5'-Methylthioadenosine Nucleosidase is Implicated in Playing a Key Role in a Modified Futalosine Pathway for Menaquinone Biosynthesis in Campylobacter jejuni

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Running Head: Modified futalosine pathway

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Menaquinone (vitamin K₂) serves as an electron carrier in the electron transport chain required for respiration in many pathogenic bacteria. Most bacteria utilize a common menaquinone biosynthetic pathway as exemplified by Escherichia coli. Recently, a novel biosynthetic pathway, the futalosine pathway, was discovered in Streptomyces. Bioinformatic analysis strongly suggests this pathway is also operative in the human pathogens Campylobacter jejuni and Helicobacter pylori. Here we provide compelling evidence that a modified futalosine pathway is operative in C. jejuni and that it utilizes 6-amino-6-deoxyfutalosine instead of futalosine. A key step in the Streptomyces pathway involves a nucleosidase called futalosine hydrolase. The closest homolog in C. jejuni has been annotated as a 5'-methylthioadenosine nucleosidase (MTAN). We have shown that this C. jejuni enzyme has MTAN activity but negligible futalosine hydrolase activity. However, the C. jejuni MTAN is able to hydrolyze 6-amino-6-deoxyfutalosine at a rate comparable to that of its known substrates. This suggests that the adenine-containing version of futalosine is the true biosynthetic intermediate in this organism. To demonstrate this in vivo, we constructed a C. jejuni mutant strain deleted for mqnA2, which is predicted to encode for the enzyme required to synthesize 6-amino-6-deoxyfutalosine. Growth of this mutant was readily rescued by the addition of 6-amino-6-deoxyfutalosine, but not futalosine. This provides the first direct evidence that a modified futalosine pathway is operative in C. jejuni. It also highlights the tremendous versatility of the C. jejuni MTAN that plays key roles in S-adenosylmethionine recycling, the biosynthesis of autoinducer molecules, and the biosynthesis of menaquinone.

INTRODUCTION

Menaquinone (vitamin K₂) is a lipid-soluble prenylated 2-methyl-1,4-naphthoquinone that plays a variety of roles in both eukaryotes and prokaryotes (Fig. 1). In humans, it serves as a cofactor that is required for the posttranslational generation of γ-carboxyglutamate residues in proteins involved in blood coagulation, bone metabolism, and vascular physiology (1). In bacteria, it plays a key role as an electron carrier in the electron transport chain required for respiration (2). While most Gram-negative bacteria, such as Escherichia coli, use menaquinone under anaerobic conditions and ubiquinone under aerobic conditions, Gram-positive bacteria and many other Gram-negative bacteria rely on menaquinone as their sole electron carrier (3,4). These include many pathogenic organisms such as
Helicobacter pylori, Campylobacter jejuni, Staphylococcus aureus, and Mycobacterium tuberculosis (5-8). In these organisms, menaquinone is required for survival. Since humans are unable to synthesize menaquinone, the bacterial enzymes responsible for the biosynthesis of this vitamin serve as viable targets for the development of antibacterial compounds (2,9).

The biosynthesis of menaquinone in E. coli has been extensively studied (10,11). It begins with the compound chorismate that is an intermediate in the shikimate pathway for the biosynthesis of aromatic compounds (Fig. 1). Five enzymes, MenB-F, generate the 1,4-dihydroxy-2-naphthoate core, and then MenA and MenG install the prenyl and methyl substituents to give menaquinone. In 2005, it was reported that various Streptomyces species lacked orthologs of the menB-menF genes, suggesting that an entirely unique biosynthetic pathway was operative in these organisms (12). Interestingly, these genes were also absent in the pathogenic bacteria C. jejuni and H. pylori, even though these organisms are known to biosynthesize menaquinone (9). Isotopic feeding studies confirmed that a unique pathway was employed in Streptomyces, and 1,4-dihydroxy-6-naphthoate (DHN) was implicated as an intermediate (Fig. 1) (13). This compound was demonstrated to recover the growth of a menaquinone auxotroph of Streptomyces coelicolor, providing strong evidence for this hypothesis. In 2008, Dairi and coworkers reported the discovery of the alternate menaquinone biosynthetic pathway, or the futalosine pathway, and identified several of the genes in either Streptomyces coelicolor or Thermus thermophilus (14). They found that MqnA was involved in the early steps of futalosine biosynthesis, presumably utilizing chorismate, phosphoenolpyruvate, and inosine as precursors (Fig. 1). The enzyme futalosine hydrolase (MqnB or futalosine nucleosidase) then cleaves the hypoxanthine ring from futalosine to generate dehypoxanthinyl futalosine (DHF). Finally, the enzymes MqnC and MqnD convert DHF into 1,4-dihydroxy-6-naphthoate (DHN). While the pathway was largely elucidated by generating S. coelicolor knock-out strains and isolating the intermediates that accumulated, in the case of futalosine hydrolase and MqnD, in vitro enzyme activity was demonstrated using recombinant T. thermophilus enzymes (15,16).

Bioinformatic analysis strongly implied that the futalosine pathway was also operative in the pathogenic organisms C. jejuni and H. pylori (9,14). These bacteria lack homologs to the men genes of E. coli and possess homologs to the mqn genes of S. coelicolor. C. jejuni is the leading cause of bacterial gastroenteritis in the developed world and has been implicated as a causative agent of the debilitating paralysis associated with Guillain-Barré syndrome (17). H. pylori causes gastritis that can lead to peptic ulceration and gastric cancer (18). Since these bacteria require menaquinone biosynthesis for survival, and since they use a biosynthetic pathway that differs from that employed by other beneficial intestinal microbiota, such as lactobacilli, these enzymes represent attractive targets for the development of specific antibacterial compounds that may exhibit minimal adverse side effects (2,9).

In this report we describe our efforts in establishing that a modified futalosine pathway is operative in C. jejuni and in identifying the hydrolase/nucleosidase that is used by this organism. We have found that unlike S. coelicolor, C. jejuni utilizes the adenine-containing version of futalosine, or 6-amino-6-deoxyfutalosine, as an intermediate in menaquinone biosynthesis. Furthermore, the enzyme responsible for the hydrolysis of the N-glycosidic bond in this organism is the 5'-methylthioadenosine nucleosidase (MTAN) that also plays roles in recycling by-products of S-adenosylmethionine (SAM)-utilizing enzymes and in the biosynthesis of autoinducer molecules. Finally, we show that a C. jejuni deletion strain lacking an mqnA homolog (herein designated mqnA2) is auxotrophic for growth on 6-amino-6-deoxyfutalosine. This strongly supports the notion that the futalosine pathway for menaquinone biosynthesis is operative in C. jejuni and that the adenine-containing intermediate is utilized instead of the hypoxanthine-containing intermediate.

**EXPERIMENTAL PROCEDURES**

**Materials and General Methods.** 5'-Methylthioadenosine and xanthine oxidase (Grade III, from bovine milk) were purchased from Sigma-Aldrich. Protein concentration was
determined by the Bradford method using bovine serum albumin as the standard (19). $^1$H NMR spectra were acquired on a Bruker AV300 NMR spectrometer. Details regarding the synthetic procedures used to make 6-amino-6-deoxyfutalosine and the corresponding $^1$H NMR spectra can be found in the supplementary information.

Cloning of a Putative 5’-Methylthioadenosine Nucleosidase (MTAN/cj0117). The cj0117 gene was amplified from C. jejuni (strain NCTC 11168) genomic DNA by PCR. Oligonucleotide primers, including overhangs for ligation-independent cloning, were: 5’-GGTATTGAGGGTCGCATGATGAAAATAGCAAT-3’ (sense) and 5’-AGAGGAGGATTAAGGCTCATATAATTCTGCACAT-3’ (antisense). The PCR product was cloned into a pET-30 Xa/LIC vector (Novagen) according to the manufacturer’s instructions. The resulting recombinant plasmid, which encodes an N-terminal His$_6$-tag on the target C. jejuni MTAN protein, was amplified in NovaBlue GigaSingles competent Escherichia coli cells (Novagen).

Overexpression and Purification of C. jejuni MTAN. The recombinant plasmid was transformed into E. coli BL21 (DE3) competent cells (Novagen), and these cells were grown on Luria-Bertani (LB) agar plates containing 30 mg/L kanamycin at 37 °C. An individual colony was used to inoculate 10 mL of LB medium containing 30 mg/L kanamycin and this culture was incubated at 37 °C with shaking at 225 rpm for 12 h. The overnight culture was poured into 500 mL of LB medium containing 30 mg/L kanamycin and incubated at 37 °C with shaking at 225 rpm until an OD$_{600}$ of 0.6-0.8 was reached. The culture was allowed to continue growing for 4 h after 70 mg/L of isopropyl $\beta$-D-galactopyranoside (IPTG) was added to induce MTAN overexpression. Cells were harvested by centrifugation at 6,000 rpm for 30 min and the pellets snap-frozen in liquid nitrogen and stored at -80 °C.

To purify MTAN, a frozen cell pellet was thawed and resuspended in 10 mL of potassium phosphate buffer (20 mM, pH 7.0). The cells were lysed twice at 20,000 psi using a French pressure cell. The cell lysate was subsequently centrifuged at 6,500 rpm for 30 min and passed through a 0.22 µm filter before affinity chromatography. A column containing 10 mL of chelating Sepharose Fast Flow resin (GE Healthcare) was charged with 20 mL of 100 mM NiSO$_4$, and then washed with 20 mL of distilled H$_2$O and 30 mL of sodium phosphate buffer (20 mM, pH 7.0, containing 0.5 M NaCl and 5 mM of imidazole). The filtered lysate was loaded onto the column and eluted with the same buffer containing increasing amounts of imidazole in a stepwise fashion (5 mM, 125 mM, and 500 mM). Fractions that eluted with 500 mM imidazole and showed absorbance at 280 nm were collected. These fractions were concentrated and buffer-exchanged into an appropriate reaction buffer using an Amicon Ultra-4 (Millipore, 10,000 MWCO) membrane filtration device at 5,000 rpm. Enzyme samples were stored at 5 °C and used within 24 h of purification.

Monitoring C. jejuni MTAN Activity with Futalosine, 5’-Methylthioadenosine and 6-Amino-6-deoxyfutalosine Using $^1$H NMR Spectroscopy. Samples of freshly purified C. jejuni MTAN (250 µg each) that had been subjected to buffer-exchange into 50 mM NaH$_2$PO$_4$/D$_2$O buffer (pD 7.0, 200 µL each) were added to solutions of 5’-methylthioadenosine, futalosine and 6-amino-6-deoxyfutalosine (3.4 mM each) in 800 µL of D$_2$O (1 mL final volume). These samples were incubated at room temperature, and $^1$H NMR spectra were acquired at timed intervals to monitor the progress of the enzymatic reaction. Spectra taken of control reactions lacking enzyme showed no change over the course of several days at room temperature.

Continuous Coupled Assay for C. jejuni MTAN Activity. The activity of C. jejuni MTAN was quantified by measuring the amount of adenine or hypoxanthine produced during the enzymatic reactions using a coupled spectrophotometric assay that employs xanthine oxidase (20-22). All kinetic assays were performed in 50 mM potassium phosphate buffer (pH 7.0, final volume 1.0 mL) containing 0.28 units of xanthine oxidase, and a variable concentration of 5’-methylthioadenosine (0.5 µM - 5 µM), 6-amino-6-deoxyfutalosine (0.5 µM - 5 µM and 150 µM), or futalosine (150 µM). Assay mixtures were
incubated at 25 °C for 10 min before enzymatic reactions were initiated by addition of a fixed amount of C. jejuni MTAN (50 ng for 5'-methylthioadenosine, and 200 ng for 6-amino-6-deoxyfutalosine or futalosine). Rates were measured by monitoring the increased absorbance at 305 nm (5'-methylthioadenosine and 6-amino-6-deoxyfutalosine) or at 290 nm (futalosine). Changes in absorbance were converted to changes in concentration using the molar absorption coefficients of 15,400 M⁻¹ cm⁻¹ for the adenine assay and 12,200 M⁻¹ cm⁻¹ for the hypoxanthine assay. Kinetic parameters were determined by fitting initial velocities to the Michaelis-Menten equation using GraFit 7.0.

Construction and Testing of a C. jejuni ∆mqnA2 Deletion Strain. cjj81176_1302, encoding a mqnA homolog we have designated mqnA2, was PCR-amplified from strain 81-176 genomic DNA using the primers mqnA2KO-1 (5'-TCA TTG TAT CAA TCA TCC ATT GAT CG-3') and mqnA2KO-2 (5'-TTG GCT CAG TTG TAG CAG ATG AAC-3') and cloning the PCR product into a commercial vector pGEM-T (Promega). Inverse PCR was performed on the generated plasmid construct using the primers mqnA2KO-3 (5'-AAA GAA TTC TTA AGA TAT ATA TGT AAA GG-3') and mqnA2KO-4 (5'-AAA GGA TCC TTA AAG CGT TTT GTA AAG-3'). The resulting amplicon and the plasmid pUC18K-2, carrying a nonpolar kanamycin resistance (kanR; aphA-3) cassette, were each digested with BamHI and EcoRI restriction enzymes (restriction site locations are underlined in the corresponding primers). aphA-3 was ligated to the PCR amplicon to form the plasmid pGEM-mqnA2::aphA-3. The E. coli derived plasmid was delivered to 81-176 by natural transformation. Colonies were isolated on Mueller-Hinton agar (Oxoid, Hampshire, England) containing 5 µg/mL trimethoprim and 10 µg/mL vancomycin (MH-TV), and supplemented with 50 µg/mL kanamycin and 100 µg/mL 6-amino-6-deoxyfutalosine. C. jejuni isolates harboring mqnA2 disrupted by the kanR cassette were confirmed via PCR and sequencing analysis. Growth analyses were performed on plates with MH-TV containing 50 µg/mL kanamycin or supplemented with 100 µg/mL 6-amino-6-deoxyfutalosine, 100 µg/mL futalosine, or 100 µg/mL menaquinone (MQ-4, Sigma-Aldrich).

RESULTS

Studies with futalosine, 5'-methylthioadenosine, and C. jejuni MTAN (Cj0117) - Our first approach towards identifying the futalosine pathway in C. jejuni involved cloning the putative futalosine hydrolase and determining whether it showed activity with synthetic futalosine. BLAST searches against the established futalosine hydrolases from S. coelicolor (SCO4327) and T. thermophilus (TTHA0556) showed only one reasonable candidate; a C. jejuni protein that was encoded by the gene cj0117 in C. jejuni 11168 (31% and 26% amino acid sequence identity, respectively). This protein had been annotated as a 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTAN). MTAN is a nucleosidase that hydrolyzes the N-glycosidic bond in either 5'-methylthioadenosine (MTA) or S-adenosylhomocysteine (SAH) and releases free adenine and 5'-methylthiouribose or S-ribosylhomocysteine, respectively (Fig. 2) (23-25). MTA is a by-product in the SAM-dependent biosynthesis of polyamines such as spermidine, and MTAN serves to recycle it back into basic metabolic pathways. SAH is a by-product of SAM-dependent methyltransferases and is converted into S-ribosylhomocysteine by MTAN. S-ribosylhomocysteine then serves as a precursor to the quorum-sensing molecule, autoinducer-2 (26). Since the cj0117 gene product shares sequence homology with this known nucleosidase, it seemed reasonable that it may function either as a futalosine hydrolase, an MTAN, or both. The latter possibility seemed reasonable since MTAN is known to show substrate promiscuity and accept either MTA or SAH, and the carboxylate of futalosine could occupy a position in the active site of MTAN that is analogous to that occupied by the carboxylate of SAH. Furthermore, very potent inhibitors of MTAN are known that contain a phenyl ring positioned in a very similar manner to that of futalosine (e.g. inhibitor 1), indicating that the active site can accommodate a substrate with this functionality (20).

We therefore cloned cj0117, expressed its gene product as a His-tagged protein, and purified the protein via ion-affinity chromatography. We
prepared futalosine using our previously described synthetic route from inosine (27). Using $^1$H NMR spectral analysis, we found that Cj0117 was active in catalyzing the hydrolysis of 5'-methylthioadenosine, but showed negligible activity (less than 5%) with futalosine under identical conditions. Thus, it appears that Cj0117 is a functional MTAN but not a futalosine hydrolase.

Studies with 6-amino-6-deoxyfutalosine and C. jejuni MTAN - The absence of an alternate candidate to serve as a futalosine hydrolase in C. jejuni led us to suspect that futalosine was not an actual intermediate in menaquinone biosynthesis in this organism. Despite this uncertainty, it still appeared that the latter steps of the futalosine pathway are operative in C. jejuni as homologs to MqnC and MqnD are present in the C. jejuni proteome. Notably, the putative MqnC (encoded by cj0462 in C. jejuni 11168) shares 44% and 41% amino acid sequence identity with the S. coelicolor and T. thermophilus enzymes, respectively. Therefore, we began to suspect that only the first steps in the futalosine pathway in C. jejuni involve an alternate intermediate. A clue to the nature of the true intermediate was found from previous mechanistic and structural studies on the E. coli MTAN. A kinetic isotope effect analysis showed that the enzyme utilizes a highly dissociative mechanism with a transition state that has considerable oxocarbenium ion character (Fig. 3) (28). Mutagenesis and structural studies have shown that there are three conserved residues that are absolutely essential for the activity of this enzyme: Glu12, Glu174 and Asp197 (29-32). Glu12 is thought to serve as a general base and to deprotonate the water that ultimately attacks the oxocarbenium ion. Glu174 forms key H-bonds to the ribose C-2' and C-3' hydroxyl groups and likely plays important roles in controlling electron density in the ribose ring and substrate positioning. Finally, Asp197 forms two H-bonds with the departing adenine and likely plays the role of a general acid catalyst. In order to analyze the potential role of these residues in futalosine hydrolysis, we performed a sequence alignment between the E. coli and C. jejuni MTANs and the S. coelicolor and T. thermophilus futalosine hydrolases (Fig. 4) (33). All three of the conserved E. coli MTAN residues aligned with their counterparts in the C. jejuni MTAN, consistent with its observed catalytic activity on 5'-methylthioadenosine. In the case of the futalosine hydrolases, the residues corresponding to Glu174 were present as anticipated, and it was not clear which residues played the role of Glu12 as the N-terminal sequences differed significantly. Most interestingly, however, was the observation that an asparagine residue was present in place of Asp197 in both of the futalosine hydrolases. This is significant since this residue interacts with the purine leaving group, and a key difference between futalosine and 5'-methylthioadenosine is that the former possesses a hypoxanthine base and the latter possesses an adenine base. It is even more compelling when one considers that hypoxanthine exists primarily as the keto tautomer whereas adenine exists as the enamine tautomer (34). This affects the H-bonding properties of the exocyclic heteroatom in the purine of futalosine so that it can only function as an H-bond acceptor. Thus, the interaction with an aspartate residue could not form the same bifunctional acceptor/donor H-bonding pattern described for MTAN catalysis. It therefore seems likely that MTAN-related nucleosidases that act on adenine-containing substrates retain the key aspartate, however, those that act on hypoxanthine-containing substrates replace it with an asparagine. An analysis of all MTAN homologs present in all sequenced genomes of Helicobacter or Campylobacter species indicated that the residue corresponding to Asp197 is always conserved. This suggested that a modified futalosine pathway is operative in C. jejuni and H. pylori in which 6-amino-6-deoxyfutalosine is used in place of futalosine.

In order to test this hypothesis it was necessary to synthesize authentic 6-amino-6-deoxyfutalosine. This was done using a similar procedure as that described for the synthesis of futalosine with the exception that the adenosine was used as the starting material (27). The 6-amino group was protected as a bis-Boc derivative and the 2', 3' hydroxyl groups were protected with an acetonide moiety to give the known compound 2 (Fig. 5). A one-pot oxidation/Wittig procedure was then used to produce alkene 3 as the trans isomer (35). Hydrogenation then provided a single isomer of compound 4. Removal of the acetonide and Boc protecting groups was achieved
by treatment with trifluoroacetic acid, and hydrolysis of the methyl ester using LiOH generated 6-amino-6-deoxyfutalosine.

With 6-amino-6-deoxyfutalosine in hand, it was possible to test whether it serves as a substrate for the C. jejuni MTAN. Monitoring an enzymatic incubation by $^1$H NMR spectroscopy (see supplemental information) confirmed that 6-amino-6-deoxyfutalosine was converted into adenine and DHFL by MTAN. This suggests that the DHFL required for menaquinone biosynthesis in C. jejuni could be provided by the action of MTAN on 6-amino-6-deoxyfutalosine. In order to assess the catalytic efficiency of this process, we employed a coupled kinetic assay for adenine release that had previously been used in monitoring MTAN kinetics with MTA (20). The nucleosidase activity with 6-amino-6-deoxyfutalosine was found to follow Michaelis Menten kinetics and the kinetic constants obtained were $k_{\text{cat}} = 0.53 \pm 0.03$ s$^{-1}$ and $K_M = 1.03 \pm 0.12$ µM (see supplemental information). We also determined the activity of the C. jejuni MTAN against MTA and obtained kinetic constants of $k_{\text{cat}} = 2.7 \pm 0.1$ s$^{-1}$ and $K_M = 0.93 \pm 0.10$ µM. The similarity of these values indicates that 6-amino-6-deoxyfutalosine is a good substrate for the C. jejuni MTAN and supports the notion that this activity may be biologically relevant. The same assay can be used to analyze for hypoxanthine release, so we reinvestigated the ability of MTAN to catalyze the hydrolysis of futalosine. Using 150 µM concentrations of substrates we found that a low level of activity could be observed with futalosine, however, it was only 2% of that observed with 6-amino-6-deoxyfutalosine.

**Studies with a C. jejuni strain deleted for mqnA2**

We next wished to provide in vivo evidence that the modified futalosine pathway for menaquinone biosynthesis is operative in C. jejuni and that 6-amino-6-deoxyfutalosine is used in place of futalosine in this organism. We anticipated that a MqnA-like enzyme in C. jejuni would be involved in the biosynthesis of 6-amino-6-deoxyfutalosine, and therefore a mutant lacking the gene encoding for this protein would grow only when supplemented with 6-amino-6-deoxyfutalosine, but not with futalosine. A search for homologs in the C. jejuni genome indicated that the gene products of cj1285c and cjj81176_1302, in C. jejuni 11168 and C. jejuni 81-176, respectively, are 99.6% identical to each other and share 26% sequence identity with MqnA from S. coelicolor. Based on our prediction that the C. jejuni MqnA-like enzyme would synthesize 6-amino-6-deoxyfutalosine instead of futalosine we designated this gene mqnA2 and constructed a deletion mutant in C. jejuni 81-176 by replacing most of the mqnA2 open reading frame with a non-polar kanamycin resistance cassette (kanR; aphA3) using double crossover homologous recombination (Figure 6A). Mutant clones were selected on standard rich medium (Mueller-Hinton; MH) plates supplemented with kanamycin and 6-amino-6-deoxyfutalosine. MH plates containing either futalosine (Figure 6B, top) or 6-amino-6-deoxyfutalosine (Figure 6B, bottom) were streaked with both wild type C. jejuni 81-176 and C. jejuni 81-176 ΔmqnA2. After 24 and 48 hours, wild type grew well on either plate, whereas growth of the ΔmqnA2 mutant was only observed in the presence of 6-amino-6-deoxyfutalosine. No growth was observed on unsupplemented plates (data not shown). This supports the hypothesis that cj1285c/cjj81176_1302 encodes for MqnA2 that is required for the biosynthesis of 6-amino-6-deoxyfutalosine and that an essential, modified futalosine pathway is operative in C. jejuni. Very weak growth of the mutant strain on the futalosine plates was observed after 72 hours incubation. This is likely due to the slow hydrolysis of futalosine catalyzed by the C. jejuni MTAN (2% of the activity observed with either MTA or 6-amino-6-deoxyfutalosine). In contrast to the work with S. coelicolor, we could not restore growth of the mutant strain with commercially available menaquinone (MQ-4, bearing a C$_{20}$-prenyl side chain, data not shown). This may be due to the insolubility of the compound and the resulting limited penetration of a very non-polar molecule across the outer membrane of the Gram-negative organism. It could also be due to a more stringent requirement for menaquinone bearing a C$_{30}$-prenyl side chain, MQ-6, that is normally utilized by C. jejuni (36).

**DISCUSSION**

This study provides the first direct evidence that a modified futalosine pathway is
operative in *C. jejuni*. A search for the gene encoding for the putative futalosine hydrolase indicated that the *C. jejuni* MTAN was the most reasonable candidate to play this role. After finding that the *C. jejuni* MTAN catalyzed the hydrolysis of 5'-methylthioadenosine, but not of futalosine, we began to suspect that the pathway in *C. jejuni* differed from that in *S. coelicolor*. An analysis of the active site architecture of the *E. coli* MTAN showed that a key aspartate residue, Asp 197, was required to act as an acid catalyst in promoting the departure of the adenine leaving group. This residue was present in all gene products annotated as MTANs in *Helicobacter* and *Campylobacter* species, but was found to be an asparagine in the two identified futalosine hydrolases from *S. coelicolor* and *T. thermophilus* (14,15). This suggested that an adenine-containing version of futalosine is the true intermediate in *Helicobacter* and *Campylobacter* species. Ultimately, we found that the *C. jejuni* MTAN could hydrolyze 6-amino-6-deoxyfutalosine, implying that this was the source of the DHFL used in menaquinone biosynthesis in this organism. The amino acid change implies that there are key differences in the mechanisms employed by the MTANs and the futalosine hydrolases. Since hypoxanthine is a better leaving group than adenine due to the more electronegative oxygen, it may not require an acid catalyst to depart; the H-bonding with an asparagine residue may be sufficient to promote catalysis. Alternatively, an as yet unidentified residue may serve as an acid catalyst in the futalosine hydrolases.

A very recent report has also demonstrated that the *H. pylori* MTAN catalyzes the hydrolysis of 6-amino-6-deoxyfutalosine, but not of futalosine (37). While *in vivo* studies and kinetics were not reported, the authors proposed that this is the true biosynthetic intermediate in that organism. Our *in vivo* studies in *C. jejuni* demonstrate that the *mqa2* gene is required for the biosynthesis of 6-amino-6-deoxyfutalosine and that this activity is essential for growth of the organism. Together with the studies on *S. coelicolor*, this strongly suggests that menaquinone is biosynthesized in *C. jejuni* and *H. pylori* via a modified futalosine pathway. This also implicates menaquinone biosynthetic enzymes as viable targets for the development of antibacterials directed against *C. jejuni* and *H. pylori*.

This work points out the truly remarkable number of roles that MTAN plays in these pathogenic bacteria. The enzyme displays a great deal of flexibility in regard to the nature of the group attached to the 5'-position of the substrate ribose moiety, yet appears to be quite specific in regard to the nature of the nucleoside base. It hydrolyzes 5'-methylthioadenosine in its role in recycling the by-products of the SAM-dependent biosynthesis of spermidine (38). It also hydrolyzes S-adenosylhomocysteine in its role in the biosynthesis of the quorum-sensing molecule, autoinducer 2, that is generated in both *C. jejuni* and *H. pylori* (39-42). Finally, as we have shown in this work it plays an essential role in the hydrolysis of 6-amino-6-deoxyfutalosine, an activity that has been implicated as a requirement for menaquinone biosynthesis. Thus, this multifunctional enzyme may serve as the Achilles' heel of these pathogens and the very potent MTAN inhibitors that are already available from work with the *E. coli* enzyme may serve as effective antibacterial agents. Given the multiple important biological roles of MTAN it may be difficult for resistance mechanisms to develop.

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FOOTNOTES

1The abbreviations used are: DHFL, dehypoxanthinyl futalosine; DHN, 1,4-dihydroxy-6-naphthoate; MTA, 5'-methylthioadenosine; MTAN, 5'-methylthioadenosine nucleosidase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

FIGURE LEGENDS

Fig. 1. Pathways for menaquinone biosynthesis in bacteria. The upper pathway shows the biosynthesis of menaquinone in *E. coli*. The middle pathway shows the futalosine pathway employed by *S. coelicolor* and *T. thermophilus*. The lower pathway shows the modified futalosine pathway employed by *C. jejuni* and *H. pylori*.

Fig. 2. Reactions catalyzed by 5'-methylthioadenosine nucleosidase (MTAN). The insert shows the structure of the potent MTAN inhibitor 1.

Fig. 3. The proposed mechanism for the reaction catalyzed by the *E. coli* MTAN and the roles of key active site residues.

Fig. 4. An alignment of the *E. coli* MTAN (EcMTAN), the *C. jejuni* MTAN (CjMTAN), the *T. thermophilus* futalosine hydrolase (TtMqnB), and the *S. coelicolor* futalosine hydrolase (ScMqnB). Accession numbers are P0AF14, Q0PC20, Q5SKT7, and Q9KXN0, respectively. Numbered residues correspond to key active site residues identified in the *E. coli* MTAN. Red residues correspond to key active site residues identified in the *E. coli* MTAN and their corresponding conserved homologs. Blue residues indicate the replacement of the *E. coli* MTAN Glu197 with Gln. Asterisks identify residues conserved in all four sequences. The alignment was performed using COBALT (33).

Fig. 5. The synthetic route used to prepare 6-amino-6-deoxyfutalosine.

Fig. 6. Genomic context of *mqnA2* and growth of WT and Δ*mqnA2* *C. jejuni* on plates supplemented with either futalosine or 6-amino-6-deoxyfutalosine. A. The genomic organization surrounding *mqnA2* showing that the gene is positioned at the end of an operon. The position of insertion of the kanamycin resistance cassette is indicated. B. Growth of Δ*mqnA2* (left) and WT (right) *C. jejuni* 81-176 on plates supplemented with either futalosine (upper) or 6-amino-6-deoxyfutalosine (lower) as a function of time.
Figure 1
Figure 2
Figure 3
**Figure 4**
Figure 5
Figure 6
5'-Methylthioadenosine nucleosidase is implicated in playing a key role in a modified fotalosine pathway for menaquinone biosynthesis in campylobacter jejuni
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