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Brucella abortus Catalase Is a Periplasmic Protein Lacking a Standard Signal Sequence

ZHENGYU SHA,[1] THOMAS J. STABEL,[2] AND JOHN E. MAYFIELD[1]*

Molecular, Cellular and Developmental Biology Program, Department of Zoology and Genetics, Iowa State University, Ames, Iowa 50011,[1] and National Animal Disease Center, USDA Agricultural Research Service, Ames, Iowa 50010[2]

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A periplasmic catalase has been purified and cloned from *Brucella abortus*. The functional enzyme is a tetramer with a subunit molecular weight of 55,000. All evidence indicates that a typical N-terminal signal sequence is not associated with the export of this protein to the periplasm.

We previously reported a method which successfully separates soluble periplasmic enzymes from soluble cytoplasmic enzymes for *Brucella abortus* and used this procedure to demonstrate that the *Brucella* Cu-Zn superoxide dismutase is a periplasmic enzyme (16). The procedure substitutes Zwittergent 3-16 for EDTA in the spheroplasting procedure commonly used with *Escherichia coli* (3). Other, previously reported methods for releasing periplasmic proteins from gram-negative bacteria, such as osmotic shock, are ineffective with *B. abortus* (16). Figure 1 shows the native gel electrophoresis of *B. abortus* whole-cell lysate and periplasmic and cytoplasmic extracts stained for catalase activity as described by Heimberger and Eisenstark (6). *B. abortus* exhibits a single band of activity in both whole-cell and periplasmic extracts and no activity in cytoplasmic extracts. A quantitative assay (1, 20) of the catalase activity in the different fractions of a typical preparation yielded 23 U/10^9 cells for total cell lysate, 17 U/10^9 cells for periplasmic extract, and no detectable activity in the cytoplasmic extract. This fractionation pattern contrasts with that of malate dehydrogenase, a soluble cytoplasmic protein. Malate dehydrogenase activity is found exclusively in the cytoplasmic extract (16). The fractionation data indicate that the *B. abortus* soluble catalase activity results from a single periplasmic enzyme. The enzyme does not react with *o*-dianisidine, suggesting it does not have significant peroxidase activity (21).

The catalase activity present in *B. abortus* periplasmic extracts was purified by liquid chromatography with successive elution from DEAE Sepharose CL-6B (50 mM potassium phosphate buffer, pH 8.5, eluted with a 0 to 0.5 M linear NaCl gradient), Sephacryl S-300 HR (5 mM potassium phosphate, pH 8.5), hydroxyapatite (1 mM potassium phosphate buffer, pH 6.8, eluted with a 1 to 400 mM linear potassium phosphate, pH 6.8, gradient), and Sephacryl S-200 HR (5 mM potassium phosphate buffer, pH 8.5). At all stages of purification, only a single peak of activity was observed. The final purified activity exhibited only a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The apparent molecular weight of the purified enzyme subunit was 59,000. Gel filtration of the native protein on Sephacryl S-300 HR yielded a native molecular weight of 240,000, suggesting that the native enzyme is a tetramer, a result which is consistent with the properties of most other reported catalases (11, 15). The visible absorption spectrum of the purified enzyme in 50 mM potassium phosphate buffer, pH 6.8, exhibits a typical catalase spectrum with a Soret peak at 407 nm and minor peaks at 501 and 628 nm (14, 15).

Two thousand plaques of a previously described *B. abortus* 19 genomic lambda library (9) were replica plated and screened with rabbit antiserum prepared against the purified enzyme. Six positive plaques were identified. DNAs from these lambda clones were isolated and digested with various restriction enzymes. The resultant restriction maps each exhibited a common region of overlap. A 9-kb *Kpn*I fragment from one of these clones containing the region of overlap was subcloned into pUC119 (18). This subclone was named pCAT9. Nondenaturing gel electrophoresis showed expression of a new catalase activity in *E. coli* TG1. pCAT9 was further subcloned, and approximately 2 kb containing the catalase coding region was sequenced by the chain termination method (13), using a combination of subclones and synthetic oligonucleotide primers. All sequences were determined with an Applied Biosystems (Foster City, Calif.) DNA sequencer and have been confirmed by a minimum of three independent runs in each direction. The sequence (Fig. 2) reveals one long open reading frame which originates at one of three closely spaced, in-frame ATG sequences. Only the middle ATG sequence has an apparent ribosome binding sequence, AGGA, located 7 bp upstream. Starting with the second ATG, the functional open reading frame would encode a protein of 498 amino acids with a calculated molecular weight of 55,000. This agrees well with the weight of 59,000 calculated from SDS-PAGE. Potential −35 and −10 promoter sequences are found upstream from the open reading frame at positions 208 and 232, respectively. A potential rho-independent transcription terminator is located 29 bp downstream of the TAG stop codon. These observations suggest an operon consisting of a single gene.

Examination of the predicted amino acid sequence both visually and with the computer algorithm of von Heijne (19) as adapted by Robert Colgrove (4) revealed no typical N-terminal signal sequence. The N-terminal amino acid sequence of the enzyme purified from the *B. abortus* periplasmic extract was determined according to the manufacturer's instructions by binding to a ProSpin polyvinylidene difluoride membrane, washing five times with high-performance-liquid chromatography (HPLC)-grade water, and loading onto an Applied Biosystems model 477A protein sequencer. Phenylthiohydantoin amino acids were separated by HPLC and detected with an Applied Biosystems model 120A analyzer. The resultant sequence was TD?PIMTTSA. This sequence is consistent with the interpretation that the second translation initiation codon

* Corresponding author. Mailing address: 2106 Molecular Biology Bldg., Iowa State University, Ames, IA 50011. Phone: (515) 294-6847. Fax: (515) 294-0345. Electronic mail address: jemayf@iastate.edu.

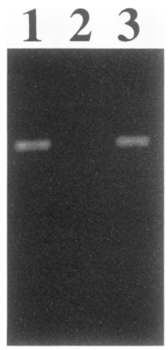

FIG. 1. Nondenaturing 6% PAGE of *B. abortus* strain 19 extracts stained to detect catalase activity. The gel buffer pH is 8.8. Lane 1, total bacterial lysate. Lane 2, cytoplasmic extract. Lane 3, periplasmic extract. The image of the stained gel was captured with a Hamamatsu C2400 CCD camera and was cropped with Adobe Photoshop, and lane markers were added with Aldus Freehand. The final figure was then printed on a Textronix Phaser IISD dye sublimation printer.

is used in vivo, assuming the terminal methionine was removed and that no N-terminal peptide was cleaved from the protein during export to the periplasm in *B. abortus.*

There are two distinct bacterial enzyme families with catalase activity which have been described. These are characterized as true catalases (EC 1.11.1.6) and catalase-peroxidases (EC 1.11.1.7). The gene families are not sequence related. True catalases are found widely among both prokaryotes and eukaryotes, while catalase-peroxidases are restricted to bacteria. *E. coli* and closely related enteric bacteria express both a true catalase, HPII, which is reported to be cytoplasmic, and a catalase-peroxidase, HPI, which is reported to be periplasmic (6). The predicted amino acid sequence of the *B. abortus* catalase exhibits 49% sequence identity and 67% sequence similarity with human kidney catalase (2), 41% identity and 61% similarity with *E. coli* HPII, and little or no homology with members of the bacterial catalase-peroxidase gene family, including *E. coli* HPI (17).

The evidence indicating export to the periplasm in the absence of a cleaved signal sequence is very unusual and is reminiscent of the import of catalase into the eukaryotic peroxisome (5, 8, 10). In a wide variety of eukaryotes, the signal required for the ATP-dependent import of catalase into the peroxisome is the amino acid sequence (Ser/Ala)-(Lys/Arg/His)-(Leu/Ile), the so-called SKL sequence located near the C terminus of the polypeptide. This sequence motif is not found near the C terminus of the *Brucella* sequence, but if a similar mechanism is active in *Brucella* species, extensive sequence divergence may have rendered the signal unrecognizable. *Pseudomonas syringae* has also been reported to express a periplasmic true catalase (7), but this enzyme apparently possesses a typical N-terminal signal sequence (6a).

The lack of evidence for more than one *B. abortus* catalase activity is also interesting. Presumably, the bacterium requires a mechanism for removal of cytoplasmic hydrogen peroxide when growing aerobically, and we can only demonstrate the

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   1 GCGGTGCACCCGCGATTGCCTCCATTTCTGCAATCTGGGCGGAGAGTGCTGGTTGTGAAACATGGGCCAGTTCGGCGGCCTTGCGAAAATGCAGGGTAGAGGCAAGCGCATCGAAATAGC

                                                                                                   -35                 -10
 121 GTATTTGACGGACTGTAAACATGATAGGAAAATCTTATCAATAATAACAAAAAAAGCAATTGGAGATTATTATTGGAATAGGTCTAATTGAGACAAATGGTTTCGCCCCGGTGTGATGTT

 241 TCCCCGTCGCCTCACGCCTTCGGTAGCGCGAATAGTAGTTTAAAACGATGAAATTGCAGGAGGATTATTCCATGACAGATCGCCCGATAATGACGACGAGTGCAGGCGCTCCGATACCGG
                                                                            M  T  D  R  P  I  M  T  T  S  A  G  A  P  I  P

 361 ACAACCAGAACTCGCTGACTGCCGGAGAGCGCGGTCCCATCCTGATGCAGGATTATCAGCTTATTGAAAAGCTCTCGCACCAGAACCGTGAGCGCATTCCCGAACGTGCGGTACATGCCA
     D  N  Q  N  S  L  T  A  G  E  R  G  P  I  L  M  Q  D  Y  Q  L  I  E  K  L  S  H  Q  N  R  E  R  I  P  E  R  A  V  H  A

 481 AGGGCTGGGGTGCTTACGGCACGCTGACCATCACTGGCGATATTTCCAGATATACCAAAGCCAAGGTTTTGCAGCCGGGTGCGCAGACGCCGATGCTGGCCCGCTTTTCCACCGTTGCCG
     K  G  W  G  A  Y  G  T  L  T  I  T  G  D  I  S  R  Y  T  K  A  K  V  L  Q  P  G  A  Q  T  P  M  L  A  R  F  S  T  V  A

 601 GCGAGCTGGGCGCTGCCGATGCCGAACGTGACGTGCGCGGCTTTGCCCTCAAATTCTACACGCAAGAAGGCAATTGGGATCTGGTTGGCAACAATACGCCGGTCTTCTTCGTGCGTGATC
     G  E  L  G  A  A  D  A  E  R  D  V  R  G  F  A  L  K  F  Y  T  Q  E  G  N  W  D  L  V  G  N  N  T  P  V  F  F  V  R  D

 721 CGCTGAAGTTCCCGGATTTCATCCATACCCAGAAGCGCCATCCCAGGACCCATCTGCGTTCCGCAACCGCCATGTGGGATTTCTGGTCGCTGTCGCCGGAAAGCCTGCATCAGGTCACGA
     P  L  K  F  P  D  F  I  H  T  Q  K  R  H  P  R  T  H  L  R  S  A  T  A  M  W  D  F  W  S  L  S  P  E  S  L  H  Q  V  T

 841 TCCTGATGTCGGATCGCGGCCTGCCGACCGATGTGCGCCATATCAACGGTTACGGCTCGCACACCTATTCGTTCTGGAACGATGCCGGTGAGCGTTACTGGGTGAAGTTCCACTTCAAGA
     I  L  M  S  D  R  G  L  P  T  D  V  R  H  I  N  G  Y  G  S  H  T  Y  S  F  W  N  D  A  G  E  R  Y  W  V  K  F  H  F  K

 961 CCATGCAGGGCCACAAGCACTGGACCAATGCGGAAGCCGAGCAGGTGATCGGCCGTACCCGTGAATCCACGCAGGAAGATCTGTTCTCGGCCATCGAGAACGGCGAATTCCCGAAGTGGA
     T  M  Q  G  H  K  H  W  T  N  A  E  A  E  Q  V  I  G  R  T  R  E  S  T  Q  E  D  L  F  S  A  I  E  N  G  E  F  P  K  W

1081 AGGTGCAGGTTCAGATCATGCCGGAACTCGACGCCGACAAGACGCCGTATAACCCCTTCGATCTTACCAAAGTATGGCCCCATGCCGACTATCCGCCGATCGATATTGGCGTGATGGAAC
     K  V  Q  V  Q  I  M  P  E  L  D  A  D  K  T  P  Y  N  P  F  D  L  T  K  V  W  P  H  A  D  Y  P  P  I  D  I  G  V  M  E

1201 TGAACCGCAACCCGGAAAACTATTTCACCGAGGTGGAGAATGCCGCCTTCTCGCCATCGAATATCGTTCCCGGCATCGGCTTTTCGCCGGACAAGATGCTCCAGGCGCGTATCTTCTCCT
     L  N  R  N  P  E  N  Y  F  T  E  V  E  N  A  A  F  S  P  S  N  I  V  P  G  I  G  F  S  P  D  K  M  L  Q  A  R  I  F  S

1321 ATGCGGACGCGCATCGCCATCGCCTCGGCACGCATTACGAAAGCATTCCGGTCAACCAGCCGAAATGCCCGGTGCATCACTATCATCGCGACGGTCAGATGAATGTCTATGGCGGCATCA
     Y  A  D  A  H  R  H  R  L  G  T  H  Y  E  S  I  P  V  N  Q  P  K  C  P  V  H  H  Y  H  R  D  G  Q  M  N  V  Y  G  G  I

1441 AGACCGGCAATCCGGATGCCTATTACGAGCCGAATTCGTTCAATGGTCCGGTCGAACAGCCATCTGCCAAGGAGCCGCCGCTGTGCATCTCCGGCAATGCGGATCGCTACAACCACCGTA
     K  T  G  N  P  D  A  Y  Y  E  P  N  S  F  N  G  P  V  E  Q  P  S  A  K  E  P  P  L  C  I  S  G  N  A  D  R  Y  N  H  R

1561 TCGGCAATGACGATTATTCACAGCCGCGCGCGCTGTTCAATCTGTTCGATGCAGCGCAGAAGCAGCGCCTGTTCTCCAATATCGCGGCTGCGATGAAGGGCGTTCCAGGCTTTATCGTTG
     I  G  N  D  D  Y  S  Q  P  R  A  L  F  N  L  F  D  A  A  Q  K  Q  R  L  F  S  N  I  A  A  A  M  K  G  V  P  G  F  I  V

1681 AGCGTCAGCTTGGCCATTTCAAGTTGATCCACCCGGAATATGAAGCCGGTGTGCGCAAGGCGCTCAAGGATGCCCATGGTTATGACGCCAACACAATTGCCTTGAACGAAAAAATTACTG
     E  R  Q  L  G  H  F  K  L  I  H  P  E  Y  E  A  G  V  R  K  A  L  K  D  A  H  G  Y  D  A  N  T  I  A  L  N  E  K  I  T

                                                 --------->     <---------
1801 CAGCGGAATAGTATATATATAATACAGTTATATTTAATATTGGTCGCCGGTTCATCCGGCGACTACTTTGTGTCTTGAATTTATATTTACCGCCTATACTGTAAGTTGTATTCTGCGTCA
     A  A  E  *

1921 GAATCAGCGGGAGGAAGCGATATGATGCTGGCGATTTTTATATTCATGACAATTGCCATG
```

FIG. 2. Nucleotide and amino acid sequences of the *B. abortus* 19 catalase gene with flanking sequences. Various potentially important sequences are underlined. These include possible −35 and −10 promoter sequences, a ribosome binding sequence, alternative ATG start codons, the TAG stop codon (*), and a downstream inverted repeat (dashed arrows). The numbers on the left indicate nucleotide coordinates.

presence of periplasmic catalase activity. Perhaps *B. abortus* relies on an unidentified peroxidase to detoxify hydrogen peroxide in the cytoplasm. The situation may be analogous to that for mammalian mitochondria which rely on glutathione peroxidase rather than catalase to remove H_2O_2 produced by respiration (12).

Nucleotide sequence accession number. The GenBank accession number for the catalase coding region sequence determined in this study is U11439.

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