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The NADPH Quinone Reductase MdaB Confers Oxidative Stress Resistance to *Helicobacter hepaticus*

Yang Hong, Ge Wang, and Robert J. Maier*

Department of Microbiology, University of Georgia, Athens, Georgia 30602, USA

Abstract

An *mdaB* mutant strain in a quinone reductase (MdaB) of *Helicobacter hepaticus* type strain ATCC51449 was constructed by insertional mutagenesis, and the MdaB protein was purified and compared to the *Helicobacter pylori* enzyme. While wild type *H. hepaticus* cells could tolerate 6% O₂ for growth, the *mdaB* strain was clearly inhibited at this oxygen level. Disruption of the gene downstream of *mdaB* (HH1473) did not affect the oxidative stress phenotype of the strain. The *mdaB* mutant was also more sensitive to oxidative stress reagents such as H₂O₂, cumene hydroperoxide, t-butyl hydroperoxide, and paraquat. All *H. hepaticus mdaB* strains isolated constitutively up-expressed another oxidative stress combating enzyme, superoxide dismutase; this is in contrast to *H. pylori mdaB* strains. *H. hepaticus* MdaB is a flavoprotein catalyzing quinone reduction using a two-electron transfer mechanism from NAD(P)H to quinone. The *H. hepaticus* enzyme specific activity was far less than for the *H. pylori* enzyme purified in the same manner.

Keywords

mdaB; quinone reductase; *Helicobacter hepaticus*; oxidative stress

1. Introduction

Helicobacter hepaticus, a microaerophilic bacterium, was first isolated from liver tissue of A/JCr mouse colonies at the U.S. National Cancer Institute [1]. A high percentage of the A/JCr mice infected with this bacterium developed chronic hepatitis and subsequently developed liver cancer. However, the primary habitat of *H. hepaticus* is the intestinal tract and the bacterium is linked to inflammatory bowel disease in immunocompromised mice. Recent studies showed that *H. hepaticus* infected mice might serve as an ideal animal model to study inflammation related carcinogenesis in humans [2,3]. The annotated genome sequence of *H. hepaticus* has been published [4].

Oxidative stress resistance is a key mechanism enabling pathogenic bacteria to survive in the host and establish persistence. Extensive studies have been conducted on oxidative stress resistance in various bacterial species including *Helicobacter pylori* [5-7], a close relative of *H. hepaticus*. An NADPH quinone reductase in *H. pylori* was identified as an oxidative stress-combating enzyme [6]. The MdaB was first identified as a “modulator of drug activity” in *E.*

*Corresponding author, Robert J. Maier, Department of Microbiology, 815 Biological Sciences Building, University of Georgia, Athens, GA 30602, Tel: +1-706-542-2323, Fax: +1-706-542-2674, Email: rmaier@uga.edu

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coli [8], and subsequently was shown to be an NADPH-specific quinone reductase that catalyzes the two-electron reduction of quinone to quinols [9]. The *H. hepaticus* genome sequence [4] reveals an MdaB homolog (HH1472) that has a significantly high percentage of identity to *E. coli* MdaB (54%) and *H. pylori* MdaB (66%), suggesting similar enzymatic properties and functions. In this study, we investigate the role of the MdaB homolog of *H. hepaticus* in oxidative stress resistance by characterizing an *mdaB* mutant. The *H. hepaticus* MdaB protein was purified; some of its enzymatic properties were characterized and compared to that of the same enzyme for *H. pylori*.

2. Results

2.1. *mdaB* mutant construction and protein expression profiles

An *mdaB* mutant was constructed to investigate the physiological role of this gene in combating oxidative stress. The *mdaB* gene of the type strain ATCC51449 was disrupted by insertion of an erythromycin resistance cassette inside the *mdaB* gene followed by transformation into *H. hepaticus* cells. PCR analysis verified the correct insertion of the antibiotic cassette in the gene (not shown). The *mdaB* mutant could be grown in microaerophilic conditions (1% O₂, 5% CO₂, balance N₂). The total protein expression profiles of the wild type and mutant cells were examined by resolving the crude cell-free extracts on a 12.5% SDS-PAGE gel (Fig. 1). A protein with a migration mass of about 26 kDa was shown to be significantly up-expressed in the mutant compared to the wild type. This protein was subsequently identified as superoxide dismutase (SodB) by N-terminal sequencing. Eight individual mutant colonies were randomly selected and examined for their protein expression profiles, and SodB was found to be up-expressed in all of these mutant strains. At the position of ~ 22 kDa (the predicted size of MdaB), there was a weak band of protein in the wild type, which appears to be missing in the mutant. However, this band could not be assigned to MdaB by N-terminal sequencing because of the low amount of protein.

2.2. Oxidative stress tolerance

Growth yields were compared for strains grown on agar plates at controlled O₂ levels. Best growth yields of the wild type *H. hepaticus* were at 1% partial pressure O₂, where the yield of the *mdaB* mutant strain was consistently about one-third that of the wild type (Table 1). At 6% O₂, the wild type cells still grew well, but the *mdaB* mutant achieved only 10% of the yield of the parent strain under this condition. Fig.2 shows the results from paper disk assays where oxidizing agent sensitivity of the strains is compared. The *mdaB* strain was clearly more sensitive than the parent strain to H₂O₂ and moderately more sensitive to cumene hydroperoxide, t-butyl hydroperoxide, and to paraquat.

In the annotated genome sequence of *H. hepaticus* [4], a conserved hypothetical gene (HH1473) is located downstream of the *mdaB* gene. To exclude the possibility that the observed phenotype of the *mdaB* mutant was due to a polar effect on the downstream gene, an *H. hepaticus* mutant strain was constructed by insertion of a chloramphenicol resistance cassette (CAT) into HH1473. *H. hepaticus* 1473:CAT strain showed a similar growth phenotype as the wild type at conditions of various O₂ concentrations (Table 1).

2.3. Purification of MdaB and identification by western blotting

The *H. hepaticus mdaB* gene was cloned into pET21a and a His-tagged version over-expressed in *E. coli* and purified. The purified MdaB protein migrated at about 21 ~ 22 kDa based on sodium dodecyl sulfate-polyacrylamide gels (Fig. 3). This result corresponded to the predicted molecular mass (21.9 kDa) of MdaB. The concentration of the purified MdaB was determined to be 12.4 mg/ml with a bicinchoninic acid protein assay kit (Pierce). Western blotting was performed using polyclonal anti-MdaB antibody against the cell extract from *H. hepaticus* wild

type and the *mdaB* strains. The assay detected an immunoreactive band migrating at the expected size for both the positive control (purified MdaB) and wild type cell extract. No such band was detected for the mutants, verifying that the efficacy of the *mdaB* strains.

2.4. Assays for NADPH quinone reductase

By conducting a conserved-domain search on the *H. hepaticus* MdaB protein, it appeared the protein contains a domain with a flavodoxin-like fold (PF02525) similar to that in *E. coli* [9] and in *H. pylori* [6]. The purified *H. hepaticus* MdaB protein showed a flavin adsorption spectrum with a major peak at 456 nm, a minor peak at 376 nm, and two shoulders at 429 and 484 nm. This spectrum is similar to those of MdaBs of *E. coli* and *H. pylori*. Different electron donors or acceptors were used to test the substrate specificity of *H. hepaticus* MdaB (Table 2). The activity was measured by the decrease of absorbance at 340 nm during NADPH or NADH oxidization. With NADPH as electron donor, the *H. hepaticus* MdaB reduced quinone or quinone-like compounds with a specific activity ranging from 0.92 to 4.6 U/mg of protein. NADH could also be used as an electron donor to *H. hepaticus* MdaB but with much lower efficiency (only about 3% of those with NADPH). This observation is in general agreement with the previous observation on MdaB in *H. pylori* (10-fold preference for NADPH over NADH). The results indicate that *H. hepaticus* MdaB is a quinone reductase specifically using NADPH as an electron donor.

The enzyme activity was determined using a coupled menadione-cytochrome *c* reduction assay that measures an increase in absorbance at 550 nm due to cytochrome *c* reduction. Using this assay, *H. hepaticus* MdaB showed an activity of 4.0 ± 0.59 μ mol of cytochrome *c* reduced per min per mg of protein. To examine whether MdaB reduces quinone by a two-electron transfer pathway, the same assay was conducted but with addition of 10 unit/ml of superoxide dismutase (SOD) (Cell Technology Inc., Mountain view, CA). The rationale for conducting this assay is that single electron-reduced menadione generates superoxide; this product in turn is capable of reducing cytochrome *c*. By adding SOD in the assay, ancillary cytochrome *c* reduction due to superoxide formation can be assessed [10]. The result was that the same level of MdaB activity was detected with or without addition of SOD.

3. Discussion

Mutant *mdaB* strains of *H. hepaticus* were studied to assess a role for MdaB in conferring oxidative stress resistance to an emerging pathogen. The *mdaB* mutants were more sensitive to high O₂ concentrations and to several oxidative stress reagents compared to the wild type. This was in spite of a concomitant up-expression of superoxide dismutase, which can be assumed to partially attenuate the oxidative stress phenotype of the strains. An *H. hepaticus* mutant strain with the disruption of the gene downstream of *mdaB* showed a similar oxidative stress phenotype as the wild type, which indicated that the O₂ sensitivity observed for the *mdaB* mutant was attributable to the loss of MdaB function. By performing assays with purified MdaB protein, we found that (like its homolog in *E. coli* and *H. pylori*) *H. hepaticus* MdaB is able to reduce a variety of quinones, and specifically uses NADPH as the electron donor.

It was reported that the expression of some *H. pylori* antioxidant proteins can be up-regulated to compensate for the loss of the target protein in the mutant [7]. In this study, *sodB* was up-expressed in the *mdaB* mutant and SodB is well-documented to be an important antioxidant enzyme [5]. Although MdaB was up-expressed in some *H. pylori* highly oxidative stress sensitive strains (i.e. double mutants), the *mdaB* *H. pylori* strains did not alter their expression of other oxidative stress-combating enzymes. Whether a regulatory response or an adaptive type spontaneous mutation, the SodB up-expression seems to be a compensatory response to the loss of MdaB in *H. hepaticus*, and underscores the adaptability of the bacterium.

The MdaB-mediated quinone reductase activity of *H. hepaticus* was much lower (by about 20 times) than its homolog in *H. pylori* even when both enzymes were purified by the identical procedure. Considering that the two enzymes are 66% homologous at the amino acid sequence level, this difference may be due to some minor structural variations at the active center. This could of course result in a large impact on substrate binding affinity or catalytic efficiency. Additional studies on structure-function relationship comparisons between the two *Helicobacter* enzymes could be useful to understand the roles of specific residues/domains conferring MdaB turnover efficiencies.

Quinones can be reduced to semiquinones by oxidoreductases via a one-electron transfer reaction or they can be competitively reduced to quinols by NAD(P)H quinone reductases [6, 9,12] via two-electron mechanism. Semiquinone radicals are potentially cytotoxic or mutagenic due to their ability to react with molecular oxygen to form superoxide radicals [13]. On the other hand, the two-electron pathway products (i.e. quinols) minimize oxidative stress as they do not have such damaging properties. Indeed, quinols have been shown to lower the levels of measured superoxide ions in the *E. coli* cell membrane [14]. Our results indicate that *H. hepaticus* MdaB catalyzes two-electron reduction of quinones to quinols, a mechanism protecting cells from stress mediated damage.

4. Materials and methods

4.1. Bacterial strains, culture, and growth Conditions

The bacterial strains used in this study include *H. hepaticus* type strain ATCC 51449, and *Escherichia coli* DH5 α (BRL) and BL21 origami (Novagen). *H. hepaticus* was cultured on Brucella agar (DIFCO) plates supplemented with 10% defibrinated sheep blood in a 37 °C incubator. The optimal atmosphere which was used for the growth is 1% O₂, 5% CO₂, with the balance of the atmosphere composed of N₂. Different oxygen levels (1% O₂, 3% O₂, and 6% O₂) were also supplied in oxidative stress resistance assays as indicated. Erythromycin (5 μ g/ml) was added to the medium for culturing the *H. hepaticus* mutants. *E. coli* was grown aerobically on Luria-Bertani plates supplemented with ampicillin (100 μ g/ml) or erythromycin (150 μ g/ml). The plates were incubated at 37 °C in air.

4.2. construction of *H. hepaticus* mutants

Based on the genome sequence of *H. hepaticus* [4], a three-step PCR was designed to generate a unique *EcoRI* restriction site inside the *mdaB* gene. Primers mdaF12 (5' CAGAGAATGCGCAGGTGGAT 3'), mdaB12 (5' TTCATCAAGTGCATTACCCTCCA 3'), mdaF22 (5' ACTTGGAATTCTCCGATTGAGGCTTTCACA 3'), and mdaB22 (5' CAATCGGAGAATTCCAAGTGAGAGAAAACA 3') were used for PCR; and the PCR fragment (1143 bp containing *mdaB* sequence) was then ligated into pGEMT vector (Promega) and the construct was used to transform *E. coli* DH5 α through electrotransformation. The cloned plasmid was then extracted from the culture (Qiagen) and an Erythromycin cassette (Ery) [11] was inserted into the unique *EcoRI* restriction site within the *mdaB* gene. The recombinant plasmid pGEMT:*mdaB*:Ery was then introduced into *H. hepaticus* by electrotransformation [15]. Allelic exchange occurred leading to the formation of the *mdaB* mutant strain. The mutant was selected on blood agar plates supplemented with Erythromycin (5 μ g/ml). Genomic DNA was prepared from the mutant clones and the disruption of the *mdaB* gene was confirmed by observing an 1140 bp size increase of PCR amplicon due to the insertion of the antibiotic cassette.

An HH1473:CAT mutant strain was constructed as follows. Primers HH1473F (5'-GCTTTGCCGAGCTTTATCT-3') and HH1473R (5'-CTTAAGCAAGAGGATTGGG-3') were used for PCR from *H. hepaticus* genomic DNA. The PCR fragment (~ 1kb) was ligated

into pGEMT vector, and the plasmid construct was transformed into *E. coli* DH5 α . A CAT cassette (0.8 kb) was then inserted into the unique BsaMI site within the gene HH1473, and introduced into *H. hepaticus* genome by electrotransformation. Disruption of the gene HH1473 in the *H. hepaticus* genome was confirmed by observing an 800 bp size increase of PCR amplicon due to the insertion of the CAT cassette.

4.3. Gel electrophoresis and protein identification using N-terminal sequencing

Bacteria were harvested from the plates and resuspended in 1 $\times$ PBS buffer containing 20 mM sodium phosphate, and 150 mM NaCl, pH 8.0. After washing once with the buffer, cells were resuspended in the same buffer and were passed twice through a French pressure cell at 138,000 kPa (SLM instruments, Inc.). The cell lysates were centrifuged at 7,500 $\times$ g for 10 min and the supernatant was transferred to a clean tube. The protein concentration of cell crude extract was determined by Bradford protein assay (Bio-Rad, Hercules, CA). Seven micrograms of cell extract was mixed with the SDS buffer and incubated at 90 $^{\circ}$ C for five minutes. Proteins were then separated on the 12.5% SDS-PAGE gel by electrophoresis for 1.5 hours at 100 V. The protein bands of interest were subjected to N-terminal sequencing after transferring and excised from a PVDF membrane (Protein sequencing lab, Georgia State University, GA).

4.4. Disk assays for oxidative stress sensitivity

BA plates were uniformly streaked with 0.1 ml of cell suspension adjusted to the OD of 0.8. Sterile 7.5 mm filter paper disks saturated with 10 μ l of each chemical (1 M H₂O₂, 0.2 M cumene hydroperoxide, 0.2 M t-butyl hydroperoxide, or 0.1 M paraquat) were placed onto the plates. The cells were cultured under microaerophilic conditions (1% O₂, 5% CO₂, balance N₂) for 72 hours before the zone of the inhibitions were measured (Fig. 2).

4.5. Oxygen sensitivity assay

Variable O₂ concentrations were created by controlled nitrogen gas flow-through in CO₂ incubator. The strains were compared for O₂ sensitivity by assessing growth on BA plates. Briefly, each cell suspension (0.1 ml) at OD of 0.8 was evenly spread onto the plates, and the plates were incubated for 72 hours at the O₂ partial pressures. The cells from plates were harvested by suspending them in 1 ml of PBS and the OD₆₀₀ was taken (or calculated from dilutions) as a measurement of growth yield.

4.6. Purification of *H. hepaticus* MdaB-6His and western blotting

A 602 bp DNA fragment containing the *H. hepaticus mdaB* gene was PCR amplified using primer pair mdaB-pET-F (5' CGCGCGGCCATATGAAAAAATTTTACTT 3'), and mdaB-pET-R (5' ATGCATGCTCGAGTTTGCCAAAAACCTTTTT 3'). The PCR product was digested with *Nde*I and *Xho*I (the recognition sequences are underlined in the primer sequences shown above) and cloned into vector pET-21a (Novagen) treated with the same restriction enzymes. The recombinant plasmid was then transformed into *E. coli* BL21 Origami competent cells (Novagen). *E. coli* cells were grown to OD₆₀₀ of in 500 ml Luria-Bertani medium supplemented with 100 μ g/ml ampicillin. Induction of MdaB was achieved by adding 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) to the medium, followed by further incubation of the cells for 2 hours. The His-tagged MdaB was purified with a nickel-nitrilotriacetic acid affinity column (Qiagen) following a procedure previously described [6]. The protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce).

Purified MdaB was used to make polyclonal anti-MdaB antibody raised in rabbits (Cocalico Biologicals, Reamstown, Pa.). Twenty micrograms of the wild type or mutant strain crude cell extracts and 0.02 μ g of purified MdaB were subjected to SDS-PAGE and electrophoretically-

transferred to a nitrocellulose membrane. Western blotting was conducted to verify the presence of MdaB in cell extracts of wild type and its absence in the mutant.

4.7. Enzyme activity assay

The enzymatic activity of MdaB protein was estimated by measuring NADPH/NADH oxidation with quinone or other electron acceptors as indicated. The reaction mixtures contained 20 mM Tris-HCl (pH 7.5), 0.2 mM NADPH or NADH, 0.1 mM quinone or other compounds as listed in Table 2, and 10 μ l of the properly diluted MdaB in a total volume of 1.0 ml. The reaction was started by the addition of the enzyme and conducted at room temperature. Enzyme activity was calculated from the decrease in absorbance at 340 nm with an absorbance coefficient of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit of activity was defined as the amount of enzyme catalyzing the oxidation of 1 μ mol of NADPH/NADH per min per mg of protein. The enzyme activity of MdaB was further determined by measuring the reduction of quinone-like artificial electron acceptors such as ferricyanide or dichlorophenolindophenol. A coupled assay of menadione-cytochrome *c* reduction was conducted as described [10]. The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 0.2 mM NADPH or NADH, 0.1 mM menadione, and 0.1 mM cytochrome *c*. Reduction of cytochrome *c* was monitored by an increase in absorbance at 550 nm ($\epsilon_{550} = 29.5 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixtures without electron acceptor or without the enzyme were used as controls.

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Fig. 1. Protein profiles of wild type and *mdaB* strains. The 12.5% polyacrylamide gel was stained with Coomassie brilliant blue. Lanes: 1, wild type cell extract; 2, *mdaB* mutant cell extract; M, protein standard (Invitrogen).

Fig. 2.

Paper disk assays for oxidative stress sensitivity assessments. Zones of inhibition were measured around the disks infiltrated with 10 μ l of the indicated reagents. Water as a control did not yield any zones of growth inhibition. Six replicate experiments were performed for each tested reagent. According to Student's t - distribution test, the mutant result for H₂O₂ is significantly different from that of the parent strain at the 99% level of confidence while the results for the other three reagents are significantly different from those of the parent strain at the 95% level of confidence.

Fig. 3. Purification progress of *H. hepaticus* MdaB (panel A) and western blot analysis (panel B). (A) Samples were resolved on the 12.5% SDS-PAGE gel and stained with Coomassie brilliant blue. Lanes: M, protein standard (Bio-Rad); 1, cell extract of non-induced *E. coli* BL21 Origami (pET-mdaB-6His); 2, cell extract of IPTG-induced *E. coli* BL21 Origami (pET-mdaB-6His) cells; 3, 10 µg of purified MdaB protein. (B) Samples were resolved on a 12.5% SDS-PAGE gel before transfer onto a nitrocellulose membrane and MdaB protein in the samples was detected using anti-MdaB antibody. Lanes: 1, 0.02 µg of purified MdaB protein; 2, 20 µg crude cell extract of *H. hepaticus* wild type; 3, 20 µg crude cell extract of *H. hepaticus* mdaB mutant.

Table 1Growth of strains in various O₂ concentrations

Strain	Growth ^a at O ₂ concentration of		
	1%	3%	6%
wild type 51449	8.0 ± 0.6	3.4 ± 0.4	2.9 ± 0.3
51449 <i>mdaB</i> :Ery	3.1 ± 0.4	2.0 ± 0.3	0.3 ± 0.1
51449 HH1473:CAT	7.5 ± 0.5	3.2 ± 0.4	2.8 ± 0.3

^aThe cells were grown on the plates for 72 hours, harvested and suspended in PBS (1ml/plate), and the OD₆₀₀ was measured. The results are the average of 3 experiments with standard deviation. According to student's t-test, the *mdaB* strain grows significantly slower than the wild type at 99% level of confidence ($p < 0.01$) at all the O₂-levels tested. There is no significant difference in growth yield between the wild type and the HH1473:CAT mutant strain.

Table 2NADPH-quinone reductase activities of purified *H. hepaticus* MdaB

Electron acceptor	Enzyme activity with following electron donor	
	NADPH	NADH
Coenzyme Q ₀	4.6 ± 0.4 ^a	0.13 ± 0.02
Coenzyme Q ₁	3.8 ± 0.2	0.09 ± 0.02
Menadione	1.7 ± 0.2	0.06 ± 0.01
1,4-Naphthoquinone	2.7 ± 0.3	0.05 ± 0.01
Ferricyanide	3.9 ± 0.6	0.22 ± 0.03
Dicholophenol-indophenol	0.9 ± 0.1	0

^a Enzyme activities were determined spectrometrically by measuring the oxidation of NADPH or NADH (decrease at 340 nm). One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADPH or NADH per minute per milligram of protein. Each value is the mean of five replicate assays with the standard deviation.