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中文摘要

爲鑑定 *Salmonella choleraesuis* 之攝鐵調節者所調節的基因 (Fur regulon)，本實驗應用攝鐵調節者之滴定法 (Fur titration assay; FURTA)，從 10,000 個克隆所組成的 *S. choleraesuis* 質體基因庫選殖由攝鐵調節者所調節的啓動子區域。其中一個 FURTA 陽性克隆 pSC4，與 *S. typhi* 負責攝鐵功能 *iroA* 基因座 (locus) 的 *iroB* 基因有同源性。選殖與定序 *S. choleraesuis* 含 *iroBCDE* cluster 和 *iroN* 基因之 *iroA* 鐵調節基因座，全長爲 9848 bp 與 *S. typhi* 之 *iroA* 基因座的核苷酸序列同源性達 97%。*S. choleraesuis* 之 *iroA* 鐵調節基因座分別由兩個操縱子組成，各有一個 Fur box。IroN 主司攝取 catechol 類螯鐵物質，與 TonB 依賴性外膜螯鐵物質接受體及非腸源性病原性大腸桿菌的推測毒力基因，$iroN_{E.\ coli}$，具同源性。以 T7 RNA 聚合酶表現 rIroN 蛋白，當培養於 20℃ 時，rIroN 表現是屬於部份可溶性蛋白。應用兔抗 rIroN 血清定位 IroN 位於外膜蛋白。田間感染 *S. choleraesuis* 之恢復豬血清含有抗體可認識 *S. choleraesuis* 三個主要鐵調節外膜蛋白分別是 FepA、IroN 和 FhuE。應用對偶基因交換技術構築 *S. choleraesuis* 之 *iroN* 基因插入突變株 (*iroN::Kan*)，突變株培養在限鐵環境裡喪失表現一 80 kDa 蛋白。進行互補試驗分析，構築含 *iroN* 基因及其上游 Fur box 之互補戴體送入突變株內，可恢復突變株喪失的功能。以腹腔內注射評估 wild type 與突變株對 BALB/c 小鼠的半致死劑量 (LD_{50})，結果無任何差異，顯示 *S. choleraesuis* 之 *iroN* 基因並非是毒力因子。

關鍵詞：豬瘟沙氏桿菌、FURTA、攝鐵基因、對偶基因交換技術, iroN 突變株

Cloning and Characterization of an Iron-Regulated Locus, *iroA*, in *Salmonella enterica* serovar Choleraesuis

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ABSTRACT

To identify genes belonging to the Fur regulon of *Salmonella enterica* serovar Choleraesuis, the Fur titration assay (FURTA) was used to screen a genomic library for Fur promoters and iron-regulated genes. Fifteen FURTA-positive clones were identified and DNA-sequence analysis showed that eleven had a Fur-binding site (Fur box), and six showed homology to the iron-regulated genes of *S. enterica* serovar Typhi and/or *E. coli*. One of these clones (pSC4) showed homology to the *iroB* gene of the *iroA* locus of *S. enterica* serovar Typhi. The *iroA* locus of *S. enterica* serovar Choleraesuis was cloned from a λ-dash library and subjected to DNA sequencing. The complete nucleotide sequence of 9,848 bp of the *iroA* locus of *S. enterica* serovar Choleraesuis, which consists of *iroB*, *C*, *D*, *E*, and *N* genes and was transcriptionally regulated by Fur, was 97% identical to that of *S. enterica* serovar Typhi. The IroN showed homology to the family of TonB-dependent outer membrane receptors and a putative virulence factor, $IroN_{E. coli}$, of the extraintestinal pathogen *E. coli*. The convalescent porcine sera contained antibodies against the three major iron-regulated outer membrane proteins of *S. enterica* serovar Choleraesuis. An insertional inactivation of the *iroN* gene of *S. enterica* serovar Choleraesuis by allelic exchange resulted in the loss of expression of the 78-Da protein. However, this mutant had an LD_{50} for mice similar to that of the parent strain when administered by the intraperitoneal route.

INTRODUCTION

Pathogenic bacteria are capable of sequestering iron within the host, from the environment, and can compete effectively with the host's iron-binding compounds, transferrin and lactoferrin, by one or more energy-dependent, iron-regulated uptake systems that secrete low molecular weight, high-affinity iron chelators termed siderophores (36, 40, 41). Transport of these siderophores occurs through iron-regulated outer membrane receptors that are specific for each ferrisiderophore complex (27). Production of those iron-uptake systems is regulated by Fur (ferric uptake regulator) and is dependent on the iron concentration. The Fur regulon — expression of the genes of which is under the control of Fur — contains genes that are involved in iron acquisition, which is essential for bacterial multiplication (15, 24, 41).

Iron seems to be particularly important in the pathogenesis of *S. enterica* serovar Choleraesuis. Kramer *et al.* directly correlated virulence with iron availability (21); virulent *S. enterica* serovar Choleraesuis more effectively captured iron from serum and had more total iron-binding capacity than an avirulent strain (21). Furman et al. found *S. enterica* serovar Typhi to be defective in enterochelin synthesis or iron-transport mutants had decreased mortality and showed marked increases in the 50% lethal dose (LD_{50}) in mice (18).

In this paper, we report that eleven FURTA-positive clones, which are homologous to that of *S. enterica* serovar Typhi and *E. coli*, were identified with the Fur titration assay and the insert DNA of one of these clones (pSC4) was used as a probe to screen a λ-dash library. The *iroA* locus from *S. enterica* serovar Choleraesuis strain 1459 was cloned, sequenced, and the gene encoding IronN, the catechol siderophore receptor, was characterized.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All bacterial strains, and plasmids used in this study are described in Table 1. Bacterial strains were maintained at –70°C until used. All

bacteria were cultured aerobically at 37°Cin Luria-Bertani (LB) broth or on LB agar plates as required. To create iron-limiting or iron-sufficient conditions, 0.2 mM 2,2'-dipyridyl or 0.1 mM $FeCl_3$ was added, respectively. The appropriate antibiotics were added to the medium in the following concentrations: ampicillin (Ap), 50 μg/mL; chloramphenicol (Cm), 35 μg/mL, kanamycin (Km), 50 μg/mL .

Recombinant DNA techniques. Chromosomal and plasmid DNA extractions, restriction endonuclease digestions, DNA ligations, plasmid transformations, and dot-blot hybridizations were performed according to standard methods (31).

Library construction and Fur titration assay (FURTA). A genomic library was constructed by using *Sau*3A partially digested *S. enterica* serovar Choleraesuis 1459 chromosomal DNA (0.5–3 or 9–20 kb) ligated to a *Bam*HI digestion, bacterial alkaline phosphatase treated pSUKS1 or λ-dash and transferred into *E. coli* DH5α or *in vitro* package as previously described (10-12). For the plasmid gene bank, a total of 10,000 independent transformants were pooled into five groups and the plasmid DNA was isolated from each group. FURTA was performed as previously described (34, 35). The *lacZ* expression was performed with MacConkey lactose plates (Difco, Detroit, MI) supplemented with 0.04 mM $FeSO_4$. The red transformants were selected and the plasmid DNA was isolated and subjected to DNA sequencing.

Cloning and expression of the recombinant IroN (rIroN) in *E. coli*. To construct an *iroN* expression clone (35), the DNA fragment containing the *iroN* coding sequence was amplified with primers iroN1 (5' AAGCTTATAACCCATCAAAACGAG 3', underline: *Hin*dIII cutting site) and iroN2 (5' GGGATCCGGAATGGGTATGA 3', underline: *Bam*HI cutting site), which generated a *Bam*HI and a *Hin*dIII cutting site and the *Bam*HI-*Hin*dIII fragment was ligated into

pRSETA with the same restriction enzyme digestion to create a pRSETAiroN and transferred into *E. coli* BE21(DE3)pLysS strain.

E. coli BL21(DE3)pLysS strain harboring pRSETAiroN was grown in LB broth to OD_{600} of 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM. Two hours after IPTG induction, the cells were harvested by centrifugation and suspended in 10 mL of Buffer A (50 mM Tris, 10 mM EDTA, 50 mM NaCl, 0.1 mM phenylmethyl sulfonylfluoride) and washed twice with Buffer A. Cells were ruptured by passage through a French pressure cell (American Instrument Co., Rochester, NY) and washed three times with Buffer A contained 1% Triton X-100 (23). The insoluble cell-fraction sediment was obtained by centrifugation and boiled in SDS-PAGE sample buffer at 100°C for 5 min. The proteins were resolved by SDS-12.5% PAGE and were visualized with Coomassie blue staining.

Preparation anti rIroN serum in rabbit. The recombinant proteins (rIroN) were visualized after staining with Coomassie Blue and the rIroN band was excised, homogenized and injected into a New Zealand female rabbit. Polyclonal antiserum to rIroN was raised as previously described (11, 12).

Preparation and analysis of outer membrane proteins. Outer membrane proteins of *S. enterica* serovar Choleraesuis strains 807 from iron-sufficient and iron-limiting media, were isolated as previously described (32). The outer membrane proteins were subjected to SDS-PAGE and Western blot analysis.

SDS-PAGE and Western blotting. The procedures for the SDS-PAGE and Western blot analysis were as previously described (7-9). For identification of rIroN expression as a fusion protein, MAb against the Xpress™ epitope (Invitrogen, Carlsbad, CA) was used as a primary

antibody. The secondary antibody, goat anti-mouse IgG-alkaline phosphatase conjugated (KPL, Gaithersburg, MD), was used at a dilution of 1:5,000. For detection of the expression of IroN in *S. enterica* serovar Choleraesuis, the rabbit anti rIroN antiserum was served as the primary antibody (1:500). The goat anti rabbit IgG-alkaline phosphatase conjugate (KPL) was used as a secondary antibody (1:5,000). To determine whether the convalescent porcine sera contain antibodies against iron-regulated outer membrane proteins, outer membrane proteins of *S. enterica* serovar Choleraesuis either from iron-sufficient or iron-limiting conditions were isolated and each used as an antigen and subjected to SDS-PAGE and Western blot analysis. Convalescent sera (1:500) from naturally infected pigs were used as a primary antibody, followed by goat anti-swine IgG-alkaline phosphate conjugated (1:5,000) (KPL) as a second antibody.

Construction of a *S. enterica* serovar Choleraesuis *iroN* mutant by allelic exchange. *S. enterica* serovar Choleraesuis *iroN* insertion mutant was constructed by allelic exchange using a suicide vector, pGP704 (24). A 1,253-bp *Hinc*II fragment containing a kanamycin cassette was isolated from pUC4K (Amersham Pharmacia, Piscataway, NJ), and ligated into the *iroN* gene at the *Sma*I site to form pBSNKan. The disrupted *iroN* determinant was isolated and cloned into the suicide vector, pGP704 to form pGPNKan. pGPNKan was transferred into *S. enterica* serovar Choleraesuis strain 807 by electroporation {18.0 kV/cm with a time constant of approximately 5 ms in a Gene pluser (Bio-Rad, Hercules, CA)}. The double crossover transformant was identified by its kanamycin-resistance and ampicillin-sensitivity and then, by dot-blot hybridization and PCR to confirm the construction.

DNA sequencing and analysis. Automated DNA sequencing was done on the Applied Biosystems Model 373A DNA system by using the T3 and T7 universal primers and primers complementary to regions already sequenced in the plasmid. The thermal cycling of the

sequencing reactions utilized the Taq DyeDeoxy™ Terminator Cycle sequencing kit. Both strands of the cloned DNA were completely sequenced. The nucleotide sequence was analyzed using the BLAST programs of the National Center for Biotechnology Information

Virulence testing in mice. The LD_{50} assays were performed by intraperitoneal inoculation of 8-week-oldBALB/c mice with serial ten-fold dilutions of the bacterial suspensions of the wild-type 807, and mutant 80521 grown in LB medium and suspended in sterile phosphate-buffered saline (PBS, pH7.4). Five mice were used per dose of bacteria. The LD_{50} was estimated by the method of Reed and Muench (29).

Nucleotide sequence accession number. The nucleotide sequences of *iroA* locus (*iroBCDE* and *N*) of *S. enterica* serovar Choleraesuis 1459 have been deposited in the GenBank under the accession number AY029471.

RESULTS

Screen for Fur-regulated promoters and sequence analyses of DNA regions identified by FURTA. Fur-regulated promoters and iron-binding proteins carried on a plasmid were transferred into *E. coli* H1717 (34), which carries *fhuF*::*lacZ*, a Fur-regulated gene fusion sensitive to changes in repressor ($Fur\text{-}Fe^{+2}$) concentration. Transformant-carrying plasmids that contain a Fur-binding site or encoding an iron-binding protein form a red colony on MacConkey lactose with $FeSO_4$ plates. Initially, a total of fifteen FURTA-positive colonies were identified. After sequence analyses, only eleven clones carrying a Fur box were identified. Plasmid pSC4 contained the 5' end of an ORF with homology to *S. typhi iroB* gene encoding a glucosyl-transferase (2). A putative Fur box matching the consensus *E. coli* Fur box (15) in seventeen of nineteen bases was present in an upstream region of the *iroB* homology of pSC4 (Table 2 and Fig. 1). One of the clones, pSC5 contained an ORF that was homologous to *E. coli*

cir, the outer membrane receptor for colicin I. A potential Fur-binding site matching the consensus sequence in fourteen of nineteen bases was located upstream of this sequence (Table 2 and Fig. 1). The insert of two FURTA-positive clones, pSC9 and pSC10, contained the promoter region and the 5' end of an ORF homologous to that of *fepBDGC*, a gene cluster encoding the binding-protein-dependent transport system of *E. coli* known to transport catechol siderophores (Table 2 and Fig. 1). Clones, pSC2 and pSC14, contained an ORF homologous to that of *fes* and *fepA*, encoding enterochelin esterase and the ferric enterochelin receptor of *E. coli*, respectively, and putative Fur boxes were identified upstream of these sequences (Table 2 and Fig. 1). The DNA sequence of the clone, pSC8, showed homology to protein 15 and to the terminase of bacteriophage P22. A putative Fur box matching the consensus sequence in thirteen of nineteen bases of *E. coli* was located in a region upstream of this sequence (Table 2 and Fig. 1). The DNA sequence of clone pSC3 showed homology to oxd-6c???, a transmembrane protein with a Fur box (Table 2 and Fig. 1). The DNA sequence of clone pSC12, showed homologous to ord-6c with a putative Fur box matching the consensus sequence in twelve of nineteen bases (Table 2 and Fig. 1). One of these clones, pSC15, showed homology to hypothetical glutaredoxin-like protein of *S. enterica* serovar Typhimurium, and the putative Fur box matched the consensus sequence in twelve of nineteen bases (Table 2 and Fig. 1). One of these clones, pSC16, which was homologous to cytochrome O ubiquinol oxidase subunit II, and had a putative Fur box matching the consensus sequence in fourteen of nineteen bases (Table 2 and Fig. 1).

Screen of the λ-dash library. The λ-dash library was screened by probing with the insert DNA from pSC4 as previously described (12). Four positive clones were identified and sequenced. Clone pLT1, which contained the whole *iroA* locus, was subject to further characterization (Fig. 2).

Nucleotide sequence analysis and predicted protein. The complete nucleotide sequence of the *iroA* locus of *S. enterica* serovar Choleraesuis strain 1459, included *iroBCDE* and *N* genes, has 9,848 bp and showed a 97% identity to that of *S. enterica* serovar Typhi. These five ORFs were designated as *iroBCDE* and *N*, but *iroN* appeared in the opposite orientation to other ORFs (Fig. 2). A putative Rho-independent transcription terminator (stem bp 7294 to 7303; loop bp 7304 to 7306; stem bp 7307 to 7316) was located on the intergenic region of the *iroE* and *iroN*. The *iroBCDE* cluster and *iroN* gene form an operon. A putative Fur box was identified at the individual upstream regions of *iroB* and *iroN* that match the *E. coli* consensus sequence.

Overexpression of recombinant IroN protein (rIroN) in *E. coli*. A 2,181-bp *iroN* coding sequence was amplified and cloned into pRSETA as previously described. This construct (pRSETAiroN) was transferred into *E. coli* and expressed as an 82-kDa rIroN that contains a 6×His Tag and a Xpress™-epitope on its N-terminal, which was specifically recognized by MAb anti Xpress™ antibody (data not shown). The partial purification of rIroN from the *E. coli* lysate was done by washing with Triton X-100 since it formed insoluble inclusion bodies. The molecular mass of the partially purified recombinant protein was found by SDS-PAGE. Rabbit anti-rIroN serum was used to localize the 78-kDa IronN of *S. enterica* serovar Choleraesuis (Fig. 3).

***In vivo* expression of iron-regulated outer membrane proteins in *S. enterica* serovar Choleraesuis.** To determine whether convalescent porcine serum contained iron-regulated outer membrane protein antibodies, three naturally infected and one normal porcine sera were used for Western blot analysis of the outer membrane proteins of *S. enterica* serovar Choleraesuis, which was cultured under the iron-limiting condition, with apparent molecular masses of 83, 78, and 69 kDa, representing FepA (4), IroN, and FhuE (17), respectively (Fig.

4).

Construction of an *iroN* mutant of *S. enterica* serovar Choleraesuis by allelic exchange (strain 80521). To introduce an insertional inactivation of *iroN* gene into the chromosome of *S. enterica* serovar Choleraesuis by allelic exchange, we constructed plasmid pGPNKan, a suicide vector containing a 1,253-bp kanamycin cassette within *iroN* . Plasmid pGPNKan was electroporated into *S. enterica* serovar Choleraesuis 807, with selection on medium containing kanamycin and ampicillin for the merodiploid state, which pGPNKan had integrated into the chromosomal *iroN* by a first crossover event. An *iroN* mutant, strain 80521, was identified by its phenotype of kanamycin-resistance and ampicillin-sensitivity after duplicate replication of the merodiploid strain without any remaining integrated plasmid. The double-crossover transformant was further proved by dot-blot hybridization and PCR. For dot-blot hybridization, the genomic DNA was prepared from both parental and mutant strains and hybridized to a probe derived from plasmid pGP704, to confirm loss of the suicide vector from the mutated allele. On dot-blot hybridization, the parental and mutant strain did not show any hybridization signal (data not shown). PCR amplification of the *iroN* mutant strain using primers iroNFurF-*Bam*HI (5' GGGATCCAGAGAGTCATATTGCAA 3', underline: *Bam*HI cutting site) and iroNFurR-*Hin*dIII (5' GAAGCTTATAACCCATCAAAACGAG 3', underline: *Hin*dIII cutting site), resulted in a 3,603-bp[h] product larger than the 2350 bp of the expected *iroN* PCR product from the wild type (data no shown).

The outer membrane proteins of the parental strain 807 and the *iroN* mutant strain 80521 under the iron-sufficient and iron-limiting conditions were compared. The parental strain, grown under iron-limiting media had apparent molecular masses of 83, 78, and 69 kDa of the three major iron-regulated outer membrane proteins. Mutant strain 80521 showed loss of the 78-kDa protein. The complete *iroN* gene, including the upstream region of Fur box, was subcloned into pACYC184 to yield pACYCiroN. Mutant strains containing pACYCiroN were

able to restore the expression of IroN that showed an apparent 78-kDa outer membrane protein under iron-limiting conditions (Fig. 5).

Virulence assay. To determine whether *iroN* has a role in the virulence of *S. enterica* serovar Choleraesuis in mice, the LD_{50} assays of the wild-type 807 and mutant 80521 were performed via intraperitoneal inoculation. The LD_{50} of both the wild type and mutant were 1.5×10^3. The result showed that *iroN* is not a virulence factor of *S. enterica* serovar Choleraesuis in mice during infection.

DISCUSSION

FURTA was developed to identify Fur-regulated genes or iron-binding proteins of Gram-negative bacteria (34, 38). Since then, this technique has been used to identify iron-regulated genes of *S. enterica* serovar Typhimurium (38) and Typhi (2). By FURTA, we identified fifteen positive clones of *S. enterica* serovar Choleraesuis from 10,000 clones in a *Salmonella* plasmid library. DNA sequencing indicated that eleven of the fifteen had a putative Fur box. Among these eleven clones, six are homologous to the iron uptake and/or iron transport genes in *S. enterica* serovar Typhi (2) and/or *E. coli* (34). The Fur regulon has been studied in *E. coli* with at least thirty-six Fur-regulated genes (34). In *S. enterica* serovar Typhimurium, the expression of at least thirty proteins is negatively regulated at the transcription level by Fur (1). Also, many of the Fur-regulated proteins were present both in *E. coli* and in *Salmonella* spp. Although we have identified only eleven genes from this study, it is expected that more Fur-regulated genes exist in *S. enterica* serovar Choleraesuis. Our results also suggest that *S. enterica* serovar Choleraesuis possesses outer membrane siderophore-receptor proteins like IroN, FepA, and Cir, which may increase the capability of obtaining iron via siderophore piracy.

A new iron-regulated *iroA* locus with *iroBCDE* and *N* genes has been identified in *S.*

enterica serovar Typhi (1, 2). In this study, we have also identified this locus with the same gene order. The DNA sequences of these genes also indicated that each showed 97% homology with *S. enterica* serovar Typhi at the nucleic acid level. IroB is a bacterial glycosyl-transferase (2), IroC is a member of the ATP-binding cassette family of transport proteins (2), IroD is an enterochelin esterase (26), IroE is similar to the amino acid sequences of an ORF located downstream of *pfeA*, the enterochelin receptor gene of *Pseudomonas aeruginosa* (16).

It is interesting that *S. enterica* serovar Choleraesuis causes disease mainly in pigs. The *iroA* locus is also almost identical to other members of *S. enterica*. IroN serves as a receptor for catecholate siderophores and the acquisition of *iroN* by *S. enterica* provided them a new protein for uptake of iron (1). It has been reported that substrates excreted by soil bacteria, such as the siderophores 2,N,6-N-bis(2,3-dihydroxybenzoyl)-L-lysine and 2,N,6-N-bis(2,3-dihydroxybenzoyl)- L-lysine amide produced by *Azotobacter vinelandii* (13) and myxochelin A (22) from *Angiococcus disciformans* were utilized by *S. enterica* through IroN receptor (1). Thus, IroN may facilitate the growth of *S. enterica* in soil that might increase the fecal oral transmission of this pathogen.

Location of IroN on the outer membrane exposes it to the host's immune system during infection. Sera from naturally recovered pigs did recognize iron-regulated outer membrane proteins. In several bacterial infections such as, *P. aeruginosa* (6), *Vibrio cholerae* (33), *Neisseria meningitidis* (5), *E. coli* (19), and *S. enterica* serovar Typhi (17), antibodies produced against iron-regulated outer membrane proteins to combat iron uptake have been shown to be present in sera from convalescent patients. Thus, acquisition of iron-uptake outer membrane protein genes in *S. enterica* introduces antigens that present as a target for the host immune system.

In this study, we have also identified other iron-uptake proteins, such as *fepA*, and *cir*. These proteins, including IroN, are able to transport various siderophores, such as enterochelin,

DBS, benzaldehyde-2,3-dihydroxybenzhydrazone, 2,N,6-N-bis (2,3-dihydroxybenzoyl)-L-lysine, 2,N,6-N-bis(2,3-dihydroxybenzoyl)-L-lysine amide and myxochelin A (1, 28). These proteins have overlapping substrate specificities the may enable them to take up enough iron under iron-limiting environments (1).

In *E. coli*, a gene homologous to IroN of *S. enterica* was also identified (30). The *E. coli*, $iroN_{E.coli}$, gene is linked to the P-pilus and FIC fimbrial gene clusters on a pathogenicity island and seems to have been acquired by *IS*1230-mediated horizontal transmission (30). It is considered to be a putative virulence gene of *E. coli* (20, 30). The contribution of siderophore-dependent iron-uptake systems to the virulence of enteric bacteria appears to vary according to the pathogen. Siderophores are reported not to be essential for shigellae to survive within the intestinal lumen or for invasion of the intestinal epithelium and intracellular survival and multiplication (25). The siderophore aerobactin appears to be a more important virulence factor for enteric bacteria than the siderophore enterobactin (14).

The siderophore enterobactin of *S. enterica* serovar Typhimurium is not required for the virulence of the pathogen (3). However, the enterochelin-mediated iron transported of pathogenicity in *S. enterica* serovar Typhi is consistent with decreased mortality and LD_{50} of mice infected with enterochelin-deficient or iron-transport deficient strains that have lost the 83- and 68-kDa iron-regulated outer membrane proteins (18). To address whether the *iroN* is a virulence factor of *S. enterica* serovar Choleraesuis in mice, an *iroN* insertional inactivation mutant was constructed by allelic exchange. However, the LD_{50} values showed no significant differences between the *iroN* mutant and its parental strain. Since several iron-uptake systems exist in *Salmonella* spp., the mutation of one system may be compensated by another system. It has been reported that *Salmonella* pathogenicity island 2 (SPI-2) mutant is avirulent in mice whereas it causes lethal morbidity and diarrhea in cattle (37, 39). Therefore, attenuation in mice is not always predictive of the degree to which a mutation will reduce pathogeneicity for other species. Whether this will be the case in pigs is unknown; further study is warranted.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristic(s)[a]	Reference or source
E. coli strains		
BL21(DE3)pLysS	F⁻ *ompT* *hsd*S_B($r_B^-m_B^-$) *gal dcm* (DE3) pLysS Cm^r; host for inducing protein expression	Invitrogen
DH5α	F⁻ *endA1hsdR17*(*rk-mk-*) *supE44t hi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*) U169 *deoR* {ϕ80 *dlac* Δ(*lacZ*)M15} λ⁻; cloning host	Laboratory stock
H1717	*araD*139 Δ(*argF-lac*) U169 *relA1 rpsL/flbB deoC ptsF rbsR aroB fhuF*:: λ*plac*Mu53	Laboratory stock
S17-1 λpir	*Prp thi recA hsdR*; chromosomal PR4-2 (*Tn1*::*ISR1 tet*::*Mu Km*::*Tn7*); λ*pir*	Laboratory stock
S. enterica serovar Choleraesuis strains		
1459	Wild-type strain isolated from swine septicemia Ap^r Km^r	This study
807	Wild-type strain isolated from swine septicemia	This study
80521	807 *iroN*::*km* km^r	This study
Plasmids		
pACYC184	4,625 bp shuttle vector; Tc^r Cm^r	New England Biolabs
pACYCiroN	2.3 kb *Hin*dIII-*Bam*HI modified *iroN* gene (including the Fur box of downstream region of the *iroA* locus) cloned into pACYC184	This study
pBluescript SK+	3.0-k cloning vector; Ap^r	Laboratory stock
pBSNKan	pBluescript SK+ with *Bam*HI-*Hin*dIII insertion of 2,181 bp *iroN* gene containing an internal insertion of 1,253 bp kanamycin cassette; Ap^r Km^r	This study
pLT1	The 9.8 kp of *Sal*I insertion of the completed *iroA* locus cloned into pHG165; Ap^r	This study
pLT2	The 2.3 kb of *Sal*I insertion cloned into pHG165; Ap^r	This study
pLT3	The 5.2 kb of *Sal*I insertion cloned into pHG165; Ap^r	This study
pLT4	The 6.8 kb of *Sal*I insertion cloned into pHG165; Ap^r	This study
pGP704	3.7 kb *oriT oriV* Ap^r	26

TABLE 1. Bacterial strains and plasmids (continued)

Strain or plasmid	Genotype or Relevant characteristic(s)[a]	Reference or source
PGPNKan	pGP704 with *SalI-XbaI* insertion of pBSNKan containing an internal insertion mutated *iroN* gene; Ap^r Km^r	This study
pRSETA	T7 promoter expression vector	Invitrogen
pRSETAiroN	2,181 bp PCR product generated from *S. enterica* serovar Choleraesuis cloned into pRSETA, His-tagged IroN	This study
pSUKS1	pSU19 with *HaeII* fragment of pBCSK+	35
pUC4K	pUC vector with kanamycin cassette	Amersham Pharmacia
Phage		
Lambda dash	red gam	8

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance.

TABLE 2. Comparison of potential Fur box found in FURTA-positive clones with the consensus sequence found by de Lorenzo *et al.* (15).

Consensus	GATAATGAT A ATCATTATC	Matches
pSC2:	GATAACTAT T TGCATTTGC	(13/19)
pSC3:	GATACTGAT T ATGGTTTAT	(13/19)
pSC4:	GATATTGGT A ATTATTATC	(17/19)
pSC5:	GCAAACAAT A ATAATTATC	(14/19)
pSC8:	GTTAATCAT A ACCGTGATA	(13/19)
pSC9:	GAAAATGAG A AGCATTAAC	(15/19)
pSC10:	GATAATCAC T ATCATTATC	(17/19)
pSC12:	GATACTGAT T ATGGTTTAT	(12/19)
pSC14:	GCAAATGCA A ATAGTTATC	(13/19)
pSC15	CTTGAAGAT A ATGGTTACC	(12/19)
PSC16	GATAATGAA A AAGGTTATG	(14/19)

Figure Legends:

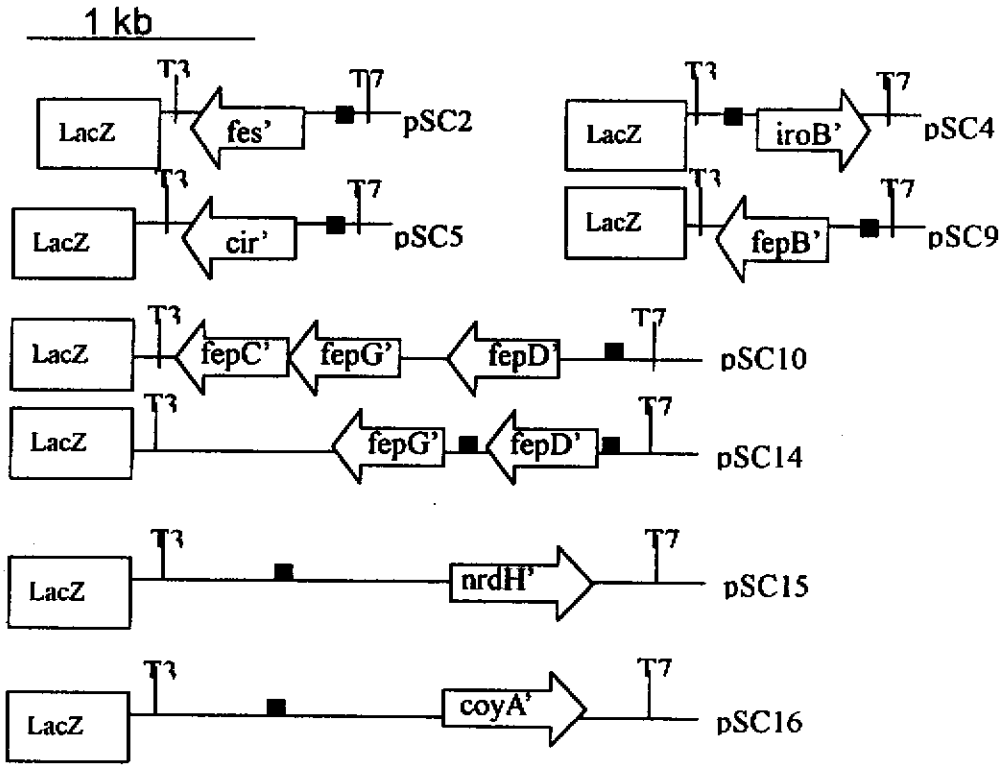
FIG. 1. FURTA-positive clones from *S. enterica* serovar Choleraesuis. Open arrows indicate ORFs. Black boxes indicate the presence of a consensus Fur box. T3 and T7 primers used for sequencing. The open arrow indicates ORFs and the director of promoter. The black boxes indicate the presence of a putative Fur box.

FIG. 2. The genetic map of *iroA* locus of *S. enterica* serovar Choleraesuis. The positions and orientations of ORFs identified by sequence analysis were indicated by open arrows. pSC4 is a FURTA-positive clone and the position and size of insert of *iroA* locus were indicated. The dense squares indicate the Fur box.

FIG. 3. SDS-PAGE and Western blot analysis of the rIroN and OMP of *S. enterica* serovar Choleraesuis probed with rabbit anti-rIroN serum. (A) SDS-PAGE analysis of the OMP profile of *S. enterica* serovar Choleraesuis grown either in iron-rich (lane 1) or iron-limiting (lane 2) conditions and the rIroN (lane 3). (B) Western blot analysis of the OMP and rIroN with rabbit anti rIroN serum. The rabbit anti-rIroN serum recognized the protein band (Lane 2 and 3). The arrowheads indicate the natural IroN (78 kDa) and the arrows indicate the rIroN (82 kDa). M, The numbers on the left indicate the position of standard marker (Bio-Rad) in kDa.

FIG. 4. Western blot analysis of the iron-regulated outer membrane proteins of *S. enterica* serovar Choleraesuis with convalescent porcine sera. The outer membrane proteins were isolated from the *S. enterica* serovar Choleraesuis which was grown in iron-limiting condition. Lanes 1, 2 and 3 (convalescent porcine sera); lane 4 (normal porcine serum). The positions of standard protein marker in kDa are shown on the left. The arrows indicate the three major iron-regulated outer membrane proteins.

FIG. 5. The outer membrane proteins (SDS-PAGE) profiles of *S. enterica* serovar Choleraesuis. Wild type (807), *iroN* mutant (80521) and 80521(pACYCiroN) were grown either in iron-sufficient medium (lanes 1, 3, and 5) or in iron-limiting medium (lanes 2, 4 and 6), respectively. Lane M provided standard protein markers (Bio-Rad) with kDa shown on the left. The IroN is indicated by an arrow.


1 kb
T3
T7
LacZ
fes'
pSC2
LacZ
iroB'
pSC4
LacZ
cir'
pSC5
LacZ
fepB'
pSC9
LacZ
fepC'
fepG'
fepD'
pSC10
LacZ
fepG'
fepD'
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LacZ
nrdH'
pSC15
LacZ
coyA'
pSC16

FIG. 1.

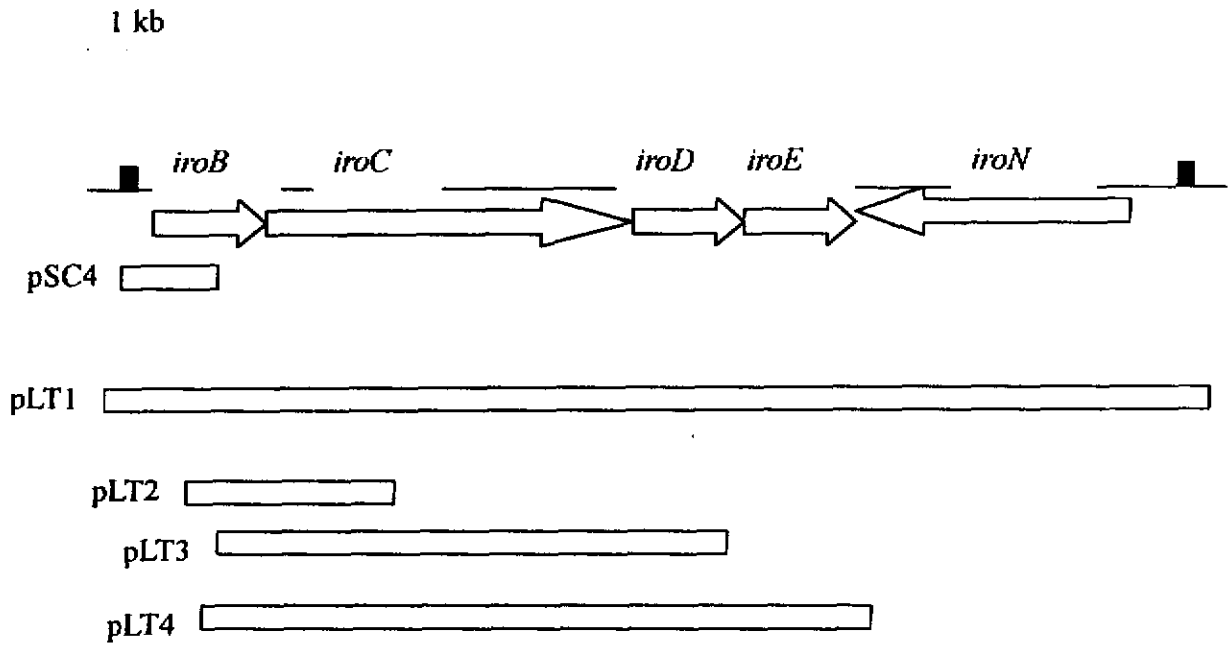

FIG. 2.

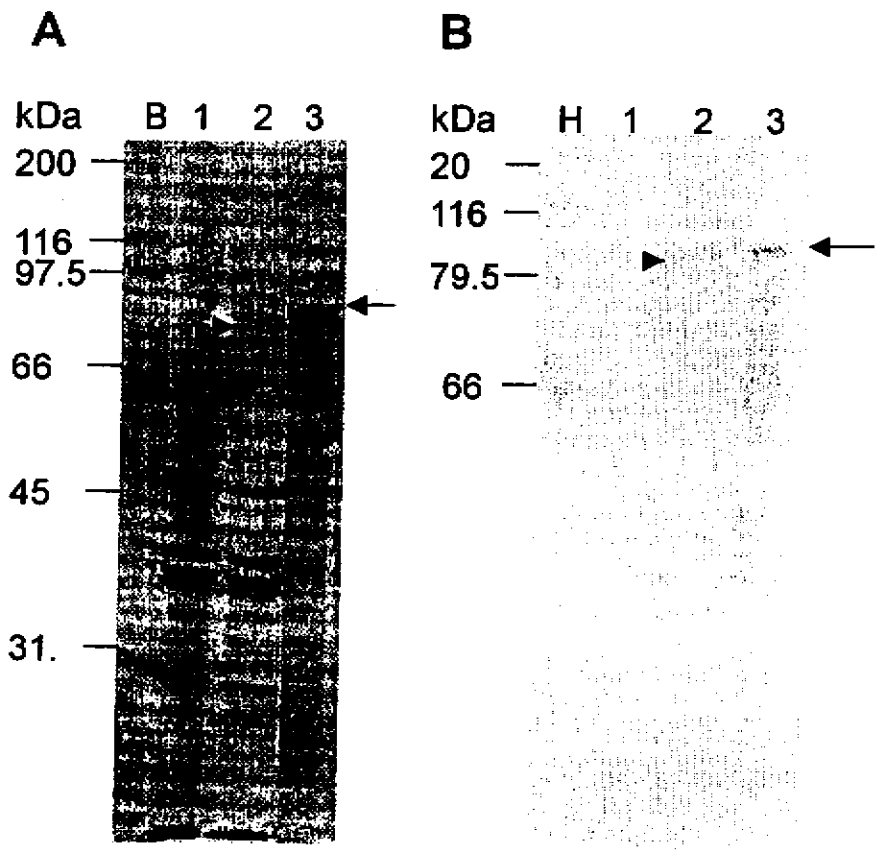

FIG. 3.

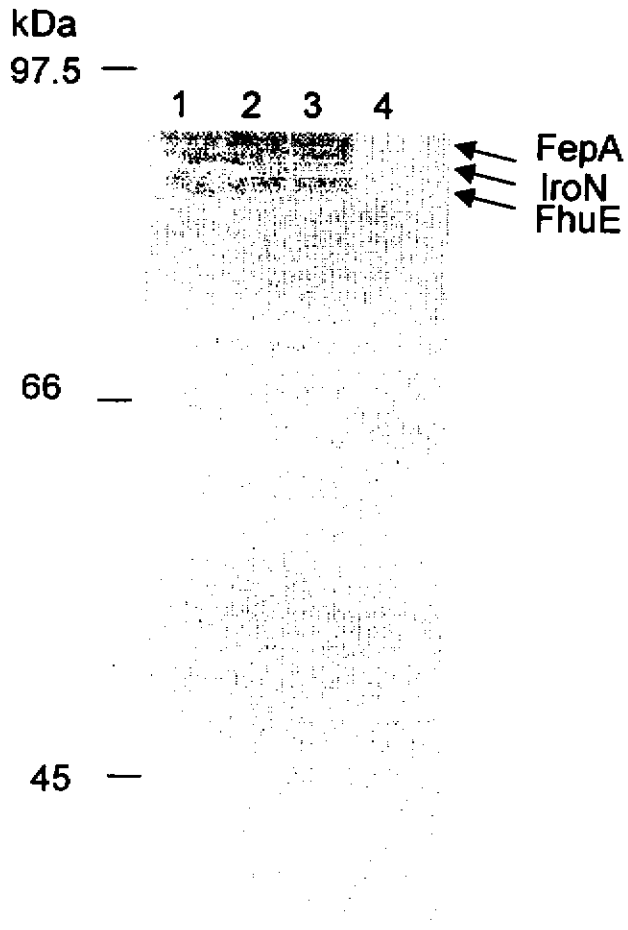

FIG. 4.

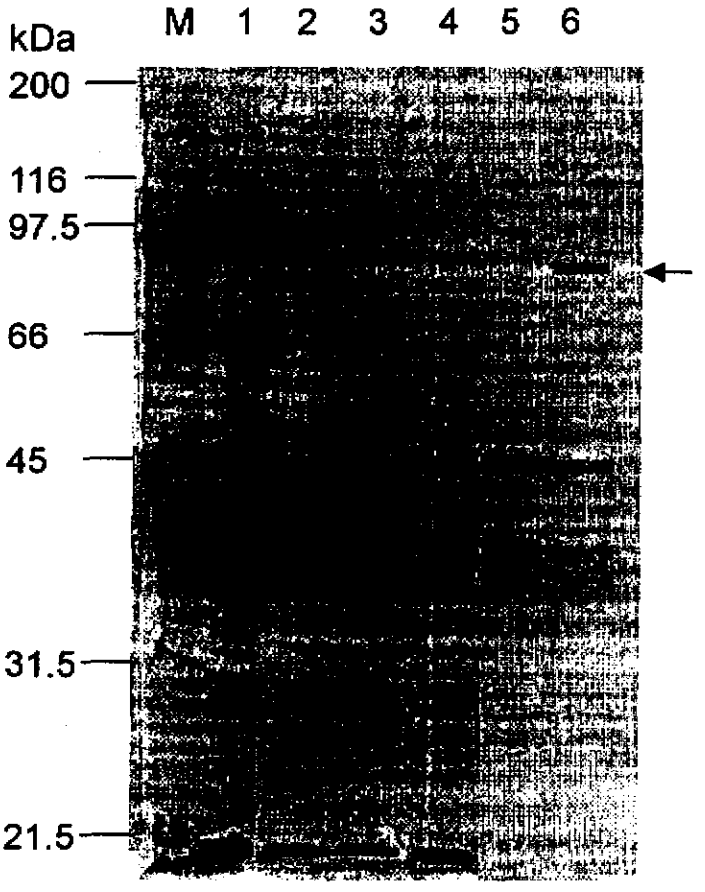

FIG.5.