THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 34, Issue of August 21, pp. 21669–21674, 1998

Printed in U.S.A.

The Bacterial Irr Protein Is Required for Coordination of Heme Biosynthesis with Iron Availability*

(Received for publication, April 21, 1998, and in revised form, June 16, 1998)

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Heme is a ubiquitous macromolecule that serves as the active group of proteins involved in many cellular processes. The multienzyme pathway for heme formation culminates with the insertion of iron into a protoporphyrin ring. The cytotoxicity of porphyrins suggests the need for coordination of its biosynthesis with iron availability. We isolated a mutant strain of the bacterium *Bradyrhizobium japonicum* that, under iron limitation, accumulated protoporphyrin and showed aberrantly high expression of *hemB*, an iron-regulated gene encoding the heme synthesis enzyme δ-aminolevulinic acid dehydratase. The strain carries a loss of function mutation in *irr*, a newly described gene that encodes a putative member of the GntR family of bacterial transcriptional regulators. Irr accumulated only under iron limitation, and turned over rapidly upon an increase in iron availability. A separate role for Irr in controlling the cellular iron level was inferred based on a deficiency in high affinity iron transport activity in the *irr* strain, and suggests that regulation of the heme pathway is coordinated with iron homeostasis. A high level of protoporphyrin accumulation is not a normal consequence of nutritional iron deprivation, thus a mechanism for iron-dependent control of heme biosynthesis may be present in other organisms.

Heme carries out a wide range of biological functions in prokaryotes and eukaryotes. Heme has long been known to be essential for respiration, oxygen metabolism, and electron transfer as the prosthetic group of hemoglobins, hydroxylases, catalases, peroxidases, and cytochromes. More recently, roles for heme as a biosensor of diatomic gases (1–3) and as a modulator of protein activity (4–6) have been described. The biosynthesis of protoheme involves seven sequential enzymatic steps from the first universal heme precursor ALA[1]; other cellular hemes arise from modifications of protoheme. Ferrous iron is inserted into protoporphyrin IX in the final step of heme biosynthesis catalyzed by ferrochelatase. Synthesis of heme presents several regulatory problems because it must be controlled in accordance with cellular function and coordinated with cellular heme levels (5, 7), hemoprotein apoprotein formation (4, 6, 8), and other factors associated with cellular differentiation (4, 9, 10).

The cytotoxicity of porphyrins has been readily demonstrated in animals (11), plants (12), and bacteria (13), which is caused by their ability to catalyze light-dependent formation of reactive oxygen species. The pathological consequences of abnormal porphyrin accumulation in humans ranges from light sensitivity to neurological disorders (11). Iron must be acquired exogenously and may be a limiting nutrient, thus a *prima facie* argument can be made for the coordination of the heme pathway with the cellular iron level to prevent protoporphyrin synthesis from exceeding iron availability. This problem has been partially addressed in mammalian erythroid cells, where the iron-regulatory proteins (reviewed in Refs. 14 and 15) inhibit translation of mRNA encoding the heme synthesis enzyme ALA synthase under low iron conditions (16, 17). However, it has not been established that this mechanism is sufficient to regulate the heme pathway as a whole or that it prevents excess protoporphyrin synthesis under iron limitation. Similarly, little is known about regulation of the heme pathway by iron in prokaryotes, and this is addressed in the present study. *Bradyrhizobium japonicum* is a good system for studying heme biosynthesis because its ability to live either as a free-living cell or as a nitrogen-fixing endosymbiont of soybean necessitates the expression and regulation of diverse systems for respiratory metabolism (18, 19). Expression of *B. japonicum hemA* and *hemB*, the genes encoding ALA synthase and ALA dehydratase, respectively, are positively affected by iron (20, 21). *hemB* is particularly responsive, with mRNA and protein levels varying over 80-fold as a function of the iron concentration in which the cells are grown (21). In the present study, we show that loss of function of a new gene called *irr* uncouples heme biosynthesis from iron availability, thereby demonstrating that the pathway as a whole is regulated by iron, and providing insight into the mechanism of control. Furthermore, this regulation is integrated with iron homeostasis.

EXPERIMENTAL PROCEDURES

Strains, Media, and Plasmids—B. japonicum LO and I110 were the parent strains used in the present work. Both strains were analyzed because strain I110 is the most commonly used strain, but strain LO can be mutagenized with Tn*5* with higher efficiency. Strain LODTM5 is a mutant derivative of strain LO that contains a transposon Tn*5* within the *irr* gene. Strain I110ek4 is a *hemH* mutant constructed as described previously (22). The *B. japonicum* strains were routinely grown at 29 °C in GSY medium (23). The medium was supplemented with kanamycin and streptomycin at 100 and 50 μg/ml for solid and liquid cultures, respectively, for strain LODTM5. Strain I110ek4 was grown with kanamycin and 15 μM hemin to fulfill its heme auxotrophy. *Escherichia coli* strains DH5α or XL1-Blue were used for propagation of plasmids and were grown in LB medium containing 100 μg/ml ampicillin.

Screening of Mutants and Isolation of the irr Gene—Strain LO was mutagenized with Tn*5* as described previously (22). Colonies were

* The work was supported by National Science Foundation Grant MCB-9722974 (to M. R. O'B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF073772.

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[1] The abbreviations used are: ALA, δ-aminolevulinic acid; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); kb, kilobase pairs.

screened for those that fluoresced under ultraviolet light on plates containing low iron medium. One protoporphyrin-accumulating strain was cultured, and the *Eco*RI restriction fragment containing the Tn*5* and flanking genomic DNA was isolated and reintroduced into the strain LO genome by homologous recombination to construct LODTM5. The position of Tn*5* in the genome of strain LODTM5 was confirmed by Southern blot analysis. Precautions were taken to avoid exposure of strain LODTM5 to light in order to prevent the formation of light-dependent reactive oxygen species catalyzed by protoporphyrin. The fluorescent compound produced by the mutant strains was purified and identified as protoporphyrin as described elsewhere (22, 24). Genomic DNA flanking the Tn*5* was used as a hybridization probe to isolate a 13-kb *Hin*dIII fragment from the wild type genome, and a 3.4-kb *Eco*RI fragment subclone contained the region mutated in strain LODTM5. The *Eco*RI fragment and subclones ligated into the broad host range plasmid pLAFR3 were tested for complementation. Although strain LODTM5 grew on plates, introduction of pLAFR3 alone into the mutant yielded pinpoint colonies that could not be cultured in liquid medium. Although unexplained, it is possible that the mutant could not bear the burden of replication of the 22-kb plasmid. Thus, we were able to use growth as a criterion for complementation as well as colony fluorescence. The nucleotide sequence of both strands of a *Sma*I-*Sac*I fragment corresponding the mutated locus of strain LODTM5 was determined, which contained a 492-bp open reading frame. The exact location of Tn*5* in the mutant was determined by sequencing of a portion of the mutated fragment in the region of the transposon.

Growth of B. japonicum Cells for Analysis—The medium used for culturing cells under iron limitation was a modified GSY medium in which 0.5 g/liter yeast extract (Difco) is used instead of 1 g/liter, and no exogenous iron source was added. The actual iron concentration of the medium was 0.3 μM as determined with a Perkin-Elmer model 1100B atomic absorption spectrometer. Cells grew well in this medium to A_{540} of 0.6, but mutant strain LODTM5 grew more slowly than the parent strain at higher cell densities. Cells were grown to A_{540} of 0.4–0.6 for all experiments except for protoporphyrin accumulation experiments, in which cells were grown to A_{540} of 0.8. For RNA and protein analysis of *hemB*, *hemH*, and *irr*, the cells were grown in medium with no added iron as the iron-limited condition or 6 μM $FeCl_3$ as the iron replete condition. For the metal-dependent protoporphyrin accumulation experiments, $FeCl_3$ was added to the final concentration indicated, and other metals were added as the chloride salt to a final concentration of 11 μM.

Analysis of mRNAs—Total RNA was prepared from cultured cells grown to mid-log phase as described previously (10). Steady state levels of *irr*, *hemB*, or *hemH* mRNA were analyzed by an RNase protection assay as described previously (10, 21) using antisense RNA probes to the respective genes. The effect of iron on the rate of *irr* mRNA synthesis was determined by transcriptional runoff analysis essentially as described previously (10).

Analysis of ALA Dehydratase and Irr protein—The presence of ALA dehydratase or Irr in whole cells or cell extracts was detected by immunoblot analysis of 10% or 15% SDS-PAGE gels using antibodies raised against the respective protein. Anti-ALA dehydratase antibodies were prepared as described previously (25). A modified *irr* gene was constructed by a polymerase chain reaction such that an *Nde*I restriction site was introduced at the second methionine codon of the open reading frame and cloned into pET3c for overexpression in *E. coli* Bl21(DE3)(pLysS). The 5′ primer used to modify the sequence was 5′-AACCATATGCTCCAGTC-3′. The M13 reverse primer complementary to pUC19 vector in which the *B. japonicum* DNA was cloned was used as the 3′ polymerase chain reaction primer. Antibodies were raised against the Irr protein derivative purified from inclusion bodies using a protocol described previously (26). For Irr turnover experiments, 1 liter of cells was grown to mid-log phase in low iron medium and then separated into two 500-ml cultures. To one culture, 6 μM $FeCl_3$ was added at time 0, and for the control an equivalent volume of buffer with no iron was added. Incubation with shaking was continued, and 25-ml samples were removed at various times after iron addition for immunoblot analysis. Autoradiograms were quantified using a Bio-Rad model GS-700 imaging densitometer in the transmittance mode and the Molecular Analyst software package, version 1.4.1.

Enzyme Activity Assays—Ferrochelatase activity was measured as the formation of mesoheme by cell extracts from ferrous iron and mesoporphyrin as described previously (22). Protoporphyrinogen oxidase activity was measured in membrane preparations as the oxidation of protoporphyrinogen to protoporphyrin as described previously (22). A unit of activity is defined as the nanomoles of product formed/h/mg of protein.

Iron Uptake Assays—Cells were grown to mid-log phase in low or high iron media, centrifuged, washed twice, and resuspended in uptake buffer to an A_{540} of 0.4. Uptake buffer contained 0.2 M MOPS, 20 mM citrate, and 2% (w/v) glucose, pH 6.8. 30 ml of cells were placed into a 125-ml flask and preincubated at 30 °C with shaking. At time 0, $^{59}FeCl_3$ was added to a final concentration of 0.05 μM (1.8 μCi). 1-ml aliquots (~125 μg of protein) were removed at various times and added to 3 ml of quench buffer precooled on ice. The quench buffer contained 0.1 M Tris, 0.1 M succinate, and 10 mM EDTA, pH 6.0. The quenched cells were collected on 0.45-μm filters presoaked in quench buffer containing 40 μM Fe-EDTA, and then counted on a LKB γ-counter.

RESULTS

Isolation of a B. japonicum Mutant That Accumulates Protoporphyrin in Iron-limited Cells—To search for mutants deregulated in iron control of heme biosynthesis, we screened a population of Tn*5*-induced mutants for colonies that accumulated protoporphyrin, as discerned by fluorescence under ultraviolet light. *B. japonicum* mutant strain LODTM5 displayed that phenotype, and the fluorescent species was purified and identified as protoporphyrin IX by the peaks of the absorption spectra of the neutral methyl ester derivative (Fig. 1*A*) and the free acid, and by thin layer chromatography (data not shown). No other porphyrins were detected. Protoporphyrin is the immediate precursor of protoheme (Fig. 1*B*), and thus the mutant was altered in some aspect of heme metabolism. In principle, protoporphyrin accumulation could be due to defects in the heme synthesis enzymes ferrochelatase or protoporphyrinogen oxidase. However, those enzyme activities were not defective in extracts of the mutant (data not shown), and *hemH* mRNA encoding ferrochelatase was expressed (see below). Furthermore, strain LODTM5 was not a heme auxotroph. The results show that the primary defect in strain LODTM5 is not a lesion in a structural gene of the heme biosynthetic pathway.

To determine whether the mutant phenotype was iron-dependent, cells were grown in media with varying concentrations of the metal. An inverse relationship was found between the quantity of protoporphyrin produced by the mutant and the iron concentration in which the cells were grown, with the highest amount found in cells grown in media with no exogenous iron added, and none detected at high iron concentrations (Fig. 1*C*). Protoporphyrin was not detected in cells of parent strain LO under any iron condition tested. When this experiment was repeated with *hemH* strain I110ek4, which accumulates protoporphyrin due to a defect in ferrochelatase (22), no decrease in the protoporphyrin level with increasing iron was observed (Fig. 1*C*). Thus, the diminution observed in strain LODTM5 was not due to the nonenzymatic chelation of iron into protoporphyrin, but rather it was specific to that mutant. The effects were also metal-specific, as seen by the failure of copper, zinc, nickel, manganese, or molybdenum to abolish protoporphyrin accumulation by strain LODTM5 (Fig. 1*D*). Thus, the aberration in heme metabolism was linked to iron availability, and the mutant phenotype is manifest under iron depleted conditions. The genetic locus mutated in strain LODTM5 was named *irr* (iron response regulator) based on these observations, and on those described below.

irr Mediates Iron Control of hemB, a Heme Biosynthesis Gene—The accumulation of protoporphyrin by strain LODTM5 in the absence of a heme deficiency strongly indicated an abnormally high synthesis of protoporphyrin by the heme pathway in iron-limited cells. We determined previously that *hemB*, the gene encoding the heme biosynthesis enzyme ALA dehydratase is regulated by iron at the mRNA level in *B. japonicum* wild type strain I110 and that accumulation is low in iron-limited cells (21). By RNase protection and immunoblot analyses, respectively, we examined the effects of iron on *hemB* mRNA and protein in wild type strains I110 and LO, and in *irr*

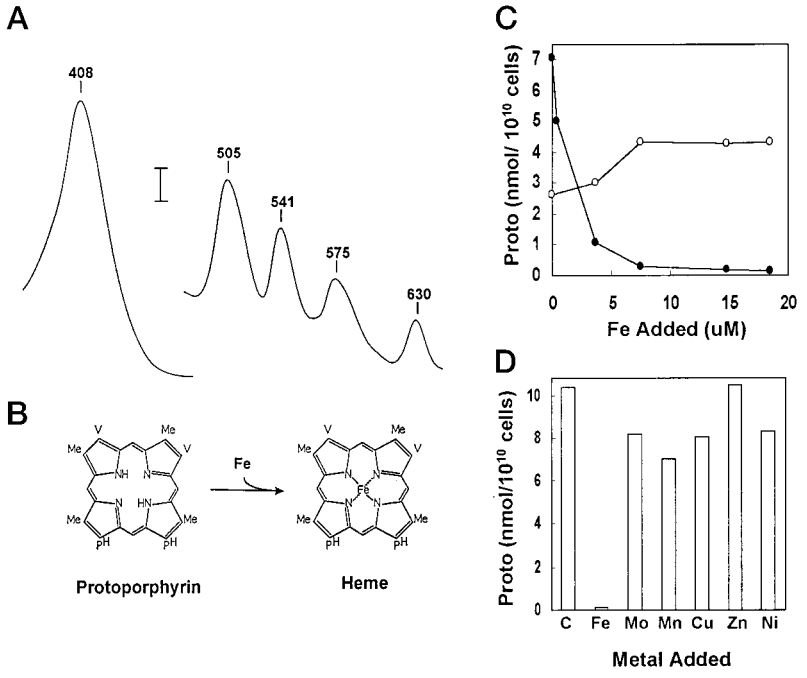

FIG. 1. **Mutant strain LODTM5 accumulates protoporphyrin in an iron-dependent manner.** *A*, the fluorescent compound produced by strain LODTM5 was purified from cells, and the absorption spectrum of its neutral methyl ester shown identifies the compound as protoporphyrin. The absorption peaks are labeled in nanometers. The *vertical bar* represents a ΔA of 0. 014 in the region from 450 to 650 nm and 0.12 from 360 to 450 nm. The absorption peaks for authentic protoporphyrin were at 408, 505, 542, 575, and 629 nm. *B*, chelation of iron into protoporphyrin to form heme (protoheme) catalyzed by ferrochelatase. Protoporphyrin is the fluorescent compound produced by strain LODTM5. *C*, effect of iron on protoporphyrin accumulation. Cultures of *irr* strain LODTM5 (*closed circles*) or *hemH* strain I110ek4 (*open circles*) were grown to A_{540} of 0.8 in the presence of varying concentrations of $FeCl_3$. Protoporphyrin was extracted from cells and quantified. *D*, effect of other metals on protoporphyrin accumulation. Strain LODTM5 cells were grown and analyzed as in *panel A* with 11 μM of the chloride salt of iron, molybdenum, manganese, copper, zinc, or nickel. The control, labeled *C*, is medium with no added metal.

strain LODTM5. As observed previously, *hemB* mRNA and protein were very low in wild type cells grown in media with no added iron, and were elevated significantly in cells grown in iron-replete medium (Fig. 2). However, mRNA and protein levels were high in strain LODTM5 even in cells grown in low iron medium, showing the loss of normal iron-dependent regulation of the gene in the mutant. Under low iron conditions, the mutant accumulated approximately 50-fold more ALA dehydratase and over 150-fold more *hemB* mRNA than was found in parent strain LO. This high expression of *hemB* by the mutant was consistent with the protoporphyrin accumulation phenotype and indicates an elevated heme pathway. Ferrochelatase catalyzes a step of the pathway subsequent to protoporphyrin formation, and therefore a regulatory system that coordinates protoporphyrin synthesis with iron availability is unlikely to control *hemH*, the gene encoding ferrochelatase. Consistent with this rationale, accumulation of *hemH* mRNA was not iron-dependent in the wild type strains, nor was its expression altered in strain LODTM5 (Fig. 2). Collectively, the results argue in favor of a role for *irr* in mediating iron control of *hemB*, and of the pathway as a whole, and support the conclusion that protoporphyrin synthesis exceeds iron bioavailability in iron-limited cells of mutant strain LODTM5.

The irr Gene Encodes a Novel Regulatory Protein That Is Absent in Mutant Strain LODTM5—To elucidate the genetic basis of the mutation in strain LODTM5, wild type DNA corresponding to that which was mutated in the *irr* strain was isolated using cloned DNA flanking the Tn*5* as a hybridization probe. A 3.4-kb *Eco*RI fragment and subclones (Fig. 3*A*) were tested for complementation of strain LODTM5 on the broad host range plasmid pLAFR3 (see "Experimental Procedures"). The smallest complementing clone tested was a 0.7-kb *Sma*I/*Bgl*II fragment containing a 492-bp open reading frame encoding a protein 163 amino acids in length (Fig. 3*A*). The Tn*5* was inserted into the 5′ portion of the *irr* gene (Fig. 3*A*). Expression of the *irr* gene region was analyzed by measuring RNA and protein accumulation in *irr*$^+$ strains LO and I110 and in the mutant (Fig. 4). RNase protection analysis showed that *irr* accumulated in wild type strains LO and I110, but not in mutant strain LODTM5 (Fig. 4*B*). In addition, no mRNA corresponding to the genomic region immediately downstream of

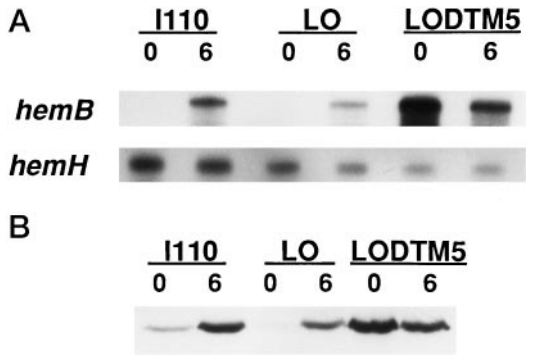

FIG. 2. **Loss of iron-dependent expression of *hemB* in *B. japonicum* mutant strain LODTM5.** *A*, RNase protection analysis of *hemB* and *hemH* mRNA from cells grown in medium supplemented with 0 or 6 μM $FeCl_3$. 2 μg of total RNA from cells grown in iron-limited or iron-replete media were analyzed per reaction. *B*, immunoblot analysis of ALA dehydratase *(hemB* product) in cells supplemented with 0 or 6 μM $FeCl_3$. 50 μg of protein were loaded per lane of a 10% SDS-PAGE gel and analyzed with anti-ALA dehydratase antibodies.

irr was detected in any strain, indicating that *irr* does not have another gene downstream of it encoded on the same transcript. Immunoblot analysis of whole cell protein of the wild type strains and of *irr* strain LODTM5 using anti-Irr antibodies showed that the wild type strains grown in low iron medium expressed an ~18-kDa protein that was absent in the mutant (Fig. 4*C*). The results show that strain LODTM5 carries a loss of function mutation in the *irr* gene, and that its phenotype is due to a lesion in that gene, and not to a polar effect of the Tn*5* mutagen. In addition, the transposon was inserted close to the 5′ end of the gene, thus it was important to establish that the mutation did not activate a truncated derivative of *irr* or a downstream gene. Finally, the data show that *irr* is a protein-encoding gene, and that Irr is involved in the negative regulation of *hemB* and of the heme biosynthetic pathway.

A data base search of protein blocks (27) suggests that Irr belongs to the GntR family of bacterial transcriptional regulators (28) based on homology to helix-turn-helix motif regions of members of that family (Fig. 3*B*). Homology searches of individual proteins revealed that Irr shares the highest homology

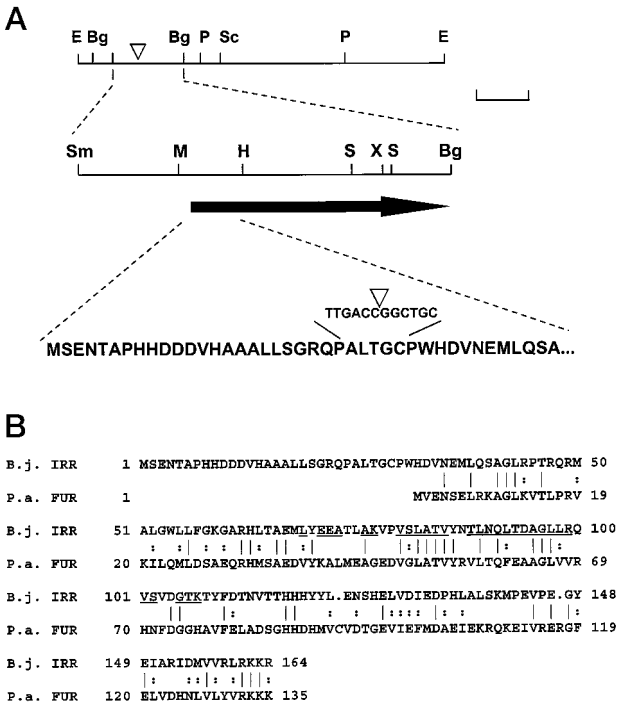

FIG. 3. **Analysis of genomic region mutated in strain LODTM5.** *A*, restriction map of the *irr* gene region. The *bar* represents 500 bp for the *upper* restriction map and 100 bp for the *lower* map. The genomic region containing the *irr* gene is marked by the restriction sites *Bgl*II (*Bg*), *Eco*RI (*E*), *Hpa*I (*H*), *Mlu*I (*M*), *Pst*I (*P*), *Sac*I (*Sc*), *Sal*I (*S*), *Sma*I (*Sm*), and *Xho*I (*X*). The *Sma*I site in the *upper* map is not unique, and therefore is marked with a *hash*, but unlabeled. The N-terminal portion of the predicted product, along with the site of Tn*5*, indicated by the *triangle*, is shown. The *arrow* represents the *irr* open reading frame. *B*, amino acid sequence comparison of *B. japonicum* Irr with *P. aeruginosa* Fur. *Solid lines* denote amino acid identity and *dotted lines* represent similar residues. The comparison was carried out using the Gap program in the Genetics Computer Group software package, version 9.0. The *underlined* region of Irr denotes the region similar to the helix-turn-helix region of the GntR family of bacterial transcriptional regulators, identified by the "Blocks" program described in the text.

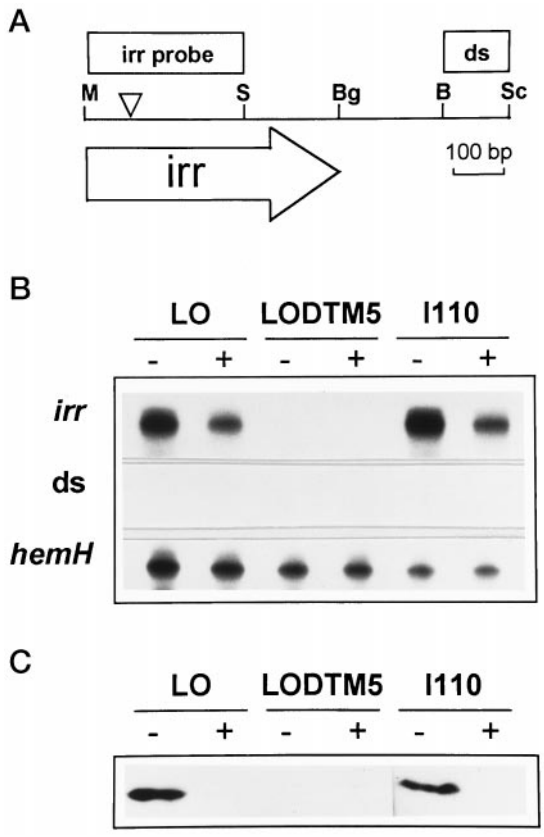

FIG. 4. **The *irr* gene is iron-regulated in wild type cells and is not expressed in mutant strain LODTM5.** *A*, the genomic region containing the *irr* gene is marked by the restriction sites *Mlu*I (*M*), *Sal*I (*S*), *Bgl*II (*Bg*), *Bam*HI (*B*), and *Sac*I (*Sc*). The *triangle* denotes the site of Tn*5* insertion. *Boxes* above the restriction map denote fragments used as templates for antisense RNA probe synthesis of *irr* and its downstream (*ds*) region used in *panel B*. *B*, RNase protection analysis of the *irr* gene, the downstream (ds) and *hemH* mRNA in cells of parent strains I110 and LO and mutant strain LODTM5. The *irr* and downstream mRNAs were analyzed using antisense probes as shown in *panel A*. *hemH* was analyzed using an antisense probe as described previously (21). Cells were grown in medium with no added iron (−) or with 6 μM $FeCl_3$ (+). 2 μg of total RNA from cells grown in iron-limited medium were analyzed per reaction. *C*, immunoblot analysis of Irr protein in strains LO, I110 and LODTM5 grown in low (−) or high (+) iron media as described in *panel B*. 50 μg of protein were loaded onto each lane of a 15% SDS-PAGE gel and analyzed with anti-Irr antibodies.

to the Fur protein from *Pseudomonas aeruginosa* (29% identity), a transcriptional regulator found in many Gram-negative bacteria involved in iron transport and other aspects of iron metabolism (29). Bacterial Fur must be bound by iron to function (30), and consequently, it is active only under iron-replete conditions. In contrast, the phenotype of the *B. japonicum irr* strain indicates that it is active under iron limitation, and data (presented below) show that Irr is not detected in cells grown in iron-rich medium. Thus, Irr and Fur cannot have the same activity and therefore are not equivalent proteins. In addition, we recently isolated a *B. japonicum fur* gene homolog by functional complementation of and *E. coli* mutant.[2] A *B. japonicum fur* strain expresses ALA dehydratase normally and does not accumulate protoporphyrin under any growth condition (data not shown). Consistent with those observations, protoporphyrin was undetectable in *E. coli fur* strain H1780 (31) grown in low or high iron media (data not shown). The results indicate that *irr* is a novel gene.

Expression of Irr Protein Is Restricted to Iron-limited Cells and Turns Over Rapidly in the Presence of Iron—Strain LODTM5 displayed a mutant phenotype only under iron limitation, suggesting that Irr function is restricted to those cells. Thus, it is likely that iron either modulates the activity of constitutively expressed Irr protein, or that the metal regulates expression of *irr* in some way. This problem was addressed by measuring *irr* mRNA and protein in cells grown in high or low iron media (Fig. 4). Immunoblot analysis showed that Irr accumulated in wild type cells grown in low iron medium, but was undetectable in those from iron-replete medium (Fig. 4*C*). This pattern was consistent with the mutant phenotypes (see Figs. 1 and 2 and below) and shows that Irr is present and active only in iron-limited cells. Unlike protein expression, however, RNase protection analysis showed that iron had only a moderate negative effect on *irr* mRNA levels, with a substantial quantity found even in iron replete cells (Fig. 4*B*). This 3-fold difference was consistent with transcriptional run-off assays demonstrating that the rate of *irr* mRNA synthesis was 4-fold greater in iron-limited cells compared with those grown in iron-rich medium (data not shown). The discrepancy between *irr* mRNA and protein strongly indicates post-transcriptional control of the *irr* gene by iron in additional to transcriptional regulation.

Addition of iron to cells grown under iron deprivation results in the rapid induction of *hemB* mRNA (21), thus *B. japonicum* responds quickly to a change in iron availability. If the assertion that Irr mediates negative control of *hemB* expression by

[2] I. Hamza and M. R. O'Brian, GenBank™ accession no. AF052295.

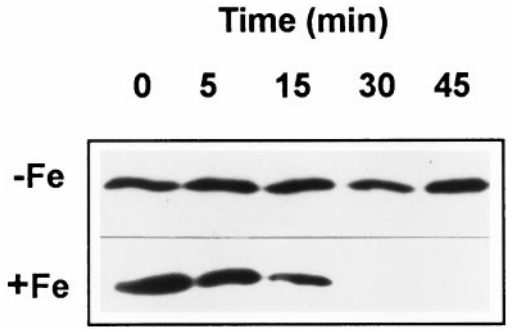

FIG. 5. **Rapid disappearance of Irr in response to iron.** Cells of strain LO were grown to mid-log phase in low iron medium to induce Irr. Subsequently, either 6 μM $FeCl_3$ (*+Fe*) or an equivalent volume of buffer containing no iron (*−Fe*) was added to the cultures at time zero. Aliquots were removed from cultures at various times and analyzed by immunoblot analysis of cell protein separated by 15% SDS-PAGE using anti-Irr antibodies. 50 μg of protein were loaded per lane.

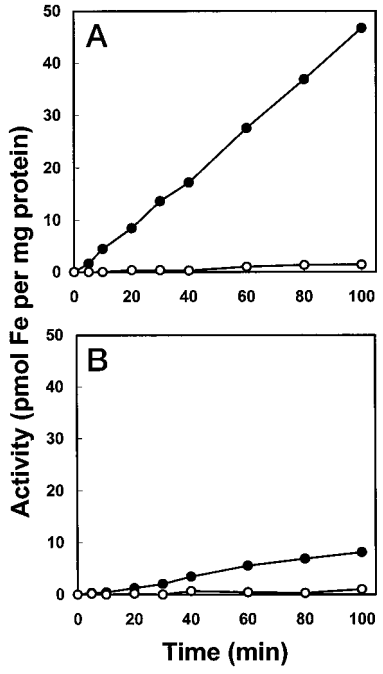

FIG. 6. **Deficiency in iron-regulated iron uptake activity in *B. japonicum* mutant strain LODTM5.** Cells were grown to mid log phase in media containing no added iron (*closed circles*) or 6 μM $FeCl_3$ (*open circles*). At time 0, 0.05 μM ^{59}Fe was added to the assay medium, and cells were harvested at various times and counted. Each time point is the average of triplicate samples, and the standard deviations were less than 10%. Uptake data for strains LO (*A*) and LODTM5 (*B*) shown were collected in a single set of experiments, but are shown in two panels for clarity of presentation.

iron is correct, then we expect a rapid loss of Irr activity upon an increase in iron availability. To address this, we monitored Irr levels in response to iron. The addition of 6 μM $FeCl_3$ iron to cells grown in low iron medium resulted in the rapid loss of Irr protein that was observed by 5 min after addition of the metal, and almost no protein remaining after 30 min (Fig. 5). This disappearance reflects a very high turnover rate for a bacterial protein (32, 33). Bacterial (34, 35) and eukaryotic (36, 37) regulatory proteins with short half-lives are specifically targeted for proteolysis under the appropriate conditions, and the observations for Irr strongly suggest a specific mechanism for its degradation. We postulate that this post-transcriptional control permits a fast response to an increase in iron availability, and that it can account for the discrepancy between *irr* mRNA and protein in iron replete cells.

B. japonicum Expresses an Inducible, High Affinity Iron Uptake Activity That Is Regulated by Irr—Iron is an essential nutrient involved in many biologic functions, but is also toxic in high concentrations. A regulatory mechanism that couples protoporphyrin synthesis with iron levels should also integrate systems necessary for iron homeostasis. Therefore, we examined the effects of iron availability on high affinity iron uptake activity in parent strain LO and mutant strain LODTM5. Wild type cells grown in iron-replete medium lacked iron transport activity using 0.05 μM ^{59}Fe (as ferric citrate) as a tracer, but uptake was induced in cells grown in low iron medium (Fig. 6*A*). This is a common response by bacteria to efficiently scavenge the metal when it is limiting. However, this activity was severely diminished in mutant strain LODTM5 (Fig. 6*B*), indicating that iron transport is under positive control in *B. japonicum* by a mechanism that involves Irr. In addition, it is plausible that the iron uptake defect in strain LODTM5 resulted in an iron deficiency that contributed to the protoporphyrin accumulation phenotype. Because iron limitation repressed *hemB* in wild type cells (Fig. 3) (21), the high expression of the gene in strain LODTM5 cannot be an indirect consequence of the iron transport defect, which should render the cell iron-deficient. We showed previously that *hemB* is expressed in iron-replete cells of a heme-defective strain (21), therefore the elevated *hemB* level in the *irr* mutant is not an indirect result of a heme deficiency created by iron limitation. The present findings suggest that Irr has dual activities to positively affect iron transport and negatively regulate *hemB*, and that the control of heme biosynthesis is coordinated with iron homeostasis.

DISCUSSION

In the present study, we provide evidence for coordination of a bacterial heme biosynthetic pathway with iron availability by a regulatory mechanism involving the newly described protein Irr. Irr accumulates in response to iron limitation by iron-dependent regulation of the *irr* gene to attenuate the heme pathway, thereby preventing protoporphyrin synthesis from exceeding iron availability. The phenotype of the *irr* mutant is important because it demonstrates that copious protoporphyrin accumulation is a consequence of uncoupling the heme pathway as a whole from iron availability, and that it can result from a single genetic lesion. Because this phenotype is not normally observed in cells, it is reasonable to extrapolate from the present work that iron control of heme biosynthesis is a general regulatory phenomenon found in other organisms. Humans with iron deficiency anemia show a 5-fold increase in blood protoporphyrin, reaching approximately 2×10^{-4} mol/mol of heme (38). This elevated level is modest by comparison with the *B. japonicum irr* mutant, which accumulates approximately 15 mol of protoporphyrin/mol of heme under iron limitation (by recalculation of the data in Fig. 1*D*). Thus, iron may regulate the heme pathway in erythrocytes to prevent severe porphyrin accumulation under iron deficiency. In bacteria, numerous genes have been recently identified that encode proteins that, like Irr, have low but significant homology to Fur, but are distinct from *bona fide fur* genes in those organisms (GenBank™ accession nos. Z82044, D84432, D90909, U76538, P32692, U58365, and U25731). Most of those genes were identified by whole genome sequencing and their functions are unknown. However, *fur*-like genes of *Vibrio cholerae* (39) and *P. aeruginosa* (40) were shown to be expressed, and the *P. aeruginosa* mutant does not have a *fur*-defective phenotype. From this, it is pertinent to ask whether bacteria other than *B. japonicum* contain Irr homologs that mediate iron control of heme synthesis.

The defective iron uptake activity in mutant strain LODTM5 indicates a link between control of the heme pathway and iron

homeostasis. The proposed roles for Irr are physiologically sensible because it allows the cell to maximize accessibility to exogenous iron when the metal is limiting as well as attenuate the heme biosynthetic pathway. By analogy, the iron regulatory proteins in mammalian erythroid cells regulate iron transport and a heme biosynthesis gene enzyme (reviewed in Refs. 14 and 15), and it will be important to determine whether that control is sufficient to modulate synthesis by the pathway as a whole. Furthermore, the identification of regulators of iron homeostasis such as bacterial Fur (29) and Aft1 from *Saccharomyces cerevisiae* (41) may provide the tools necessary to assess the relationship between iron transport and heme biosynthesis in those organisms.

Acknowledgment—We thank Dr. K. Hantke for *E. coli* strains.

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