

IroN Functions as a Siderophore Receptor and Is a Urovirulence Factor in an Extraintestinal Pathogenic Isolate of *Escherichia coli*

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IroN was recently identified in the extracellular pathogenic *Escherichia coli* strain CP9. In this study experimental evidence demonstrating that IroN mediates utilization of the siderophore enterobactin was obtained, thereby establishing IroN as a catecholate siderophore receptor. In a mouse model of ascending urinary tract infection the presence of *iroN* contributed significantly to CP9's ability to colonize the mouse bladder, kidneys, and urine, evidence that IroN is a urovirulence factor. However, growth in human urine *ex vivo* and adherence to uroepithelial cells *in vitro* were equivalent for an isogenic mutant deficient in IroN (CP82) and its wild-type parent (CP9). Taken together, these findings establish that IroN is a siderophore receptor and a urovirulence factor. However, uncertainty exists as to the mechanism(s) via which IroN contributes to urovirulence.

Extraintestinal pathogenic *Escherichia coli* (ExPEC) organisms possess genes encoding diverse extraintestinal virulence factors that enable them to cause infections outside the gastrointestinal tract (10, 15). The concentration of iron (Fe) is limited in sites of extraintestinal infection in large part due to host factors that reduce its availability. As a result, Fe acquisition is a critical need for pathogens that must grow within a host. Strains of ExPEC, like strains of *E. coli* in general, possess multiple Fe acquisition mechanisms, which include siderophore/siderophore receptor systems (5). Our laboratory has been studying the ExPEC strain CP9 (O4/K54/H5) (20). We recently described a new gene (*iroN*), which was identified by virtue of having increased expression in human urine, ascites, and blood (19). IroN expression was shown to be Fe regulated, and a homology search based on putative peptide sequence suggested that IroN was a siderophore receptor. Molecular epidemiologic evidence from several studies has demonstrated an increased prevalence of *iroN* among urinary tract infection isolates relative to fecal isolates (2, 19). Taken together, this evidence suggests that IroN functions as a siderophore receptor and is a virulence factor, at least for urinary tract infection. In this study, the following hypotheses were tested: (i) IroN functions as a catecholate siderophore receptor, (ii) IroN is a urovirulence factor, (iii) the mechanism by which IroN contributes to urovirulence is via Fe acquisition, and (iv) IroN is also a virulence factor in infections outside the urinary tract.

IroN was identified in the ExPEC strain CP9, which has been previously described in detail (9, 20). CP82 (lacking *iroN*) is an isogenic, *TnphoA*-generated derivative of CP9 (19). *iroN* plus 150 nucleotide bases 5' and 80 nucleotide bases 3' to *iroN*

(2,645 bp) was cloned via PCR-mediated amplification (forward, 5' ATATATGGATCCATTTATCTTGTGAGGGA TTG 3', reverse, 5' GCGCGCAAGCTTCCACTAAACACT GCCCGCTTT 3') based on the *iroN* sequence (19) and ligated into pET28a T7/his-tag expression vector (Novagen, Madison, Wis.). *iroN* was confirmed to be identical to that in strain CP9 by bidirectional DNA sequencing. The clone or vector alone was subsequently electroporated into ORN172 and RWB18-60. Expression was confirmed by preparing outer membrane protein extracts from ORN172/pET28a:*iroN* and analyzing the samples after separation on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% acrylamide) gel (data not shown).

Enterobactin utilization was assessed via a cross-feeding assay. The enterobactin-producing strain MC4100 (4) was streaked down the middle of Tris-succinate minimal media plate (8), from which Fe was chelated with dipyriddy (final concentration, 0.1 mM). RWB18-60/pET28a:*iroN*, a strain that expressed IroN as its sole enterobactin receptor (1, 12) was streaked at a 90° angle to the MC4100 streak and observed for growth after incubation at 37°C overnight.

Comparative protein sequence analysis was done by using several *E. coli* siderophore receptors, including FepA (P05825), IroN_{*E. coli*} (AF135597), IroN_{*Salmonella*} (IroN_{*Sal*}) (AJ000635), IreA (AF320691), FhuA, which transports ferrichrome bound Fe (P06971), and FecA, which transports ferric citrate (20150925) (GenBank accession numbers are given in parentheses). Amino acid residue numbers were derived from proteins that included the signal sequence. The "plug" domain used for comparison consisted of 142 amino acid residues beginning at the TonB box within each protein. The experimentally defined plug domains for FepA (residues 34 to 175), FhuA (residues 40 to 181), and FecA (residues 56 to 197) (3, 7, 13) were used as the basis for defining the putative plug

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domain from $\text{IroN}_{E. coli}$ (residues 33 to 174), IroN_{Sal} (residues 34 to 175), and IreA (residues 32 to 172).

Dual infection (competition) experiments were used to assess growth in urine *ex vivo*. CP9 and CP82 were grown separately overnight in 2 ml of human urine (pooled from eight individuals, filter sterilized, and stored at -80°C prior to use) and diluted in fresh urine the next day to achieve a starting concentration of approximately 1.0×10^2 to 1.0×10^3 CFU/ml of both CP9 and CP82. The titer of CP82 was determined from the enumeration from Luria-Bertani (LB) medium plus kanamycin (40 $\mu\text{g}/\text{ml}$), and the titer of CP9 was established by subtracting the titer of CP82 from the total bacterial titer enumerated from LB medium. Alternatively, if the calculated titers of CP9 and CP82 were similar, colonies from the LB medium plates were replica plated onto LB medium plus kanamycin to determine the relative proportion of each strain.

Mouse urinary tract infection experiments (female mice, 18 to 22 g, 8 to 12 weeks old) were done by using an ascending, atraumatic mouse model of urinary tract infection as described previously (17). CP9 and CP82 were grown overnight in M9 minimal medium (14) containing 0.5% glucose and treated with Chelex to remove any trace Fe present (M9-glucose-Chelex-treated) prior to challenge to ensure expression of IroN (19). Forty-eight hours postchallenge, urine, bladder, and kidneys were harvested and bacterial titers were determined. In initial experiments, CP9 and CP9.82 were evaluated individually in the mouse urinary tract infection model. With this experimental design there was no clear difference in the ability of each of these strains to colonize mouse urine, bladder, and kidneys. Therefore, to minimize the impact of mouse-to-mouse variation and to maximize the sensitivity for identifying differences between strains, dual infection (competition) experiments were done as described earlier (18). The enumeration of CP9 versus CP82 was accomplished as described for *ex vivo* urine competition experiments. After adjustment for the ratio of CP9 to CP82 in the inoculum suspension (i.e., the input ratio), proportional differences ($>1\times$, $>3\times$, $>10\times$) between CP9 and CP82 (categorical variable analysis) as recovered from postmortem cultures were calculated for each culture on a per mouse basis, thereby enabling each mouse to serve as its own control. Median CFU per milliliter (for urine) and median CFU/organ (bladder and kidneys) for CP9 and CP82 were also compared as continuous variables on a per mouse basis.

Adherence assays were performed essentially as described earlier (23). Human bladder cells (ATCC HTB-1 J82) were grown to confluency in 24-well plates (Nalge Nunc International Corp., Naperville, Ill.). Derivatives of ORN172 (a nonadherent *E. coli* K-12 strain) (26) that either expressed IroN (ORN172/pET28a::*iroN*) or did not express IroN (ORN172/pET28a) were grown overnight in iron-chelated Minimum M9 media with 0.1% Casamino Acids plus kanamycin (Amresco, Solon, Ohio) at 37°C , subcultured the next day, grown to mid-log phase, and centrifuged and resuspended in iron-chelated, serum-free minimal essential medium plus kanamycin to a concentration of approximately 5×10^5 cells/ml. Medium was removed from 24-well plates, and 0.2 ml of bacterial suspension was seeded into appropriate wells. Each strain was tested in triplicate for each time point. Time points were 15, 30, 60, and 90 min. Plates were centrifuged at $1,172 \times g$ for 3 min at room temperature and then incubated at 37°C . At designated

time points, supernatants containing nonadherent bacteria were removed from wells. Wells were washed (by gently pipetting up and down 3 or 4 times) with media. Bacteria associated with bladder cells were harvested by adding 0.5 ml of 0.25% trypsin (Gibco, Grand Island, N.Y.) to each well, incubating at 37°C for 5 min and transferring the mixture of bacteria and bladder cells from triplicate wells to a single tube. An additional rinse of the well (0.5 ml of minimal essential medium) removed any remaining bacteria and bladder cells and was combined with the previously harvested mixture of bacteria and bladder cells. The harvested bacteria associated with bladder cells were centrifuged to remove the trypsin, resuspended in 3 ml of phosphate-buffered saline, and the bacterial titer was enumerated via serial 10-fold dilutions.

The rat granuloma pouch was created as previously described (21). A dual infection (competition) approach was also used in this infection model. CP9 and CP82 were grown overnight in M9-glucose-Chelex-treated medium, and an approximately equal inoculum of each strain was injected into the pouch. Starting bacterial titers within the pouch ranged from 1.0×10^3 to 1.0×10^4 CFU/ml. Aliquots of pouch fluid were removed at 0, 3, 6, and 24 h, and bacterial titers were determined. Proportional differences between CP9 and CP82, the measured endpoint, were analyzed on a per rat basis, thereby enabling each rat to serve as its own control.

IroN is a functional siderophore receptor. An enterobactin cross-feeding assay was used to test whether IroN , at least in part, functions as a siderophore receptor. The sole enterobactin receptor expressed by strain RWB18-60/pET28a::*iroN* was IroN (1, 12). When RWB18-60/pET28a::*iroN* was grown on an Fe-deficient medium next to MC4100, a strain which secretes the catechol siderophore enterobactin, RWB18-60/pET28a::*iroN* was able to utilize this secreted siderophore and grow (data not shown). In contrast, RWB18-60/pET28a, which does not express IroN , was unable to grow (data not shown). This finding demonstrated that IroN was able to function as a siderophore receptor and that enterobactin was a cognate siderophore.

Comparison of the putative $\text{IroN}_{E. coli}$ plug domain to those of other Fe transport receptors. Crystal structure data from Fe transport receptors FepA (which transports enterobactin), FhuA (which transports ferrichrome bound Fe), and FecA (which transports ferric citrate) have defined the structure of these proteins as a monomeric 22-stranded β barrel, occluded by a plug domain. The plug domain is critical for ligand binding and transport (3, 7, 13). FepA, IroN_{Sal} , and (in this study) $\text{IroN}_{E. coli}$ have been shown to be able to utilize enterobactin. Although $\text{IroN}_{E. coli}$ has a relatively high degree of homology to IroN_{Sal} (82% identity, 91% similarity), FepA possesses far less homology (52% identity, 69% similarity). Given that all three of these receptors are able to utilize the siderophore enterobactin, it was hypothesized that there exists a greater degree of homology between the putative ($\text{IroN}_{E. coli}$, IroN_{Sal}) or established (FepA) plug domains within each of these siderophore receptors. CLUSTAL analysis supported this hypothesis (Table 1.) The homology between the putative $\text{IroN}_{E. coli}$ plug domain and those of IroN_{Sal} and FepA was significantly greater than the overall homology between these proteins. In contrast, the homology between the putative $\text{IroN}_{E. coli}$ plug domain and those of FecA and FhuA was un-

TABLE 1. Deduced protein sequence identity and similarity of *IroN*_{*E. coli*} and its putative plug domain to the Fe transport proteins *IroN*_{*Sal*}, FepA, IreA, FecA, and FhuA

Organism	Homologue	Complete protein		Plug domain ^a			
		% Identity ^b (ratio)	% Similarity ^b (ratio)	% Identity (ratio)	<i>P</i> value ^c	% Similarity (ratio)	<i>P</i> value ^d
<i>Salmonella enterica</i>	<i>IroN</i> _{<i>Sal</i>}	82 (593/725)	91 (661/725)	92 (130/142)	0.001	98 (139/142)	0.003
<i>E. coli</i>	FepA	52 (375/725)	68 (492/725)	71 (101/142)	<0.001	82 (116/142)	<0.001
<i>E. coli</i>	IreA	27 (197/725)	43 (312/725)	45 (64/142)	<0.001	62 (88/142)	<0.001
<i>E. coli</i>	FecA	21 (152/725)	39 (282/725)	16 (23/142)	>0.10	30 (43/142)	0.06
<i>E. coli</i>	FhuA	18 (127/725)	35 (251/725)	18 (25/142)	>0.10	37 (52/142)	>0.10

^a As defined by the crystallographic structure for FepA (3), FecA (7), and FhuA (13) (see text).

^b Determined by CLUSTAL alignments.

^c Compared to the percent identity of the complete protein from the same strain (Fisher's exact test).

^d Compared to the percent similarity of the complete protein from the same strain (Fisher's exact test).

changed or tended to be less than the overall homology between these proteins. The homology between the putative *IroN*_{*E. coli*} plug domain and that of IreA was significantly greater than the overall homology between these proteins but lower than the comparative homologies for *IroN*_{*Sal*} and FepA. Whether IreA is able to utilize enterobactin has not yet been determined. Therefore, the greater degree of conservation of the plug domain of *IroN*_{*E. coli*}, *IroN*_{*Sal*}, and FepA relative to the entire protein is consistent with the ability of each of these receptors to transport enterobactin.

IroN is a urovirulence factor. The role of *IroN* in urovirulence was assessed in an ascending, atraumatic mouse model of urinary tract infection via dual infection (competition) experiments. Overall, in three independent experiments both categorical variable (proportional) and continuous variable (median CFU per milliliter or per organ) analyses, CP9 outcompeted CP82 for every parameter of urine, bladder, and renal colonization ability (Table 2.) The median concentration of CP9 was significantly greater in urine ($P = 0.0004$) and bladder ($P = 0.0001$) than that of CP82, and there was a trend towards

significance in the kidneys ($P = 0.109$) (Table 2). Likewise, a statistically significant predominance of CP9 over CP82 was seen at the >1-fold and >3-fold ratios for urine ($P < 0.01$), bladder ($P < 0.01$), and kidneys ($P < 0.02$) and at the >10-fold ratio for urine ($P < 0.01$) and kidneys ($P < 0.05$) (Table 2).

The growths of CP9 (wild type) and CP82 are similar in human urine ex vivo. To gain insight into the mechanism by which *IroN* contributes to urovirulence, the growth of CP82 and that of its wild-type parent CP9 were compared in human urine ex vivo. In view of the demonstration that *IroN* was a functional siderophore receptor, it seemed logical that Fe acquisition should be its functional role in urinary tract infection. However, previous comparisons of the ex vivo growths of CP82 and CP9 in separate human urine samples failed to demonstrate a growth difference between these strains (19). To maximize the sensitivity for identifying potential growth differences between CP82 and CP9 in human urine, these experiments were repeated by using a dual infection (competition) experimental design. Despite optimizing conditions, again a growth difference in human urine between these strains could not be demonstrated (data not shown). Although this finding did not exclude Fe acquisition as the mechanism by which *IroN* contributes to urovirulence, it prompted consideration of other possible mechanisms.

IroN does not affect the adherence of CP9 to bladder epithelial cells in vitro. The possibility that *IroN* might be multifunctional and contribute to virulence by serving also as an adhesin was suggested by the observation that an *IroN* homologue, Iha, which was recently identified in a Shiga toxin-producing strain of *E. coli* (O157:H7), conferred adherence to HeLa cells (24). To test the hypothesis that *IroN* is an adhesin and to exclude the confounding effect of other adhesions, adherence to bladder epithelial cells was assessed for ORN172/pET28a, an adherence-deficient *E. coli* construct (26), and ORN172/pET28a:*iroN*, which expresses *IroN* on its surface. Adherence was evaluated under conditions in which *IroN* expression is known to occur (Fe depletion) (19). Expression of *IroN* did not enhance the ability of ORN 172 to adhere to bladder epithelial cells in vitro (data not shown).

The expression of *IroN* does not affect growth in the rat granuloma pouch model. To determine whether *IroN* contributes to virulence in an extraintestinal site of infection other than the urinary tract, dual infection (competition) experiments were performed with the rat granuloma pouch model,

TABLE 2. Virulence of CP9 (wild type) and CP82 in a mouse urinary tract infection model

Specimen and infecting strain	Median CFU/g or CFU/ml ^a	No. of samples (%) for which concn of strain exceeded that of the other:		
		>1-fold	>3-fold	≥10-fold
Bladder ($n = 48$)				
CP9	1.3×10^4	41 (85)	22 (46)	6 (13)
CP82	4.0×10^{3b}	7 (15) ^f	3 (6) ^f	1 (2) ^h
Urine ($n = 44$) ^c				
CP9	2.0×10^4	32 (73)	23 (52)	17 (39)
CP82	6.1×10^3	11 (25) ^f	6 (14) ^f	4 (9) ^f
Kidney ($n = 48$)				
CP9	5.3×10^1	25 (52)	20 (42)	17 (35)
CP82	2.8×10^{1e}	10 (21) ^g	7 (15) ^g	6 (13) ⁱ

^a Data were corrected for input ratios prior to statistical analysis.

^b $P = 0.0001$ (Wilcoxon rank sum test).

^c Urine specimens could not be obtained from four animals.

^d $P = 0.0004$ (Wilcoxon rank sum test).

^e $P = 0.109$ (Wilcoxon rank sum test).

^f $P < 0.01$ (McNemar's test).

^g $P < 0.02$ (McNemar's test).

^h Not significant (McNemar's test).

ⁱ $P < 0.05$ (McNemar's test).

which mimics an intra-abdominal abscess (6). Three independent experiments were performed, in which a total of 20 rats were challenged with a mixed inoculum containing approximately 10^3 to 10^4 CFU of both CP9 (wild type) and its isogenic *IroN*-deficient derivative (CP82), and the ratio of CP9 to CP82 in granuloma pouch fluid was determined at 0, 3, 6, and 24 h. Although some animal-to-animal variability occurred, overall no consistent or statistically significant competitive advantage was observed for either strain (data not shown).

In this study we obtained novel experimental evidence for the protein homology-based hypothesis that *IroN* is a catecholate siderophore receptor and for the epidemiologically derived inference that *IroN* is a urovirulence factor. Our findings leave uncertainty as to the mechanism(s) through which *IroN* contributes to urovirulence and as to whether *IroN* also contributes to extraintestinal virulence at sites other than the urinary tract.

Establishing that *IroN* is able to utilize the siderophore enterobactin is consistent with data that demonstrated that a siderophore receptor in *Salmonella* (*IroN*_{Sal}), which is highly homologous to *IroN* in *E. coli* (82% identity, 91% similarity) (19), is also able to mediate uptake of enterobactin as well as the siderophore corynebactin (16). On the other hand, *FepA*, the cognate siderophore receptor for enterobactin, possesses only 52% identity and 68% similarity with *IroN*_{*E. coli*} and is unable to mediate uptake of corynebactin but is able to utilize the siderophore myxochelin C. Comparison of the plug domain, which is critical for Fe binding and transport, of *FepA* with the putative plug domains of *IroN*_{*E. coli*} and *IroN*_{Sal} (Table 1) reveals a much higher degree of conservation among these domains than within the entire proteins, thereby making the functional overlap in the ability of *FepA*, *IroN*_{*E. coli*} and *IroN*_{Sal} to mediate the transport of enterobactin more consistent with the structural biology of these proteins. Despite the somewhat promiscuous interactions of these siderophore receptors with enterobactin, specificity is retained in the transport of myxochelin C (*FepA*) and corynebactin (*IroN*_{Sal}) (16). Whether *IroN*_{*E. coli*} is able to transport these or other nonenterobactin siderophores remains to be determined.

IroN was shown to be a urovirulence factor in dual infection experiments using an atraumatic, ascending mouse urinary tract infection model. This finding is consistent with the epidemiologic evidence that *IroN* is important for urovirulence (2, 19). An increasing body of data indicates that siderophore receptors and/or putative siderophore receptors are important for maximal urovirulence. *IreA*, a putative siderophore receptor that was also identified from CP9, was previously shown to be a urovirulence factor (18). Likewise, the outer membrane receptors for heme transport (*ChuA*), the aerobactin transport (*IutA*), and the ability to synthesize aerobactin and enterobactin have been shown to contribute to urovirulence in the extraintestinal pathogenic strain CFT073 (25). Taken together, these experimental findings support the hypothesis that Fe acquisition is necessary for urovirulence.

Previous data from our laboratory demonstrated that human urine is an Fe-limited environment (19). Therefore, it seemed logical that if the mechanism by which *IroN* contributes to urovirulence is Fe acquisition, then the growth of the *IroN*-deficient strain CP82 may be diminished in human urine *in vivo* compared to the growth of its wild-type parent. However,

a growth difference between CP9 and CP82 could not be demonstrated in human urine in either single or dual infection experiments. Interestingly, in a previous study no difference in growth in human urine was demonstrated when strain CFT073 was compared with its mutant derivatives deficient in aerobactin or heme transport, despite the demonstrated *in vivo* competitive disadvantage of these mutants (25). One explanation for these results may be that *IroN*-mediated Fe acquisition occurs in association with host cells, either extracellularly (when adherent) or possibly intracellularly (11). Alternatively, *IroN* may have contributed to urovirulence via a mechanism that did not involve Fe acquisition.

To test this hypothesis of *IroN* as an adhesin, the role of *IroN* in adherence to bladder epithelial cells was evaluated. A precedent for this concept was provided by *Iha*, which was identified from a Shiga toxin-producing intestinal pathogenic strain of *E. coli* (O157:H7) and, although homologous to several siderophore receptors at the protein level, was shown to be an adhesin (24). However, within the limitations of the *in vitro* adherence assay used in this report, a role for *IroN* in mediating adherence to the human bladder cells (ATCC HTB-1 J82) could not be demonstrated. It remains possible that different results may have been obtained with the use of another cell line. Therefore, although it remains logical to presume that *IroN* contributes to urovirulence via Fe acquisition, neither this mechanism (nor an alternative mechanism) has yet been experimentally established.

Many pathogens, including extraintestinal isolates of *E. coli*, possess multiple Fe acquisition systems. The model pathogen (CP9) being studied by our laboratory expresses the siderophore receptors *IroN* (19) and *IreA* (18) and possesses genes for *FyuA* (J. Johnson, personal communication) and *FepA* (T. Russo, personal communication). It is likely that other siderophore receptors and/or Fe acquisition systems are also expressed in this pathogen, by analogy to *E. coli* K-12 and other ExPEC (CFT073) (5, 25). A variety of hypotheses have been generated to explain this apparent redundancy. These include the following: (i) insurance in the event that one system becomes dysfunctional due to (a) a random mutation or nonrandom hypermutation that occurs in Fe-regulated genes because of Fe limitation within the host (27) or (b) antibody-mediated inactivation specific for one receptor, but not another; (ii) different systems enable the pathogen to acquire Fe from different host sources (e.g., heme versus transferrin) (25); (iii) the ability to acquire Fe by siderophore piracy (relevant in a polymicrobial milieu) (16); and (iv) different systems are site specific (e.g., urinary tract versus bloodstream). To test the latter hypothesis, a pathogenic role for *IroN* outside the urinary tract was assessed. The rat granuloma pouch model was utilized, since it mimics an intra-abdominal abscess, an infection in which ExPEC often participate. However, in contrast to the urinary tract infection model, in the granuloma pouch model no difference in growth and survival between CP9 (wild type) and CP82 (lacking *iroN*) was observed. Fluid in the rat pouch contains red blood cells, which are hemolyzed after challenge with CP9 and derivatives (data not shown), presumably due to the hemolysin that CP9 produces. Despite this, the granuloma pouch environment is still Fe limited, as demonstrated by the increased expression of *IroN* observed within the pouch relative to LB medium (T. Russo, personal communi-

cation). The observed absence of growth impairment in the granuloma pouch model with the *iroN* mutant supports the hypotheses that alternative iron acquisition systems, i.e., either nonsiderophore (e.g., heme transport) systems or siderophore systems other than *iroN*, are sufficient for iron acquisition in this setting.

Extraintestinal infections due to *E. coli* are common, cause significant morbidity and mortality, and are costly to our healthcare system. The development of an effective vaccine that could prevent even some of these infections would be medically important and likely cost-effective. An ideal vaccine candidate needs to be surface exposed, be broadly prevalent among clinical extraintestinal isolates of *E. coli*, possess epitopes that are conserved, and elicit a protective immune response. Other desirable characteristics include increased expression at the site of infection and a role in the pathogenesis of disease. The expression of *iroN* in various human body fluids and its prevalence among clinical extraintestinal isolates of *E. coli* have been previously demonstrated (2, 19). In this report, a role for *iroN* in the pathogenesis of urinary tract infection, the most common type of extraintestinal *E. coli* infection, was established. Studies of whether immunization with *iroN* can elicit a protective immune response are under way.

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