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) were found in seemingly dissimilar brain ankyrin (products of proteins of lower and higher eukaryotes that regulate the cell identity to brain ankyrin) were found in seemingly dissimilar closely related repeats (30 to 35% copies with 30 to 35% identity between the repeats (reference 5 and references therein). Closely related repeats (30 to 35%) are part of a small operon whose transcription is induced dramatically by H\(_2\)O\(_2\), and controlled by the global transactivator OxyR. Interestingly, unlike the spherical nature of ankyrin-deficient erythrocytes, the cellular morphology of an ankB mutant was identical to that of wild-type bacteria, yet the mutant produced more membrane vesicles. The mutant also exhibited a fourfold reduction in KatB activity and increased sensitivity to H\(_2\)O\(_2\), phenotypes that could be complemented in trans by a plasmid constitutively expressing ankB. Our results suggest that AnkB may form an antioxidant scaffolding with KatB in the periplasm at the cytoplasmic membrane, thus providing a protective lattice work for optimal H\(_2\)O\(_2\) detoxification.

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) were found in seemingly dissimilar proteins of lower and higher eukaryotes that regulate the cell cycle in yeast (e.g., products of cdc10 and SWI6) and are involved in intercellular signaling during development and cell differentiation of Caenorhabditis elegans (products of lin-12, glp-1, and fem-1), Drosophila (Notch), or Xenopus (Xotch) (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
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 *rpoA* 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 in the DNA Core Facilities in the Department of Molecular Genetics, Biotechnology and Microbiology at the University of Cincinnati College of Medicine or in the Department of Microbiology at the University of Colorado Health Sciences Center. Sequence analyses were performed using either MacVector 6.5.1 (Eastman Chemical Co., New Haven, Conn.), Sequencher 3.1 (Gene Codes Corp., Ann Arbor, Mich.), or Gene Runner (Hastings Software, Inc.) software. Amplified DNA fragments were purified using either the Bluestrip program provided by the National Center for Biotechnology Information (1) or the Align Plus 3 Global Alignment Program (Sci-Ed Software, Durham, N.C.). Potential membrane-spanning domains (MSDs) of AnkB and other bacterial ankyrin-like proteins were determined using TOP-PRED II (57) or the SOSUI program (http://www.tuat.ac.jp/~mitaku/adv_sosui/submit.html).

Manipulation of recombinant DNA and RNA. Plasmid DNA was transformed into *E. coli* DH5α-MCR (Gibco-BRL, Gaithersburg, Md.) or SM10 (48). S-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (40 μg/ml) was routinely added to agar medium to detect the presence of insert DNA. Restriction endonucleases, alkaline phosphatase (AP), Klenow fragment, T4 DNA polymerase, and T4 DNA ligase were used as specified by the vendor (Gibco-BRL). Plasmid DNA was isolated using miniprep kits from Qiagen. DNA fragments used for cloning or in the construction of radiolabeled probes were recovered from agarose gels using SeaPlaque low-melting-point agarose (FMC BioProducts, Rockland, Maine) or with the GeneClean II kit (BIO 101, Inc., La Jolla, Calif.). RNA was isolated by the hot-phenol method and analyzed by RNase protection assays as described in detail elsewhere (3). Radiolabeled riboprobes were generated from cloned DNA fragments (Table 1) using an in vitro run-off transcription system (Promega), and excess probe was hybridized to 20 μg of total RNA.

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)-ctgAggGgTCgGTTGCTGGGAgGTG (and BamHI)-gga TcCAGATACTGCCGAGCCG (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 pCRIII (Invitrogen), sequenced, and directionally cloned as an XhoI-BamHI fragment into pET14b (Novagen). The resulting plasmid, pET14b-ankB, allowed T7-inducible expression of AnkB containing a frameshift tag His, tag at the C terminus and was used to generate constructs containing the *ankB* gene minus the first 12 codons and lacking the last two codons of the amplified gene (Ndel-GCTgAgGgCGtGCTGGGAgGTG and (BamHI)-GGgGcCGaGCAGCTGGTcGCCG) and (NdeI)-GCTgAgGgCGtGCTGGGAgGTG and (BamHI)-GGgGcCGaGCAGCTGGTcGCCG). The resulting plasmid, pET23a linearized with Ndel and NotI. 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* DH5α-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 μg/ml to an optical density at 600 nm (OD600) of 0.5. At this point, the synthesized 17-mers were induced by the addition of 0.5 mM β-mercaptoethanol (β-ME) and the cultures were grown to an OD600 of 0.80 (at 80°C in a 1.1 suspension of overnight-grown culture and 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.

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containing gentamicin (75 μg ml⁻¹) and irgasan (Ciba-Geigy) (50 μg ml⁻¹) as a counteseletive agent. Several colonies were grown to late logarithmic phase in L broth, and serial dilutions were spread onto L agar containing gentamicin (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 ΔankB deletion mutant was obtained as follows. A 2.7-kb fragment containing the katB-ankB region was PCR amplified using the primers CTTGGAACGTCGCAAGTCACTGAC and GGCTCAGAAAGCTGACGCGAAG and cloned into pCRII. A 1.75-kb SfiI fragment containing most of the katB and ankB genes was excised and replaced by a 1.2-kb Gm² cartridge by blunt-end ligation after filling in the ends with Klenow enzyme. A 2.6-kb Blunt-end ligation after filling in the ends with Klenow enzyme. A 2.6-kb fragment of the resulting plasmid, pCRII-ΔkatB ΔankB, was cloned into the Smal site of pEX100T, yielding the donor plasmid pEX100T::Gm mutant 18.

**TABLE 1. Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or characteristics</th>
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<td>HB101</td>
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<td>H. Boyer</td>
</tr>
<tr>
<td>DH5a</td>
<td>F− lacZAM15 recA1 hisD17 supE44 Δ(lacZYA-argF)</td>
<td>Bethesda Research Laboratories</td>
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<td>SM10</td>
<td>Km²; mobilizer strain</td>
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</tr>
<tr>
<td>BL21(DE3)</td>
<td>F− dcmompT hisSD galA (DE3); T7 polymerase gene under control of the lacUV5 promoter</td>
<td>53</td>
</tr>
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<td><em>E. coli</em> strains</td>
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<td></td>
</tr>
<tr>
<td>PAO1 ΔkatB ΔankB</td>
<td>Gm²; deletion of 0.526 kb of the ankB gene</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 ΔkatA ΔankB</td>
<td>Gm²; deletion of 0.526 kb of the ankB gene in a PAO1 ΔkatA background</td>
<td>This study</td>
</tr>
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<td>Gm²; katA::Tc mutant</td>
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</tr>
<tr>
<td>PAO1 katB ΔankB</td>
<td>Gm²; katB::Gm mutant</td>
<td>18</td>
</tr>
<tr>
<td>PAO1 katB ΔankB</td>
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<td>This study</td>
</tr>
<tr>
<td>PAO1 radA</td>
<td>Gm²; radA::Gm mutant</td>
<td>This study</td>
</tr>
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<td><strong>Plasmids</strong></td>
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<td>Ap²; extended polyn linker pUC derivative</td>
<td>Stratagene</td>
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<td>pKS/ankB</td>
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<td>This study</td>
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<td>pUCP22</td>
<td>Ap²; broad-host-range extended polyn linker pUC derivative</td>
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<td>pPHO7</td>
<td>Ap²; broad-host-range phoA alkaline phosphatase fusion plasmid</td>
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<td>pPZ30</td>
<td>Ap²; broad-host-range lacZ-based promoter probe vector</td>
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<td>pUCGM</td>
<td>Gm²; pUC19 plus S80-b aacC1 cassette</td>
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</tr>
</tbody>
</table>

*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); Ap², ampicillin resistance; Cm², chloramphenicol resistance; Gm², gentamicin resistance; Km², kanamycin resistance.
shown that OxyR, a 34-kDa transactivator, responds to H\textsubscript{2}O\textsubscript{2} by activating the transcription of katB and ankB (39), T. T-33-amino-acid inverted repeat that could represent the transcriptional terminator for the katB-ankB operon.

placed on ice for 20 min, followed by two passages through a French pressure cell at 1,200 lb/in\textsuperscript{2} at 4°C. The cell debris was removed by centrifugation at 5,500 x 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 x 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 second centrifugation stage involved layering the membrane fractions on 9 ml of 52% sucrose, 9 ml of 58% sucrose, 9 ml of 64% sucrose, and 3 ml of 70% sucrose, each in T buffer. The membranes were again separated by centrifugation in an SW28 swinging bucket rotor at 130,000 x g for 17 h at 4°C. The purified cytoplasmic and outer membrane fractions were concentrated by dropwise collection and dialyzed exhaustively against T buffer for 17 h at 4°C. The purity of each cellular fraction was gauged by measuring enzymatic activities specific for the cytoplasm, periplasm, and inner membrane. Stripping of AP activity from cytoplasmic membrane preparations was accomplished by incubating the membrane preparations with 1 mM H\textsubscript{2}O\textsubscript{2} for 10 min at 4°C. The membranes were then separated by centrifugation in an SW28 swinging bucket rotor at 130,000 x g for 17 h at 4°C. The pellet was solubilized in 2% Triton X-100 in T buffer and assayed for AP activity as described below. Glucose-6-phosphate dehydrogenase, a cytoplasmic marker, was assayed by monitoring the reduction of dichlorophenol-indophenol as previously described (12). Catalase activity was determined using an extinction coefficient of 43.6 mM. One unit of activity is that which degrades 1 \mu M H\textsubscript{2}O\textsubscript{2} per min at 23°C. Nondenaturing polyacrylamide gels (5%) were stained for catalase activity according to the method of Wayne and Diaz (58).

**RESULTS**

**Sequence analysis downstream of the** *P. aeruginosa* katB, ankB, and radA** genes: 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 H\textsubscript{2}O\textsubscript{2} 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 possesses the 33-amino-acid tandem, nonidentical ank repeats. These repeats are based upon the consensus ank repeat motif put forth by Bennett (36). AnkB (PaAnkB), not surprisingly, is similar to AnkF (PsAnkF, in the related plant pathogenic species R. leguminosarum; accession no. AJ243305) and PfAnkB in that they are followed by a proteolytic proline-threonine-box (hinge (32)). Interestingly, PaAnkB and PfAnkB, but not PsAnkF and PsAnkB, possess an RGD motif, which, in eukaryotes, is responsible for physical interaction with integrins, structural proteins that play a role in the homing and action of immune cells. Other bacterial ALPs (some of which are not listed in Fig. 2) from V. cholerae (http://www.tigr.org/) (VcAnkB), S. argillaceus (U43537) (SaAnk), C. viinosum (17) (CvAnkA), and S. liquefaciens (21) (SlPhlB, for phospholipase A1-related ALP) possessed longer putative signal sequences (25 to 28 residues), while the ALP from R. leguminosarum (AJ243305) (R1Ank) contained a very short signal peptide. These ALPs from V. cholerae and C. viinosum are similar to the ALP from R. leguminosarum, which possesses a very short signal peptide.

FIG. 2. Alignment of the deduced amino acids from genes coding for bacterial ankyrins PaAnkB (P. aeruginosa; accession no. U59457), PsAnkF (P. syringae; U16026), CvAnkA (C. viinosum; 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.

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N-terminal MSD of 12 residues. 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) (TMRGWILAGLL-LAALAAQAEMV), 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* PAO1. 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(xDE3) without their predicted MSDs. Figure 4A demonstrates purified pET23a-AnkB. Gorina and Pavletich revealed that the secondary structure of an ank repeat in protein 53BP2, 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 53BP2 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 ankyrins 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 differences 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 2). 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. 4. Overexpression (A) and circular dichroism analysis (B) of recombinant AnkB proteins. (A) E. coli BL21(DE3) harboring pET23-ankB-480 was grown aerobically in L broth to mid-logarithmic phase and treated with 1 mM IPTG for 3 h at 37°C. After Ni$^{2+}$-nitrilotriacetic acid purification, purified protein was separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and the gel was stained with Coomassie blue R-250. Lane 1, molecular mass standard; lane 2, 15 gm l$^{-1}$ in 10 mM sodium phosphate (pH 7.0) at 23°C.
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-
Bacterial ALPs differ dramatically from their eukaryotic counterparts in that they contain only the ank repeats, which we believe to be involved in protein-protein interactions (5). Bacterial ALPs should, therefore, lack the structural role in the cell, similar to the function of prototypical erythrocyte spectrin-binding ankyrin. Examination of transmission electron micrographs of the ankB mutant confirmed this assumption (Fig. 6). Due to the polycistronic nature of katB-ankB and the conservation of this operon 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 H$_2$O$_2$. Indeed, AnkB appears to play a role in the response of *P. aeruginosa* to H$_2$O$_2$, because an ankB mutant was more sensitive to it than wild-type organisms (Fig. 7). Furthermore, the enhanced H$_2$O$_2$ 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 cytoplasmic 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 H$_2$O$_2$ (e.g., F$_1$F$_0$-ATPase [55]). H$_2$O$_2$ must first enter a protein channel leading to the heme catalytic site of the catalase molecule (47). Without entering this channel, the H$_2$O$_2$ is free to damage cellular components, especially sensitive respiratory chain components and DNA (16). Thus, AnkB may position or anchor KatB so that its H$_2$O$_2$ channel is in the optimal orientation for H$_2$O$_2$ entry. Alternatively, AnkB may serve to stabilize KatB, allowing it to persist longer and function better upon exposure of bacteria to H$_2$O$_2$.

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 H$_2$O$_2$ degradation, thereby increasing cellular turgor pressure. Although unexplained, such cell swelling has been shown in *E. coli* (38) and in mitochondria treated with H$_2$O$_2$ or agents that generate it (30). Upon H$_2$O$_2$ degradation by catalase activity and activity gel staining were monitored in cell extracts. Bars: 1, katA plus pUCP22; 2, katA plus pankB; 3, katA ankB plus pUCP22; 4, katA ankB plus pankB. Error bars indicate standard errors of the means.

**FIG. 8. Absence of AnkB causes a decrease in KatB activity.** Bacteria were grown aerobically overnight in L-broth medium containing 0.35 mM paraquat at 37°C. Catalase activity and activity gel staining were monitored in cell extracts. Bars: 1, katA plus pUCP22; 2, katA plus pankB; 3, katA ankB plus pUCP22; 4, katA ankB plus pankB. Error bars indicate standard errors of the means.

**FIG. 9. Quantitative effect of AnkB on KatB activity.** Bacteria were grown aerobically overnight in M9F medium containing 0.35 mM paraquat at 37°C. Catalase activity and activity gel staining were monitored in cell extracts. Bars: 1, katA plus pUCP22; 2, katA plus pankB; 3, katA ankB plus pUCP22; 4, katA ankB plus pankB. Error bars indicate standard errors of the means.
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 by H₂O₂ 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.).

REFERENCES

4. Beveridge, T. J. 2000. The anion exchanger and Na⁺/H⁺ antiporter (to D.J.H.) and DK-50749 (to K.M.B.) and Cystic Fibrosis grant work.