Identification of Brucella abortus OxyR and Its Role in Control of Catalase Expression

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We report the cloning and sequencing of the Brucella abortus oxyR homolog and provide evidence that the transcription product of this gene binds to the B. abortus catalase promoter region. A gene replacement/deletion Brucella oxyR mutant exhibits increased sensitivity to prolonged exposure to \( \text{H}_2\text{O}_2 \) and is unable to adapt to \( \text{H}_2\text{O}_2 \) in the environment.

Nearly all aerobic organisms express one or more hydroperoxidases that detoxify metabolically generated hydrogen peroxide. Organisms that survive phagocytosis must also avoid being killed by active oxygen intermediates produced by the phagocyte. This is generally accomplished by some combination of direct detoxification of peroxides and superoxide and/or suppression of phagocyte activity. Brucella abortus has a single hydroperoxidase, a true catalase, which is expressed in the periplasm (17). In previous studies (10, 11), it was shown that Brucella catalase is regulated at the transcriptional level.

In a preliminary study (10), primer extension indicated that transcription of the catalase gene begins at nucleotide 221 on the published catalase gene sequence (17). Immediately upstream are reasonable −10 and −35 promoter sequences, TAATTG and TGGAGA, respectively. In Fig. 1 we show that this region also contains a four-part sequence motif characteristic of OxyR binding sites (24).

To determine if proteins bind specifically to the catalase promoter region, we performed a gel mobility shift assay with \( B. \) abortus soluble extract and a 350-bp PCR product containing sequences upstream of the catalase gene. The procedure followed that given in Current Protocols in Molecular Biology (2) modified for \( B. \) abortus

To confirm this result, we performed a Southwestern blot. In this procedure, proteins separated by gel electrophoresis are transferred to a membrane and probed with a labeled DNA fragment. The detailed procedure is described in Current Protocols in Molecular Biology (2). Two \( B. \) abortus polypeptide bands were revealed (Fig. 2A) when the blot was incubated with the same labeled probe used for the gel shift experiment. The higher-molecular-mass band (35 kDa) did not appear on blots incubated with the labeled probe plus a 10-fold excess of unlabeled probe (Fig. 2B). This result suggests specific binding by a 35-kDa \( B. \) abortus polypeptide.

The region upstream of the catalase promoter was sequenced revealing an open reading frame with 43% nucleotide sequence identity with \( E. \) coli oxyR. The gene is oriented so that the promoters of the catalase and \( B. \) abortus oxyR genes most likely overlap. The predicted start codons are separated by 169 nucleotides, and potential promoter sequences for both genes are present. The open reading frame predicts a 317-amino-acid polypeptide with a molecular mass of 35 kDa.

To confirm a regulatory function for this open reading frame, an insertion inactivation \( B. \) abortus oxyR mutant was made by gene replacement (25). In this construction, 9 bp defined by two closely spaced \( \text{Hind} \)III sites near the middle of the gene were replaced by a 1.4-kb DNA fragment containing the kanamycin resistance gene from \( Tn5 \). Both ends of the replacement were confirmed by DNA sequencing. The plasmid was introduced into \( B. \) abortus by electroporation, and kanamycin-resistant, ampicillin-sensitive colonies were selected. Successful double recombinants were confirmed by PCR and Southern blotted (data not shown). The mutant selected for further study was designated S19oxyR−.

Sensitivity of S19oxyR− to hydrogen peroxide was tested by a halo assay. A 5-mm-diameter filter paper disc was placed in the center of a \( B. \) abortus-seeded agar plate. Ten microliters of 30% \( \text{H}_2\text{O}_2 \) was loaded onto each disc. Following incubation at 37°C, the diameter of the clear halo surrounding each disc was measured to the nearest millimeter. The halo for wild-type S19 \( B. \) abortus averaged 36 mm, whereas the halo for the oxyR mutant averaged 57 mm. This indicates that the mutant exhibits increased sensitivity to hydrogen peroxide.

To test whether the mutant is able to adapt to hydrogen peroxide, wild-type and S19oxyR− strains were exposed to \( 30\% \text{H}_2\text{O}_2 \), and the diameter of the clear halo surrounding each disc was measured to the nearest millimeter. The halo for wild-type S19 \( B. \) abortus averaged 36 mm, whereas the halo for the oxyR mutant averaged 57 mm. This indicates that the mutant exhibits increased sensitivity to hydrogen peroxide.

<table>
<thead>
<tr>
<th>Consensus</th>
<th>ATAG</th>
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FIG. 1. Comparison of known OxyR binding sites to sequences in the \( B. \) abortus catalase promoter region. The numbers between the sequences indicate the number of nucleotides between the motif sites. Underlined nucleotides match the consensus sequence (24) listed at the top. The fractions of matched nucleotides are at the right. oxyRS, \( E. \) coli; katG, \( E. \) coli; ahpC, \( S. \) enterica serovar Typhimurium; gorA, \( E. \) coli; Mu mom, bacteriophage Mu; bacat, \( B. \) abortus catalase gene; boxyR, \( B. \) abortus oxyR.
peroxidase (HPI) in E. coli katE catalyzed by OxyR and is unrelated by sequence to the product of B. abortus catalase (HPII-like) in E. coli katG (17). This suggests a close relationship between these bacteria and diverse eukaryotic organisms observed today.

The oxyR gene family is widespread among prokaryotes (4, 6, 13, 15, 18) and has been extensively studied in E. coli and Salmonella (1, 3, 5, 21, 22, 24), where it plays an important role in adaptation to hydrogen peroxide. Nearly all known oxyR genes share overlapping promoters with other genes (8, 16). In each situation the paired gene typically is different.

The single B. abortus catalase gene is homologous to E. coli katE (17). E. coli has a second enzyme, the peroxidase (encoded by katG) HPI, with catalase activity. This enzyme is regulated by OxyR and is unrelated by sequence to the product of katE or to Brucella catalase. Thus, oxyR regulates periplasmic catalase (HP II-like) in B. abortus and periplasmic catalase-peroxidase (HPI) in E. coli (7, 12, 17). This suggests a conserved OxyR function of protecting the cell from external hydrogen peroxide even when the specific genes regulated are different.

Data in the literature indicate that regulated catalase activity plays an important role in the life of intracellular pathogens. If the true catalases arose in the eukaryotic lineage and were later “expropriated” by various prokaryotes, as suggested by phylogenetic analysis (14), then it is interesting to contemplate the process by which a foreign gene was introduced into a bacterial cell and subsequently became fully integrated into the metabolic machinery. It is not known when the transfer to the B. abortus lineage occurred or from what organism. Catalase activity is reported for other species of Brucella, so the event probably predates the genus. The known catalase sequence most closely resembling the Brucella sequence is reported from Sinorhizobium melloti (9, 19). The high sequence similarity implies that the primary transfer event occurred prior to the divergence of Sinorhizobium and Brucella. Many members of this branch of the alpha subdivision of the Proteobacteria (20, 25) live in close relationship with eukaryotic cells, either as pathogens or as symbionts. We speculate that the acquisition of catalase enzymatic activity and its subsequent regulation may have played a key role in the evolution of the various close relationships between these bacteria and diverse eukaryotic organisms observed today.

**Nucleotide sequence accession number.** The sequence determined in this study has been assigned GenBank accession no. U081286.

### Table 1. Adaptation experiment in liquid culture

<table>
<thead>
<tr>
<th>Exposure time (min)</th>
<th>% Survival in 30 mM H2O2 of:</th>
<th>Wild type with:</th>
<th>S19oxyR− with:</th>
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**FIG. 2.** Southwestern blot. B. abortus soluble extract was separated on a sodium dodecyl sulfate–12% (wt/vol) polyacrylamide gel and transferred to nitrocellulose. Membranes were incubated with 5 × 10⁵ cpm of 32P-labeled catalase promoter probe without (A) or with (B) specific competitor DNA. Arrow, approximate size of the larger band.

**REFERENCES**