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The *Bradyrhizobium japonicum fegA* Gene Encodes an Iron-Regulated Outer Membrane Protein with Similarity to Hydroxamate-Type Siderophore Receptors

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Iron is important in the symbiosis between soybean and its nitrogen-fixing endosymbiont *Bradyrhizobium japonicum*, yet little is known about rhizobial iron acquisition strategies. Analysis of outer membrane proteins (OMPs) from *B. japonicum* 61A152 identified three iron-regulated OMPs in the size range of several known receptors for Fe(III)-scavenging siderophores. One of the iron-regulated proteins, FegA, was purified and microsequenced, and a reverse genetics approach was used to clone a *fegA*-containing DNA fragment. Sequencing of this fragment revealed a single open reading frame of 750 amino acids. A putative N-terminal signal sequence of 14 amino acids which would result in a mature protein of 736 amino acids with a molecular mass of 80,851 Da was predicted. FegA shares significant amino acid similarity with several Fe(III)-siderophore receptors from gram-negative bacteria and has greater than 50% amino acid similarity and 33% amino acid identity with three bacterial receptors for hydroxamate-type Fe(III)-siderophores. A dendrogram describing total inferred sequence similarity among 36 TonB-dependent OMPs was constructed; FegA grouped with Fe(III)-hydroxamate receptors. The transcriptional start site of *fegA* was mapped by primer extension analysis, and a putative Fur-binding site was found in the promoter. Primer extension and RNA slot blot analysis demonstrated that *fegA* was expressed only in cells grown under iron-limiting conditions. This is the first report of the cloning of a gene encoding a putative Fe(III)-siderophore receptor from nitrogen-fixing rhizobia.

Rhizobia live in the soil or engage in a nitrogen-fixing symbiosis with a suitable legume host plant. Each environment presents unique challenges with respect to the acquisition of essential nutrients such as iron. Although iron is the fourth most abundant element in the earth's crust, it is extremely insoluble at neutral pH under aerobic conditions and is predominantly found as precipitated, oxyhydroxide polymers (34, 57). Therefore, as free-living soil microorganisms, rhizobia must have a way to solubilize iron as well as a way to compete for this nutrient with other organisms present in the rhizosphere. In planta, iron is likely to be tightly chelated in various storage forms, because free iron catalyzes the production of potent oxidants that damage biomolecules (for example, see reference 80). In addition, iron has been shown to be a pathogenicity factor (22), so rhizobia must have mechanisms for accessing iron which is generally unavailable to invading pathogens.

As a starting point in the study of rhizobial iron acquisition, investigators have established that some rhizobial strains can produce siderophores, Fe(III)-specific ligands with a high affinity for iron (reviewed in reference 33; see also references 14, 15, 23, 41, 48, 52, 61, 68, 69, 78, and 82). At present, we can generalize and say that siderophore production, when present, is strain specific. That is, there does not appear to be any particular siderophore made by all rhizobia. For example, *Rhizobium meliloti* DM4 produces a carboxylate-type siderophore (76), whereas *R. meliloti* 1021 produces a dihydroxamate-type siderophore (62). We can also generalize that the ability to produce siderophores seems to be more widespread among rhizobial species than among bradyrhizobial strains. This may reflect the evolution of bradyrhizobia in the acid soils of the tropics, where iron is generally more available than in neutral or high-pH soils. A final generalization we can make is that a number of rhizobial and bradyrhizobial strains release citric acid as a siderophore under iron-deficient growth conditions (15, 36, 48). Many fungi also release hydroxy acids under conditions of iron deficiency (90). Although there is definitely a competitive advantage to producing siderophores, the ability to utilize siderophores which are produced by other organisms can also provide a clear benefit (13). For example, *Pseudomonas aeruginosa* synthesizes and uses two of its own siderophores but has also been demonstrated to utilize the heterologous bacterial siderophores enterobactin, aerobactin, and ferrioxamine B and a number of siderophores produced by other pseudomonads (20).

In order to utilize a siderophore, an organism must have a siderophore-specific iron uptake system composed of four main components: a high-affinity outer membrane receptor, the inner membrane-anchored TonB protein, a periplasmic binding protein, and several inner membrane-associated proteins (reviewed in reference 11). The genes encoding these proteins are coordinately derepressed under conditions of iron deficiency and are negatively regulated by the product of the *fur* (ferric uptake regulation) gene (reviewed in reference 34). Gram-negative bacteria selectively control the entry of iron into the cell at the outer membrane through Fe(III)-siderophore receptors, presumably because the large size of Fe(III)-siderophore complexes exceeds the limits of the outer membrane pores (reviewed in reference 37). In *Escherichia coli*, cell surface-exposed protein loops of the outer membrane receptors have been demonstrated to bind specific Fe(III)-siderophore complexes, which are subsequently internalized via a nonspecific channel region of the receptor (43, 70). Although there have been a number of reports of iron-regulated outer membrane proteins (OMPs) in rhizobia (14, 23, 28, 41, 61, 68, 69), most simply state that such proteins were observed and that they may serve as Fe(III)-siderophore receptors.

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Reigh and O'Connell (68) correlated the presence of specific iron-repressible OMPs with the production and release of specific siderophores, suggesting that Fe(III)-siderophore transport in rhizobia may be similar to transport in *E. coli* and other gram-negative bacteria.

Our work with the rhizobial soybean endosymbiont *Bradyrhizobium japonicum* focuses on strain 61A152, which releases citric acid as a siderophore under conditions of iron deficiency (36) and is also able to utilize Fe(III) from two fungal siderophores, rhodoturulate and ferrichrome (63). In order to more fully characterize iron uptake in *B. japonicum* 61A152, we have purified the three major iron-regulated OMPs from this strain. We report here on the cloning and characterization of the *fegA* gene, which encodes one of the iron-regulated OMPs from 61A152. The *fegA* gene is iron regulated, has a presumptive Fur-binding site in its promoter, and encodes a protein with similarity to hydroxamate-type siderophore receptors. This is the first report of the cloning and sequencing of a gene encoding a siderophore receptor from any rhizobia.

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, calf intestinal alkaline phosphatase, and Klenow fragment of DNA polymerase were purchased from New England Biolabs (Beverly, Mass.). Nonidet P-40 was from Calbiochem (La Jolla, Calif.). *Taq* polymerase was from Perkin-Elmer (Foster City, Calif.) Avian myeloblastosis virus reverse transcriptase was from Promega (Madison, Wis.). [α-^{32}P]dCTP, [γ-^{32}P]ATP, and [α-^{35}S]dATP were purchased from Dupont/NEN (Boston, Mass.). Unless otherwise stated, chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Strains, plasmids, phage, and growth conditions. Bacterial strains, plasmids, and phage used in this study are listed in Table 1. *E. coli* cultures were grown at 37°C in Luria-Bertani broth (5) supplemented with 80 μg of ampicillin per ml when necessary. *E. coli* cultures grown for lambda plating were supplemented with maltose (0.2% final concentration) and $MgSO_4$ (10 mM final concentration), and phage infections were performed according to standard procedures (5). *B. japonicum* cells were cultured at 30°C with shaking in minimal medium (filter-sterilized mannitol was added to a 0.2% final concentration after autoclaving) (36). Cells were initially cultured in minimal medium supplemented with $FeCl_3$ to a final concentration of 10 μM (from a filter-sterilized stock of 70 mM $FeCl_3$ in 0.1 N HCl) and then diluted into fresh iron-free minimal medium. Cultures then either were supplemented with $FeCl_3$ to a final concentration of 10 μM or were not supplemented with iron. After one cycle of growth in iron-free minimal medium, cells to be subjected to iron starvation were again diluted into fresh iron-free minimal medium to ensure iron limitation. Precautions were taken to minimize the iron content of both the culture vessels and the medium. Glassware was washed with 1 N HCl and then rinsed extensively with distilled water.

OMP preparation, analysis, and purification. OMPs were isolated from *B. japonicum* cells by using the nonionic detergent Nonidet P-40, as originally described for bacteroids (6). Briefly, bacterial outer membranes are resistant to solubilization by Nonidet P-40 because of a low protein-to-lipid ratio, while other bacterial membranes are solubilized. Protein concentrations were estimated with the BCA assay (Pierce, Rockford, Ill.). Seventy-five micrograms of protein per lane was run on sodium dodecyl sulfate (SDS)–8.6% polyacrylamide gel electrophoresis (PAGE) gels in 1× SDS electrophoresis running buffer (5) at 30 mA with prestained protein molecular weight standards (Sigma Chemical Co.). After electrophoresis, the gels were stained with Coomassie brilliant blue and photographed. Two hundred fifty-microgram quantities of isolated OMPs were loaded into wells made by a curtain comb in an SDS–8.6% PAGE gel. The gel was run, and proteins were visualized by negative staining (8). This method allowed visualization of proteins without permanently fixing them within the gel. Appropriate bands were excised, and protein-containing slices of acrylamide were electroeluted in an Isco electrophoretic concentrator according to the manufacturer's instructions (Isco Inc., Lincoln, Nebr.). Collected fractions were concentrated in Centricon 30 microconcentrators (Amicon, Danvers, Mass.) for 15 min at 6,000 × *g*. Protein concentrations were determined, and 250 ng of each protein fraction was run on 8.6% minigels. The gels were silver stained (54), and proteins were examined for purity. Gel purification and concentration steps were repeated until silver staining revealed a single sharp band of the proper molecular weight.

Preparation of antibodies and immunoblotting. Polyclonal antiserum against protein 61A2 was raised in rabbits (75), and immunoglobulins were precipitated with ammonium sulfate and affinity purified (79). Proteins were transferred to reinforced nitrocellulose (0.45-μm pore size; BA-S 85; Schleicher and Schuell, Keene, N.H.) by electrophoresis (81). The transfer was performed at a constant current (400 mA) for 60 min in a buffer containing 25 mM Tris and 192 mM glycine (pH 8.3) with a Genie electroblotting device (Idea Scientific Co., Minneapolis, Minn.). Nitrocellulose sheets were then blocked in 0.15 M NaCl–10 mM Tris (pH 7.5)–25% gelatin (warmed to allow gelatin to go into solution) and processed for detection of antigen-antibody complexes with alkaline phosphatase-conjugated secondary antibodies according to the manufacturer's instructions (Promega).

TABLE 1. Bacterial strains, phage, and plasmids used

Strain, phage, or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
DH5α	*hsdR17 endA1 thi-1 gyrA96 relA1 recA1 supE44* Δ-*lacU169* (ϕ80d*lacZ*ΔM15)	Gibco-BRL[a]
XL1-Blue	*recA1 endA1 gyrA96 thi hsdR17* (r_K^- m_K^-) *supE44 relA1* λ^- *lac* [F′ *proAB lacI*^{q}ZDM15 Tn*10* (Tc^r)]	Stratagene
B. japonicum		
61A152	Nitrogen-fixing *Glycine max* (soybean) symbiont	Nitragin Co.[b]
USDA 110d	Small colony derivative of USDA 110; nitrogen-fixing soybean symbiont	35
Phage		
Lambda ZAP	Ap^r; λ insertion vector used for making the *B. japonicum* 61A152 genomic library	Stratagene
Plasmids		
pBJ142	Ap^r, Tc^r; contains 7.5-kb ribosomal DNA operon from *B. japonicum* USDA 110d	73
pBluescript SK^+	Ap^r; P_{lac} *lacZ′*, T7p, T3p, ColE1 origin, f1 origin	Stratagene
pBluescript SK^-	Ap^r; P_{lac} *lacZ′*, T7p, T3p, ColE1 origin, f1 origin	Stratagene
p*fegA*SK	Ap^r; 5.0-kb *Bam*HI-*Pst*I genomic clone containing part of *fegA* and approx 3.0 kb of sequence upstream of *fegA*	This study
pOMPPCR	Ap^r; *fegA*-containing 5.1-kb *Eco*RI genomic clone in SK	This study

[a] Gaithersburg, Md.
[b] Milwaukee, Wis.

Amino acid analysis. Purified FegA protein (100 pmol) was electroblotted onto an Immobilon P^{SQ} polyvinylidene fluoride membrane according to the manufacturer's instructions (Millipore, Bedford, Mass.). Initial Edman degradation analysis was determined with a Model 476 A Gas Phase Protein Sequencer (Applied Biosystems Inc., Foster City, Calif.) at the Dartmouth College Molecular Biology Core Facility. Data indicated that the protein was blocked at the N terminus. Consequently, 100-pmol samples of blotted, purified FegA protein were sent to the Wistar Protein Microsequencing Facility (Philadelphia, Pa.) and subjected to tryptic digestion followed by high-pressure liquid chromatography (HPLC) separation. Two of the separated peptides were subjected to Edman degradation. Sequence data were generated for two peptide fragments. A fragment with a molecular mass of 1,924 Da had the sequence GINFLPYQGTVT NAPFGK, and a fragment with a molecular mass of 1,764 Da had the sequence DTANQADLDNQLEYR.

DNA manipulations and PCR. Restriction enzyme digestions, DNA ligations, transformation of *E. coli* with plasmid DNA, *E. coli* plasmid DNA isolation, and PCRs were performed according to standard procedures (5). Genomic DNA was isolated according to the method of Adams et al. (2). Genomic DNA of *B. japonicum* 61A152 was used as a template for PCR, with degenerate oligonucleotides corresponding to internal peptide fragments of FegA. Primers 1765 reverse [5′-G(C/T)TG(G/A)TT(A/G)TCIAG(A/G)TCIGC(C/T)TG-3′] and 1924 forward [5′-GGCATCAACTTCCTGCCGTA(C/T)CAGGG-3′] (synthesized on an Applied Biosystems Inc. DNA/RNA Synthesizer, model 392) amplified a 307-bp DNA fragment. The amplification protocol used was as follows: 94°C, 1.5 min; 65°C, 2 min; and 72°C, 4 min. This cycle was repeated 25 times and

was followed by 8 cycles of 94°C, 1 min; 50°C, 1 min; and 72°C, 2 min. $MgCl_2$ (2 mM) gave optimum results for PCR. A Lambda ZAP library, consisting of *B. japonicum* 61A152 genomic DNA partially digested with *Eco*RI and ligated into λ arms, was constructed according to the manufacturer's instructions (Stratagene, La Jolla, Calif.).

Southern and slot blot conditions. Nitrocellulose membranes were used for plaque and filter lifts, and reinforced nitrocellulose BA-S 85 membranes were used for Southern blots and RNA slot blots. Filters were treated by sequential soaking with denaturation (1.5 M NaCl, 0.5 M NaOH) and neutralization (1.5 M NaCl, 0.5 M Tris-HCl, pH 8) buffers for 2 min each, washing with 2× SSPE (0.36 M NaCl, 0.02 M Na_2HPO_4, 0.002 M $Na_2EDTA \cdot 2H_2O$ [pH 7.7])–0.1% SDS, and UV cross-linking in a Stratalinker (Stratagene). Gels to be used for Southern analysis were blotted and treated according to standard procedures (5), with the exception that 20× SSPE was substituted for 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) in all solutions. RNA slot blotting was performed according to standard procedures (5), using a Minifold II apparatus (Schleicher and Schuell), except that RNA was denatured in 6.15 M formaldehyde–10× SSC. DNA probes were labeled by random priming (24). The single 307-bp band amplified by PCR was purified from an 0.8% agarose gel, labeled, and used as a probe for the 61A152 Southern blot and for screening the Lambda ZAP library. Library filters and Southern blots were prehybridized at room temperature, hybridized at 65°C in aqueous hybridization solution (5), and washed at room temperature in 5× SSPE–0.1% SDS (two times for 15 min each) followed by 0.1× SSPE–0.1% SDS (once for 15 min). To allow for reuse, Southern blots were stripped in 1× SSPE–0.1% SDS at 90°C for 15 min, with four changes of solution. Low-stringency Southern blots were hybridized at 50°C and washed at room temperature in 5× SSPE–0.1% SDS (two times for 15 min each). RNA slot blots were prehybridized and hybridized at 42°C in 0.01 M PIPES (piperazine-*N*,N′-bis(2-ethanesulfonic acid)–0.8 M NaCl–0.01% *N*-lauroylsarcosine–0.01% Ficoll 400–0.01% polyvinylpyrrolidone–0.01% bovine serum albumin–200 μg of hydrolyzed salmon sperm DNA per ml–50% formamide. Membranes were washed in 0.1× SSC–0.1 mg of sodium PP_i–0.5 mg of Sarkosyl per ml four times for 30 min each at 50°C. To allow for reuse, the filters were stripped in slot blot washing buffer at 90°C for 30 min, with two changes of solution. As a control for RNA loading, slot blots were stripped and reprobed with a 7.5-kb *Bam*HI fragment encoding 16S and 23S rRNA (73).

Isolation of a clone containing sequences upstream of the *fegA* gene. Sequence analysis indicated that the 5.1-kb insert from the initial isolate from the 61A152 genomic library ended at an *Eco*RI site 110 bp upstream of the *fegA* gene. Hybridization analysis using a ^{32}P-labeled 1,561-bp *Not*I-*Eco*RI fragment from within the coding region of the *fegA* gene as a probe demonstrated that a *B. japonicum* 61A152 *Bam*HI-*Pst*I genomic fragment of approximately 5 kb contained sequences upstream of the *fegA* gene. Consequently, a *Bam*HI-*Pst*I minilibrary of genomic DNA was constructed. DNA was double digested with *Bam*HI and *Pst*I, precipitated, and run on an 0.8% agarose gel. DNA in the size range of 4 to 6 kb was cut from the gel, eluted with spin columns, precipitated, resuspended in ligase buffer, and ligated according to standard procedures (5) with similarly cut pBluescript SK^+ vector DNA. DNA was transformed into *E. coli* DH5α cells, white colonies were struck onto fresh plates, and filter lifts were performed. Clones were screened for the proper insert by hybridization with the ^{32}P-labeled 1,561-bp *Not*I-*Eco*RI fragment from the *fegA* gene. A clone with a 5-kb insert was identified and partially sequenced. DNA sequence analysis confirmed that the *fegA* gene, including upstream regions, was included within the clone.

DNA sequencing and computer analysis. Nucleotide sequencing was performed by automated sequencing of both DNA strands of clones with nested primers on an Applied Biosystems model 373A sequencer (Dartmouth College Molecular Biology Core Facility), using a Ready Reaction Terminator Cycle Sequencing Kit with AmpliTaq DNA polymerase FS (Perkin-Elmer). Sequencing reactions run alongside primer extension reactions were performed with a Sequenase kit (U.S. Biochemicals, Cleveland, Ohio). Sequence comparisons, database searches, and manipulations were performed with BLAST (3, 29) and Genetics Computer Group (GCG) software (27). Parameters used for the PILEUP program were as follows: gap weight, 3.0; gap length weight, 0.1. The FegA signal peptidase cleavage site was predicted with the PSORT program coded by K. Nakai (http://psort.nibb.ac.jp/) with the weight-matrix method (83). Membrane-spanning regions of FegA were predicted with the program TopPredII, developed by M.-G. Claros, based on the algorithm of von Heijne (85). The GCG program PILEUP was used to generate the dendrogram on the basis of sequence similarities of TonB-dependent receptors. Indicated figures were generated with the programs Adobe Photoshop 3.0 (Adobe Systems, Inc., Mountain View, Calif.) and Canvas 3.5.3a (Deneba Systems, Inc., Miami, Fla.).

Mapping of the *fegA* transcription initiation site by primer extension. Ten picomoles of the oligonucleotide primer 5′-GATGCCTGCGGTACCACTGCG AAA-3′ (corresponding to complementary nucleotides 293 to 270 in Fig. 4) were 5′ end labeled according to standard procedures (5), except that the amount of [γ-^{32}P]ATP was doubled. Completed labeling reaction mixtures were passed through Sephadex G-25 mini spin-columns for removal of unincorporated ATP. Primer extension reactions were performed based on a protocol modified from reference 38 and carried out as follows. Lyophilized total RNA (10 μg) (16) from *B. japonicum* 61A152 was resuspended in 4 μl of annealing mix (2 μl of 1 mM EDTA, 0.5 μl of 10× annealing buffer [0.5 M Tris-Cl {pH 8.3}, 0.6 M NaCl, 0.1 M dithiothreitol], 1.5 μl of end-labeled oligonucleotide). Annealing mixtures were heated to 60°C for 3 min, placed in a dry-ice–ethanol bath for 1 min, and allowed to cool on ice until thawed. One microliter of a 2 mM dATP–dCTP–dGTP–dTTP mix (in 1× reaction buffer [annealing buffer plus 60 mM Mg-acetate]) and 2 μl of 1:20 avian myeloblastosis virus reverse transcriptase diluted in 1× reaction buffer were added to annealing mixtures, and extension reactions were carried out at 47°C for 30 min. Completed reactions were desalted on Sephadex G-50 mini spin-columns. Loading buffer (4 μl) from a U.S. Biochemicals Sequenase kit was added to terminate the reactions, and reactions were run alongside sequencing reactions on an 8% acrylamide–Tris-borate-EDTA sequencing gel (5).

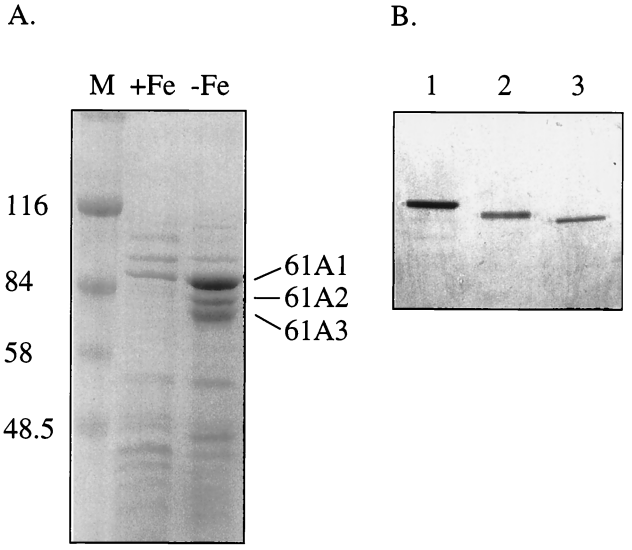

FIG. 1. Iron-regulated OMPs prepared from *B. japonicum* 61A152. (A) Coomassie blue-stained SDS-PAGE gel of OMPs prepared from cultures grown in the presence (+Fe) or in the absence (−Fe) of 10 μM $FeCl_3$. Seventy-five micrograms of protein was loaded in each lane. Three proteins which are highly induced under low-iron conditions are indicated and labeled 61A1 through 61A3. M, molecular weight standards; sizes (in kilodaltons) are indicated on the left. (B) Silver-stained SDS-PAGE gel of purified iron-regulated OMPs. Two hundred fifty nanograms of purified protein was loaded in each lane. Lane 1, 61A1; lane 2, 61A2; lane 3, 61A3. This figure was generated using the programs Adobe Photoshop and Canvas.

Phosphor image analysis. All radiography and densitometry were performed with a PhosphorImager model 425E apparatus (Molecular Dynamics, Sunnyvale, Calif.).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned the accession number U61401.

RESULTS AND DISCUSSION

Analysis of iron-regulated OMPs. In order to examine OMP profiles in relation to the iron status of *B. japonicum* cells, cultures of strain 61A152 were grown under iron-deficient and iron-sufficient growth conditions. OMPs were prepared from the cultures, and proteins were run on SDS-PAGE gels (Fig. 1A). Coomassie blue-stained gels showed that several new OMPs were present in membrane preparations from *B. japonicum* 61A152 cells grown under conditions of iron deficiency compared with cells grown under conditions of iron sufficiency. As numerous iron-regulated OMPs in the size range of 75 to 85 kDa serve as Fe(III)-siderophore receptors for other bacteria (56), the three highly expressed 61A152 iron-regulated OMPs in the 80-kDa size range (61A1 to 61A3 in Fig. 1A) were presumed to be potential siderophore receptors. Such receptors would serve to import siderophore-bound iron into iron-starved *B. japonicum* cells.

The three most highly expressed iron-regulated OMPs, 61A1, 61A2, and 61A3, were purified through a series of suc-

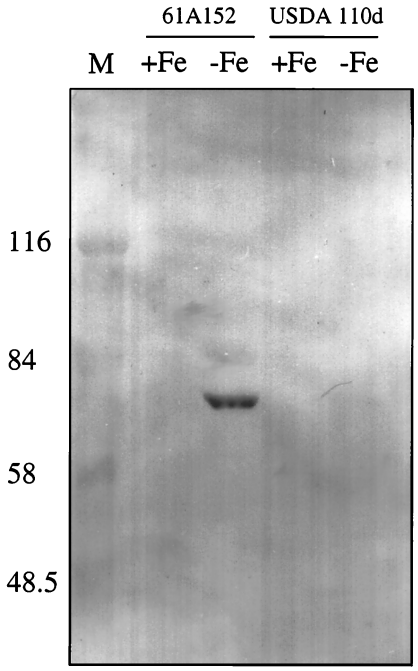

FIG. 2. Western blot of *B. japonicum* OMPs isolated from strains 61A152 and USDA 110d using polyclonal antibodies raised against the 61A2 protein. An SDS-PAGE gel with OMPs prepared from cultures of each strain grown in the presence (+Fe) or in the absence (−Fe) of 10 μM $FeCl_3$ was electroblotted onto nitrocellulose. Antibodies raised against the iron-regulated OMP 61A2 were used as a probe. Secondary antibodies conjugated to alkaline phosphatase allowed color development on the filter. M, prestained molecular weight standards; sizes (in kilodaltons) are indicated on the left. This figure was generated using the programs Adobe Photoshop and Canvas.

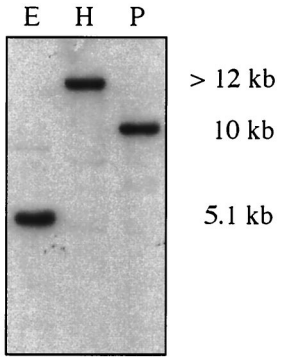

FIG. 3. Southern blot of *B. japonicum* 61A152 genomic DNA restriction digests hybridized with the radiolabeled PCR product generated from primers designed from 61A2 protein microsequence data. Total genomic DNA (10 μg) was cut with various restriction enzymes, separated by electrophoresis in an 0.8% agarose gel, and blotted onto nitrocellulose. The radiolabeled PCR-generated product of 307 bp was hybridized with the resulting Southern blot. Approximate DNA fragment sizes are indicated on the right. E, *Eco*RI; H, *Hin*dIII; P, *Pst*I. This figure was generated using the programs Adobe Photoshop and Canvas.

cessive gel separation and isolation steps (Fig. 1B). We chose to focus on the iron-regulated OMP protein 61A2, which was approximately 79 kDa in size. Polyclonal antibodies were generated against this protein and Western blotting (immunoblotting) was performed (Fig. 2). Included on the blot were OMPs prepared from *B. japonicum* USDA 110d, because most of the genetic and molecular research on *B. japonicum* is done with this strain, and it was of interest to see whether an OMP in this strain would be recognized by the antibody to 61A2. Western blot staining patterns demonstrated that the 61A2 protein was not expressed in *B. japonicum* USDA 110d and was only detected in membrane preparations from iron-deficient cultures of strain 61A152. This difference between the two strains is surprising, in light of the fact that transport assays with ^{55}Fe-labeled Fe(III)-siderophore compounds have demonstrated that the compounds which can be transported by 61A152 and USDA 110d are identical (63).

Cloning of the gene encoding 61A2. In order to facilitate the cloning of the gene encoding the iron-regulated OMP 61A2, a partial amino acid sequence was determined for the purified 61A2 protein. Initial microsequencing attempts to determine the N-terminal sequence of 61A2 protein by using Edman degradation indicated that the protein was blocked at the N terminus. 61A2 protein was then subjected to tryptic digestion followed by HPLC separation, and two of the ensuing peptide fragments were microsequenced. Oligonucleotide primers were designed based on the amino acid sequence obtained from microsequencing (primer 1765 reverse was 32-fold degenerate and primer 1924 forward was 2-fold degenerate). These primers amplified a 307-bp band when used in PCRs with *B. japonicum* 61A152 genomic DNA. Southern blot analysis of various restriction digests of *B. japonicum* 61A152 genomic DNA confirmed that the PCR fragment was indeed amplified from *B. japonicum* genomic DNA (Fig. 3). The 307-bp PCR product was used to screen a *B. japonicum* 61A152 genomic library, and a Lambda ZAP clone with a 5.1-kb *Eco*RI insert was identified. This clone (designated pOMPPCR) hybridized to the same bands as did the 307-bp PCR product when used as a probe against a Southern blot of various restriction digests of *B. japonicum* 61A152 genomic DNA (data not shown).

Nucleotide sequence and sequence analysis of the gene encoding 61A2. Southern blot analysis of various restriction digests of the pOMPPCR plasmid hybridized with the radiolabeled 307-bp PCR fragment indicated that the region of homology between the two was localized to an *Nco*I-*Eco*RI DNA fragment of approximately 2.4 kb. The nucleotide sequence was determined for this DNA fragment (Fig. 4), and only a single open reading frame (ORF) large enough to encode the 61A2 protein (corresponding to nucleotides 235 to 2488 in Fig. 4) was found. The methionine beginning at nucleotide position 235 is the initiator for the ORF and would lead to the production of a translation product in agreement with the size of the 61A2 protein; none of the other Met codons internal to the ORF would lead to a translation product of sufficient size. This ORF encodes a protein of 750 amino acids, and the deduced protein has a calculated molecular mass of 82,241 Da. The deduced amino acid sequence of the ORF includes sequences identical to those determined by microsequencing of the 61A2 protein (amino acids 302 to 319 and 393 to 407 in Fig. 4). The proteolytic enzyme trypsin, which cleaves specifically on the carboxyl side of arginine or lysine, was used to generate the peptide fragments of 61A2 for microsequencing. Accordingly, one of the 61A2 microsequenced peptides is immediately preceded by an arginine, and the other is preceded by a lysine. The translational initiation codon of 61A2 is preceded by a putative ribosome-binding site (222 5′-GAAC AG-3′ 227 [30]). The N-terminal amino acids of the deduced protein are characteristic of a gram-negative bacterial leader peptide (84), and a signal peptidase cleavage site was predicted to be after alanine 14 (indicated by an arrow in Fig. 4), using the program PSORT. Cleavage after Ala-14 would lead to a mature protein of 736 amino acids, with a calculated molecular

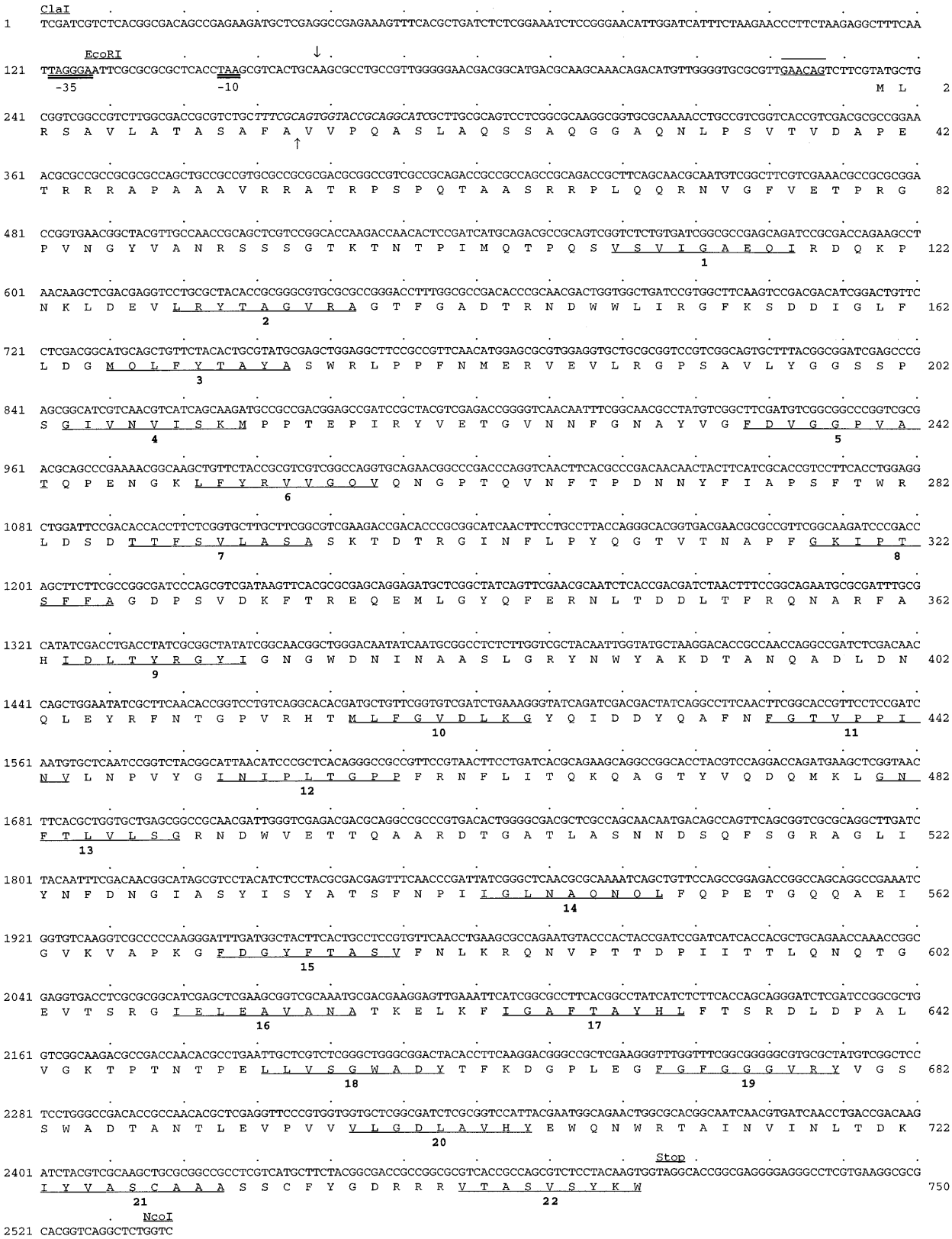

FIG. 4. Nucleotide and deduced amino acid sequences of a 2,538-bp *Cla*I-*Nco*I DNA fragment containing the *fegA* gene. Single-letter codes for the deduced amino acid sequence are indicated beneath each nucleotide codon. The transcriptional start site is marked with a downturned arrow, and the putative signal sequence cleavage site is marked with an upturned arrow. Amino acids 302 to 319 and 393 to 407 correspond to peptides determined by protein microsequencing. Potential membrane-spanning regions are underlined and numbered. A potential ribosome-binding site is overlined, and putative −10 and −35 promoter sequences are double underlined.

TABLE 2. Amino acid similarities and identities among FegA and related siderophore receptor proteins[a]

Protein	% Similarity or identity with:					
	FegA	FhuA[b]	FhuE[c]	FctA[d]	PupA[e]	FoxA[f]
FegA		35.5	23.9	33.6	24.6	33.2
FhuA	**53.7**		22.8	38.0	25.6	34.7
FhuE	**48.3**	**45.1**		25.6	37.1	23.8
FctA	**51.5**	**56.5**	**47.5**		24.5	36.4
PupA	**48.6**	**47.5**	**58.5**	**46.2**		25.6
FoxA	**55.2**	**56.0**	**46.6**	**54.1**	**48.7**	

[a] Sequences were compared using the GCG program GAP. Non-boldface values are percent identity; boldface values are percent similarity.
[b] FhuA is the *E. coli* Fe(III)-ferrichrome receptor (18).
[c] FhuE is the *E. coli* receptor for Fe(III)-coprogen and Fe(III)-rhodoturulic acid (71).
[d] FctA is the *Erwinia chrysanthemi* Fe(III)-chrysobactin receptor (unpublished; GenBank accession no. X87967).
[e] PupA is the *P. putida* Fe(III)-pseudobactin receptor (10).
[f] FoxA is the *Y. enterocolitica* Fe(III)-ferrioxamine receptor (7).

mass of 80,851 Da, which agrees well with the size of 61A2 predicted by SDS-PAGE.

The gene encoding the 61A2 protein has a G+C content of 62%, corresponding to the high G+C content of the *B. japonicum* genome (61 to 65% [42]). A study which compared the G+C contents of 45 *B. japonicum* genes showed that genes involved in nodulation and nitrogen fixation had G+C contents of approximately 58%, whereas genes not directly involved in nodulation and nitrogen fixation had G+C contents of approximately 65% (67). Codon usage in the 61A2 ORF is consistent with codon usage seen in *B. japonicum* genes previously cloned and examined (the codon table was constructed by using all 44 *B. japonicum* structural genes which were on 15 November present in GenBank 1995 [88]).

The deduced amino acid sequence encoded by the 61A2 ORF was compared with sequences in the GenBank, EMBL, PIR-Protein, and SWISS-PROT databases, by using the BLASTX program (3, 29), and showed significant similarity to numerous siderophore receptor proteins. The gene encoding the iron-regulated OMP 61A2 was therefore designated *fegA* [Fe(III)-siderophore-gathering OMP). Similarities between FegA and proteins encoding receptors for hydroxamate-type siderophores were the most significant and are presented in Table 2.

Predicted features of the FegA protein. A number of structural features predicted for the FegA protein are similar to those which have been determined for other bacterial OMPs. The 10 most C-terminal amino acids in the vast majority of OMPs analyzed have hydrophobic residues at positions 3 (with tyrosine preferred), 5, 7, and 9 from the C terminus and have an aromatic, hydrophobic amino acid at position 1 (77). This C-terminal region has been demonstrated to form a membrane-spanning β-sheet which is necessary and sufficient for incorporation of OMPs into the outer membrane (77). The 10 C-terminal amino acids of FegA (RVTASVSYKW) fit the consensus pattern for a membrane-anchoring β-sheet (Fig. 5). The FegA protein is predicted to have 22 membrane-spanning, amphipathic β-sheets (Fig. 4), similar in number to those predicted for other Fe(III)-siderophore receptor proteins (e.g., FepA has 29 [70], FoxA has 30 [7], and FpvA has 26 [64] regions predicted to form β-sheets). The numerous membrane-spanning amphipathic β-sheets found in Fe(III)-siderophore receptor proteins are predicted to form membrane-spanning β-barrel structures which are seen in outer membrane receptor proteins (58). In contrast to the hydrophobic β-helix-rich structures of integral membrane proteins of the cytoplasmic membrane, OMPs are thought to have numerous amphipathic β-sheets to facilitate their export across the cytoplasmic membrane (58). Once OMPs have moved through the periplasm, interactions with lipopolysaccharides which are unique to the

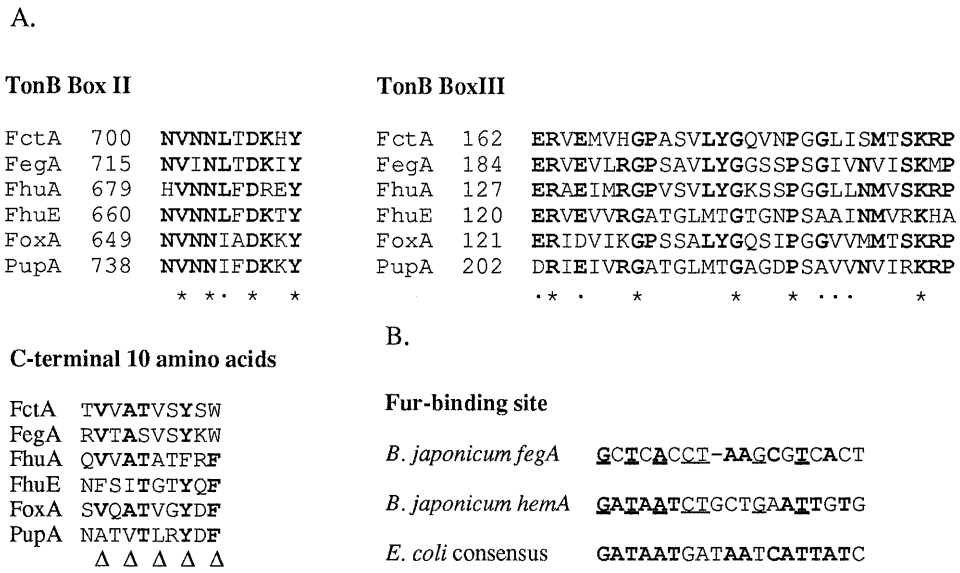

FIG. 5. (A) Amino acid sequence alignments of regions found in TonB-dependent siderophore receptors and the *B. japonicum* FegA protein. Proteins aligned are FctA from *Erwinia chrysanthemi* (unpublished; GenBank accession no. X87967), FhuA from *E. coli* (18), FhuE from *E. coli* (71), FoxA from *Y. enterocolitica* (7), and PupA from *Pseudomonas putida* (10). Sequences were aligned using the GCG programs PILEUP and PRETTY. Numbers to the left of the sequences indicate the positions of the first residue of the indicated region in the mature (actual or predicted) protein. Asterisks denote residues which are identical in all six proteins; dots denote conserved amino acids present in all six proteins; and triangles denote hydrophobic residues present in all six proteins. Boldface letters represent residues present in the same position in four of six proteins. (B) Alignments of the *E. coli* Fur-binding site consensus sequence (21) and putative Fur-binding sites from *B. japonicum* genes *fegA* and *hemA* (60). Nucleotides in boldface type are identical between either *B. japonicum* sequence and the *E. coli* consensus; nucleotides which are underlined are identical in *fegA* and *hemA*.

outer membrane are thought to lead to the formation of the stable β-barrel configuration (74).

Sequence comparisons among receptor proteins dependent on the periplasm-spanning TonB protein have identified three major regions of amino acid conservation, and these regions have been termed the TonB box (also known as Box I), Box II, and Box III (modified from reference 51). Amino acids within the TonB box (Box I) of outer membrane receptor proteins are hypothesized to contact the TonB protein directly (e.g., see references 40 and 72); a subsequent conformational change in TonB is thought to allow internalization of the ligand that is bound to the receptor. In the majority of TonB-dependent proteins, the TonB box (Box I) is found near the N terminus of the receptor (4), within the first membrane-spanning domain (e.g., see reference 7); the placement of TonB box (Box I) sequences within membrane-spanning regions of OMPs suggests that TonB interacts with the OMPs within the membrane channel region. FegA does not have a TonB box (Box I)-like sequence near the N terminus but does have two regions containing T-X-X-V-X-A, a motif conserved among the majority of TonB boxes analyzed (66). Both of these potential TonB box (Box I) sequences are found within putative membrane-spanning sequences of FegA (membrane-spanning regions 2 and 7, which are located 118 and 274 amino acids from the predicted N terminus of the mature protein, respectively [Fig. 4]). As more sequence data have become available, the idea of the invariant N-terminal position for the TonB box (Box I) has broken down; TonB boxes are found as far as 66 to 70 amino acids from the N terminus of mature proteins in the TonB-dependent Fe(III)-siderophore receptors PbuA (53), PupA (10), PupB (45), and FpvA (64). Therefore, it is likely that one of the T-X-X-V-X-A regions found within FegA serves as a TonB box (Box I) for the protein. The functions of the highly conserved Box II and Box III in TonB-dependent proteins are currently unknown. FegA shows particularly strong similarity to Box II and Box III (Box II, amino acids 715 to 724; Box III, amino acids 184 to 213 [Fig. 5]), which are found in FegA in positions corresponding to the positions of these boxes in other TonB-dependent proteins. The presence in FegA of conserved sequences found in all TonB-dependent proteins suggests that a TonB-like system is present in *B. japonicum.*

The amino acid sequence of FegA was aligned to the deduced amino acid sequences of all the TonB-dependent receptor proteins within the sequence databases, using the program PILEUP, and a dendrogram describing total inferred sequence similarities was generated (Fig. 6). The receptors for transferrin binding protein 1 (or A) and lactoferrin grouped together, as did receptors for transferrin binding protein 2 (or B). The three major subfamilies of siderophore receptors were apparent as groups in the dendrogram: receptors for hydroxamate-containing siderophores (FcuA, PbuA, PupB, FpvA, PupA, FhuE, FhuA, FctA, and FoxA), receptors for citrate or citrate-containing siderophores (FecA and RumA), and receptors for catecholate/phenolate-containing siderophores (FyuA, IrpC, BtuB, FepA, PfeA, BfeA, CirA, IrgA, IutA, and ViuA). As expected from DNA sequence analysis, FegA grouped most closely with receptors for hydroxamate-containing siderophores.

Originally, three subfamilies of TonB-dependent receptors had been defined based on sequence similarities, those for hydroxamate-, catecholate/phenolate-, and citrate-containing siderophores. As receptors for more siderophores have been isolated and studied, the distinctions among some of these classes have broken down, and new subfamilies have been designated. Siderophores which do not fit into the original structural classes often have hybrid structures which contain, for example, both catecholate and hydroxamate moieties (as seen in the case of anguibactin). Despite the fact that the majority of TonB-dependent OMPs with specificity for the same or similar ligands have sequence similarity, a few studies have demonstrated that this is not always the case. For example, the *P. aeruginosa* FptA receptor for the phenolate-containing siderophore pyochelin is similar to siderophore receptors for hydrophilic hydroxamates and has no significant similarity to receptors for hydrophobic phenolate/catecholate-containing siderophores (4). Similarly, FcuA, the *Yersinia enterocolitica* receptor for the hydroxamate ferrichrome, is most similar to the *Vibrio anguillarum* FatA receptor, despite the fact that the FatA ligand anguibactin has a very dissimilar structure (44). Thus, despite the fact that FegA consistently groups with receptors for hydroxamate-type siderophores based on sequence similarity, the natural ligand(s) for this receptor remains to be determined.

Isolation of a clone with DNA sequence upstream of the *fegA* gene. Because the *Eco*RI site at the 5′ end of the fragment encoding *fegA* was only 110 bp upstream of the putative Met initiation codon, a genomic clone that had more upstream sequence was isolated from the Lambda ZAP genomic library (p*fegA*SK). Approximately 1.5 kb of DNA sequence was determined from this clone, including 200 bp overlapping with the pOMPPCR clone. Because the genes for some outer membrane Fe(III)-siderophore receptors are found in operons encoding multiple components of the membrane-periplasm transport complex (e.g., *fhuA* [18] and *fecA* [65]), we examined DNA sequences flanking the *fegA* gene. Sequence data spanning the region from 1.3 kb upstream of the *fegA* start site of translation to 250 bp downstream of the stop codon demonstrated that *fegA* is unlikely to be in an operon.

Regulation of *fegA*. The transcriptional initiation site for *fegA* was mapped by primer extension analysis (Fig. 7) using a primer within the coding region of *fegA* and total RNA prepared from *B. japonicum* 61A152 cultures grown in the presence or in the absence of 10 μM $FeCl_3$. The start site of transcription is an adenine residue 77 bp upstream from the methionine start codon. A primer extension product was present only in reactions performed with total RNA prepared from cells which had been starved for iron, demonstrating that *fegA* is regulated at the level of steady-state mRNA accumulation. Slot blots of total RNA isolated from cultures grown either in the presence or in the absence of 10 μM $FeCl_3$ were probed with a radiolabeled 2.0-kb *Kpn*I-*Bgl*II DNA fragment from within the coding region of *fegA* and confirmed that *fegA* expression is regulated by iron at the level of mRNA accumulation, with iron affecting either initiation of transcription or mRNA stability (data not shown).

Bacterial RNA polymerases have specificity for particular gene promoters based on the σ subunit of the polymerase holoenzyme that serves as a recognition factor for transcription initiation (reviewed in reference 26). In *E. coli* and other gram-negative bacteria, σ^{70} is the major promoter recognition factor. An alternative σ factor, σ^{54}, which was originally described as playing a role in the control of genes involved in nitrogen metabolism, is used in rhizobia for recognition of promoters of nitrogen fixation genes (26). In published studies, the majority of *B. japonicum* genes which have had their promoters mapped and analyzed have the "−12/−24"-type promoter sequences recognized by σ^{54}. The region upstream of the *fegA* gene shows limited similarity to σ^{70} promoters from *E. coli* (49) and *B. japonicum* (87), and putative −10 and −35 regions are indicated in Fig. 4.

The ferric uptake regulation (Fur) protein is a global regulator which is involved in control of gene regulation in numer-

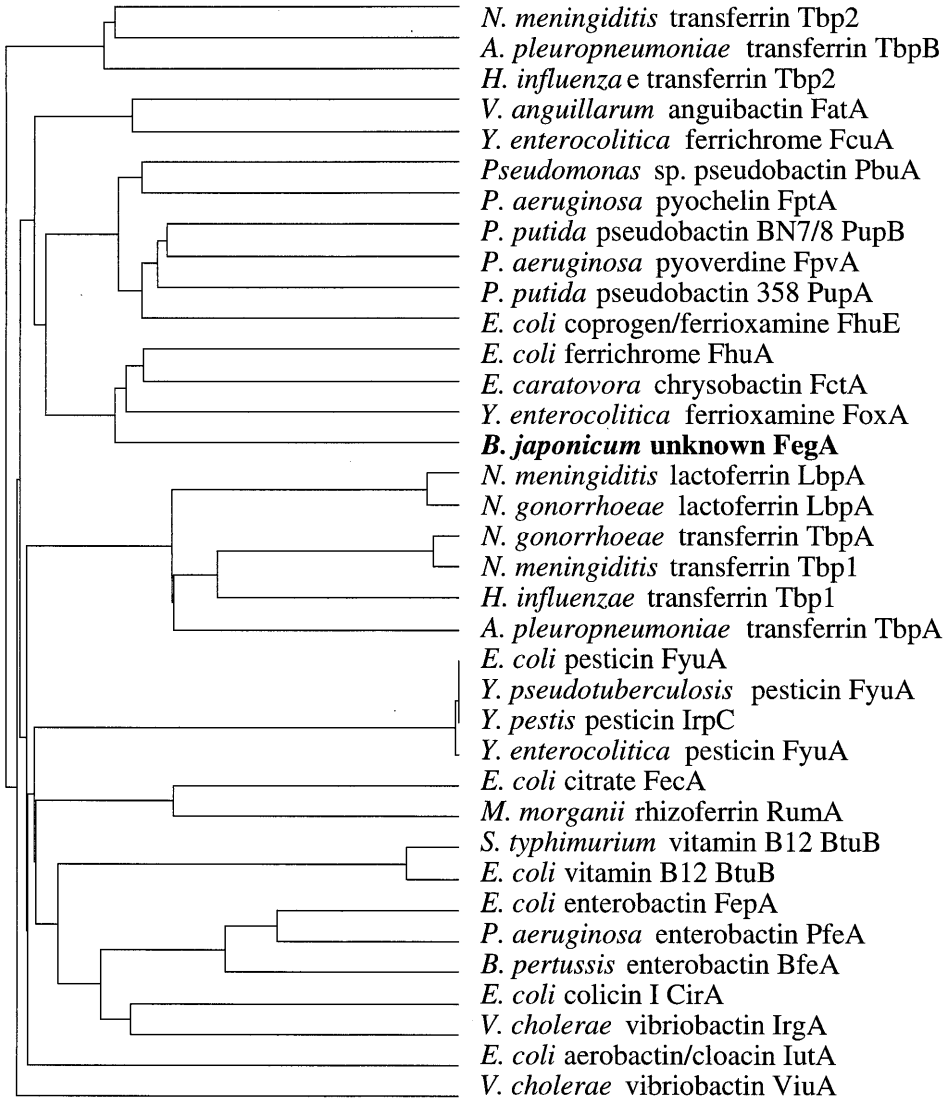

FIG. 6. Dendrogram showing total inferred sequence similarities among deduced amino acid sequences of TonB-dependent receptors of gram-negative bacteria. The tree was constructed using the GCG program PILEUP. The sequences compared were as follows: *Neisseria meningitidis* Tbp1 and Tbp2 (unpublished; GenBank accession no. Z15130) and LbpA (unpublished; GenBank accession no. X79838); *Actinobacillus pleuropneumoniae* TbpA (19) and TbpB (32); *Haemophilus influenzae* Tbp1 and Tbp2 (50); *V. anguillarum* FatA (a); *Y. enterocolitica* FcuA (44), FoxA (7), and FyuA (66); *Pseudomonas* sp. PbuA (53); *P. aeruginosa* FptA (4), FpvA (64), and PfeA (20); *P. putida* PupA (10) and PupB (45); *E. coli* FhuE (71), FhuA (18), FecA (65), FyuA (unpublished; GenBank accession no. Z38065), BtuB (39), FepA (51), CirA (55), and IutA (46); *E. chrysanthemi* FctA (unpublished; GenBank accession no. X87967); *Neisseria gonorrhoeae* LbpA (9) and TbpA (17); *Yersinia pseudotuberculosis* FyuA (unpublished; GenBank accession no. Z35107); *Yersinia pestis* FyuA (25); *Morganella morganii* RumA (47); *Salmonella typhimurium* BtuB (86); *Bordetella pertussis* BfeA (unpublished; GenBank accession no. U13950); *Vibrio cholerae* IrgA (31) and ViuA (12).

ous bacterial species and represses the transcription of a variety of genes involved in iron uptake (89). When iron is plentiful in bacterial cells, Fe(II) binds to a Fur homodimer, and the complex binds to a highly conserved inverted repeat motif in the promoter regions of iron-regulated genes (the Fur box), thereby blocking their transcription. Because *fegA* showed iron regulation, we looked for a Fur box upstream of the gene. Overlapping the potential −10 region of the *fegA* promoter is a putative Fur box with an imperfect inverted repeat which is 48% identical (when a gap of 1 bp is introduced) to the *E. coli* Fur-binding consensus sequence (Fig. 5) (21). The location of a Fur box overlapping the −10 promoter region has been seen in numerous genes for Fe(III)-siderophore receptors (e.g., *irpC* [25], *fecA* [65], *fhuE* [71], *fptA* [4], *fyuA* [66], *pfeA* [20], *rumA* [47], and *viuA* [12]). The presence of a putative Fur box, taken together with data demonstrating the regulation of *fegA* mRNA accumulation by iron, suggests that there is a system of iron regulation in *B. japonicum* cells similar to the Fur system seen in other bacteria. However, the promoter region of the *B. japonicum hemA* gene also has a putative iron box which is 52.6% identical to the *E. coli* iron box consensus sequence (Fig. 5) (60). Deletion analysis of the promoter of *hemA* demonstrated that the region containing the Fur box-like sequence is not necessary for iron regulation of *hemA* (59).

Distribution of *fegA*-like sequences among gram-negative bacteria. It was of interest to determine whether sequences similar to the putative *B. japonicum* Fe(III)-siderophore receptor-encoding gene *fegA* could be found in other genera of gram-negative bacteria. *Eco*RI restriction digests of genomic DNA isolated from *B. japonicum* (strain USDA 110d), a cow-

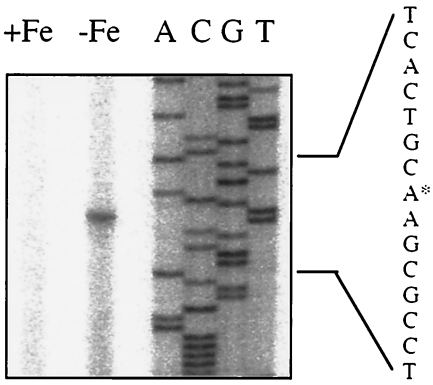

FIG. 7. Mapping of the transcription initiation site of *fegA* by primer extension. A radiolabeled oligonucleotide primer complementary to bases 293 to 270 (5′ to 3′) within the coding region of *fegA* (Fig. 4) was used with 10 μg of total RNA isolated from *B. japonicum* cells grown either in the presence (+Fe) or in the absence (−Fe) of 10 μM $FeCl_3$. Primer extension reaction products were electrophoresed alongside the products of dideoxy chain termination sequencing reactions performed with the same primer (lanes A, C, G, and T); the DNA sequence was determined for the antisense strand. The coding (sense) strand sequence is indicated on the right, and the initiation site is marked with an asterisk. This figure was generated using the programs Adobe Photoshop and Canvas.

pea *Rhizobium* sp., *Rhizobium leguminosarum*, *Rhizobium trifolii*, *P. aeruginosa*, *Pseudomonas stutzeri*, and *E. coli* (DH5α) were subjected to Southern blot analysis under conditions of low stringency. The blot was probed with a radiolabeled 2.1-kb *Kpn*I-*Bgl*II fragment from within the coding region of *fegA*. Bands of approximately 6.5 and 5.0 kb were detected in DNA isolated from *B. japonicum* USDA 110d and in the cowpea symbiont, respectively (data not shown). No hybridization signal was observed with genomic DNA from any of the other bacteria analyzed. *B. japonicum* USDA 110d and the cowpea *Rhizobium* sp. are more closely related to *B. japonicum* 61A152 than the other organisms analyzed.

Summary. Analysis of the *B. japonicum fegA* gene suggests that it belongs among the ranks of a growing number of cloned genes encoding TonB-dependent Fe(III)-siderophore receptor proteins. The iron-regulated promoter of the *fegA* gene should serve as a valuable tool, and *cis* analysis of the region will aid in the study of iron regulation of gene expression in *B. japonicum*. The presence of a putative Fur box within the promoter suggests that *fegA* might be useful in the isolation of a *B. japonicum fur* gene. Additionally, because transport assays have demonstrated that *B. japonicum* 61A152 has the ability to use several structurally different Fe(III)-siderophore compounds as iron sources (63), multiple Fe(III)-siderophore receptors would be expected to be present. On the basis of molecular mass and regulation by iron, the iron-regulated OMPs 61A1 and 61A3 would be good candidates for additional siderophore receptors. Future investigations similar to the studies with FegA will address the role of these proteins in the iron metabolism of *B. japonicum.*

The presence of over a hundred known structures for siderophores and a large variety of Fe(III)-siderophore uptake systems reflects the importance of iron to bacteria. Studies on the iron acquisition systems of rhizobia should ultimately lead to the elucidation of the mechanisms by which these bacteria secure the iron necessary for carrying out the nitrogen fixation process.

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