Ankyrins are structural proteins in human erythrocytes and brain that bridge the spectrin exoskeleton to the cytoplasmic surface of the plasma membrane (5). They are composed of three domains: (i) an N-terminal membrane-binding domain, (ii) a spectrin-binding domain, and (iii) a C-terminal domain with an apparent regulatory function (5). Integral membrane proteins that associate with ankyrin both in vivo and in vitro include the band III anion exchanger (15), Na⁺/K⁺-ATPase (15), and multiple sodium channels (51, 52). The protein-binding N-terminal domain harbors a series of 33-amino-acid tandem repeats (herein termed ank repeats) that extend over 740 residues. The tandem repeat motif is present in 22 contiguous copies with 30 to 35% identity between the repeats (reference 5 and references therein). Closely related repeats (30 to 35% identity to brain ankyrin) were found in seemingly dissimilar proteins with 30 to 35% identity between the repeats (references 2, 9, 19, and 50 and references therein). Subsequently identified ankyrin-like proteins (ALPs) include transcription factors (e.g., GABP-β and NF-κB), toxins (e.g., black widow spider venom), enzymes (e.g., rat liver-specific glutaminase), and a viral host range factor (Vaccinia hr gene product) (listed in references 5 and 50); a protein-tyrosine kinase in Hydra vulgaris (14); and the Chlorella virus long terminal repeat gene product (GenBank accession no. D14469). Two ALPs were also identified in the higher plant Arabidopsis thaliana (GenBank accession no. M82883), one of which was implicated in membrane transport (GenBank accession no. X62907). So far, more than 150 genes possessing ank repeats have been reported in eukaryotic systems (GenBank search, May 2000). Due to the success in whole genome sequencing, however, genes encoding ankyrin homologs found most recently reside in bacteria.

The first bacterial ALP-encoding gene (phiB), from Serratia liquefaciens, was not recognized as such (21) until Bennett (5) identified an ank repeat consensus sequence (-G-TA/PLM/H-AA-GH---V/A-LL--GAD-N/D-D-). According to various
databases, bacterial ALPs have been identified in several actinobacteria (Streptomyces verticillus [13], Streptomyces argillaceus [U43537], Streptomyces coelicolor cosmid 6D7 [AL133213]), two spirochetes (Treponema pallidum [AE001254] and Deinococcus radiodurans [AE002034 and AE001863]), two cyanobacteria (Anabaena sp. strain PCC 7120 [X95645] and Synechocystis sp. strain PCC 6803 [D09000]), and several prokaryotes (S. liquefaciens [21], Chromatium vinosum [17], Rhizobium leguminosarum [AJ243395], Rickettsia prowazekii [AJ235273], Vibriocholerae [http://www.tigr.org], two Eubacteria species [AE57897 and AF153716], and the four species of fluorescent pseudomonads, i.e., Pseudomonas aeruginosa [U59457], Pseudomonas fluorescens [U83332], Pseudomonas putida KT2440 [http://www.tigr.org], and Pseudomonas syringae [A2][AF133262 and AF133263]). Interestingly, unlike eukaryotic anly or ALPs, bacterial ALPs seem to belong to divergent operons: bloomycin and myrhimycin antibiotic resistance in S. verticillus (13) and S. argillaceus (U43537), respectively; periplasmic flavocytochrome c and cytoplasmic tetraheme cytochrome c in S. vinosum (17); and a catalase with proposed periplasmic and cytoplasmic locations in P. syringae (32) and P. fluorescens (U83328). The anlyerin gene in V. cholerae (http://www.tigr.org) is also downstream of a gene encoding a type I bacterial catalase. A putative open reading frame (ORF) up-stream of a gene encoding a histidinol phosphate aminotransferase, an enzyme required for ethanol tolerance, was found in Acetobacter pasteurianus (DDBJ accession no. D14440) (54). The ALP of S. liquefaciens, whose gene is located downstream of the gene encoding periplasmic phospholipase A1, has a putative regulatory function regarding phospholipase activity (21). Taken together, the bacterial ALP genes are located in close proximity to genes encoding proteins involved in either (i) nutrient acquisition and uptake or (ii) tolerance or resistance to antibiotics, starvation, or oxidative stress.

In this study, we demonstrate the first functional characterization of a bacterial ALP, AnkB, in P. aeruginosa. AnkB was found to be a cytoplasmic membrane-periplasmic protein whose expression is increased upon exposure of bacteria to H2O2. AnkB was also found to be essential for optimal resistance to H2O2, which we believe is in part due to its ability to bind to and stabilize KatB, a type I bacterial catalase.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** All P. aeruginosa and Escherichia coli strains used in this study are listed in Table 1 and were maintained on Luria (L) agar (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl) or M9 minimal medium (6 g of Na2HPO4, 3 g of KH2PO4, 1 g of NH4Cl, 0.5 g of NaCl, 1 mM MgSO4, 7H2O, and 0.2% glucose [per liter]) plates, with each medium solidified with 15 g of Bacto agar per liter. All strains were stored indefinitely at –80°C in a 1:1 suspension of overnight-grown culture and either 25% glycerol or 10% skim milk.

**Growth conditions.** All bacteria were grown from single-colony isolates or overnight cultures in L broth or M9 minimal medium. Liquid cultures were grown at 37°C with shaking at 300 rpm or on a roller wheel at 70 rpm unless otherwise indicated. Culture volumes were 1/10 of the total Erlenmeyer flask volume to ensure proper aeration.

**Cloning and sequence analysis of ankB.** Steps involved in the cloning of the P. aeruginosa PA01 ankB and ralA genes are described in Results. DNA sequencing was performed on both strands using the PRISM Dye Deoxy Terminator Cycle Sequencing Kit and analyzed on an ABI model 373A DNA sequencer. Oligonucleotides for DNA sequencing reactions and PCR analysis were synthesized at the DNA Core Facilities in the Department of Molecular Genetics, at Michigan (http://www.tigr.org) is also downstream of a gene encoding a type I DNA polymerase (U83328). The ankyrin gene in P. syringae (32) and P. fluorescens (DDBJ accession no. D14440) (54).

**Overexpression of recombinant AnkB in E. coli.** Overexpression of recombinant AnkB as a His6-tagged protein in E. coli was performed using the T7 promoter-T7 RNA polymerase system (53). A 0.53-kb fragment containing the ankB gene minus the first 19 codons comprising its signal sequence was amplified using primers (XhoI)-ctcGAGGTCGATGGTGGTGGAGT and (BamH)gga TCGAAGACTAGCCCGCAAGGC (bases in the XhoI and BamHI restriction sites are underlined, and nonmatching bases near the 5' end are in lowercase type). This PCR product was cloned into pcRII (Invitrogen), sequenced, and directionally cloned as an XhoI-BamHI fragment into pET14b (Novagen). The resulting plasmid, pET14b-ankB, allowed the inducible expression of AnkB containing the sequence. A frame His tag, tag A at the C terminus spliced by a linker and untagged cloned as an Nhel-NorI fragment into pET23a linearized with Nhel and NorI.

In the resulting plasmid, pET23- ankB, the AnkB protein was fused in frame to a carboxy-terminal His tag encoded by pET23a. Recombinant plasmids were first selected in E. coli DH5a-MCR and then transformed into E. coli BL21 (DE3), which harbors a single genomic copy of the T7 RNA polymerase gene under control of the lacUV5 promoter. These bacteria were grown in 1 liter of L broth containing ampicillin at 100 mg/ml to an optical density at 600 nm (OD600) of 0.5. At this point, the system of 17 polymerase was induced by the addition of 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG), and the cells were allowed to grow for an additional 3 h at 37°C. Recombinant AnkB proteins were then purified using a nickel-nitrictriatoic acid column as specified by the manufacturer (Qiagen).

**Construction of ankB::blaM fusion plasmid.** For construction of a translational ankB::blaM fusion plasmid, the N-terminal region of ankB was PCR amplified to create a 551-bp fragment harboring the entire ankB gene. This fragment was cut with NruI, and the 162-bp 5' fragment containing the predicted MSD and 3' terminus of the predicted translation start site of ankB was ligated into the Smal site of pKM1 (10). For construction of a translational ankB::phoA fusion plasmid, a 174-bp 5' fragment of the ankB gene containing an AffIII restriction site was first cloned into a T/A vector of pKSL (J) (35). The ankB fragment was then digested from this plasmid and cloned into the NcoI-Smal sites of pEX3 (H. P. Schweizer), pPH7, a broad-host-range phoA-containing plasmid encoding AP (24), first was cut with BsmI, blunted, and then excised with PstI. The resulting phoA gene was then ligated to pEX-ankB that had been previously digested with EcoRI (blunted) and used for directional cloning of ankB in frame with phoA. The resulting construct fused the first 58 amino acids of AnkB with PhoA. Translational fusions of ankB to the lacZ reporter gene were constructed as follows. PCR products were generated with primer (BamH)-gagCTCGAGATGCTGCATGTCACCATG and containing a BamHI site and either primer TACAA GGTGACAGGCGACT (0.16 kb upstream), primer GCGACGGAGCTGATTAG CTGTC (0.47 kb upstream), or primer CTGGGAACTGCGCATGCA (1.9 kb upstream). The PCR products were cloned into pCR1 and directionally ligated as EcoRI-BamHI fragments into pPZ01 linearized with EcoRI and BamHI, yielding pPZ-ankB-600, pPZ-ankB-470, and pPZ-ankB-1900, respectively.

**Construction of isogenic P. aeruginosa ankB and katB ankB mutants.** To construct a ΔankB deletion mutant, the DNA sequences flanking ankB were PCR amplified using primers GACGCGGAGCTGATCATGTT and (KpnI)-gttaCGGTGTCGATCATGCTG (0.408-kb ankB upstream fragment) and primers (XhoI)-gttaATGCTGCCGCGACCATGCATGTT and (HindIII) aaGCTCAACCTGCGCTGCAGCA (0.334-kb ankB downstream fragment). These PCR products were cloned into pCR1, sequenced, and cloned as an EcoRI-KpnI ankB upstream fragment and an XbaI-HindIII ankB downstream fragment on either orientation to a 1.7-kb aac1 (Gm) cartridge in pUC-Gm, yielding pUCankB::Gm. A 2.8-kb PvuII fragment of pUCankB::Gm containing the ΔankB::Gm construct was cloned into the Smal site of the suicide vector pEX100 (46), and the resulting pEX100-ΔankB::Gm was then transformed into E. coli B, which then was selected on a biparental plate mating (16, 37°C, with P. aeruginosa PA01 or with PA01 ΔkatA::Tc (35). The mating mixture was plated on brain heart infusion agar
At this point, organisms were treated with 350 mkatB-ankB strains of P. aeruginosa transfer (RK2); Ap r, ampicillin resistance; Cm r, chloramphenicol resistance; Gm r, gentamicin resistance; Km r, kanamycin resistance. A PAO1 DkatA::Gm mutant was obtained as follows. A PAO1 DkatA::Gm mutant This study

Table 1. Strains and plasmids used in this study

| Strain or plasmid | Genotype or characteristics* | Source or reference |
|-------------------|-----------------------------|---------------------|
| **E. coli strains** |                             |                     |
| HB101             | prosA2 leuB6 thi-1 lacY1 hisD R hsdM recA13 supE44 rpsL20 | H. Boyer Bethesda Research Laboratories |
| DH5α              | lacZAM15 recA1 hsdR17 supE44 Δ(lacZYA argF) |                     |
| SM10              | Km r; mobilizer strain | 48                  |
| BL21(DE3)         | F' dcm ompT hsdS gal λ (DE3); T7 polymerase gene under control of the lacUV5 promoter | 53                  |
| **P. aeruginosa strains** |                             |                     |
| PAO1              | Prototrophic, wound isolate | 28                  |
| PAO1 ΔankB::Gm    | Gm r; deletion of 0.526 kb of the ankB gene | This study |
| PAO1 katA::Tc     | Gm r; katA::Tc mutant | 35                  |
| PAO1 ΔkatA ΔankB  | Gm r; deletion of 0.526 kb of the ankB gene in a PAO1 ΔkatA background | This study |
| PAO1 katB::Gm     | Gm r; katB::Gm mutant | 18                  |
| PAO1 ΔkatB ankB::Gm | Gm r; deletion of 1.75 kb of the katB-ankB locus | This study |
| PAO1 radA         | Gm r; radA::Gm mutant | This study |
| **Plasmids**      |                             |                     |
| pBluescript KS (+/-) | Ap r; extended polylinker pUC derivative | Stratagene |
| pKS/T/A           | Ap r; T/A vector using the EcoRV site for cloning | 35                  |
| pKS-ankB          | Ap r; 174-bp 5' fragment of ankB in EcoRV site | This study |
| pUCP22            | Ap r; broad-host-range extended polylinker pUC derivative | 44                  |
| pUCP22-ankB       | pUCP22 containing a 0.852-kb EcoRI-HindIII fragment with the ankB gene under lac promoter control | This study |
| pET14b            | Expression vector; Ap r | Novagen |
| pET14b-ankB       | pET14b containing ankB with an amino-terminal His tag under T7 promoter control | This study |
| pET23a            | Expression vector; Ap r | Novagen |
| pET23a-ankB       | pET23a containing ankB with a carboxy-terminal His tag under T7 promoter control | This study |
| pEX30             | Ap r; broad-host-range expression vector |                     |
| pEX30-ankB        | Ap r; pEX30 with a 193-bp ApIII-SmaI ankB fragment within the Ncol-SmaI sites | This study |
| pEX30-ankB:phoA   | Ap r; pEX30-ankB with a 2.6-kb phoA-containing fragment of pPHO7 fused to ankB | This study |
| pEX100T           | Ap r; oriT mob sacB gene replacement vector | 46                  |
| pEX100T-ΔankB::Gm | pEX100T carrying a 2.8-kb ΔankB::Gm gene study replacement construct |                     |
| pEX100T-ΔkatB ankB::Gm | pEX100T carrying a 2.6-kb ΔkatB ankB::Gm gene study replacement construct | This study |
| pKM1              | Ap r; broad-host-range blaM β-lactamase fusion plasmid | 10                  |
| pPHO7             | Ap r; broad-host-range phoA alkaline phosphatase fusion plasmid | 24                  |
| pPZ30             | Ap r; broad-host-range lacZ-based promoter probe vector | 43                  |
| pPZ-ankB:1900     | pPZ30 containing a 1.9-kb fragment of the katB-ankB region including the katB promoter | This study |
| pPZ-ankB:470      | pPZ30 containing a 0.47-kb fragment of the ankB upstream region | This study |
| pPZ-ankB:160      | pPZ30 containing a 0.16-kb fragment of the ankB upstream region | This study |
| pUCGM             | Gm r; pUC19 plus 850-bp gusA cassette | 45                  |

* Abbreviations used for genetic markers were as described by Holloway et al. (29), mob, mobilization site (ColEl); Tra”, conjugative phenotype; oriT, origin of transfer (RK2); Ap r, ampicillin resistance; Cm r, chloramphenicol resistance; Gm r, gentamicin resistance; Km r, kanamycin resistance.

containing gentamycin (75 µg ml⁻¹) and irgasan (Ciba-Geigy) (50 µg ml⁻¹) as a counterselective agent. Several colonies were grown to late logarithmic phase in L broth, and serial dilutions were spread onto L agar containing gentamycin (75 µg ml⁻¹) and sucrose (5%). Chromosomal DNA from individual colonies was evaluated for deletion of the ankB gene by PCR and Southern blot analysis (data not shown). A PAO1 ΔkatB ankB deletion mutant was obtained as follows. A 2.7-kb fragment containing the katB-ankB region was PCR amplified using the primers CTGGAAGCTTGGCTAATGCG and GCCTCAACCCTGCCAGC and cloned into pCRII. A 1.75-kb SfiII fragment containing most of the katB and ankB genes was excised and replaced by a 1.2-kb Gm-I cartridge by blunt-end ligation after filling in with Klenow enzyme. A 2.6-kb ProII fragment of the resulting plasmid, pCRII-ΔkatB ankB::Gm, was cloned into the SfiI site of pEX100T, yielding the donor plasmid pEX100T-ΔkatB ankB::Gm for a mating as described above.

**Cell fractionation: periplasm, cytoplasm, and cytoplasmic membrane.** Bacteria were grown aerobically in 1 liter of L broth at 37°C until the O₂, reached 0.6. At this point, organisms were treated with 350 µM paraquat (Sigma) for 1 h to stimulate katB-ankB transcription prior to harvesting the bacteria by centrifugation at 10,000 × g for 10 min. The pellet was washed twice in ice-cold 10 mM Tris-HCl-30 mM MgCl₂ (pH 7.3) (Tris-Mg) and resuspended in 1/25 the volume of the same buffer. Chloroform (15 µl/ml of buffer) was then added, and the cells were incubated on ice for an additional 15 min followed by dilution with an additional 1 ml of buffer. The bacteria were pelleted by centrifugation at 10,000 × g for 10 min at 4°C, and the supernatant was further subjected to centrifugation at 150,000 × g for 2 h at 4°C to remove potential contaminating membranes. Finally, the periplasmic preparation was stored on ice. The bacteria were washed again, resuspended in 5 ml of Tris-Mg, and disrupted by sonication with a Heat Systems-Ultrasonioc (Farmingdale, N.Y.) model W-225 sonicator equipped with microtip at output setting 5 at 4°C. Cell debris and membrane fractions were clarified by centrifugation at 35,000 × g for 1 h at 4°C. The supernatant was designated the cytoplasmic fraction.

For preparation of cytoplasmic membrane proteins, the above-described growth conditions were employed. The pellet of paraquat-stimulated bacteria was resuspended in 10 ml of 10 mM Tris-HCl (pH 7.5) (T buffer), containing 20% sucrose, treated with 0.5 µg of DNase I (Gibco) and RNase (Sigma) per ml, and incubated for 15 min at 22°C with periodic agitation. The suspension was
placed on ice for 20 min, followed by two passages through a French pressure cell at 1,200 lb/in² at 4°C. The cell debris was removed by centrifugation at 5,500 × g for 10 min at 4°C. The supernatant containing the membranes was subjected to a two-stage sucrose gradient centrifugation. The first stage involved layering 10 ml of membranes on 14 ml of 50% sucrose and 14 ml of 70% sucrose in T buffer. The membranes were then separated by centrifugation in an SW28 swinging-bucket rotor at 130,000 × g for 17 h at 4°C. Cytoplasmic membranes (the top red band) and outer membranes (the bottom white band) were collected by dropwise collection and diluted to 7 ml in cold T buffer–20% sucrose. The negatively stained bacteria were carbon- and Formvar-coated 200-mesh TEM grid, stained for 15 s with 2% uranyl acetate, and blotted dry. The JASCO J-710 spectropolarimeter calibrated with (wt/vol) uranyl acetate, and the results were expressed as international units with an extinction coefficient of 43.6 mM. One unit of activity is that which degrades 1 mM p-nitrophenol through hydrolysis of 1 mM p-nitrophenyl phosphate (Sigma) (20). AP activity in bacterial colonies on L-agar plates containing 10 mM potassium phosphate (pH 7.0) to inhibit endogenous AP activity was monitored using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (40 g/ml) in the medium. Sucinate dehydrogenase, a cytoplasmic membrane marker, was assayed by monitoring the production of o-nitrophenyl-β-D-galactopyranoside.

![FIG. 1. Gene map of the ∼2-kb insert of pSM1 containing the katB, ankB, and radA genes. The functions of the gene products are also given. We have previously shown that OxyR, a 34-kDa transactivator, responds to H₂O₂ by activating katB and ankB (39). T, 3-amino-acid inverted repeat that could represent the transcriptional terminator for the katB-ankB operon.](Image)

RESULTS

Sequence analysis downstream of the *P. aeruginosa* katB gene: identification of ankB, encoding an ALP, and radA, a DNA repair protein. In a previous study, we cloned and characterized the *katB* gene, encoding a 228-kDa tetrameric catalase (11). The *katB* gene was recently found to be under the control of the global transactivator OxyR (39), and its transcription is markedly induced upon exposure to H₂O₂ or the redox-cycling agent paraquat (11). DNA sequence analysis downstream of the *katB* locus revealed a small, 549-bp ORF (Fig. 1). This ORF, ankB, is predicted to encode a protein of 183 amino acids with a monomeric molecular mass of 19,360 Da and a pI of 5.55. The deduced amino acid sequence demonstrated the highest similarity with genes harboring ank repeats in other bacteria (13, 17, 21, 32) and various ankyrin or ALP genes in eukaryotes (5) (see below). When we sequenced further downstream of the *ankB* locus, we discovered a large inverted repeat spanning 33 bp (Fig. 1). We then identified a gene downstream of the inverted repeat that was 70% identical to the *radA* gene of *E. coli* (49). The *radA* gene in *E. coli* encodes a protein that repairs DNA damaged (alkylated) by gamma irradiation. However, because virtually nothing is known of the function of ALPs in bacteria, we chose to focus our efforts on the functional characterization of *P. aeruginosa* AnkB.

Amino acid comparison of AnkB with other bacterial ALPs. Ankyrins are proteins that are characterized by 33-amino-acid
ank repeats that are thought to represent an ancient motif that has evolved to allow for functional diversity without compromising specificity (5). Each of the five bacterial ALPs in Fig. 2 possess the 33-amino-acid tandem, nonidentical ank repeats. These repeats are based upon the consensus ank repeat motif put forth by Bennett using the erythrocyte ank repeat consensus sequence (5). Proposed signal sequences are indicated by a double underline. The conserved ank repeat sequences for erythrocyte ankyrin are given below the selected bacterial ALP sequences. RBC, erythrocyte consensus ank repeat sequence.

| ALP   | Sequence | Comment |
|-------|----------|---------|
| CvAnkA | MDPIRTSRLAPPLLASSALLAGSSV | - |
| PaAnkB | N--D--G-TPLH-AA--GH----V--LL--GAD- | - |
| PsAnkB | N--D--G-TPLH-AA--GH----V--LL--GAD- | - |
| CvAnkA | DAPHQFGKTALMFAVEGDDLETVALLSRGADEV | - |
| SlPhlB | N--D--G-TPLH-AA--GH----V--LL--GAD- | - |
| SvAnkA | WILAGWLLAALAOQ---------GEVHG | - |
| PaAnkB | CVQDAGKYGTLAYYHGHGGAVQLELLEAGADP | - |
| PsAnkB | NIRDQGTYGLIAYYHGDQVRLIDAGAP | - |
| CvAnkA | EADKQGATALLIAADQCGQAVETALGLAGQ | - |
| SlPhlB | AAALGNSALLTIAHLNDLYRLLLAEGAMN | - |
| SvAnkA | TDARDWSSAIDRAGRQCVVARRALLAGADP | - |
| RBC   | N--D--G-TPLH-AA--GH----V--LL--GAD- | - |
| PaAnkB | DQNGAQOTAAMYAALPKREELLQASARGADL | - |
| PsAnkB | NLTNAGOTAAMYAALPKRTVEYKLTDDGADL | - |
| CvAnkA | NQDRKGGTALMEAVADNHELIDRLIAAGART | - |
| SlPhlB | TLDSDLRGPPLHIAAKNKRTWCCCRCPGPH | - |
| SvAnkA | ASGPAAARPRTWEPYCRYPVVLARLYWPREE | - |
| RBC   | N--D--G-TPLH-AA--GH----V--LL--GAD- | - |
| CvAnkA | P* | - |
| SlPhlB | VFDATVPEWRDFCERDELAFAPDDFDLVP | - |

FIG. 2. Alignment of the deduced amino acids from genes coding for bacterial ankyrins PaAnkB (P. aeruginosa; accession no. U59457), PsAnkB (P. syringae; U16026), CvAnkA (C. violaceum; L13419), SlPhlB (S. liquefaciens; P18954), and SvAnkA (S. verticillus; L26954). The 33-amino-acid tandem repeats (underlined) were revealed by using the Ank motif of conserved residues (boldface) as identified by Bennett using the erythrocyte ank repeat consensus sequence (5). Proposed signal sequences are indicated by a double underline. The conserved ank repeat sequences for erythrocyte ankyrin are given below the selected bacterial ALP sequences. RBC, erythrocyte consensus ank repeat sequence.
The ALPs of S. verticillus (SvAnkA and SvAnkB) possessed only two 33-amino-acid ank repeats. All other identified bacterial ALPs contain a putative cleavage site following an MSD either in the middle or close to the C terminus, or they do not contain a hydrophobic region long enough to incorporate into the membrane.

AnkB is a periplasmic protein: AnkB-β-lactamase and AnkB-alkaline phosphatase protein fusion analysis. Using the membrane topology program TopPred 3.0, it was predicted that the cytoplasmic N terminus of P. aeruginosa AnkB (3 amino acids) is followed by a 20-amino-acid stretch that is predicted to be the hydrophobic inner-MSD (for a von Heijne schematic, see Fig. 3A) (TMRGWILAGLLLAALAAQAEVH), with the remaining portion of the protein (being highly hydrophilic) predicted to reside in the periplasm. The ALPs of other selected gram-negative bacteria, including C. vinosum, S. liquefaciens, and other Pseudomonas species, are also predicted to span the cytoplasmic membrane (Fig. 3A). To test whether the predicted cytoplasmic membrane location was correct, we constructed AnkB-BlaM and AnkB-PhoA protein fusions linking the C terminus of AnkB with both reporters (Fig. 3B). E. coli and P. aeruginosa harboring an ankB-BlaM fusion plasmid were resistant to ampicillin (E. coli) or carbenicillin (P. aeruginosa). Organisms expressing AnkB-PhoA were found to hydrolyze the alkaline phosphatase substrate BCIP in L-agar plates. IM, inner membrane; OM, outer membrane. (C) AP activity in cellular fractions of P. aeruginosa ankB harboring pEX30-ankB-phoA. Bar 1, cytoplasm; bar 2, periplasm; bar 3, cytoplasmic membrane; bar 4, outer membrane.

FIG. 3. Cellular localization of AnkB in P. aeruginosa. (A) Predicted cytoplasmic membrane organization of P. aeruginosa AnkB bacterial ankyrin-like proteins from P. syringae, P. fluorescens, S. liquefaciens, and C. vinosum based upon the positive-inside-rule algorithm developed by von Heijne (57). For the P. aeruginosa AnkB protein, the large number 1 indicates the predicted single MSD. N, N terminus; C, C terminus; LL, loop length; KR, number of lysine and arginine residues; KR Diff, positive charge difference. (B) Schematic diagram of AnkB–β-lactamase and AnkB-PhoA protein fusions in both E. coli and P. aeruginosa PA01. In both cases, organisms expressing AnkB–β-lactamase were resistant to ampicillin (E. coli) or carbenicillin (P. aeruginosa). Organisms expressing AnkB-PhoA were found to hydrolyze the alkaline phosphatase substrate BCIP in L-agar plates. IM, inner membrane; OM, outer membrane. (C) AP activity in cellular fractions of P. aeruginosa ankB harboring pEX30-ankB-phoA. Bar 1, cytoplasm; bar 2, periplasm; bar 3, cytoplasmic membrane; bar 4, outer membrane.

Overexpression of AnkB in E. coli: AnkB secondary structure is predominantly α-helical. To obtain some preliminary structural analysis of AnkB, we overexpressed and purified two recombinant AnkB proteins with N-terminal (pET23a-AnkB) and C-terminal (pET14b) His6-tagged fusions in E. coli BL21(λDE3) without their predicted MSDs. Figure 4A demonstrates purified pET23a-AnkB. Gorina and Pavletich revealed that the secondary structure of an ank repeat in protein S3BP2, which binds to the p53 tumor suppressor, consists of an L-shaped structure with a β-turn and 2 α-helices (22). Circular dichroism spectropolarimetric analysis of recombinant AnkB-23a suggested that AnkB is ~60 to 70% α-helical (Fig. 4B). This structure is consistent with the 66% α-helical nature of the ank repeats of the S3BP2 protein (22).
Polycistronic nature of katB and ankB: regulation by H$_2$O$_2$. To determine if katB and ankB are part of a small operon, RNase protection assays were performed. Figure 5 demonstrates that transcription of both katB and ankB is stimulated by paraquat in a concentration-dependent fashion. The transcriptional start site was found to be a G 227 bp upstream of the katB start codon. Furthermore, transcription of katB-ankB is dependent upon OxyR, since no katB and very little ankB transcript could be detected in an oxyR mutant. These results were also confirmed using ankB::lacZ reporter fusion studies (data not shown).

Phenotypes of a P. aeruginosa ankB mutant. (i) Normal cell size and shape. Humans with hereditary spherocytosis (HS) suffer from an ankyrin deficiency. Erythrocytes from individuals with HS lack deformability and stability (40) and are unable to pass through capillaries, resulting in hemolytic anemia and hypersensitivity to osmotic lysis. This disorder has been reproduced in nb/nb (normoblastosis, ankyrin-deficient) mice (7), which have a severe hemolytic anemia throughout life (41). In these settings, it is predicted that a loss of ankyrin from the lipid bilayer causes a reduction in the critical surface area/volume ratio, leading to a shift in the morphology of erythrocytes from discoidal to spherical. Thus, there is a definitive structural role for ankysins in erythrocytes. In contrast to the case for HS erythrocytes, the ultrastructure of wild-type and ankB mutant bacteria was observed by TEM and no significant differences in overall cell shape were found (Fig. 6). However, the ankB mutant produced more membrane vesicles than wild-type bacteria (Fig. 6B). This implies that a fundamental difference exists between the surfaces of the wild type and the ankB mutant and that their ability to package periplasmic constituents in natural membrane vesicles has changed (i.e., the ankB mutant has more packaging potential). The difference in quantities in membrane vesicles has been confirmed by thin sections (6).

(ii) Enhanced sensitivity to H$_2$O$_2$. Because we found that ankB is part of a small operon with katB, we postulated that its gene product may play a role in resistance to H$_2$O$_2$. To test this hypothesis, the wild type and ankB, katB, and katB ankB mutants were screened for H$_2$O$_2$ sensitivity. As shown in Fig. 7, an ankB mutant was only slightly more susceptible to H$_2$O$_2$ than wild-type organisms (bars 2). However, when the mutant was pretreated with a sublethal dose of H$_2$O$_2$, which activates the katB-ankB operon, sensitivity was increased dramatically (bars 3 versus bars 1 [shaded bars]). Bars 6 demonstrate that provision of a plasmid that allows for constitutive expression of ankB restored wild-type resistance regardless of H$_2$O$_2$ pretreatment. The katB (bars 3) and katB ankB (bars 4) mutants were equally susceptible to H$_2$O$_2$, and more so than the ankB mutant. Interestingly, provision of ankB alone to the katB ankB mutant dramatically helped these organisms resist H$_2$O$_2$ (bars 8 relative to bars 7).

(iii) Absence of AnkB decreases KatB activity. Because of the close proximity of katB and ankB, their organization in a small operon, and the enhanced H$_2$O$_2$ sensitivity of the ankB mutant, we postulated that AnkB could play a role in KatB function. To test this hypothesis, the catalase isozyme profiles of several mutant organisms were examined. Fig. 8 (left panel) shows that the KatB activities of the ankB mutant (lane 2) and a katA ankB (lane 5) mutant are significantly reduced relative to that of wild-type bacteria (lane 1). When transcription of katB was stimulated by the addition of paraquat, there was a robust increase in KatB activity in the wild type (right panel, lane 1) and especially in the katA mutant (right panel, lane 3). The catalase activity band produced in the katA mutant that migrated to the same R$_f$ as KatA could be another, previ-
FIG. 5. RNase protection assays indicate that katB and ankB comprise an operon and are regulated by OxyR. Riboprobes specific for the katB promoter (katB rp) and for the katB-ankB overlapping region (katB-ankB rp) were used to detect the corresponding transcripts in P. aeruginosa PAO1 or oxyR mutant total RNA isolated during the exponential growth phase in aerobic M9 minimal medium. Paraquat (PQ) was added to final concentrations of 10 and 100 μM 1 h prior to harvest as indicated. Also shown are the digested probes in the absence of any P. aeruginosa RNA as a control. A DNA sequencing reaction was run in parallel and served as a size marker. Numbers on the left are base pairs.
ously undiscovered catalase in *P. aeruginosa*, although analysis of the recently completed *P. aeruginosa* genome suggested otherwise (data not shown). We now know that this paraquat-inducible catalase band is one of the alkyl hydroperoxide reductases, AhpA, that possesses weak catalase activity (39). The KatB activities of the *ankB* mutant (Fig. 8, right panel, lane 2) and a *katA ankB* mutant (right panel, lane 5) were still reduced relative to that of wild-type bacteria (right panel, lane 1).

(iv) **Quantitative effect of AnkB on KatB activity.** To quantify the effect of AnkB on KatB activity, we examined catalase activity in *katA* and *katA ankB* mutants that had been treated with paraquat in stationary-phase culture, where the only catalase activity that can be detected and quantified spectrophotometrically is KatB. As shown in Fig. 9, the KatB activity of a paraquat-treated *katA* mutant is ~21.5 U/mg (bar 1). Provision of *ankB* in trans to the *katA* mutant had no effect on KatB activity (bar 2). Interestingly, KatB activity in a *katA ankB* mutant was reduced fourfold (bar 3) relative to that of the *katA* mutant and was fully complemented by providing *ankB* in trans (bar 4). There was no observation of the AhpA activity band under these conditions.

**DISCUSSION**

The major catalase gene of *P. aeruginosa*, *katA*, encoding a constitutive 170-kDa heteromultimer, is positively regulated by iron (35) and maximally expressed in stationary phase, in part through a process of cell-to-cell communication known as quorum sensing (27). Thus, it is not surprising that KatA contributes significant protection against H$_2$O$_2$ in both planktonic and biofilm cultures (26, 27, 35).

In contrast to *katA*, which is minimally responsive to H$_2$O$_2$, we found in this study that the *katB-ankB* operon is transcribed dramatically in its presence and requires the global transactivator OxyR (25, 39). When we discovered *ankB* downstream of *katB*, we immediately classified its gene product as an ALP because it possessed the characteristic 33-amino-acid ank re-
ankB plus pUCP22; 6,
plates. Filter paper disks (7 mm) impregnated with 8.8 M H2O2 were placed on
100-fold in 7 ml of M9F 0.6% top agarose kept at 37°C and poured onto M9F
bars) relative to control bacteria (open bars). The suspensions were diluted
aerobic incubation at 37°C. Bars: 1, PAO1; 2, 
the top agar surface. Zones of growth inhibition were measured after a 24-h
involved in protein-protein interactions (5). Bacterial ALPs
their importance as cytosolic H2O2 scavengers (28). ALPs contained in the
p. aeruginosa
ankyrin. Examination of transmission electron micrographs of the
mutant confirmed this assumption (Fig. 6). Due to the polycistronic nature of
and the conservation of
organism in the pseudomonads and other proteobacteria such as V. cholerae, we postulated that AnkB might belong to a group of evolutionarily related proteins with a novel, unrecognized function(s), one of which could contribute toward protection against H2O2. Indeed, AnkB appears to play a role in the response of P. aeruginosa to H2O2, because an ankB mutant was more sensitive to it than wild-type organisms (Fig. 7). Furthermore, the enhanced H2O2 sensitivity of an isogenic katB ankB mutant did not change when only katB was provided in trans (39). Although unproven, the nearly fourfold reduction in KatB activity in the ankB mutant suggests that there could be a physical interaction between the two proteins. We found KatB activity in the cytoplasm, periplasm, and cytoplasmic membrane (data not shown). Because AnkB is a cytosolic membrane protein whose bitopic integration into the inner membrane ultimately causes its ank repeat domain to reside in the periplasm, we postulated that one function of AnkB may be to bind KatB near inner membrane targets that are sensitive to H2O2 (e.g., F1F0-ATPase [55]). H2O2 must first enter a protein channel leading to the heme catalytic site of the catalase molecule (47). Without entering this channel, the H2O2 is free to damage cellular components, especially sensitive respiratory chain components and DNA (16). Thus, AnkB may position or anchor KatB so that its H2O2 channel is in the optimal orientation for H2O2 entry. Alternatively, AnkB may serve to stabilize KatB, allowing it to persist longer and function better upon exposure of bacteria to H2O2.

An alternative hypothesis is that AnkB may reinforce the cytoplasmic membrane and prevent crippling of the proton motive force. Microscopic oxygen bubbles could be produced upon H2O2 degradation, thereby increasing cellular turgor pressure. Although unexplained, such cell swelling has been shown in E. coli (38) and in mitochondria treated with H2O2 or agents that generate it (30). Upon H2O2 degradation by cata-
lase, oxygen gas nuclei could be stabilized and even grow in the bacteria at hydrophobic sites. With the production of gas at a rate that saturates the cytoplasm, gas bubbles could readily appear, be stabilized by lipid and/or protein adsorption, and take up considerable volume inside a cell, thereby creating a turgor pressure (31). Thus, AnkB could serve to stabilize the inner membrane against swelling due to the mounting intracellular pressure built up by $H_2O_2$ degradation. Both hypotheses are being tested experimentally.

ACKNOWLEDGMENTS

The first three authors contributed equally toward completion of this work.

This work was supported by Public Health Service grants AI-40541 (to D.J.H.) and DK-50749 (to K.M.B.) and Cystic Fibrosis grant HASSET98PO (to D.J.H.).

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