene encoding the *E. coli* single-stranded-DNA-binding protein RecF (35). The 5′-end-labelled synthetic oligonucleotide CCACTGAGCTG TTATCCGC, which is complementary to the *sodC* DNA sequence from position 453 to 471, was used in a primer extension experiment with avian myeloblastosis virus reverse transcriptase and total cellular mRNA as a template to locate the transcriptional start site 30 nucleotides upstream of the *sodC* start codon (Fig. 3). In the −10 region, but not in the −35 region, an excellent match to the *E. coli* consensus for RNA polymerase binding was found.

5′ to the *sodC* promoter region and on the strand opposite that encoding *sodC*, an open reading frame was found, running back to the beginning of the region of DNA sequenced, with high similarity (67% over 61 amino acid residues) to the *E. coli* gene *asd*, which encodes aspartate semialdehyde dehydrogenase (22). Intriguingly, we found the same gene order in the genital pathogen *Haemophilus ducreyi* (EMBL accession number X98737), but not in relation to any other bacterial *sodC* that we have cloned (31).

The deduced amino acid sequence of the *A. pleuropneumoniae sodC* product was compared with the sequences of other bacterial Cu,Zn SODs (Fig. 4). As in all other cases in which this has been determined, the N-terminal portion resembles a classic leader peptide, suggesting that the protein is exported. Beyond this, towards the C terminus, the match percentage at the amino acid level with SodC from *Haemophilus parainfluenzae* was 75%. The environment of the catalytically important Cu^{2+} and structurally important Zn^{2+} ions, defined by six histidines and an aspartate that act as ligands to the divalent cations; the arginine residue, which is important in the electrostatic guidance of the superoxide radical anion to the active site; and two cysteines, which are involved in intramolecular bridging (7, 53), are all highly conserved in Cu,Zn SODs, and they are all present in the deduced amino acid sequence of *A. pleuropneumoniae* SodC.

Expression of cloned *sodC* in *E. coli* and the cellular localization of Cu,Zn SOD. The cloned *A. pleuropneumoniae sodC* gene was inactivated by insertional mutagenesis with a gene car-

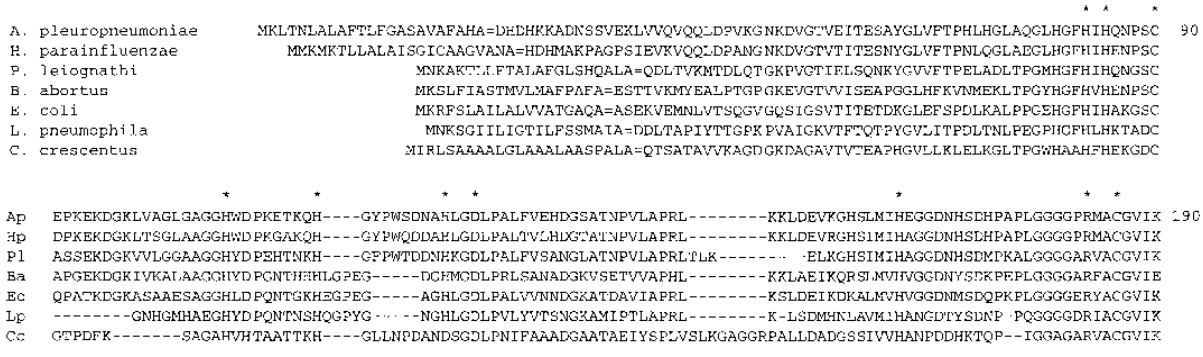

FIG. 4. Amino acid sequence alignment of bacterial Cu,Zn SODs. Residues in the *A. pleuropneumoniae* sequence are numbered at the end of each line. Leader peptides are shown, separated by equal signs from sequence of mature Cu,Zn SOD. Hyphens are used to align residues known to be of structural and functional importance (indicated by asterisks). Non-*A. pleuropneumoniae* sequences are from references 4, 24, 28, and 48 to 50.

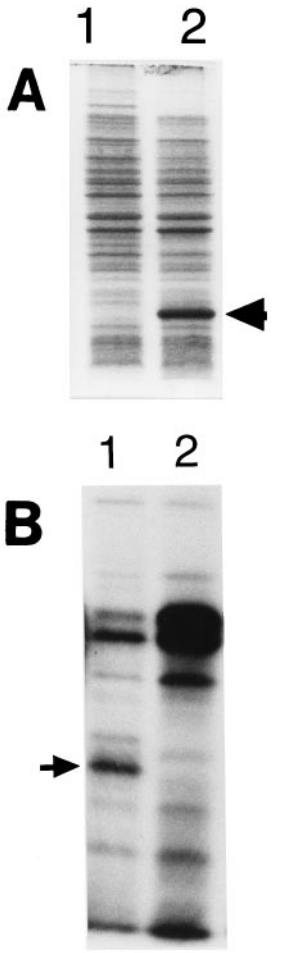

FIG. 5. Proteins expressed in *E. coli* minicells. (A) Visualization with Coomassie brilliant blue R: minicell proteins (10 μg) from *E. coli* AA10 harboring plasmid pBluescript (lane 1) or pJSK157 (lane 2). The arrow indicates abundant 18-kDa *A. pleuropneumoniae* protein expressed from pJSK157. (B) Visualization by autoradiography after 12.5% SDS-PAGE: minicell proteins (10 μg) from *E. coli* AA10 harboring plasmid pJSK157 (lane 1) or pJSK160 (lane 2). The arrow indicates labeled 18-kDa protein.

tridge encoding aminoglycoside phosphotransferase (pJSK160), and gene expression was then studied in the *E. coli* minicell-producing strain AA10. After denaturing PAGE, an approximately 18-kDa protein encoded by the *A. pleuropneumoniae* DNA in pJSK157 (Fig. 5) was found in abundance in the minicell extract, but it was not found in minicells harboring the pBluescript vector alone (Fig. 5A) or pJSK160 (Fig. 5B); the protein's size agrees well with the predicted molecular mass of 17.86 kDa for the processed protein.

E. coli QC779 was used to demonstrate SOD activity expressed from cloned *A. pleuropneumoniae* DNA. Extracts of this strain grown to mid-exponential phase contain no detectable SOD activity under the assay conditions employed in this work. After transformation with pJSK157, pJSK160, or the plasmid vector pBluescript, whole-cell extracts were examined as described previously (Fig. 6). An intense band of SOD activity with the same electrophoretic mobility as the Cu,Zn SOD band of wild-type *A. pleuropneumoniae* was found with pJSK157, while no detectable activity was found in QC779 alone or when this strain was transformed with either of the other plasmids, demonstrating that the *sodC* homolog identified in this work was responsible for the activity observed.

The N-terminal 23-amino-acid sequence of SodC, with a central hydrophobic domain preceding the tripeptide Ala-His-Ala, typical of a leader peptide peptidase cleavage site (38), and characteristically followed by several charged residues, suggests that *A. pleuropneumoniae* Cu,Zn SOD, like other bacterial examples of the enzyme, is extracytoplasmic. After growth of *A. pleuropneumoniae* in liquid culture, different subcellular compartments of this microorganism were examined for their SOD content. No activity was found in the membrane fraction or in a concentrated culture supernatant, suggesting that if exported, Cu,Zn SOD ends up in the periplasm. In experiments using established preparative methods described for *E. coli* (23) and *Haemophilus influenzae* (41), it proved impossible to recover pure periplasmic contents from *A. pleuropneumoniae*; unacceptable levels of contamination with cytoplasmic constituents, as judged by marker enzymes (glucose-6-phosphate dehydrogenase for cytoplasm and alkaline phosphatase for periplasm) resulted (data not shown). The indirect genetic approach of Broome-Smith and Spratt (8) was therefore used to define the putative leader peptide experimentally and to establish a periplasmic location for *E. coli*-expressed *A. pleuropneumoniae* Cu,Zn SOD. A set of nested translational fusions of 5′ *sodC* sequence to the promoterless *bla* gene was generated (Table 1) and used to transform *E. coli* DH5α. In-frame fusions at the predicted signal peptidase cleavage site (fusion 5) and at positions further towards the C terminus (fusions 8 to 10) conferred the ampicillin-resistant phenotype corresponding to the presence of a functional leader peptide. One transformant contained an in-frame fusion encoding a protein with only the first three amino acids of the putative leader peptide present (fusion 1). This transformant could grow on ampicillin-containing medium only when a large inoculum was used, indicating that the version of β-lac-

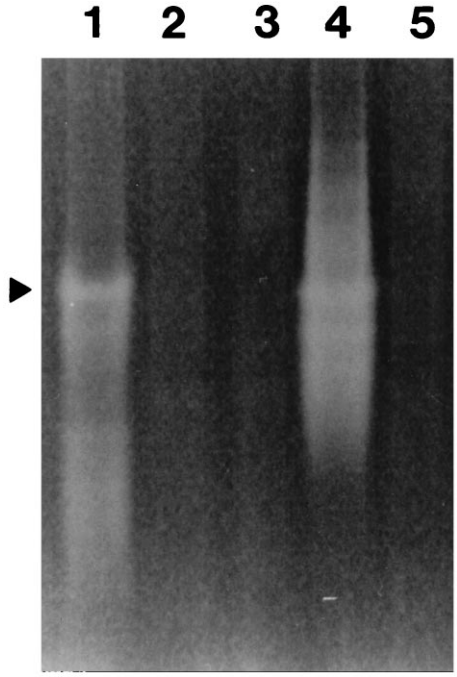

FIG. 6. SOD from *A. pleuropneumoniae* and plasmid constructions in *E. coli*. As in Fig. 2, SOD activity, indicated by the arrowhead, is represented by a decolorized zone. Lanes contain 20 μg protein from extracts of strains as follows: lane 1, *A. pleuropneumoniae* 1421; and lanes 2 to 5, *E. coli* QC779 harboring no plasmids (lane 2) or pBluescript (lane 3), pJSK157 (lane 4), or pJSK160 (lane 5).

TABLE 1. Characteristics of nested translational fusions of 5′ *sodC* sequence to promoterless *bla* gene

Fusion no.	Nucleotide at fusion junction[a]	Frame	Resistance to 100-mg/liter ampicillin demonstrated by:		Inferred location of fusion protein
			Confluent colonies	Single colonies	
1	375	In	Yes	No	Cytoplasm
2	416	Out	No	No	NA[b]
3	422	Out	No	No	NA
4	424	Out	No	No	NA
5	435	In	Yes	Yes	Periplasm
6	436	Out	No	No	NA
7	446	Out	No	No	NA
8	447	In	Yes	Yes	Periplasm
9	459	In	Yes	Yes	Periplasm
10	483	In	Yes	Yes	Periplasm

[a] First nucleotide of *sodC* sequence in the fusion (Fig. 3).
[b] NA, not applicable.

tamase synthesized in these circumstances was not exported to the periplasm. No transformants with out-of-frame fusions (e.g., fusions 2 to 4 and 6 to 7) could grow on ampicillin-containing medium either as single colonies or when a large inoculum was used.

The possibility that Cu,Zn SOD might be exposed during the course of porcine infection was tested serologically. Recombinant Cu,Zn SOD from *E. coli* AA10 minicells was blotted to a nylon membrane and exposed in a Western hybridization procedure to convalescent-phase sera from pigs infected experimentally with *A. pleuropneumoniae* serotype 3 (kindly provided by J. Lida, Royal Veterinary College, London). This demonstrated a strong band of reactivity at just below 20 kDa, consistent with recognition of Cu,Zn SOD (Fig. 7). Control serum obtained from gnotobiotically derived, barrier-sustained piglets (also kindly provided by J. Lida) did not show a similar band of reactivity.

DISCUSSION

In contrast to Fe and Mn SODs, which are well established as being important in protecting the bacterial cytosol from free-radical damage, bacterial Cu,Zn SOD is a comparatively newly described enzyme, in the past considered to be a curiosity to be found only in a few unusual species. Its widespread occurrence has been emphasized in the last few months in a cluster of accounts identifying the enzyme and, in many cases, reporting the cloning of part or all of its gene, *sodC*, in a wide range of organisms occupying widely disparate ecological niches, most recently *Legionella pneumophila*, *Salmonella typhimurium*, and *E. coli* (9, 24, 50). Initial suggestions have been offered as to the part that this form of the enzyme may play in bacterial biology (28, 44, 50), but it remains unresolved whether it has either a peripheral or a central role in pathogenic behavior.

A. pleuropneumoniae has two forms of SOD, characterized here by inhibitor studies as the conventional cytosolic enzyme Mn SOD (SodA) and Cu,Zn SOD (SodC). Qualitative assessment of activity after electrophoretic separation in nondenaturing gels suggests that both are expressed during exponential- and stationary-phase growth. *sodA* expression was found to be susceptible to regulation by O_2 and Fe^{2+} concentration; the enzyme was functionally active when *A. pleuropneumoniae* was grown aerobically with or without Fe restriction, but it was active in anaerobically grown organisms only under iron-restricted conditions. This regulatory pattern differs from that found for *sodA* in the *Haemophilus-Actinobacillus-Pasteurella* group organism *H. influenzae* (29), rather resembling that found in *E. coli* (21). No comparable regulatory effect or variation of expression in relation to phase of growth could be demonstrated for *sodC* in *A. pleuropneumoniae*, in which it therefore appears to differ from the homologous gene recently described in *E. coli*. In this enteric organism, Cu,Zn SOD production is induced during exponential-phase growth, accumulating to the point of ready detection only during stationary phase (5, 24). As a first step in exploring its regulation, the transcriptional start site of *A. pleuropneumoniae sodC* was identified by primer extension. TACAAT in the −10 region is an excellent match to the *E. coli* consensus for RNA polymerase binding, but in contrast, TTATT at −35 is a poor match to the corresponding *E. coli* consensus; if *A. pleuropneumoniae* promoter structures resemble those of *E. coli*, this would suggest that *sodC* is regulated rather than constitutively expressed (39). However, Frey et al. (20) have found that while the *A. pleuropneumoniae* cytotoxin operon *apxI* has −10 and −35 promoter regions similar to the *E. coli* consensus, its expression is modulated by calcium, suggesting that analogy between organisms can be pushed too far. More *A. pleuropneumoniae* promoters will have to be defined before any reliable predictions can be made about transcriptional activity in this organism.

In addition to an apparent difference in gene expression in relation to phase of growth, the thermal instability of the *E. coli* enzyme reported by Benov and Fridovich (5) is not a feature of *A. pleuropneumoniae* Cu,Zn SOD. In contrast to these authors' observation that activity of the *E. coli* enzyme is lost after heating to 55°C for 15 min, *A. pleuropneumoniae* Cu,Zn SOD activity appears undiminished after incubation at

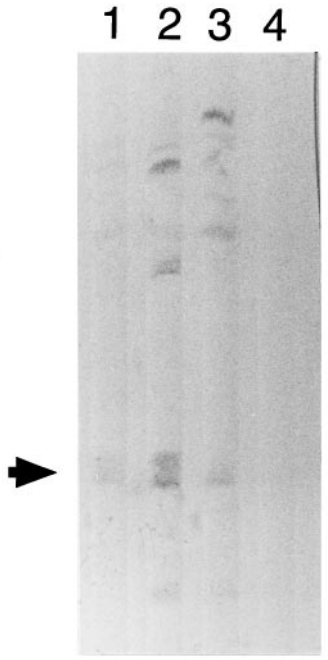

FIG. 7. Western blotting of *A. pleuropneumoniae* Cu,Zn SOD with pig sera. Lanes contain the proteins described in the legend to Fig. 5A, lane 2, blotted in a multichannel device with sera from different pigs convalescent from experimental pleuropneumonia (lanes 1 to 3) or a gnotobiotic piglet (lane 4). The arrow indicates prominent reactivity of sera with an 18-kDa protein in lanes 1 to 3.

65°C for 1 h, a step incorporated into the preparative procedure.

Taken together, these observations suggest that the structure and function of Cu,Zn SOD in *A. pleuropneumoniae*, other members of the *Pasteurellaceae*, and other aerobic colonists and pathogens of the upper and lower respiratory tract (28, 30, 32) may differ in significant respects from the enzyme in enteric organisms (24) or in others found in substantially different environments, such as an intracellular niche (e.g., *Brucella abortus* [4] and *L. pneumophila* [50]) or in fresh water (e.g., *L. pneumophila* [50] and *Caulobacter crescentus* [44]). In particular, the functions proposed for the enzyme in a bacterial colonist of one environment—for example, to protect undefined factors accumulating from oxidative stress in *L. pneumophila* during stationary phase (50) or to dismutate environmentally (or perhaps photolytically) derived O_2^- in *C. crescentus* (44)—not apply in these very different species. Comparison after alignment of all published bacterial Cu,Zn SOD sequences (Fig. 4) identifies many likely structurally significant differences between the *C. crescentus* sequence and the others, as previously noted (7, 30), but also highlights possibly significant differences between the upper respiratory tract colonists and some of the others, e.g., a variant loop domain between conserved ligands His-117, His-126, and Asp-129. While it will be interesting to explore the potential functional importance of residues in such positions by site-directed mutagenesis, the practical limitations of this approach are obvious, and it is likely that real insight into structure-function relationships must await elucidation of the structure of this and other bacterial forms of the enzyme. This should soon be achieved. The *Photobacterium leiognathi* Cu,Zn SOD structure has been elucidated (personal communication cited in reference 50), and the study of the *A. pleuropneumoniae* Cu,Zn SOD is well advanced (crystals have been grown and structural data have been acquired to a resolution of 2.5Å [0.25 nm] [18]).

Like all other examples of bacterial Cu,Zn SODs in which subcellular localization has been established, the *A. pleuropneumoniae* enzyme appears to be periplasmic. While the results of β-lactamase fusion experiments presented here strictly establish only that the Cu,Zn SOD is exported to the periplasm when expressed in *E. coli*, its similarity to all other examples in the possession of a typical leader peptide (Fig. 4) taken with the incomplete fractionation data make the conclusion reasonable. In this site, it has the potential to modulate the course of host-parasite interaction by dismutating exogenous superoxide and offers a potential target for modulation of the course of infection by vaccines.

Survival advantage may be conferred on pathogens by an ability to deal with host cell-derived superoxide. One possibility relevant to *A. pleuropneumoniae* is that the action of phagocytic cells might be abrogated. Liggett and Harrison (34) found that formation of necrotic and hemorrhagic lung lesions induced by *A. pleuropneumoniae* was caused by an intense inflammatory reaction characterized by an exudation of neutrophils. *A. pleuropneumoniae* can induce a respiratory burst in neutrophils (and alveolar macrophages) in vitro before these host cells are killed, an effect mediated by ApxI and ApxII (27). A similar phenomenon in vivo would result in foci of infection in which bacteria are bombarded with reactive oxygen species, including superoxide, with the bactericidal activity to which the *A. pleuropneumoniae* Cu,Zn SOD, like the enzyme in *C. crescentus*, might confer resistance. Another possibility is that the product of superoxide dismutation, hydrogen peroxide, may interfere with mucociliary clearance of bacteria from the upper respiratory tract and so have a role in the early stages of *A. pleuropneumoniae* disease. Dom et al. (14) have shown that *A. pleuropneumoniae* associates with cilia on the respiratory epithelium. Superoxide generated by localized inflammation resulting from superficial infection might, through the action of bacterial Cu,Zn SOD, lead to production of H_2O_2, which in a catalase-negative strain (and *A. pleuropneumoniae* strains are variable in their expression of catalase [10]) can diffuse freely across the bacterial outer membrane to reach a local concentration sufficient to inhibit ciliary beating (17). To move forward with these and other hypotheses on what—if any—role Cu,Zn SOD may play in the virulence of *A. pleuropneumoniae* or any other organism, defined mutants are needed. Until recently, the immense difficulty in their construction in *A. pleuropneumoniae* has completely barred progress. However, thanks to the recent success of Jansen et al. (27) and Mulks and Buysse (37) in defining new tools for site-directed mutagenesis in some serotypes of this organism (although serotype 3 is still problematic), there is at last the prospect of advancement in the assessment of infection of the natural host by *sodC* mutants in comparison to that by the wild type.

Finally, could Cu,Zn SOD be a target for vaccine development? Experiments with *Nocardia asteroides*, which also synthesizes a superoxide dismutase exposed to the external environment, have shown that antibodies which bind and inactivate the enzyme attenuate the course of chronic infection in mice (2), suggesting a potential therapeutic role. However, in this organism the enzyme is probably disposed on the bacterial surface. Western blotting data (Fig. 7) suggest that Cu,Zn SOD is exposed to the immune system in the course of porcine pleuropneumonia. In this it would resemble the homologous enzyme in *B. abortus*, to which a major antibody response is directed (4). However, the protective nature of this response is uncertain, and puzzling, contradictory data have been obtained from studies of the pathogenic behavior of $sodC^+$ and *sodC* mutant isogenic strains of *B. abortus* in their interaction with mice and murine macrophages (33, 54). It is difficult to imagine that an antibody directed against a periplasmic protein has a significant role in preventing disease. Rather, any protective immune response involving Cu,Zn SOD may relate to cell-mediated immunity, as suggested by Tabatabai and Pugh (52). However, conclusions reached with regard to any role for Cu,Zn SOD in the generation of immunity against *B. abortus*, the cause of chronic intraphagocytic infection, may not be applicable to very different pathogens, like *A. pleuropneumoniae*, which causes acute respiratory infection, so further investigation seems justified.

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