

## Riboflavin Biosynthesis Is Associated with Assimilatory Ferric Reduction and Iron Acquisition by *Campylobacter jejuni*<sup>∇</sup>

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One of the pathways involved in the acquisition of the essential metal iron by bacteria involves the reduction of insoluble Fe<sup>3+</sup> to soluble Fe<sup>2+</sup>, followed by transport of Fe<sup>2+</sup> to the cytoplasm. Flavins have been implicated as electron donors in this poorly understood process. Ferrous iron uptake is essential for intestinal colonization by the important pathogen *Campylobacter jejuni* and may be of particular importance under low-oxygen conditions. In this study, the links among riboflavin biosynthesis, ferric reduction, and iron acquisition in *C. jejuni* NCTC11168 have been investigated. A riboflavin auxotroph was generated by inactivation of the *ribB* riboflavin biosynthesis gene (Cj0572), and the resulting isogenic *ribB* mutant only grew in the presence of exogenous riboflavin or the riboflavin precursor diacetyl but not in the presence of the downstream products flavin adenine dinucleotide and flavin mononucleotide. Riboflavin uptake was unaffected in the *ribB* mutant under iron-limited conditions but was lower in both the wild-type strain and the *ribB* mutant under iron-replete conditions. Mutation of the *fur* gene, which encodes an iron uptake regulator of *C. jejuni*, resulted in an increase in riboflavin uptake which was independent of the iron content of the medium, suggesting a role for Fur in the regulation of the as-yet-unknown riboflavin transport system. Finally, ferric reduction activity was independent of iron availability in the growth medium but was lowered in the *ribB* mutant compared to the wild-type strain and, conversely, increased in the *fur* mutant. Taken together, the findings confirm close relationships among iron acquisition, riboflavin production, and riboflavin uptake in *C. jejuni*.

The bioavailability of iron is recognized to be a significant factor which potentially limits bacterial growth and depends on environmental conditions, particularly pH and oxygen tension, which affect iron solubility and oxidation state (1). Soluble ferrous iron (Fe<sup>2+</sup>) is readily available to bacteria and, in gram-negative bacteria, requires only transport across the inner membrane (7, 19). However, in the presence of oxygen at a pH of  $\geq 7$ , ferrous iron is rapidly converted into ferric iron (Fe<sup>3+</sup>), which is almost completely insoluble at a pH of  $\geq 7$ . In mammalian tissues, iron is also complexed into hemoglobin, stored intracellularly in ferritin, or chelated by transferrin in serum and by lactoferrin at mucosal surfaces (40). The resulting free-iron concentration is far too low to support bacterial growth, and consequently, bacteria have evolved different mechanisms to acquire host-complexed iron compounds, including siderophore production and outer-membrane transport mechanisms and the production of ferric reductases (1, 9, 40).

One process linked with the formation of ferrous iron is ferric reduction by the action of flavin-cofactored ferric reductases (9, 29), which reduce flavins using reduced nicotinamide

nucleotides as hydride donors, with the reduced flavins subsequently being used to reduce Fe<sup>3+</sup> (15, 29). In a number of pathogenic bacteria, ferric (flavin) reductases have been found to be secreted extracellularly (4, 10, 11, 35), but in general, these proteins are poorly characterized. A close interrelationship of flavin status and iron uptake has been suggested in a wide range of organisms, including yeasts (14), higher plants (30), and mammals (25). A role for ferric reduction and riboflavin in iron uptake was previously suggested for several pathogenic bacteria, including the gastric pathogen *Helicobacter pylori*, which produces substantially more riboflavin under iron-limited conditions than under iron-replete conditions (6, 13, 41). In addition, ferrous iron uptake was shown to be required for virulence of *H. pylori* (36).

*Campylobacter jejuni* is a major cause of food poisoning in the developed parts of the world (39), and its sequelae include Guillain-Barré syndrome, an acute demyelinating disease of the peripheral nervous system (31). *C. jejuni* is normally found in the avian gut and cecum, and this environment is thought to be low in oxygen, favoring the formation of ferrous iron. While mechanisms for the acquisition of ferric iron complexes (heme, siderophores) by *C. jejuni* have been described previously (23, 27), it was recently demonstrated that ferrous iron uptake also plays an important role in avian gut colonization by *C. jejuni* (22).

In view of the importance of ferrous iron uptake in gut colonization by *C. jejuni*, we have initiated a study investigating the possible role of flavins and ferric reduction in iron uptake in this organism. Analysis of the genome sequence of *C. jejuni* strain NCTC11168 (17, 24) indicated the presence of two genes

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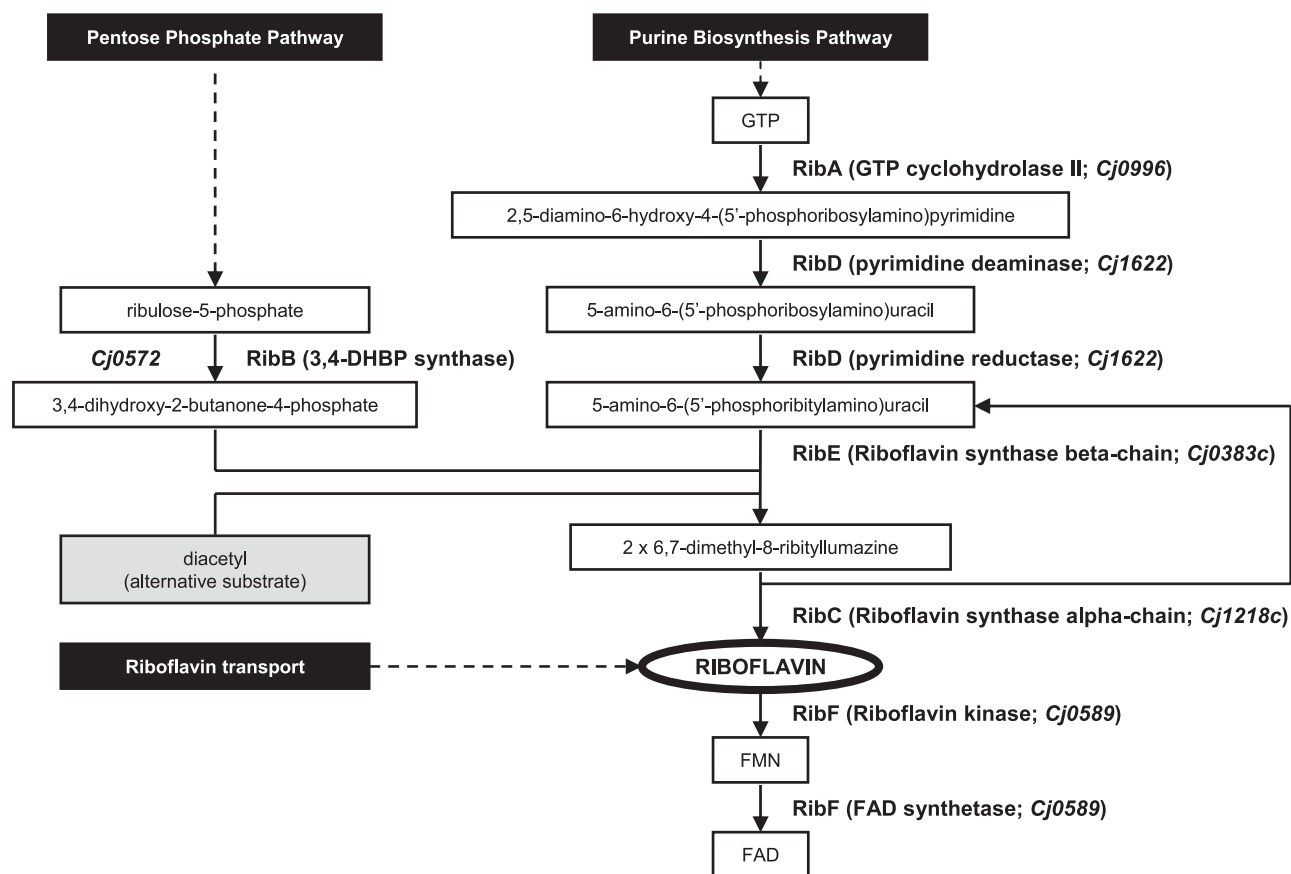


FIG. 1. Overview of the different enzymatic steps in the riboflavin biosynthetic pathway resulting in the biosynthesis of riboflavin and the coenzymes FAD and FMN. Two distinct branches of the pathway interact at the formation of 3,4-dihydroxy-2-butanone-4-phosphate (DHBP), which undergoes condensation with 5-amino-6-(5'-phosphoribitylamino)uracil to yield the riboflavin precursor 6,7-dimethyl-8-ribityllumazine. One molecule of riboflavin is formed from one molecule of GTP and two molecules of ribulose-5-phosphate in a series of enzyme-catalyzed reactions. The enzymes and corresponding genes listed are those generally found in *E. coli* (2, 37). The Cj gene numbers given with the RibA, RibD, RibE, RibC, and RibF steps are based on the annotation of the *C. jejuni* genome sequence (17, 24) and require experimental validation. The mechanism whereby the DHBP analogue diacetyl can complement a *ribB* deficiency is indicated by the gray box.

(Cj0572 and Cj0996), both designated *ribA* and both putatively encoding orthologs of GTP cyclohydrolase II, the enzyme that mediates the initial step of riboflavin biosynthesis from GTP (Fig. 1). The Cj0572 protein has also been annotated as a bifunctional RibBA protein, which suggests that it also encodes RibB activity, which is the enzyme 3,4-dihydroxy-2-butanone-4-phosphate synthase that furnishes a four-carbon unit for incorporation into the riboflavin molecule (Fig. 1). In this study, it is demonstrated that the Cj0572 gene encodes the RibB enzyme of *C. jejuni*, that there are close links between riboflavin synthesis and ferrous and ferric iron acquisition by *C. jejuni*, and that these pathways are associated with the major iron-responsive regulator Fur.

#### MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** *C. jejuni* strain NCTC11168 was obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London, United Kingdom. The *fur* mutant AV17 (NCTC11168 *fur::Kan*) was described previously (18, 33). *Campylobacter* strains were maintained and grown at 37°C in a microaerophilic cabinet (Don Whitley, Shipley, United Kingdom) with a constant gas supply (10% CO<sub>2</sub>, 5% O<sub>2</sub>, 85% N<sub>2</sub>). *C. jejuni* strains were routinely cultured on Skirrow agar (Oxoid) and subcultured every 48 h onto fresh plates. The chemically defined tissue culture

medium MEM $\alpha$  (Invitrogen) contains riboflavin (0.27  $\mu$ M) but does not contain any added iron source (3) and supports the growth of *C. jejuni* (3, 18, 34). Fifty milliliters of standard MEM $\alpha$  or of MEM $\alpha$  without riboflavin (Invitrogen) was routinely supplemented with 20  $\mu$ M FeCl<sub>3</sub> and 20 mM HEPES and shaken at 200 rpm under microaerophilic conditions. *Escherichia coli* TOP10 (Invitrogen) was used as the host for cloning experiments and was routinely grown at 37°C in Luria-Bertani medium (28). When indicated, growth media were supplemented with kanamycin or ampicillin to final concentrations of 50  $\mu$ g/ml and 25  $\mu$ g/ml, respectively.

**Construction of riboflavin auxotrophs by disruption of Cj0572.** The Cj0572 gene of *C. jejuni* strain NCTC11168 was amplified with primers 0571forward and 0573reverse (Table 1) and cloned into pGEM-T<sub>easy</sub> (Promega). A BamHI restriction site was introduced by inverse PCR (42) with primers 0572Bamforward and 0572Bamreverse (Table 1). This inverse PCR also deleted a 502-bp internal fragment of the Cj0572 gene, which was subsequently interrupted by insertion of the kanamycin resistance gene from pJMK30 (34) into this unique BamHI site. The resulting plasmids, pCj0572CK and pCj0572KC, were subsequently introduced into *C. jejuni* NCTC11168 by electroporation (33). Two colonies derived from independent transformations were tested, and both colonies gave identical results in all experiments. Correct allelic replacement of the wild-type Cj0572 gene with the interrupted version was confirmed by PCR with combinations of the primers 0570forward, 0574reverse, CKRout, and CKFout (Table 1).

**Chemicals.** Unless stated otherwise, laboratory chemicals were purchased from Sigma Aldrich (Poole, Dorset, United Kingdom). Restriction enzymes were obtained from Promega (Southampton, United Kingdom). Oligonucleotide primers were synthesized by Sigma-Genosys (Pampisford, Cambridge, United Kingdom).

TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5' → 3')
0571forward.....	GGAAAGTCTAAGCGCCCAAG
0573reverse.....	AAATTAAGTCTTTTGCCATCAACACT
0572Bamforward <sup>a</sup> .....	TGTGGATCCTTATAGAATATCGCCT AAAACATGA
0572Bamreverse <sup>a</sup> .....	TGTGGATCCATCTTCTGCATCTACCA TAACAAGCAT
0570forward.....	GGTGAGGGAAGATTTTAGTCG
0574reverse.....	CTATAAGCCGTAGCTAGACC
CKFout .....	GGTTCGCTGGGTTTATCGGAT
CKRout.....	AGATGTCTAAAAAGCTTGGGA

<sup>a</sup> Primer contains a 5' extension with a BamHI restriction site (underlined).

**Riboflavin uptake.** The measurement of riboflavin uptake was adapted from the assay of Fe<sup>3+</sup> uptake described previously (26). Late-exponential-phase cultures of strains grown in MEM $\alpha$  lacking riboflavin or in MEM $\alpha$  with additional riboflavin supplementation were harvested and washed twice in 50 mM phosphate buffer, pH 7.4. The cells were resuspended in 50 mM phosphate buffer (pH 7.4) to an optical density at 600 nm of 0.8, and 200  $\mu$ l of this suspension was then added to 1 ml of standard MEM $\alpha$ . Samples were mixed and left to incubate microaerobically at 37°C for 1 to 2 h. The assay was initiated by adding [G-<sup>3</sup>H]riboflavin (9,874 Bq; Tocris Cookson Ltd., Bristol, United Kingdom). Samples (200  $\mu$ l) were withdrawn at 1, 5, and 10 min and washed twice with 0.5 ml of 50 mM phosphate buffer (pH 7.4). The pellet was resuspended in 100  $\mu$ l of Triton X-100 solution (0.5%, vol/vol, in water), mixed with 5 ml of a Quicksafe A scintillant-water cocktail (containing 1 part of scintillant to 10 parts of water, by volume [Zinsser Analytic, Maidenhead, Berks, United Kingdom]), and shaken vigorously. Radioactivity was determined by scintillation counting with radioactively labeled stock solutions as standards.

**Fe<sup>3+</sup> and Fe<sup>2+</sup> uptake.** Iron uptake was measured by an adaptation of previously described methods (26, 36). A stock solution of Fe<sup>2+</sup> was prepared by diluting 10 mM FeCl<sub>3</sub> 10-fold in 1 M ascorbate, and a stock solution of Fe<sup>3+</sup> was prepared by diluting 10 mM FeCl<sub>3</sub> 10-fold into 1 M sodium citrate. <sup>55</sup>FeCl<sub>3</sub> (Perkin-Elmer, Waltham, MA) was added such that each assay mixture subsequently contained 7,750 Bq (the specific radioactivity therefore varied with the Fe<sup>2+</sup> or Fe<sup>3+</sup> concentration). Labeled Fe<sup>3+</sup> and Fe<sup>2+</sup> solutions were prepared and kept overnight at 4°C before use. Nitrilotriacetate (1.2 mM), which prevents nonspecific binding of iron to the bacterial membrane (16), was mixed with MEM $\alpha$  (1 part of nitrilotriacetate solution to 10 parts of medium, by volume) and kept overnight at 37°C before use. Late-exponential-phase cultures were harvested and washed twice in 50 mM phosphate buffer, pH 7.4, and the cells were resuspended in 50 mM phosphate buffer, pH 7.4, to an optical density at 600 nm of 0.8. Two hundred microliters was added to 1.1 ml of the nitrilotriacetate-MEM $\alpha$  solution and incubated microaerobically at 37°C for 1 to 2 h. The assay was initiated by adding the cell suspension in nitrilotriacetate-MEM $\alpha$  to the labeled Fe<sup>2+</sup> or Fe<sup>3+</sup> solution and mixing it thoroughly. Samples (200  $\mu$ l) were withdrawn at 1, 5, and 10 min and treated as described above for measurement of riboflavin uptake.

**Measurement of Fe<sup>3+</sup> reduction.** Fe<sup>3+</sup> reduction activity was determined as described previously (36).

**Measurement of protein concentrations.** Protein concentrations of cell extracts were determined by a modified Lowry method (21) with bovine serum albumin as the standard.

## RESULTS

**Inactivation of the *C. jejuni* Cj0572 gene results in auxotrophy for riboflavin.** The requirement of *C. jejuni* NCTC11168 for riboflavin was investigated by two independent means, first by inactivation of genes putatively involved in riboflavin biosynthesis and second by investigation of growth kinetics of *C. jejuni* supplemented with different levels of exogenous riboflavin. Two *C. jejuni* genes annotated as *ribA* orthologs (Cj0572

and Cj0996, respectively) (17, 24) were targeted by insertional inactivation with a kanamycin resistance cassette. In view of the predicted auxotrophy for riboflavin, growth media used for isolation of these mutants were supplemented with riboflavin to final concentrations of 5, 50, and 500  $\mu$ M.

Mutants containing a kanamycin resistance cassette in the Cj0572 gene were readily isolated on media containing high concentrations of riboflavin. Colonies were only recovered on Skirrow agar or blood agar containing >5  $\mu$ M riboflavin, with the highest numbers of colonies appearing on plates containing 50  $\mu$ M and 500  $\mu$ M riboflavin. While mutants in Cj0572 were readily isolated with the antibiotic resistance cassette in either transcriptional orientation, we were unable to isolate Cj0996 mutants, despite repeated attempts (data not shown). This suggests that either the Cj0996 gene product or proteins encoded by genes in the proximity of Cj0996 may be essential for *C. jejuni*.

**The *C. jejuni* Cj0572 gene encodes a RibB orthologue.** The growth kinetics of the *C. jejuni* Cj0572 mutant at different levels of riboflavin supplementation was determined with the defined tissue culture medium MEM $\alpha$  (3). Neither of two independent Cj0572 mutants was able to grow satisfactorily in the absence of added Fe<sup>3+</sup> in MEM $\alpha$  and required supplementation with FeCl<sub>3</sub> to a final concentration of 20  $\mu$ M (data not shown). Even in the presence of 50  $\mu$ M or 500  $\mu$ M riboflavin, the Cj0572 mutant grew slowly compared to the wild-type strain (Fig. 2A). Growth of the Cj0572 mutant was optimal with riboflavin supplementation to 50 and 500  $\mu$ M, and growth was significantly reduced with 5 and 0.5  $\mu$ M riboflavin (Fig. 2B). Finally, the presence of the riboflavin precursor molecule diacetyl, an analogue of 3,4-dihydroxy-2-butanone-4-phosphate (2), allowed growth of the Cj0572 mutants and thus could substitute for riboflavin (Fig. 2C). In contrast, neither flavin mononucleotide (FMN) nor flavin adenine dinucleotide (FAD) could support growth (data not shown). The effect of diacetyl supplementation strongly suggests that the Cj0572 gene encodes a RibB orthologue (Fig. 1), and hence we have annotated the Cj0572 gene as *ribB* and its mutant as an *ribB* mutant in the remainder of this report.

***C. jejuni* possesses a riboflavin uptake activity that is influenced by mutation of the *fur* gene.** The auxotrophy of the *ribB* mutant indicated that the cells were able to assimilate riboflavin. Furthermore, even the wild-type strain showed a small but consistent stimulation of growth in response to riboflavin (Fig. 2A). Riboflavin assimilation was studied directly by measuring the uptake of tritiated riboflavin in the wild-type strain and the *ribB* mutant (Fig. 3). To investigate the relationship between iron acquisition and riboflavin, we also included a *fur* mutant lacking the ferric uptake regulator (Fur), the main iron-responsive regulator of *C. jejuni* (18, 23, 34). Riboflavin uptake activities were comparable in all of the strains, although values were found to be higher in the *fur* mutant than in the other strains (Fig. 3). In the wild-type strain, and to a lesser degree in the *ribB* mutant, riboflavin uptake was lower under iron-replete conditions.

**Riboflavin auxotrophy does not alter ferric or ferrous iron uptake.** The effects of riboflavin status upon cellular iron uptake were studied by measuring the uptake of radiolabeled ferrous and ferric iron in cells grown under iron-limited and iron-replete conditions (1  $\mu$ M and 40  $\mu$ M FeCl<sub>3</sub> in MEM $\alpha$ ,

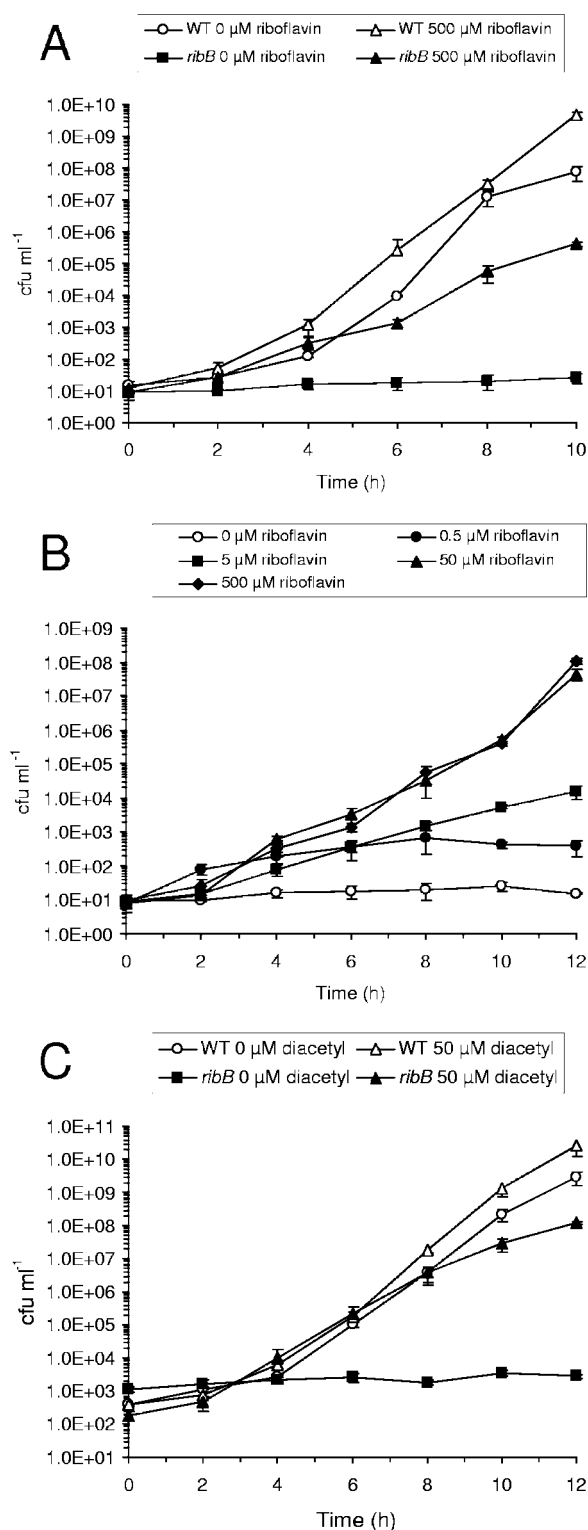


FIG. 2. Mutation of the *Cj0572* gene in *C. jejuni* NCTC11168 results in riboflavin auxotrophy, which can be overcome by supplementation with exogenous riboflavin or the riboflavin precursor diacetyl. (A) Growth curves of the wild-type (WT) strain and the *ribB* (*Cj0572*) mutant in riboflavin-free MEM $\alpha$  with 20  $\mu$ M FeCl $_3$ , with and without supplementation with 500  $\mu$ M riboflavin. (B) Growth curves of the *ribB* (*Cj0572*) mutant strain in riboflavin-free MEM $\alpha$  supplemented with 20  $\mu$ M FeCl $_3$  and a range of riboflavin concentrations. (C) Growth curves of the *C. jejuni* NCTC11168 wild-type strain (WT) and *ribB* mutant in response to

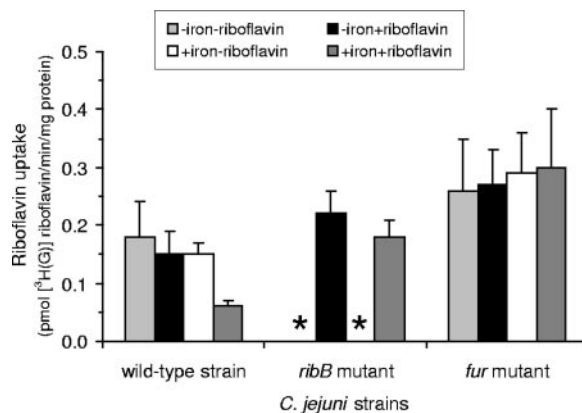


FIG. 3. Riboflavin uptake by *C. jejuni* NCTC11168 is iron and riboflavin responsive and is increased in the *fur* mutant. Shown are rates of tritiated [ $G$ - $^3$ H]riboflavin uptake in the *C. jejuni* wild-type strain, *ribB* mutant, and *fur* mutant. Cultures were grown in riboflavin-free MEM $\alpha$  under iron-limited (1  $\mu$ M FeCl $_3$ ; indicated as -iron) and iron-replete (40  $\mu$ M FeCl $_3$ , indicated as +iron) conditions with and without supplementation with 50  $\mu$ M riboflavin (indicated as -riboflavin and +riboflavin). Data shown are from two independent experiments performed in triplicate. The *ribB* mutant could not be tested in medium without riboflavin because of the riboflavin auxotrophy; the absence of data for these samples is indicated by asterisks.

respectively). The wild-type strain and the *fur* mutant were also grown with and without 50  $\mu$ M riboflavin (Fig. 4), but this was not possible with the *ribB* mutant because of its requirement for exogenous riboflavin. It was found that the values for Fe $^{3+}$  uptake were at least as large as those for Fe $^{2+}$ , particularly in the case of the *fur* mutant, where the Fe $^{3+}$  uptake values were about twice those of Fe $^{2+}$  uptake and around two- to fourfold greater than those of Fe $^{3+}$  uptake in the wild-type strain (Fig. 4). However, there was no appreciable difference between the *ribB* mutant and the wild-type strain with respect to either Fe $^{2+}$  uptake or Fe $^{3+}$  uptake, regardless of the level of Fe $^{3+}$  under which the cells were grown (Fig. 4). There was therefore no evidence from these studies of any influence of riboflavin status on the uptake of Fe $^{3+}$  or Fe $^{2+}$  per se.

**Ferric reduction activity requires riboflavin synthesis and is increased in a *fur* mutant.** The lack of an observable effect of riboflavin status upon the uptake of Fe $^{3+}$  or Fe $^{2+}$  was unsurprising. It was more likely that there would be an effect upon Fe $^{3+}$  reduction (15, 29). Accordingly, Fe $^{3+}$  reduction activity was compared among the wild-type strain, the *fur* mutant, and the *ribB* mutant (Table 2). Rates of ferric iron reduction were generally twice as high in the *fur* mutant than in the wild-type strain, whereas rates of ferric iron reduction were significantly reduced in the *ribB* mutant, which is surprising since the *ribB* mutant was grown in medium supplemented with riboflavin to a final concentration of 50  $\mu$ M. Fe $^{3+}$  reduction activity was not substantially affected by the level of Fe $^{3+}$  supplied in the growth medium. Lowering iron availability from medium levels

exogenous diacetyl. Cultures were grown in MEM $\alpha$  without riboflavin, supplemented with 20  $\mu$ M FeCl $_3$  at 37°C, with or without 50  $\mu$ M diacetyl. Data shown are from two independent growth experiments, each performed in triplicate. Error bars indicate standard deviations.

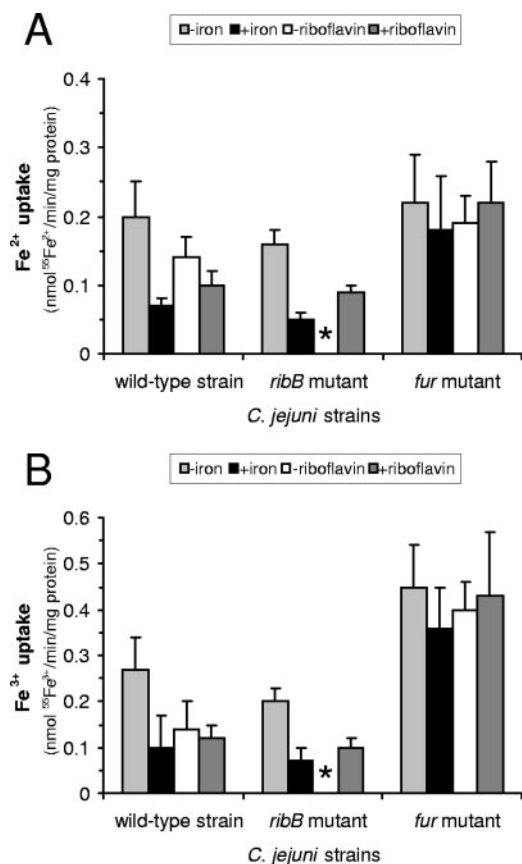


FIG. 4. Ferric and ferrous iron uptake by *C. jejuni* NCTC11168 is iron and riboflavin responsive and is increased in the *fur* mutant. Rates of <sup>55</sup>Fe<sup>2+</sup> (A) and <sup>55</sup>Fe<sup>3+</sup> (B) uptake in the wild-type, *ribB* mutant, and *fur* mutant strains. Cultures were grown in iron-limited (1  $\mu$ M FeCl<sub>3</sub>; indicated as -iron) and iron-replete (40  $\mu$ M FeCl<sub>3</sub>, indicated as +iron) MEM $\alpha$  or in standard (20  $\mu$ M FeCl<sub>3</sub>) MEM $\alpha$  without and with additional supplementation with 50  $\mu$ M riboflavin (indicated as -riboflavin and +riboflavin). Data shown are from two independent experiments performed in triplicate. The *ribB* mutant could not be tested in MEM $\alpha$  without added riboflavin because of the riboflavin auxotrophy; the absence of data for this sample is indicated by an asterisk.

(20  $\mu$ M FeCl<sub>3</sub>) to low levels (1  $\mu$ M FeCl<sub>3</sub>) increased ferric reduction activity by about 15% in both the wild-type strain and the *fur* mutant (data not shown).

## DISCUSSION

The riboflavin biosynthetic pathway remains, in principle, a potential target for new antibacterial strategies (5), but aside from this, the possibility that riboflavin availability influences bacterial growth and competitiveness, particularly through effects on iron assimilation, attracts investigation. This is perhaps especially so in the case of food-borne pathogens such as *C. jejuni*, which, in principle, might produce riboflavin endogenously or, alternatively, might assimilate it from the host environment, including the gut luminal contents. In this study, we have investigated the processes involved in the initiation of the riboflavin biosynthesis pathway in *C. jejuni* by identification of the *ribA* (Cj0996) and *ribB* (Cj0572) genes. The ability of the *ribB* mutant to grow in the presence of diacetyl instead of

riboflavin demonstrates that Cj0572 encodes a 3,4-dihydroxy 2-butanone-4-phosphate synthase, analogous to the *ribBA* gene of *H. pylori* (6, 13, 41). There is thus far no physiological evidence that the Cj0572 gene encodes a bifunctional RibBA enzyme. Similar findings have been reported for the riboflavin biosynthesis pathway in the related bacterium *H. pylori* (13).

The results presented here show that *C. jejuni* strain NCTC11168 can grow independently of exogenous riboflavin (Fig. 2A). It is also able to take up riboflavin from the medium, and exogenous riboflavin stimulated the growth of the wild-type and *ribB* mutant strains (Fig. 2). Riboflavin transport was confirmed directly by demonstrating the uptake of tritiated riboflavin, and rates of riboflavin uptake were somewhat higher in the *ribB* mutant than in the wild-type strain (Fig. 3). This suggests the possibility that the uptake mechanism—whatever this may be—may be responsive to the cellular demand for riboflavin and to the capability of the cell to fulfill it. In addition, riboflavin uptake in the *fur* mutant was found to be generally greater than in the wild type or the *ribB* mutant and unaffected by growth under riboflavin supplementation or under iron-replete conditions, suggesting an involvement of *fur* in the mechanism. Genes encoding specific bacterial riboflavin transporters (*ypaA*, *ribU*, *pnuX*, *impX*, and *rftN*) have been identified in a number of bacteria, and expression of *ypaA* was found to be regulated by an *RFN* element, in concert with regulation of the expression of the *rib* operon (37); the RibU, YpaA, and PnuX riboflavin transport proteins of *Lactococcus lactis*, *Bacillus subtilis*, and *Corynebacterium glutamicum*, respectively, have recently been studied in detail (8, 12, 38). The genome sequences of *C. jejuni*, however, do not encode orthologs of these riboflavin transporter systems (17, 20, 24). It will therefore be of considerable interest to investigate further the biochemical and genetic properties of riboflavin uptake in *C. jejuni*. The results presented here also clearly show that neither FMN nor FAD supports the growth of the *ribB* mutant, suggesting that neither compound was taken up appreciably by the cells or that neither compound is hydrolyzed to riboflavin itself extracellularly.

Uptake systems potentially responsible for the assimilation of Fe<sup>3+</sup> and Fe<sup>2+</sup> in *C. jejuni* have been characterized, and it seems likely that their relative contributions will depend upon the particular iron sources available, the redox potential, and other environmental and physiological variables (18, 22, 23, 26, 32). Irrespective of the form in which iron is assimilated, there is a probable role for ferric (flavin) reductases, acting intracellularly in the case of the uptake of iron as Fe<sup>3+</sup> or extracellularly (or periplasmically) in the case of uptake as Fe<sup>2+</sup>, for

TABLE 2. Fe<sup>3+</sup> reduction activity in *C. jejuni* NCTC11168 and its *ribB* and *fur* mutants

Strain	Mean Fe <sup>3+</sup> reduction (nmol min <sup>-1</sup> mg protein <sup>-1</sup> ) $\pm$ SEM <sup>a</sup>		
	Whole culture	Lysed cell pellet	Culture supernatant
Wild type	0.58 $\pm$ 0.01	0.38 $\pm$ 0.03	0.10 $\pm$ 0.01
<i>ribB</i> mutant	0.14 $\pm$ 0.00	0.05 $\pm$ 0.00	0.05 $\pm$ 0.00
<i>fur</i> mutant	1.10 $\pm$ 0.01	0.80 $\pm$ 0.01	0.58 $\pm$ 0.01

<sup>a</sup> Standard errors are for triplicate determinations on three sets of data.

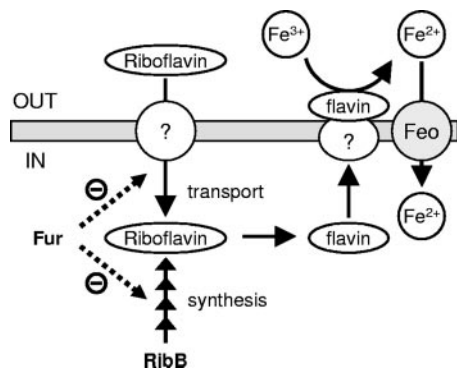


FIG. 5. Graphic representation of our hypothetical model illustrating the proposed links among riboflavin biosynthesis, assimilatory ferric reduction, and iron acquisition in *C. jejuni*. Riboflavin biosynthesis via RibB (Fig. 1 and 2) or riboflavin uptake (Fig. 3) allows the activation of an as-yet-unknown flavin/ferric reductase, which mediates extracellular or membrane-bound reduction of ferric iron ( $\text{Fe}^{3+}$ ) to ferrous iron ( $\text{Fe}^{2+}$ ) (Table 2) (9, 15, 29). Ferrous iron may subsequently be transported by the Feo ferrous iron transporter system (Fig. 4) (22, 36). The ferric uptake regulator Fur is depicted as a negative regulator both of riboflavin synthesis and of an uncharacterized riboflavin uptake mechanism (Fig. 3 and 4; Table 2). The dashed lines indicate that it is not known whether the observed phenotypes are due to direct or indirect regulation by Fur.

example, via the action of the FeoB transporter (22, 36). Riboflavin auxotrophy or (in the wild-type strain) supplementation with exogenous riboflavin had little effect on the uptake of  $\text{Fe}^{2+}$  or of  $\text{Fe}^{3+}$ , but riboflavin auxotrophy was associated with diminished  $\text{Fe}^{3+}$  reduction activity. As the amount of riboflavin available to the *ribB* mutant was much greater than that available to the wild-type cultures (probably because of the supplementation with exogenous riboflavin), a simple insufficiency of riboflavin could not have been responsible for this diminished activity in the auxotrophs. Although riboflavin provided exogenously was sufficient to support the growth of the auxotrophic cultures, it did not support ferric (flavin) reductase activity to the levels of the wild-type strain (Table 2). This suggests that exogenous supplementation with riboflavin can only partially complement the riboflavin auxotrophy of the *ribB* mutant. This could be for a variety of potential reasons, including repression of the synthesis of ferric (flavin) reductase in the absence of a functioning riboflavin biosynthetic pathway, or conversely, high levels of exogenous riboflavin might act to repress the synthesis of ferric (flavin) reductase. The identity of the putative ferric reductase is still unknown, and there are unfortunately no obvious candidates present in the *C. jejuni* genome sequence (17, 24).

Our work unequivocally supports the links among riboflavin status, ferric reductase activity, and iron assimilation in *C. jejuni*, and these relationships are altered in the absence of the Fur iron-responsive regulator (Fig. 5). We have demonstrated riboflavin uptake by this organism and have shown that this process too is connected to regulation by Fur. Exactly how riboflavin uptake might be regulated in response to the cellular demand for riboflavin and to its role in iron assimilation is an intriguing question now demanding further investigation.

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