JOURNAL OF BACTERIOLOGY, Apr. 2010, p. 1890–1901 Vol. 192, No. 7
0021-9193/10/$12.00 doi:10.1128/JB.01638-09

Functional Analysis of the RdxA and RdxB Nitroreductases of *Campylobacter jejuni* Reveals that Mutations in *rdxA* Confer Metronidazole Resistance▽†

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Received 17 December 2009/Accepted 25 January 2010

***Campylobacter jejuni* is a leading cause of gastroenteritis in humans and a commensal bacterium of the intestinal tracts of many wild and agriculturally significant animals. We identified and characterized a locus, which we annotated as *rdxAB*, encoding two nitroreductases. RdxA was found to be responsible for sensitivity to metronidazole (Mtz), a common therapeutic agent for another epsilonproteobacterium, *Helicobacter pylori*. Multiple, independently derived mutations in *rdxA* but not *rdxB* resulted in resistance to Mtz (Mtz^r), suggesting that, unlike the case in *H. pylori*, Mtz^r might not be a polygenic trait. Similarly, Mtz^r *C. jejuni* was isolated after both *in vitro* and *in vivo* growth in the absence of selection that contained frameshift, point, insertion, or deletion mutations within *rdxA*, possibly revealing genetic variability of this trait in *C. jejuni* due to spontaneous DNA replication errors occurring during normal growth of the bacterium. Similar to previous findings with *H. pylori* RdxA, biochemical analysis of *C. jejuni* RdxA showed strong oxidase activity, with reduction of Mtz occurring only under anaerobic conditions. RdxB showed similar characteristics but at levels lower than those for RdxA. Genetic analysis confirmed that *rdxA* and *rdxB* are cotranscribed and induced during *in vivo* growth in the chick intestinal tract, but an absence of these genes did not strongly impair *C. jejuni* for commensal colonization. Further studies indicate that *rdxA* is a convenient locus for complementation of mutants in *cis*. Our work contributes to the growing knowledge of determinants contributing to susceptibility to Mtz (Mtz^s) and supports previous observations of the fundamental differences in the activities of nitroreductases from epsilonproteobacteria.**

Nitroreductases form a large family of enzymes whose physiological roles have been implicated or proposed to function in diverse processes, such as the generation of nitrogen sources for metabolism, degradation of potentially toxic nitro compounds, vitamin and bioluminescence production, redox balancing, and oxidative stress responses (20, 31, 32, 35, 41, 43, 58). These enzymes can been subdivided into two main categories based on characteristics of their reductive processes, including the mechanism of electron transfer and sensitivity to oxygen. Type I (O_2-insensitive) nitroreductases catalyze a sequential two- or three-step reduction of the nitro group on heterocyclic compounds via paired-electron transfer to produce either hydroxylamine or amino derivatives. Type II (O_2-sensitive) nitroreductases catalyze a single-electron reduction of heterocyclic nitro compounds that is reversible in the presence of oxygen (40). Nitroreductases are common in bacteria, with a given bacterial species often containing multiple paralogs that presumably reduce different substrates. The genes encoding nitroreductases have received intense study due to their unusual nature in degrading or transforming xenobiotic chemicals. Consequently, these enzymes have become attractive candidates for bioremediation processes, and some are utilized in cancer chemotherapies (27, 49). However, the nitroreductases are also a puzzling class of enzymes, because the natural substrates for most remain unknown and these proteins likely did not evolve to exclusively manipulate xenobiotic compounds.

Metronidazole (Mtz) has been used in multidrug therapy for *Helicobacter pylori* infections due to production of factors that convert this 5-nitroimidazole product to a toxic form (1, 45, 53). Therapeutic failure with Mtz has been predominantly associated with mutations occurring in one of two genes of *H. pylori* encoding the nitroreductases RdxA and FrxA. A previous biochemical study characterized the Mtz reductase activity of RdxA (37). Even though RdxA was capable of reducing other nitro compounds under aerobic conditions, the enzyme was unable to reduce Mtz. However, under anaerobic conditions, RdxA was shown to catalyze the reduction of Mtz, and its specific activity for this reaction was 60-fold greater than that of the NfsB nitroreductase of *Escherichia coli* under similar conditions. In addition, this work revealed that RdxA exhibited a potent NADPH oxidase activity not appreciated in other nitroreductases. Not only did this study demonstrate a direct reduction of Mtz by a nitroreductase, but results from this work implied that RdxA of *H. pylori* possessed novel biochemical properties relative to other nitroreductases.

Like *H. pylori*, *Campylobacter jejuni* is a Gram-negative bacterium belonging to the epsilonproteobacteria class. *C. jejuni* is a common commensal bacterium of the intestinal tracts of wild and agriculturally significant animals, especially poultry. In contrast, *C. jejuni* causes acute diarrhea in humans, ranging from a mild enteritis to a bloody diarrheal syndrome, and is

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† Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 29 January 2010.

one of the most prevalent causes of food-borne gastritis (4, 5, 34, 38). Additionally, postinfectious sequelae can develop in a small percentage of patients following a *C. jejuni* infection. One major complication is Guillan-Barré syndrome, a temporary and partial paralysis of the peripheral nervous system (21).

Many individuals with *C. jejuni* enteritis resolve the infection without therapeutic treatment. If antibiotics are administered, fluoroquinolones or macrolides, such as ciprofloxacin or erythromycin, are common drugs of choice, with therapeutic use of Mtz for *C. jejuni* infections being unconventional. However, Mtz-resistant (Mtz^r) *C. jejuni* isolates have been recovered from humans and animals. In agriculture, 19 to 92% of *C. jejuni* isolates from avian species (including chickens and turkeys) and 6 to 20% of isolates from lambs, sheep, and cows were Mtz^r (13, 47). One study also demonstrated that 62% of *C. jejuni* clinical isolates from humans were Mtz^r (47). These data are curious, since these *C. jejuni* isolates would have likely developed Mtz^r during infections in the absence of selection.

Because susceptibility to Mtz (Mtz^s) was also found in isolates associated with each host in studies described above and since *C. jejuni* is closely related to *H. pylori*, we hypothesized that *C. jejuni* may produce a nitroreductase to reduce Mtz to its toxic form, leading to Mtz^s. In this report, we identify and characterize the gene required for Mtz^s in *C. jejuni*. Mutations in this gene, encoding a putative nitroreductase, but not in a downstream paralog were linked to the development of Mtz^r, indicating that Mtz^r in *C. jejuni* appears to be linked to mutation of only one nitroreductase. Supporting our findings, we provide evidence that a proportion of Mtz^r isolates of *C. jejuni* are due to spontaneous errors during DNA replication in the absence of Mtz exposure, resulting in a variety of mutations. Biochemical analysis of these *C. jejuni* nitroreductases demonstrated that these proteins had potent NADPH oxidase activity and could reduce Mtz under anaerobic conditions. These results, along with previous biochemical analysis of *H. pylori* RdxA (37), demonstrate that the nitroreductases of epsilonproteobacteria have unique characteristics in comparison to other bacterial counterparts.

MATERIALS AND METHODS

Strains and culture conditions. *C. jejuni* strain 81-176 is a clinical isolate that is able to colonize the intestinal tract of chicks and cause disease in human volunteers (3, 18, 28). DRH212 is a streptomycin-resistant (Sm^r) derivative of 81-176 with an *rpsL*^{Sm} allele (17). *C. jejuni* strains were routinely grown from frozen stocks on Mueller-Hinton (MH) agar containing trimethoprim (TMP) under microaerobic conditions (10% CO_2, 5% O_2, 85% N_2) at 37°C for 48 h, followed by reinoculation onto MH agar containing TMP for growth for 16 h. Antibiotics for *C. jejuni* strains were used at the following concentrations: TMP, 10 μg ml^{-1}; cefoperazone, 30 μg ml^{-1}; chloramphenicol, 15 μg ml^{-1}; and Mtz, 0, 0.2, 0.5, 1, 2.5, 5, 10, 25, 50, 100, and 200 μg ml^{-1}. All *C. jejuni* strains were stored in 85% MH broth–15% glycerol at −80°C. *Escherichia coli* DH5α, XL-1 Blue, and BL21(DE3) were grown in Luria Bertani (LB) agar or broth with antibiotics when needed at the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 15 μg ml^{-1}; Mtz, 10 μg ml^{-1}. All *E. coli* strains were stored in 80% LB broth–20% glycerol at −80°C.

Identification of Mtz^r transposon mutants. A *C. jejuni* 81-176 transposon library constructed with the *darkhelmet* transposon, a *mariner*-based transposon, was grown as described above (16). Bacteria were resuspended and diluted in MH broth to an optical density at 600 nm (OD_{600}) of 0.4. The culture was diluted 1:5,000, and 100 μl was spread on two MH agar plates containing either 5, 10, 25, or 50 μg ml^{-1} Mtz. Serial dilutions were also spread on MH agar containing TMP to determine the bacterial density of the culture. Plates were incubated for 4 days at 37°C under microaerobic conditions. At least one Mtz^r colony was obtained per plate, with a total of nine Mtz^r isolates recovered. The location of the transposon in each mutant was determined as previously described (16).

Construction of *rdxA* and *rdxB* mutants in *C. jejuni* 81-176. Insertional inactivation of *rdxA* or *rdxB* was accomplished by first amplifying 1.6-kb fragments containing *rdxA* or *rdxB* with approximately 500 nucleotides of flanking DNA sequence from *C. jejuni* 81-176 genomic DNA by PCR with primers containing 5′ BamHI sites (9). After the DNA fragments were cloned into BamHI-digested pUC19 to create pDAR473 (containing *rdxA*) or pDRH2879 (containing *rdxB*), PCR-mediated mutagenesis was used to create an internal EcoRV restriction site in each gene. pDAR475 contains an EcoRV site in *rdxA* by an A222G mutation, and pDAR476 contains an EcoRV site within *rdxB* by a G293C mutation. These plasmids were used to insert a SmaI-digested *cat-rpsL* cassette into the EcoRV site of *rdxA* (creating pDAR505) or *rdxB* (creating pDAR609) (17). pDAR505 or pDAR609 was electroporated into *C. jejuni* 81-176 Sm^r (DRH212) to create DAR521 (81-176 Sm^r *rdxA*::*cat-rpsL*) or DAR562 (81-176 Sm^r *rdxB*::*cat-rpsL*), respectively.

Analysis of colonization capacity of *C. jejuni* mutants. One-day-old White Leghorn strain Δ chicks were orally infected with wild-type or mutant *C. jejuni* 81-176 strains as previously described (2, 18). Briefly, fertilized chicken eggs (SPAFAS) were incubated for 21 days at 37.8°C with appropriate humidity and rotation in a Sportsman II model 1502 incubator (Georgia Quail Farms Manufacturing Company). Approximately 12 to 36 h after hatching, chicks were orally infected with 100 μl of phosphate-buffered saline (PBS) containing approximately 10^2 or 10^4 CFU of wild-type or mutant *C. jejuni* strains. To prepare strains for infection, bacteria were grown from frozen stocks as described above. Bacteria were resuspended from plates and diluted to the appropriate inoculum in PBS. Dilutions of the inocula were plated to determine the number of bacteria in the inoculation dose. At day 7 postinfection, chicks were sacrificed and the cecal contents were recovered. After suspension in PBS, serial dilutions were spread on MH agar containing TMP and cefoperazone to determine the number of CFU per gram of cecal contents. Statistical analysis of results from colonization experiments was performed by the Mann-Whitney U test.

Isolation of Mtz^r mutants after *in vitro* and *in vivo* growth. Seven Mtz^s *C. jejuni* 81-176 isolates were identified, and low-passage frozen stocks of each were prepared. To determine the rate of generation of Mtz^r during *in vitro* growth, each strain was grown from frozen stocks as described above. After suspension of each culture to an OD_{600} of 1.0, serial dilutions were spread on MH agar containing TMP (to determine total CFU per ml) or 10 μg ml^{-1} Mtz to recover Mtz^r isolates. After growth for 5 days at 37°C under microaerobic conditions, the number of bacteria recovered on MH agar with TMP or Mtz was determined. Twelve random Mtz^r isolates from each culture were saved, and the *rdxAB* operon was PCR amplified from each and sequenced.

Isolation of Mtz^r mutants after commensal colonization of the chick ceca was performed by orally gavaging six 1-day-old White Leghorn strain Δ chicks with 100 μl PBS containing approximately 10^4 CFU of Mtz^s *C. jejuni* 81-176. An aliquot of the inoculum was spread on MH agar containing 10 μg ml^{-1} Mtz to confirm spontaneous Mtz^r *C. jejuni* were not present in the inoculum. Chicks were given food and water *ad libitum* and were not administered Mtz during the course of infection. At day 7 postinfection, chicks were sacrificed and the bacteria from the ceca were recovered. Serial dilutions were plated on MH agar containing TMP and cefoperazone (to determine the total *C. jejuni* load in the cecum of each chick) or MH agar containing TMP, cefoperazone, and 10 μg ml^{-1} Mtz (to determine the number of Mtz^r *C. jejuni* CFU in the cecum of each chick). Sixty-four Mtz^r isolates were recovered. The *rdxAB* operon was PCR amplified from each isolate and sequenced.

Determination of MIC of Mtz for *C. jejuni* strains. MICs of Mtz for various *C. jejuni* strains were determined by the method of Jeong et al. (23). Briefly, *C. jejuni* strains were grown from frozen stocks under microaerobic conditions for 48 h at 37°C. Strains were reinoculated on MH agar and grown for another 16 h. Bacteria were suspended and diluted to an OD_{600} of 1.0. Tenfold serial dilutions were made, and 10 μl of each dilution was spotted onto MH agar containing Mtz at the following concentrations: 0, 0.2, 0.5, 1, 2.5, 5, 10, 25, and 50 μg ml^{-1}. After growth for 3 days at 37°C under microaerobic conditions, the MIC was determined by examining the lowest Mtz concentration resulting in a 10-fold decrease in CFU compared to growth on MH agar without Mtz.

Mtz-induced killing and mutation assays. Wild-type Mtz^s *C. jejuni* 81-176 was grown on three sets of MH agar plates from frozen stocks for 48 h at 37°C under microaerobic conditions. The strains were then restreaked on MH agar and grown for another 16 h. Bacteria were suspended and diluted to an OD_{600} of 1.0 in 24 ml of MH broth to give three independent cultures. The cultures were split into two volumes, with one volume receiving 10 μg ml^{-1} Mtz. The cultures were incubated under microaerobic conditions at 37°C for 6 h. At time zero, 2, 4, and 6 h after addition of Mtz, the number of viable bacteria in cultures with or

without Mtz were determined by plating 10-fold serial dilutions on MH agar containing TMP. In addition, the number of Mtz^r bacteria at each time point was determined by plating dilutions on MH agar containing 10 μg ml^{-1} Mtz. Plates were incubated for up to 5 days at 37°C under microaerobic conditions before determining the number of viable CFU per ml of culture.

The ability of Mtz to induce mutations to generate Sm^r was analyzed in strain 81-176 *rpsL*^{Sm} *cjj0275*::*cat-rpsL* (LKB635). To construct this strain, *81176_cjj0275*, a gene that has been proposed to be involved in flagellar motility (17), was first PCR amplified from the *C. jejuni* 81-176 chromosome with primers containing 5′ BamHI restriction sites, resulting in a 2.9-kb fragment containing the gene. The amplified DNA was then cloned into BamHI-digested pUC19 to create pDRH1962. PCR-mediated mutagenesis was then performed to create an MscI site within the coding sequence of *cjj0275*, resulting in pDRH1975. After digestion with MscI, a SmaI-digested *cat-rpsL* cassette was then ligated into the plasmid to interrupt *cjj0275* to generate pDRH2024 (17). This plasmid was then used to electroporate DRH212 (81-176 *rpsL*^{Sm}), resulting in LKB635.

Methods similar to the experiment described above to test for Mtz-induced mutations and killing with wild-type *C. jejuni* 81-176 were performed with LKB635 to determine the extent of killing by addition of Mtz. In addition, the number of viable Sm^r bacteria was determined by plating 10-fold serial dilutions of cultures with or without Mtz at time zero, 2, 4, or 6 h after exposure to Mtz on MH agar containing 2 mg ml^{-1} Sm.

Expression and purification of RdxA and RdxB. The coding sequences of *rdxA* and *rdxB* from codon 2 to the stop codon were PCR amplified from *C. jejuni* 81-176 genomic DNA with primers containing in-frame 5′ BamHI restriction sites and cloned into pGEX-4T-2, creating fusion proteins of glutathione *S*-transferase (GST) to the N terminus of RdxA (pDAR538) or RdxB (pDAR558). Plasmids were transformed into BL21(DE3) for protein expression and purification. For purification of GST, BL21(DE3)/pGEX-4T-2 was used. For induction of proteins, 500 ml LB with ampicillin was inoculated with a 1:40 dilution of overnight culture and grown at 37°C to an OD_{600} of 0.5. After induction with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 100 μM for 3 h, bacteria were recovered, washed once in cold PBS, and then resuspended in 30 ml PBS. After lysis by passage four times through an EmusliFlex-C5 cell disrupter (Avesin) at 15,000 to 20,000 lb/in^2, GST-tagged proteins were purified from the soluble fraction with glutathione Sepharose 4B by gravity flow according to the manufacturer's instructions (GE Healthcare). For purification of native RdxA and RdxB, GST-tagged proteins bound to the Sepharose slurry were resuspended in 2 ml 1× PBS and transferred to a microcentrifuge tube to which 50 U of thrombin (GE Healthcare) was added. Cleavage of the GST tag occurred by overnight incubation at 37°C. Thrombin was removed from purified RdxA and RdxB using benzamidine Sepharose 6B according to the manufacturer's instructions (GE Healthcare). Protein concentrations for purified GST-RdxA, RdxA, GST-RdxB, RdxB, and GST were determined by Bradford assay (Bio-Rad), and purity was examined by SDS-PAGE. Similar to other flavin proteins, purified RdxA and RdxB and their fusions were yellow in color (43).

Antiserum generation and immunoblotting analysis. The coding sequence of *rdxB* from codon 2 to the penultimate codon was PCR amplified from *C. jejuni* 81-176 genomic DNA with primers containing in-frame 5′ and 3′ BamHI restriction sites. The DNA fragment was inserted into BamHI-digested pQE30 (Qiagen) to create pDAR520 and transformed into XL1-Blue. For protein induction, 500 ml LB with ampicillin was inoculated with a 1:40 dilution of overnight growth, and the culture was grown at 37°C to an OD_{600} of 0.5. The culture was induced with 1 mM IPTG for 4 h, and the bacterial pellet was recovered. Bacteria were lysed as described above, and His_6-RdxB was purified from the soluble fraction using nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) according to the manufacturer's instructions. GST-RdxA was purified using glutathione Sepharose 4B according to the manufacturer's instructions. GST-RdxA and His_6-RdxB were used to immunize mice by standard procedures for antiserum generation by a commercial vendor (Cocalico Biologicals).

Immunoblotting analyses were performed by growing *C. jejuni* strains from frozen stocks as described above. Bacteria were suspended from plates and diluted to an OD_{600} of 0.8. Whole-cell lysates (WCLs) were prepared by pelleting 1 ml of bacteria, washing once with PBS, and then resuspending the bacteria in 50 μl of SDS-PAGE loading buffer. For total membrane preparations, 5 ml of culture prepared as described above was pelleted and washed once in 10 mM HEPES, pH 8.0. Bacteria were sonicated, and total membranes were recovered as the insoluble material as previously described (2). Ten or 20 μl of WCL preparations were separated by SDS-PAGE for detection of RdxA and RdxB, respectively, and proteins were then transferred to an Immobolin-P membrane. Membranes were incubated with a 1:1,000 dilution of M100 anti-RdxA antiserum or a 1:500 dilution of M95 anti-RdxB antiserum for 2 h, washed, and then incubated with a 1:12,500 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse antiserum (Bio-Rad) for 1 h. For detection of FlhB in total membranes, the membrane pellet recovered from 5 ml of culture for each strain was resuspended in 50 μl of SDS-PAGE loading buffer. After SDS-PAGE, proteins were transferred to a membrane and incubated overnight at 4°C with 1:1,000 dilution of Rab476 anti-FlhB antiserum (25), followed by incubation for 4 h at room temperature with a 1:7,500 dilution of HRP-conjugated goat anti-rabbit antiserum (Bio-Rad).

Biochemical analysis of RdxA and RdxB. Aerobic reductase or oxidase activities of proteins for various substrates were analyzed as described by Sisson et al. (45). Briefly, the initial rates of activity of purified proteins were determined spectrophotometrically at 25°C in a 1-ml reaction volume containing 50 mM Tris-HCl (pH 7.5), 0.1 mM NADPH, and 0.1 mM substrate. Reactions were initiated by the addition of 10 μg of enzyme, and absorbance readings were measured every 15 s for 5 min by a Beckman DU 530 spectrophotometer. Anaerobic measurement of Mtz reduction was also performed according to the method of Olekhnovich et al. (37). Briefly, reaction mixtures (50 mM Tris [pH 7.5], 0.1 mM NADPH, 0.1 mM substrate, 25 mM glucose, and 6 U ml^{-1} of both glucose oxidase and catalase) were allowed to undergo anaerobic generation for 5 min. Purified proteins (10 μg) were added to initiate reactions, and absorbance readings were examined as described previously (37). Blank cuvettes did not contain Mtz. The reduction of substrates was measured at the indicated wavelengths, and specific activity for each was calculated from the initial rate, extinction coefficient, and protein concentration: nitrofurazone at 400 nm (E = 12.6 mM^{-1} cm^{-1}), nitrofurantoin at 420 nm (E = 12 mM^{-1} cm^{-1}), and furazolidone at 400 nm (E = 18.8 mM^{-1} cm^{-1}). The oxidation of NADPH was measured at 340 nm (E = 6.22 mM^{-1} cm^{-1}). Endogenous NADPH oxidase activity for each substrate tested was also determined and was used to correct the specific activities calculated for those substrates under aerobic conditions (37). Assays were performed in duplicate from two independent batches of purified protein.

Analysis of flavin cofactor binding to the GST-RdxA and GST-RdxB fusion proteins was determined by the method of Zenno et al. (57). Purified GST, GST-RdxA, and GST-RdxB were boiled for 20 min and then centrifuged for 20 min at 10,000 × g. Supernatants were analyzed by thin-layer chromatography (TLC) using cellulose polyethyleneimine (PEI) sheets and a solvent system of 0.4 M K_2HPO_4 and 0.7 M boric acid. Control preparations of flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and riboflavin were run alongside supernatants. Spots were visualized by UV.

Transcriptional analysis of the *rdxAB* operon. Reverse transcriptase PCR (RT-PCR) was performed to analyze cotranscription of *rdxA* and *rdxB*. DRH212 (81-176 Sm^r) was grown from freezer stocks as described above. RNA was isolated using an RNAeasy minikit with the RNAprotect bacterial reagent (Qiagen). After DNase treatment to remove genomic DNA, RNA was reverse transcribed into cDNA in the presence and absence of Superscript II reverse transcriptase (Invitrogen). The products of the cDNA synthesis reactions were used in PCRs to amplify regions internal to *rdxA* or *rdxB* or spanning *rdxA* and *rdxB*. Products from the cDNA synthesis reactions in the absence of RT served as a negative control to verify that no amplification originated from possibly contaminating genomic DNA. Genomic DNA was used as a positive control in PCR to verify amplification and size of generated fragments.

Primer extension reactions were carried out to determine the transcriptional start site of *rdxA*. RNA was prepared as described above and used in a RT reaction with the primer *rdxA PE* (5′-TCCACTTTCTAAGATAAAAT-3′). The same primer was used in a sequencing reaction with pDAR473. Products from the sequencing reaction were run alongside the product of the RT reaction on a 6% sequencing gel to align the cDNA product to the sequencing ladder. The gel was dried and exposed to a Phosphorimager cassette and visualized using the ImageQuant software program.

For real-time RT-PCR analysis, three 1-day-old chicks were orally gavaged with 10^4 CFU of DRH212 (81-176 Sm^r). At day 7 postinfection, chicks were sacrificed and the cecal contents were recovered. RNA from the cecal contents containing *C. jejuni* was isolated using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA from *in vitro*-grown bacteria was isolated from DRH212 as described above. RNA was DNase treated (GenHunter) and diluted to a final concentration of 50 ng μl^{-1}. Real-time RT-PCR analysis was performed with a 25-μl volume in 1× Sybr green PCR master mix (Applied Biosystems), 0.2 μM forward and reverse primers, and 2.5 μg RNA. RT-positive samples also contained 0.1 μl of Multiscribe reverse transcriptase (Applied Biosystems). Reactions were performed on a 7500 real-time PCR system (Applied Biosystems) under the following conditions: 42°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Results were normalized using *gyrA* or 16S rRNA as a control, and analysis was performed using the ΔΔ*CT* method. The level of gene expression from *in vitro*-grown samples was calibrated to 1.

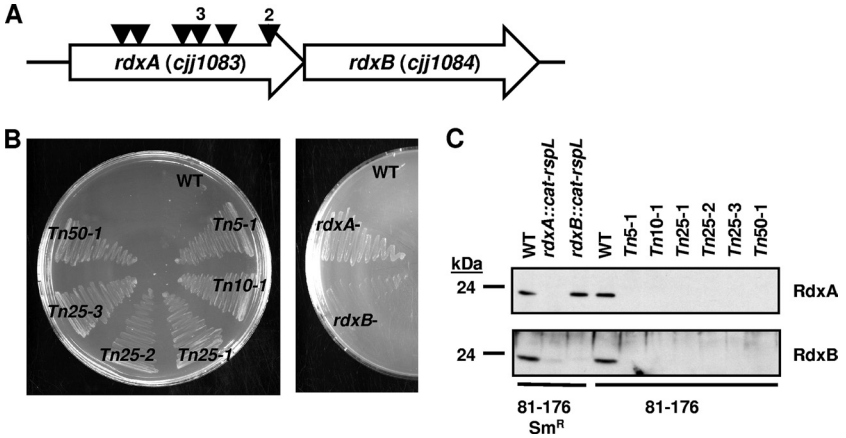

FIG. 1. Mtz^s and Mtz^r phenotypes and production of RdxA and RdxB in *C. jejuni* transposon and site-directed mutants. (A) Identification of Mtz^r *darkhelmet* transposon mutants of *C. jejuni* 81-176. Triangles represent locations of the *darkhelmet* transposons within *rdxA*. Numbers above triangles indicate the numbers of mutants identified with transposon insertions at identical locations, suggesting that these mutants were likely siblings. (B) Mtz^s and Mtz^r phenotypes of wild-type *C. jejuni* 81-176 (WT), isogenic *darkhelmet* transposon mutant strains (left), and wild-type 81-176 Sm^r (WT) and isogenic site-directed mutant strains (right) after 48 h of growth on MH agar containing 10 μg ml^{-1} Mtz. (C) Immunoblot analysis of RdxA and RdxB production in whole-cell lysates of wild-type *C. jejuni* 81-176 or 81-176 Sm^r strains and derived transposon or site-directed mutants. For panels B and C, strains include wild-type DRH212 (81-176 Sm^r; WT producing RdxA and RdxB), DAR521 (81-176 Sm^r *rdxA*::*cat-rpsL*, which produces neither RdxA nor RdxB), DAR562 (81-176 Sm^r *rdxB*::*cat-rpsL*, which produces only RdxA), and wild-type 81-176 (WT).

Complementation of *C. jejuni* mutants in *cis*. A 1.2-kb fragment containing *flhB* with its native promoter was PCR amplified from genomic DNA of *C. jejuni* 81-176 with primers to create 5′ and 3′ SmaI restriction sites. After digestion with SmaI, the PCR product was cloned into the EcoRV site in *rdxA* in pDAR475 to create pDRH3031. Similarly, a SmaI-digested *cat* cassette encoding chloramphenicol acetyltransferase from pRY109 was inserted into the EcoRV site in *rdxA* in pDAR475 to create pDRH3033 (55). Plasmids were electroporated into SNJ471 (81-176 Sm^r Δ*flhB* [25]) or DRH1827 (81-176 Sm^r Δ*astA* Δ*flhB flgDE2*::*nemo* [19]). Transformants were recovered on MH agar containing 10 μg ml^{-1} Mtz. Mtz^r colonies appeared after 4 to 5 days of growth at 37°C under microaerobic conditions. For identification of transformants with *rdxA*::*cat*, 80 to 200 colonies were patched on MH agar containing 10 μg ml^{-1} chloramphenicol. One or two Mtz^r colonies were identified that were also resistant to chloramphenicol, and colony PCR was performed to confirm the *rdxA*::*cat* mutation.

DRH3107 (81-176 Sm^r Δ*flhB rdxA*::*flhB*) was recovered by stabbing Mtz^r transformants after electroporation of SNJ471 with pDRH3031 in MH motility medium containing 0.4% agar. After 24 h of incubation under microaerobic conditions at 37°C, one motile transformant was identified. An agar plug containing the motile bacteria was vortexed in 1 ml MH broth, and dilutions were plated on MH agar containing 10 μg ml^{-1} Mtz. Colonies were then verified by colony PCR to confirm the *rdxA*::*flhB* mutation.

DRH3057 (81-176 Sm^r Δ*astA* Δ*flhB flgDE2*::*nemo rdxA*::*flhB*) was recovered by electroporating DRH1827 with pDRH3031 and then plating transformants on MH agar containing 10 μg ml^{-1} Mtz and 35 μg ml^{-1} 5-bromo-4-chloro-3-indolyl sulfate, the chromogenic substrate for AstA. After incubation for 5 days at 37°C under microaerobic conditions, one transformant displayed a blue-colony phenotype, indicating complementation of expression of the *flgDE2-astA* transcriptional fusion. Colony PCR was performed to confirm the *rdxA*::*flhB* mutation.

Motility assays and arylsulfatase assays to monitor expression of flagellar genes. Motility phenotypes of *C. jejuni* wild-type, mutant, and complemented strains were assessed as previously described (26). Strains were grown from frozen stocks as described above, suspended in MH broth to an OD_{600} of 0.8, and stabbed into semisolid MH motility agar by using a sterilized inoculating needle. The plates were incubated for 30 h at 37°C under microaerobic conditions and then visualized for motility. Wild-type, mutant, and complemented strains containing *flgDE2*::*nemo*, a transcriptional fusion of *astA* to the *flgDE2* operon, were grown from frozen stocks as described above. Arylsulfatase production from the transcriptional fusions in these strains was measured by a previously published method (14, 19, 56).

RESULTS

Mutation of *rdxA* is responsible for Mtz^r. For ease in communicating our findings, we propose annotation of the *C. jejuni* 81-176 gene *cjj81176_1083* as *rdxA* and *cjj81176_1084* as *rdxB*, which were not annotated in the genome sequence of this strain (9). Our proposed annotation counters that of another *C. jejuni* strain (NCTC11168), for which *cj1064* (*cjj81176_1083* in the 81-176 genome) was annotated as a gene encoding a nonfunctional protein due to a frameshift mutation and *cj1066* (*cjj81176_1084* in the 81-176 genome) was annotated as *rdxA* (9, 39). As described in detail below, we found that the function of Cjj81176_1083 rather than Cjj81176_1084 was similar to that of RdxA of *H. pylori* in regard to being the determinant for Mtz^s, and our proposed annotation better reflects these findings. Because the coding sequence of *cjj81176_1084* has homology to type I nitroreductases and the gene is immediately downstream and in an apparent operon with *cjj81176_1083* (see below, Fig. 1A) (9), we propose to annotate *cjj81176_1084* as *rdxB*.

In preliminary studies, *C. jejuni* strain 81-176 was unable to grow on MH agar containing more than 5 μg ml^{-1} Mtz, suggesting that this strain may contain a gene encoding a protein that converts Mtz to a toxic form, thus resulting in Mtz^s. We used a transposon mutant library of *C. jejuni* strain 81-176 made with the *darkhelmet* transposon, a *mariner*-based transposon, to identify mutants with transposon insertions interrupting such a gene to result in Mtz^r (16). Nine Mtz^r transposon mutants were recovered on MH agar containing Mtz ranging from 5 to 50 μg ml^{-1}. All mutants had a *darkhelmet* insertion in *rdxA* (*cjj81176_1083*), with six independent insertions identified in the gene (Fig. 1A). *rdxA* is organized on the *C. jejuni* genome upstream of *rdxB* (*cjj81176_1084*) with the stop codon of *rdxA* overlapping the start codon of *rdxB*, sug-

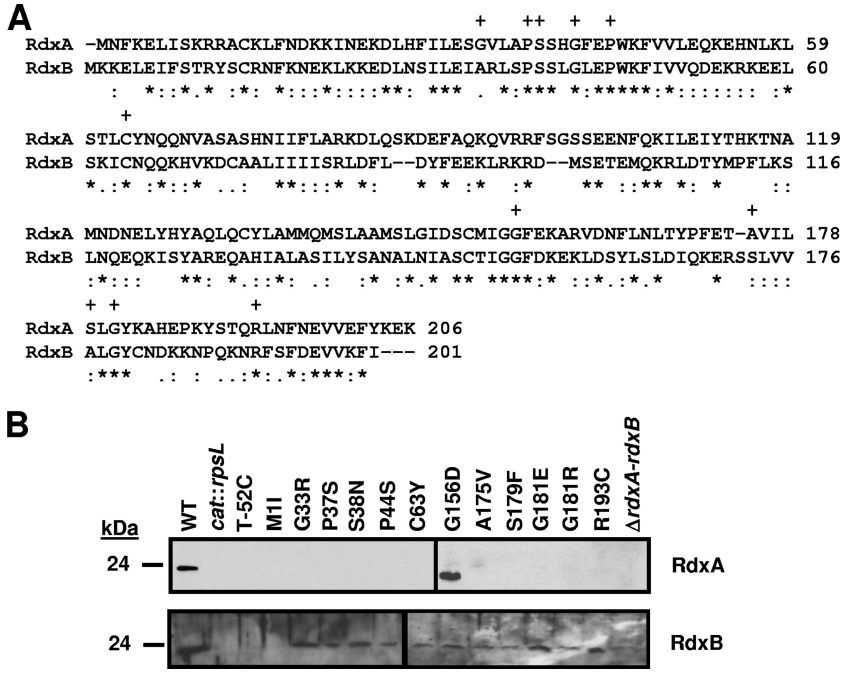

FIG. 2. Comparison of RdxA and RdxB amino acid sequences and production of RdxA and RdxB in *in vitro*- and *in vivo*-isolated Mtz^r *C. jejuni* 81-176 mutants. (A) ClustalV alignment of the amino acid sequences of RdxA and RdxB from *C. jejuni* 81-176. Asterisks (*) indicate identical residues, semicolons (:) indicate highly conserved residues, and periods (.) indicate weakly conserved residues. Plus signs (+) above residues of RdxA indicate residues changed by point mutations in Mtz^r *C. jejuni* isolates after *in vitro* or *in vivo* growth. (B) Immunoblot analysis of RdxA and RdxB production in whole-cell lysates of Mtz^r *C. jejuni* 81-176 isolates with point mutations in *rdxA* isolated after *in vitro* or *in vivo* growth. The wild-type strain is 81-176 Sm^r (DRH212) (WT), and the *cat*::*rpsL* mutants used in the immunoblots are DAR521 (81-176 Sm^r *rdxA*::*cat-rpsL*) and DAR562 (81-176 Sm^r *rdxB*::*cat-rpsL*), respectively. Most Mtz^r isolates are identified by the type of change occurring in the protein sequence after spontaneous mutation. T-52C refers to an Mtz^r isolate with a nucleotide change occurring 52 bases upstream of the *rdxA* start codon. Δ*rdxA-rdxB* refers to an Mtz^r isolate with a deletion of DNA encompassing the 3′ end of *rdxA* through the 5′ end of *rdxB*.

gesting an operonic construction and cotranscription. Both *rdxA* and *rdxB* encode putative nitroreductases, which are similar in size (201 to 206 amino acids) and amino acid sequence (34% identity and 60% similarity across the entire sequences) (Fig. 2A). RdxA and RdxB show a general level of similarity to a variety of type I nitroreductases, with conservation of key residues and putative structures for biochemical activity (as reviewed in reference 43).

All 81-176 *darkhelment* transposon mutants and a reconstructed 81-176 Sm^r *rdxA*::*cat-rspL* mutant promoted growth on MH containing 10 μg ml^{-1} Mtz, unlike the wild-type strain 81-176 or 81-176 Sm^r (DRH212) (Fig. 1B). However, production of both RdxA and RdxB was eliminated by interruption of *rdxA* with these transposons or the *cat*::*rpsL* cassette (Fig. 1C), hindering confirmation that *rdxA* is the determinant responsible for Mtz^s and that mutation solely of *rdxA* contributed to Mtz^r in these mutants. We next investigated if interruption of *rdxB* contributed to Mtz^r. Disruption of *rdxB* did not affect production of RdxA and did not change the Mtz^s phenotype of the parental *C. jejuni* wild-type strain (Fig. 1B and 1C).

As a more quantitative and sensitive method to verify that mutation solely of *rdxA* contributed to Mtz^r, we determined the MIC of Mtz for growth of wild-type and mutant *C. jejuni* strains. The MIC of Mtz for wild-type *C. jejuni* 81-176, 81-176 Sm^r, and 81-176 Sm^r *rdxB*::*cat-rpsL*, which produces RdxA but not RdxB (Fig. 1C), was 2.5 μg ml^{-1}, demonstrating that a lack of RdxB did not result in an increase in Mtz^r. In contrast, a spontaneous Mtz^r mutant isolated during *in vitro* growth (see below) with an *rdxA*(*A175V*) mutation that lacked production of RdxA but produced RdxB displayed a MIC of Mtz of 50 μg ml^{-1} (Fig. 2B and data not shown). In addition, all nine 81-176 *darkhelmet* transposon mutants that failed to produce RdxA and RdxB displayed a MIC of Mtz of 50 μg ml^{-1}. These combined results suggest that Mtz^r appeared to be due to mutation of *rdxA*. Furthermore, mutation of *rdxB* did not contribute to an increase in the MIC of Mtz in the presence or absence of RdxA at least as analyzed in this strain, 81-176.

Mtz^r can be generated in a stepwise process through accumulated mutations in various alleles of *H. pylori*, with each mutation allowing for growth in the presence of increasing levels of Mtz (1, 23, 24). We attempted to recover additional mutants in the *rdxA* mutant background that allowed for growth on higher concentrations of Mtz. We used the *rdxA*(*A175V*) mutant described herein, which failed to produce RdxA but produced wild-type RdxB and grew in the presence of 50 μg ml^{-1} Mtz. Several attempts were made to isolate colonies of the *rdxA*(*A175V*) mutant on media containing 100 and 200 μg ml^{-1} Mtz, but we were unable to obtain any colonies after incubation of bacteria for several days. These findings suggest that *rdxA* is the major determinant influencing growth in the presence or absence of Mtz, and other mutations that may allow growth at higher concentrations of Mtz could not be identified by this approach or are lethal to the bacterium.

Mtz^r is generated during *in vitro* or *in vivo* growth due to diverse mutations within *rdxA*. Mtz^r occurs in *H. pylori* during *in vitro* growth and *in vivo* growth in humans due to mutations mainly within *rdxA* (22, 24, 30, 48). We examined if Mtz^r isolates could be recovered after *in vitro* or *in vivo* growth of *C. jejuni* in the absence of selection. After growth on MH agar without Mtz, Mtz^r *C. jejuni* from seven independent cultures was isolated at a rate of $1.06 \times 10^{-6} \pm 0.32 \times 10^{-6}$. Sequencing of *rdxA* from 12 randomly selected isolates from each culture revealed that at least 87% of the isolates were derived independently from each other, with the remainder possibly representing siblings.

We next determined if Mtz^r could be generated during *in vivo* growth of *C. jejuni* in chicks, a natural host for the bacterium to promote a harmless, commensal colonization. Six 1-day-old chicks were orally gavaged with wild-type Mtz^s *C. jejuni* 81-176, and *C. jejuni* bacteria from the ceca were recovered at day 7 postinfection on selective agar with or without 10 μg ml^{-1} Mtz. Mtz^r *C. jejuni* was isolated from the cecum of each chick, with all isolates from five of the six chicks arising from independent mutations (see Table S1 in the supplemental material). Compared to the number of *C. jejuni* bacteria that colonized the chick ceca, the best approximation for the rate of Mtz^r was determined to be $8.62 \times 10^{-6} \pm 0.34 \times 10^{-6}$.

Mtz^r in both the *in vitro*- and *in vivo*-isolated mutants was due to many different types of mutations, including point, frameshift, insertion, and deletion mutations, occurring mostly within *rdxA* (see Tables S2 and S3 in the supplemental material). Approximately 44 to 50% of the *in vivo*- or *in vitro*-grown isolates, respectively, had lost or gained one or two nucleotides within the putative promoter region of *rdxA* or within the coding sequence causing frameshifts. Seventeen to 35% had transition or transversion mutations, mostly occurring in *rdxA*, resulting in either premature stop codons or amino acid changes that truncated, inactivated, or destabilized the protein (Fig. 2B). One transition mutation in the putative promoter of *rdxA* changed T-52 relative to the start codon, which prevented production of not only RdxA but also RdxB (Fig. 2B), suggesting that the two genes likely share a promoter and are cotranscribed. Two transition mutations resulted in coding sequences creating the RdxA(M1I) and RdxA(A175V) proteins, which were not detected in the mutants (Fig. 2B). RdxA(A175V) was likely an unstable protein, whereas RdxA(M1I) was may not have been translated due to alteration of the start codon. Curiously, RdxB was not detected in the *rdxA*(*M1I*) mutant, suggesting that the mutation may have caused instability of a putative bicistronic mRNA for *rdxAB* or lack of translation of the entire transcript. An additional missense mutation caused a mutant to produce RdxA(G156D), which was produced at wild-type levels but likely was enzymatically inactive (Fig. 2B).

The remaining 18 to 32% of Mtz^r isolates had insertion or deletion of DNA segments of at least 7 and up to 902 nucleotides in length within *rdxA* or the *rdxAB* operon to cause frameshifts in the *rdxA* coding sequence (see Tables S2 and S3 in the supplemental material). Mutants with DNA insertions in *rdxA* resulted from a direct duplication of a DNA segment to create a two-unit heteropolymeric nucleotide repeat of 7 to 179 nucleotides in length. For the mutants that had lost 22 to 902 bp of DNA within *rdxA* or *rdxAB*, the event leading to deletion of the DNA usually occurred at an apparently random site within the gene or locus. However, the DNA that was lost in three of the mutants was flanked by identical DNA repeats 8 to 12 nucleotides in length. These DNA repeats may have influenced a recombination event leading to excision of the intervening DNA. Only one *in vivo*-grown Mtz^r isolate did not possess a mutation within *rdxAB*, and the mutation leading to Mtz^r remains unidentified.

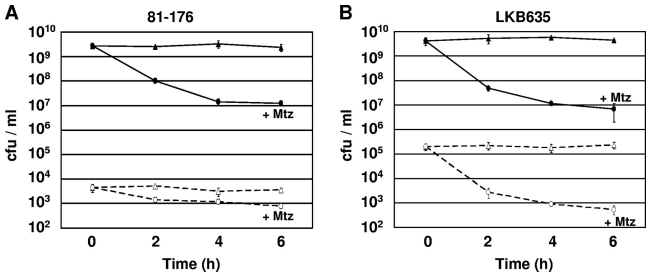

FIG. 3. Analysis of Mtz-induced killing and mutation over time. (A and B) Mtz^s wild-type *C. jejuni* 81-176 or 81-176 Sm^r *cjj0275*::*cat-rpsL* (LKB635) was grown and then not exposed (triangles) or exposed (circles) to 10 μg ml^{-1} Mtz over the course of the assay. At each time point, bacteria were plated on MH agar containing TMP (closed symbols, solid lines or 10 μg ml^{-1} Mtz (open symbols, dashed lines)) (A) or MH agar containing TMP (closed symbols, solid lines) or 2 mg ml^{-1} Sm (open symbols, dashed lines) (B). The average number of viable bacteria at each time point is reported as the number of CFU per ml. Each assay was performed in triplicate, and the standard deviation is indicated by bars.

Of note, a small collection of *C. jejuni* strains in our laboratory was analyzed for Mtz^s. We found that those strains that were Mtz^s appeared to contain an intact *rdxA* allele with only one or two amino acid changes, suggesting that a functional RdxA protein was produced. Strains that were Mtz^r all had multiple point mutations and frameshift mutations in *rdxA*, corroborating data of our studies with *C. jejuni* 81-176 that disruption of *rdxA* contributes to Mtz^r (data not shown).

Due to the reported mutagenic nature of reduced Mtz (46), Mtz^r isolates we recovered after *in vitro* and *in vivo* growth could represent two populations: (i) those that spontaneously mutated *rdxA* by DNA replication errors prior to Mtz exposure to result in Mtz^r or (ii) those that are Mtz induced once plated on agar containing 10 μg ml^{-1} Mtz. To help distinguish the origins of these two possible populations, we exposed *in vitro*-grown cultures of Mtz^s wild-type *C. jejuni* 81-176 to 10 μg ml^{-1} Mtz and compared the number of Mtz^r isolates generated over time to those without Mtz. We detected a 225-fold decrease in viability of 81-176 over a 6-h period with exposure to Mtz (Fig. 3A). Examination of the Mtz^r population after exposure to Mtz for 2 h indicated that 31% of this population was Mtz^r prior to any Mtz exposure (Fig. 3A; compare time 2 h with time zero h) and suggested that approximately one-third of the total Mtz^r isolates were generated by spontaneous mutation through DNA replication errors in the absence of Mtz. Furthermore, we did not detect any increase in the number of Mtz^r isolates with exposure to Mtz over time (Fig. 3A).

We next examined if Mtz exposure caused nonspecific mutation at a second locus to result in an increase in the number of antibiotic-resistant mutants. To this end, we used strain LKB635, a *C. jejuni* 81-176 derivative that has a wild-type *rpsL* allele and a recessive streptomycin-resistant (Sm^r) *rpsL* allele

TABLE 1. Reductase and oxidase activities of RdxA and RdxB and respective GST fusions for various substrates

Activity type	Substrate (acceptor)	Sp act (μmol/min/mg of protein) ± SE[a]				
		GST-RdxA	RdxA[b]	GST-RdxB	RdxB[b]	GST
Oxidase	NADPH (O_2)	2.4 ± 0.50	2.1 ± 0.01	0.3 ± 0.04	1.25 ± 0.01	<0.01
Reductase	Mtz, anaerobic	0.18 ± 0.07	0.19 ± 0.02	0.03 ± 0.01	0.06 ± 0.01	<0.01

[a] Specific activities were calculated as the means of results from two assays from two independent purifications of the protein ± standard errors.
[b] GST-RdxA or GST-RdxB was treated with thrombin to cleave the fusion proteins and release the free nitroreductase protein. Thrombin was removed from the nitroreductases by benzamidine Sepharose 6B.

(*rpsL*^{Sm}). Analogous to mutations in *rdxA* that result in Mtz^r, any type of disruption to the wild-type *rpsL* allele leading to aberrant protein production will result in Sm^r. We incubated this strain in the presence and absence of Mtz and then monitored viability and generation of Sm^r over time. Similar to the results with exposure of 81-176 to Mtz, we detected a 600-fold reduction in viability over 6 h (Fig. 3B). In addition, we did not detect an increase in Sm^r isolates over time with Mtz exposure (Fig. 3B). Taken together, these results suggest that many of the Mtz^r isolates recovered after *in vitro* or *in vivo* growth were due to spontaneous DNA replication errors rather than strictly being induced by the mutagenic potential of Mtz.

RdxA and RdxB reduce nitro compounds. *In vitro* biochemical analysis of RdxA was performed to provide evidence that the protein reduces Mtz, which would corroborate our findings that production of RdxA in *C. jejuni* is responsible for Mtz^s. Additional nitro compounds, such as those that have been shown to be modified by RdxA and FrxA of *H. pylori*, were also used to more fully characterize *C. jejuni* RdxA as a potential nitroreductase (45). Since RdxB is also a putative nitroreductase, we analyzed the reductase activity of this protein as well.

We first cloned *rdxA* and *rdxB* into an expression vector in *E. coli* BL21(DE3) to create N-terminal GST fusion proteins. After induction and purification, both GST-RdxA and GST-RdxB protein preparations, but not GST alone, were yellow in color, suggesting that the flavin cofactor was bound to the fusion proteins. The flavin cofactor was determined to be FMN by protein denaturation followed by TLC (data not shown). Further analysis in *E. coli* BL21(DE3), which is normally Mtz^r, on medium containing 10 μg ml^{-1} Mtz showed that expression via induction of GST-RdxA or GST-RdxB rendered *E. coli* more Mtz^s, indicating that our recombinant proteins were active.

A potential problem of using GST fusion proteins for biochemical characterization of enzymes is that the GST component could artificially contribute to the activity under investigation. To ensure that the nitroreductase component of these fusions was largely responsible for enzymatic activity, the GST component from each fusion was removed by thrombin-mediated cleavage. The recovered nitroreductases were almost identical to the native proteins in *C. jejuni* with just the initial methionine replaced with a glycine and serine residue.

RdxA, RdxB, and the respective uncleaved GST fusion proteins were then examined for their ability to oxidize NADPH using oxygen as an electron acceptor or reduce Mtz, nitrofurazone, furazolidone, and nitrofurantoin using NADPH as an electron donor. As shown in Table 1, RdxA, RdxB, and the respective GST fusion proteins oxidized NADPH. Because the activities of RdxA and RdxB were similar with the respective GST fusion proteins and GST alone did not promote oxidation of NADPH, we conclude that these activities were specific to the nitroreductases. Furthermore, RdxA demonstrated a higher specific activity of approximately 2- to 8-fold over that of RdxB. This potent oxidase activity is in line with what has been observed with RdxA of *H. pylori* (37).

Assays to ascertain the reductase activities of these enzymes were initially performed under aerobic conditions. These experiments revealed that RdxA and RdxB were able to reduce all nitro compounds (data not shown). However, when we accounted for the specific endogenous NADPH oxidase activity present for each nitro compound, we could not demonstrate any significant reductase activity for any of the compounds tested, including Mtz, under aerobic conditions. As reported by Olekhanovich et al. (37), reduction of Mtz by RdxA of *H. pylori* occurred only under strict anaerobiosis, because trace amounts of molecular oxygen inhibited reduction. Considering these findings, we tested RdxA and RdxB for Mtz reduction under anaerobic conditions. We observed Mtz reduction under these conditions that was specific for the nitroreductases and not due to GST. Again, RdxA and GST-RdxA demonstrated 3- to 6-fold higher activity than RdxB and GST-RdxB, possibly corroborating our genetic analysis that RdxA influences Mtz^s and Mtz^r in *C. jejuni*. Furthermore, these data support previous conclusions of the different enzymatic characteristics of nitroreductases of epsilonproteobacteria (as demonstrated by *H. pylori* RdxA [37]) compared to those of other bacteria.

***rdxAB* is a cotranscribed operon induced during *in vivo* growth.** The genomic organization of *rdxA* and *rdxB* with the coding sequences of the genes overlapping suggests a cotranscribed operon. Inactivation of *rdxA* with transposons or antibiotic resistance-encoding cassettes and spontaneous mutation of the *rdxA* promoter region all resulted in the absence of RdxB, also suggesting cotranscription of these genes (Fig. 1C and 2B). By using reverse transcriptase PCR (RT-PCR), we detected an mRNA transcribing both *rdxA* and *rdxB*, verifying that these genes are cotranscribed (Fig. 4A). Primer extension analysis revealed that the start site of transcription for *rdxA* (and perhaps *rdxAB*) was located at T-28 relative to the start codon of *rdxA* (Fig. 4B). A putative −10 binding site for σ^{70} was located upstream of this site, suggesting T-28 as the most likely start site of transcription.

Preliminary real-time RT-PCR analysis suggested that the *rdxAB* operon was not highly expressed during *in vitro* growth of *C. jejuni* (data not shown). We considered if this operon may be preferentially expressed *in vivo* during commensal colonization of the ceca of chicks. One-day-old chicks were infected with *C. jejuni* 81-176 Sm^r (DRH212), and the chicks were sacrificed at day 7 postinfection. Cecal contents were isolated,

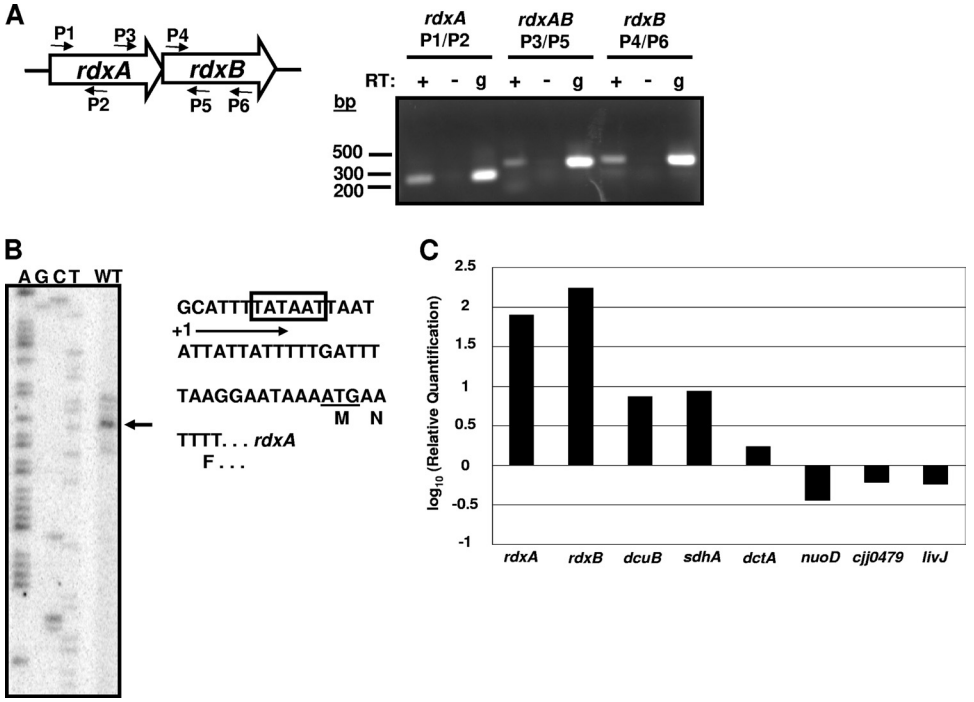

FIG. 4. Analysis of expression of the *rdxAB* operon during *in vitro* and *in vivo* growth. (A) Examination of cotranscription of the *rdxAB* operon. Left, diagram of the *rdxAB* locus and locations of primers used for reverse transcriptase PCR (RT-PCR) analysis. Right, DNA fragments generated after RT-PCR analysis. Three sets of PCRs were performed, each using the indicated primer pair. Each PCR set included a reaction mixture containing cDNA generated from *C. jejuni* 81-176 RNA after *in vitro* growth (RT +), a reaction of the same RNA incubated in the absence of RT (RT −) to verify the absence of DNA in the RNA preparation, and a positive-control reaction using genomic DNA (g) as a template for amplification of a DNA product with the primers used. (B) Primer extension analysis to identify the transcriptional start site for *rdxAB*. Left, the arrow indicates the most prominent start site of transcription next to the sequencing ladder generated with the same primer used for the primer extension reaction. Right, partial sequence of the promoter region for *rdxAB*. The box indicates the proposed −10 binding site for σ^{70}, +1 indicates the start site of transcription, and the start codon is underlined. The amino acids of the protein are indicated below each codon. (C) Semiquantitative real-time RT-PCR analysis of the level of expression of *rdxA* and *rdxB* during *in vivo* and *in vitro* growth. Expression analysis was also performed on other genes shown and described in the text as controls for detecting specific induction of *in vivo* expression. Values indicated are the $\log_{10}$-fold increase or decrease in expression relative to expression of *in vitro*-grown samples.

and RNA was extracted. In comparison to *in vitro*-grown DRH212, expression of *rdxA* and *rdxB* were approximately 75- to 175-fold higher *in vivo*, respectively (Fig. 4C). We also analyzed expression of other genes to determine if enhanced expression of *rdxA* and *rdxB* was specific for *in vivo* growth. A previous study examining expression of genes of *C. jejuni* strain 11168 after a 12-h infection of chicks compared to results for *in vitro*-grown cultures found no differential expression of *dctA* and *nuoD* and a 19- to 32-fold-increased expression of *dcuB* and *sdhA*, respectively, *in vivo* (54). We found similar trends in expression of these genes in our analysis (Fig. 4C). Additionally, we included analyses of expression of two additional genes, *livJ* and *cjj0479*, identified in a previous study as ones required for optimal colonization of chicks (18). Both of these genes did not show evidence of increased expression *in vivo* (Fig. 4C). Thus, augmented expression of *rdxA* and *rdxB* was specific for *in vivo* growth, although the precise mechanism behind this increased expression remains unknown at this time.

Considering these data, we speculated that RdxA or RdxB may be necessary for wild-type levels of colonization of the chick ceca. One-day-old chicks were infected with approximately 100 or 10^4 CFU of wild-type *C. jejuni* 81-176 or 81-176 mutants lacking RdxA, RdxB, or both. For these experiments, we used 81-176 *rdxB*::*cat-rpsL* as a mutant lacking production of RdxB but unaffected for production of RdxA (data not shown). Due to the inability to generate an insertionally inactivated *rdxA* mutant that did not affect production of RdxB, we took advantage of one of the Mtz^r isolates recovered after *in vitro* growth, *rdxA*(*A175V*). This mutant did not produce RdxA but was unaffected for production of RdxB (Fig. 2B). For an *rdxAB* double mutant, we took advantage of another Mtz^r isolate recovered after *in vitro* growth, which lacked a portion of DNA from nucleotide 601 of *rdxA* through nucleotide 620 of *rdxB* (see Table S3 in the supplemental material). This strain produced neither RdxA nor RdxB (Fig. 2B).

At 7 days postinfection, the levels of wild-type and mutant *C. jejuni* strains in the ceca were determined. In chicks infected with approximately 10^4 CFU of *C. jejuni* strains, only the *rdxA* mutant showed an average colonization defect of approximately 5-fold, which was not statistically significant (Fig. 5). When the inoculum was reduced to approximately 100 CFU, the *rdxA* mutant was present at a lower level in four out of six chicks than the wild-type strain in respective infected birds. These levels resulted in an average colonization defect of approximately 1,200-fold compared to the wild-type strain, which was marginally significant ($P < 0.05$, two-tailed Mann-Whitney

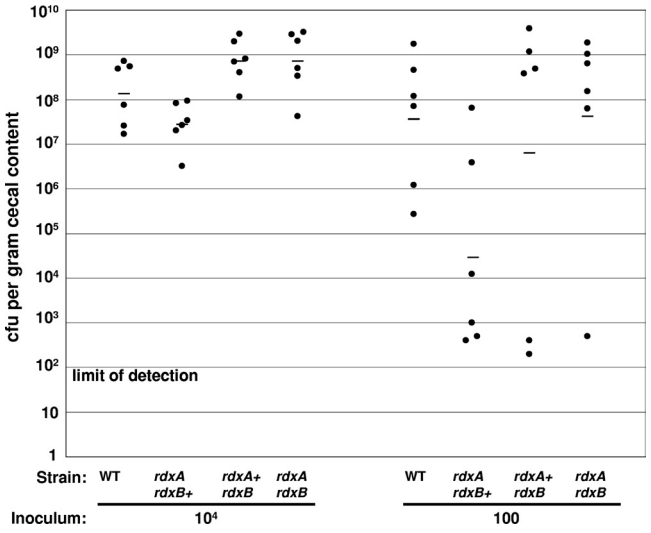

FIG. 5. Commensal colonization capacity of wild-type *C. jejuni* 81-176 and 81-176 mutant strains. One-day-old chicks were orally gavaged with 3.5×10^3 to 1.16×10^4 CFU (to approximate an inoculum of 10^4 CFU) or 11 to 85 CFU (to approximate an inoculum of 100 CFU) of wild-type *C. jejuni* 81-176 or 81-176 derivatives lacking production of RdxA, RdxB, or both RdxA and RdxB. At seven days postinfection, the number of *C. jejuni* CFU in the cecal contents of each chick was determined. Each dot represents the load of *C. jejuni* in the cecum of an individual chick. The geometric mean of the bacterial loads from each set of chicks is denoted by the horizontal bar. The status of *rdxA* and *rdxB* is indicated for each strain analyzed. Statistical analysis was performed using the two-tailed Mann-Whitney U test ($P < 0.05$).

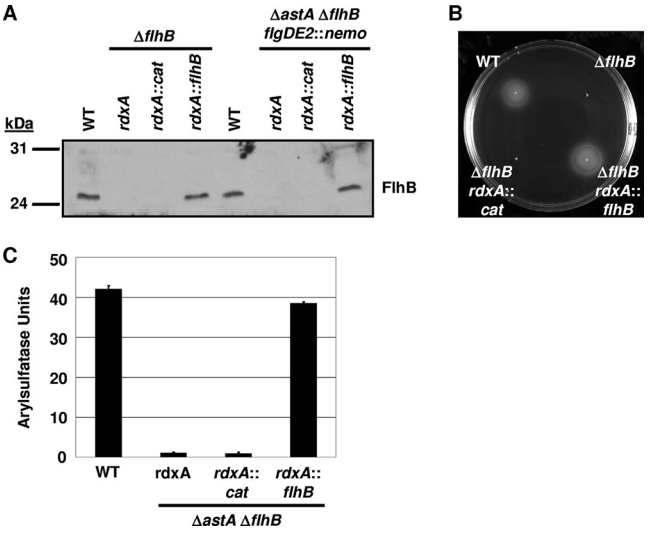

FIG. 6. *rdxA* as a locus for genomic complementation of mutants in *C. jejuni* in *cis*. (A) Immunoblot analysis of FlhB production in wild-type, mutant, and complemented strains of *C. jejuni*. Total membranes from wild-type *C. jejuni* strains and respective mutant or complemented derivatives were analyzed for production of FlhB. Strains (from left to right) include DRH212 (wild-type 81-176 Sm^r), SNJ471 (81-176 Sm^r Δ*flhB*), DRH3067 (81-176 Sm^r Δ*flhB rdxA*::*cat*), DRH3107 (81-176 Sm^r Δ*flhB rdxA*::*flhB*), DRH533 (wild-type 81-176 Sm^r Δ*astA flgDE2*::*nemo*), DRH1827 (81-176 Sm^r Δ*astA* Δ*flhB flgDE2*::*nemo*), DRH3056 (81-176 Sm^r Δ*astA* Δ*flhB flgDE2*::*nemo rdxA*::*cat*), and DRH3057 (81-176 Sm^r Δ*astA* Δ*flhB flgDE2*::*nemo rdxA*::*flhB*). (B) Flagellar motility phenotypes of wild-type *C. jejuni* 81-176 Sm^r (DRH212) derivatives. Motility was assessed 30 h after inoculation in MH motility agar and incubation at 37°C under microaerobic conditions. Strains include DRH212, SNJ471, DRH3067, and DRH3107. (C) Arylsulfatase assays for analysis of expression of *flgDE2*::*nemo*, encoding an *flgDE2-astA* transcriptional fusion in *C. jejuni* 81-176 Sm^r Δ*astA flgDE2*::*nemo* derivatives. The results are from a typical assay with each strain tested in triplicate. The values reported for each strain are the average arylsulfatase activity ± standard deviation. Strains include DRH533, DRH1827, DRH3056, and DRH3057.

U test). In no instances did we detect significant colonization defects of the *rdxB* mutant or the *rdxAB* double mutant, even though RdxA was absent in the latter strain. These results suggest that the *rdxA* mutant may have a dose-dependent colonization defect; however, this defect is not recapitulated in a mutant lacking both *rdxA* and *rdxB*.

***rdxA* as a site for *cis* complementation of mutants in *C. jejuni*.** The *rdxA* locus of *H. pylori* has been used as a site on the chromosome for insertion of genes to express in *cis* for complementation of mutants (6, 33). The advantage of this method is that Mtz^r can be used as a marker to identify transformants containing *rdxA* interrupted with the gene for *cis*-chromosomal complementation. We expected that a similar complementation and selection strategy could be used to complement *C. jejuni* mutants, which would be an additional tool beneficial for the *C. jejuni* field.

To test *rdxA* for accepting genes for the purpose of complementation in *cis*, we inserted DNA containing the putative promoter and coding sequence of *flhB*, which encodes a component of the flagellar export apparatus, into *rdxA* (17, 19, 25). A Δ*flhB* mutant of *C. jejuni* lacks flagellar biosynthesis and motility, due in part to the lack of expression of σ^{54}-dependent flagellar genes encoding many components of the flagellar basal body and hook (17, 19, 25). *rdxA* of two different *C. jejuni flhB* mutants, SNJ471 (81-176 Sm^r Δ*flhB*) and DRH1827 (81-176 Sm^r Δ*astA* Δ*flhB flgDE2*::*nemo*), was replaced with *rdxA*::*flhB* to test the ability of this construct to complement the mutants for restoration of motility and expression of the σ^{54}-dependent *flgDE2* operon, encoding two flagellar-hook-associated proteins. As a control, we also transformed these mutants with *rdxA* interrupted by *cat*, encoding chloramphenicol acetyltransferase (55). Approximately 1 in 100 Mtz^r colonies contained *rdxA* interrupted with the complementing wild-type *flhB* allele. As shown in Fig. 6A, wild-type and complemented strains containing *rdxA*::*flhB* but not *rdxA*::*cat* produced equal amounts of FlhB in the total membrane fraction of the strains.

We then tested the strains for restoration of motility and σ^{54}-dependent flagellar gene expression. We observed that the Δ*flhB* mutant containing *rdxA*::*flhB* was restored for motility to wild-type levels but an isogenic mutant with *rdxA*::*cat* was not (Fig. 6B). Similarly, expression of the σ^{54}-dependent flagellar operon *flgDE2* as analyzed by a *flgDE2-astA* transcriptional fusion (encoded by *flgDE2*::*nemo*) was restored to wild-type levels in the Δ*flhB* mutant with *rdxA*::*flhB* but not in the mutant containing *rdxA*::*cat* (Fig. 6C). These results indicated the functionality of using *rdxA* as a site for chromosomal insertion of genes for *cis* complementation of *C. jejuni* mutants.

DISCUSSION

Despite the almost ubiquitous presence of nitroreductases in diverse bacterial species, the biological function and the natu-

ral substrate of most nitroreductases are unknown. These enzymes, however, have been shown to have useful applications in industry and human medicine by reducing certain xenobiotic compounds. In this study, we found the RdxA and RdxB nitroreductases of *C. jejuni* can reduce Mtz, a prodrug that is used in therapeutic treatment of the closely related bacterium *H. pylori* and certain parasitic infections (53). Production of wild-type RdxA but not RdxB was associated with Mtz^s, presumably due to *in vivo* conversion of Mtz to a toxic form that is lethal to *C. jejuni*. Even though we demonstrated the ability of RdxA to reduce Mtz *in vitro*, Mtz is a xenobiotic compound and likely not the natural substrate for RdxA. Thus, we suspect that RdxA and also RdxB function in other biological processes in *C. jejuni* whose pathways and natural substrates have yet to be elucidated.

In *H. pylori*, Mtz^r is typically due to a loss of RdxA or production of catalytically inactive RdxA (11). Mutation of a gene encoding a second nitroreductase, FrxA, has been shown to contribute to Mtz^r in some strains (22–24, 30, 48). RdxA and RdxB of *C. jejuni* are paralogs of RdxA and FrxA of *H. pylori* but are as similar to each other as they are to other bacterial nitroreductases. Contrary to findings of some studies in *H. pylori*, we found that mutation of only *rdxA* contributed to Mtz^r in *C. jejuni* strain 81-176. Transposon mutagenesis of *C. jejuni* identified six independent insertions in *rdxA*, but none in *rdxB*, leading to Mtz^r. Furthermore, all spontaneous Mtz^r isolates generated during *in vitro* or *in vivo* growth had mutations in *rdxA* or *rdxAB*, but none had only a mutation in *rdxB*. An exception was one Mtz^r isolate for which the location of the mutation could not be identified. These data indicate that elimination of wild-type RdxA resulted in Mtz^r.

To confirm that mutation of *rdxB* did not contribute to Mtz^r in *C. jejuni* 81-176, we constructed a mutant that produced wild-type RdxA but lacked RdxB. The MIC of Mtz for this *rdxB* mutant was equal to that of the Mtz^s parental wild-type strain (approximately 2.5 μg ml^{-1}). Additionally, mutations that eliminated RdxA and RdxB did not increase the MIC value relative to that for a Mtz^r strain lacking only RdxA (approximately 50 μg ml^{-1}). *In vitro* biochemical analysis revealed reduction of Mtz by both proteins, suggesting that RdxB could contribute to Mtz^s. However, the specific activity of RdxB for Mtz was 3- to 6-fold lower than that of RdxA, which may not be physiologically significant in *C. jejuni* 81-176 to promote conversion of Mtz to a toxic form for Mtz^s in the absence of RdxA. It is possible that in other *C. jejuni* strains, RdxB may influence Mtz^r, and this possibility is currently under examination. Although we could not show a role for RdxB in Mtz^r in strain 81-176, we believe that the major determinant influencing growth of this strain in the presence or absence of Mtz is *rdxA*.

In *H. pylori*, it has been shown that Mtz^r is largely due to mutations within *rdxA*, and additional mutations in *frxA* and other genes can cause a stepwise increase in the MIC of Mtz for the mutant derivatives (1, 23, 24). In our hands, a *C. jejuni* 81-176 derivative lacking production of wild-type RdxA had a MIC of Mtz of 50 μg ml^{-1}. We attempted to isolate mutants in this background that were able to grow on higher concentrations of Mtz, hopefully identifying other genes potentially mutated that would contribute further to Mtz^r, similar to what has been observed in *H. pylori*. However, we were never able to isolate a mutant that could grow on Mtz above 50 μg ml^{-1}. The lack of isolation of such a mutant suggests one of the following possibilities: (i) mutation of a gene that would contribute to growth on higher levels of Mtz, like those identified in *H. pylori*, may be lethal when generated in *C. jejuni*; (ii) Mtz at concentrations higher than 50 μg ml^{-1} may inhibit growth of *C. jejuni* by a mechanism independent of reduction by a bacterial nitroreductase; or (iii) an exhaustive screen, more intensive than the one used in the current study, may be required to find rare, spontaneous mutants in the *rdxA* mutant background that allow for growth on concentrations of Mtz higher than 50 μg ml^{-1}. These findings possibly suggest that the mechanisms of Mtz^r show some deviations from the multiple ones in *H. pylori* that allow for growth in the presence of this prodrug.

Olekhnovich et al. have recently characterized the biochemical activity of *H. pylori* RdxA for reduction of Mtz (37). Significant findings from this work included the finding that RdxA of *H. pylori* demonstrated a 60-fold higher activity than the NfsB nitroreductase of *E. coli* under strict anaerobic conditions. Additionally, RdxA was found to possess a strong NADPH oxidase activity. These data imply that the *H. pylori* RdxA nitroreductase and possibly other nitroreductases from epsilonproteobacteria may have unique biochemical and functional properties. As such, we found that the *C. jejuni* nitroreductases could also reduce Mtz *in vitro* only under anaerobic conditions and demonstrated a robust NADPH oxidase activity. Future work could reveal interesting findings by examining nitroreductases of other epsilonproteobacteria to discern if these enzymes universally share characteristics that differentiate them from those of other bacteria.

Another possibly interesting technique we discovered that may be of interest to the nitroreductase field is the use of GST fusions to purify and analyze the enzymology of nitroreductases. In the case of RdxB, we could easily purify a GST fusion protein from *E. coli* that contained the flavin cofactor and demonstrated enzymatic activity. Removal of GST by a thrombin-mediated cleavage enhanced enzymatic activity. For RdxA, the GST fusion protein appeared to contain a specific activity comparable to that of native RdxA. Thus, the use of GST fusion proteins may be a convenient method for purification of soluble, stable, and active nitroreductases.

In our analyses, we found a specific increase in expression of *rdxA* and *rdxB* during *in vivo* growth in the chick ceca, implying that these genes may be involved in colonization processes. Therefore, we assessed the ability of mutants lacking RdxA, RdxB, or both RdxA and RdxB to promote commensal colonization of the chick ceca. An *rdxB* mutant did not demonstrate an appreciable colonization defect. An Mtz^r mutant lacking RdxA did not show a significant colonization defect when administered at an inoculum of 10^4 CFU. At an inoculum of 100 CFU, we did observe a lower colonization rate for four of six chicks compared to that for chicks infected with wild-type bacteria. Confusing these data is the observation that the possible colonization defect due to a lack of RdxA was not maintained in a mutant lacking both RdxA and RdxB, which colonized at wild-type levels. Currently we cannot provide an explanation for this discrepancy. A limited contribution of RdxA to commensalism may not be surprising considering the prevalence of Mtz^r *C. jejuni* isolated from both wild and agriculturally significant birds (13, 47), presumably due to a loss of

RdxA. Thus, the loss of *rdxA* most likely does not significantly hinder the ability of the bacterium to colonize avian hosts in nature. The possibility remains that RdxA or RdxB could be important for infection of humans to promote disease or in maintaining viability in the environmental reservoirs for transmission to humans and animals. Testing of these hypotheses will require development of better *in vitro* and *in vivo* model systems.

During analysis of the *rdxAB* locus, we frequently identified Mtz^r isolates after *in vitro* or *in vivo* growth of *C. jejuni* in the absence of selection. Examination of such mutants revealed that point and frameshift mutations, in addition to insertion and deletion of DNA fragments up to approximately 0.9 kb in length, mainly within *rdxA*, were responsible for generation of Mtz^r. These results possibly suggest that these mutations are generated randomly in *C. jejuni* during normal growth. However, since Mtz is a mutagen (46), these mutations could have been induced by exposure to Mtz rather than having been generated spontaneously through DNA replication errors. Over a series of experiments, we determined that a significant proportion of the population of *C. jejuni* (at least 31%) developed Mtz^r due to spontaneous mutation and that Mtz did not contribute to increased Mtz^r in wild-type *C. jejuni* or Sm^r in an appropriate *C. jejuni* mutant over time. While these experiments are not perfect in their execution, they provide evidence that *C. jejuni* does undergo spontaneous mutation of *rdxA* resulting in Mtz^r.

Some of the spontaneous mutations that resulted in disruption of *rdxA* to give the Mtz^r phenotype are likely caused by the absence in *C. jejuni* of a complete methyl-directed mismatch repair (MMR) system consisting of MutL, MutH, and MutS, usually present in other bacteria (9, 10, 39). MMR normally corrects base pair mismatches and insertions or deletions of small pieces of DNA, usually under four nucleotides in length (29). Instead, *C. jejuni* produces only MutS, but the protein is a member of the MutS2 family, of which a homolog in *H. pylori* has been shown to prevent oxidative damage to DNA (8, 50). Loss of MMR would presumably contribute to some mutations, such as point and small frameshift mutations, within *rdxA*, causing Mtz^r. The lack of MMR may also contribute to a high degree of phase and antigenic variation of important virulence and colonization factors, such as lipooligosaccharide (LOS) glycosylation, capsule production, and flagellar motility (7, 12, 36, 42, 44, 51, 52). Recently we identified *flgS* and *flgR*, encoding the sensor kinase and response regulator of a two-component system required for expression of σ^{54}-dependent flagellar genes, as two genes undergoing phase variation (15, 16). However, analysis of spontaneous revertants of *flgS* and *flgR* "OFF" variants restored for flagellar motility revealed that not all mutations in the "ON" revertants correctly repaired the original lesion in *flgS* or *flgR*. Instead, second-site suppressor mutations and intragenic recombinations, including insertion of repeated DNA sequences and deletions of DNA, were detected in *flgS* and *flgR* to create functional mutant proteins that restored flagellar gene expression and motility (15, 16). These mutations that occurred in *flgS* are similar to the ones contributing to alterations in *rdxA*, conferring Mtz^r (16). Since the revertant *flgS* alleles were generated in the absence of Mtz, these findings are indirect, corroborative evidence that genetic variability occurs often and randomly to affect production of many phenotypes of *C. jejuni*, including flagellar motility and Mtz^r.

Through the contribution of mutation of *rdxA* leading to Mtz^r, we were able to complement mutants of *C. jejuni* by a very similar approach that has been useful in *H. pylori* research (6, 33). By interrupting *rdxA* with *flhB* in *flhB* mutants of *C. jejuni*, we showed the functionality of *cis* complementation of motility mutants by recovery of Mtz^r transformants. We expect this type of complementation to be useful in complementing a wide range of mutants in the bacterium. Thus, we have added another genetic tool to the *C. jejuni* field that is likely to assist the molecular analysis of *C. jejuni*.

In this study, analysis of the *rdxAB* locus has revealed various interesting aspects of the biology of *C. jejuni*, ranging from DNA mutation, antibiotic resistance, and biochemistry of metabolic enzymes. Furthermore, we provide supportive evidence that the biochemical mechanisms of Rdx proteins of *C. jejuni* and *H. pylori* demonstrate characteristic anaerobic reduction of Mtz, as well as potent oxidation of NADPH, which may be common to those of epsilonproteobacteria and are intrinsically different from those of the nitroreductases of enteric bacteria. In addition, continued exploration of *C. jejuni* RdxA or RdxB in other potential infection models or assays may reveal a natural substrate for these nitroreductases and a biological function in infection, environmental survival, or transmission.

ACKNOWLEDGMENTS

This work was supported by NIH grant R01 AI065539 and by National Research Initiative grants 2006-35201-17382 and 2009-35201-05039 from the USDA Cooperative State Research, Education, and Extension Service Food Safety Program.

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