The *Francisella* pathogenicity island protein IglA localizes to the bacterial cytoplasm and is needed for intracellular growth

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**Abstract**

**Background:** *Francisella tularensis* is a gram-negative, facultative intracellular bacterium that is the etiological agent of tularemia. *F. novicida* is closely related to *F. tularensis* but has low virulence for humans while being highly virulent in mice. IglA is a 21 kDa protein encoded by a gene that is part of an *iglABCD* operon located on the *Francisella* pathogenicity island (FPI).

**Results:** Bioinformatics analysis of the FPI suggests that IglA and IglB are components of a newly described type VI secretion system. In this study, we showed that IglA regulation is controlled by the global regulators MglA and MglB. During intracellular growth IglA production reaches a maximum at about 10 hours post infection. Biochemical fractionation showed that IglA is a soluble cytoplasmic protein and immunoprecipitation experiments demonstrate that it interacts with the downstream-encoded IglB. When the *iglB* gene was disrupted IglA could not be detected in cell extracts of *F. novicida*, although IglC could be detected. We further demonstrated that IglA is needed for intracellular growth of *F. novicida*. A non-polar *iglA* deletion mutant was defective for growth in mouse macrophage-like cells, and in cis complementation largely restored the wild type macrophage growth phenotype.

**Conclusion:** The results of this study demonstrate that IglA and IglB are interacting cytoplasmic proteins that are required for intramacrophage growth. The significance of the interaction may be to secrete effector molecules that affect host cell processes.

**Background**

*Francisella tularensis* is the etiological agent of the severe, febrile disease tularemia. Although there have been rare isolates of *F. tularensis* in Australia, tularemia is mainly a disease of the Northern hemisphere that is spread by blood-sucking mosquitoes, flies, and ticks or acquired from contact with infected animals such as rabbits, rodents, and beavers [1]. Occasionally, local outbreaks of tularemia are associated with contact or consumption of contaminated natural water. In addition, *F. tularensis* is potentially a threat as a bioterrorist agent due to its high infectivity and lethality when inhaled. *F. novicida* is highly related at the DNA level to *F. tularensis*, and serves as a model organism since it is very virulent in mice while being avirulent in humans.

*F. tularensis* is a gram-negative, facultative intracellular bacterium capable of survival and replication in macrophages [2]. A common virulence strategy of intracellular pathogens is to favorably modulate the intracellular milieu of hosts for their own benefit. In *Legionella pneumophila* a type IV secretion system (T4SS) delivers effectors...
that allow the pathogen to replicate in ribosome-studded phagosomes that fail to fuse with lysosomes [3,4]. *Salmonella enterica* relies on a pathogenicity island-encoded type III secretion system (TTSS) to modify phagosome biogenesis [5,6], including inhibition of phago-lysosomal fusion [7] and the NADPH oxidase-mediated killing by host cells [5]. Other intracellular pathogens, such as *Listeria monocytogenes*, degrade the phagosomal membrane and escape into the cytoplasm to replicate freely [8]. *F. tularensis* initially resides in a phagosome which accumulates some late endosome markers. After about four hours most *F. tularensis* cells escape the phagosome and grow in the cytoplasm. [2,9-11]. Although an intact *iglC* gene is required for intramacrophage growth.

**IglA** is a cytoplasmic protein that interacts with IglB, and this simplifies the construction of mutants. Further, it was demonstrated that this gene cluster encodes components of a proposed type VI secretion system (T6SS) in *Vibrio cholerae* [24].

In light of the emerging role of IAHP/T6SS in the secretion of proteins we re-examined the ORFs in the FPI to determine if components of a type VI secretion system may be present. Three essential components of a T6SS are a protein with an IcmF-motif and two linked genes that correspond to *iglA* and *iglB*. A BLASTP search revealed that an IcmF region was found as part of the C-terminal third of PdpB which aligned with the corresponding regions of proteins belonging to the IcmF conserved orthologous group (COG3516) with an E-value of $7 \times 10^{-5}$. The identification of IglA and IglB as members of COGs is much clearer. IglA has strong identity to members of COG3516 (E-value of $2 \times 10^{-20}$) and IglB has strong identity with COG3517 (E-value of $2 \times 10^{-102}$). Remarkably all of the relatives of *iglAB* are organized in the same order, and are always adjacent to each other on the chromosome. The *iglAB* genes together with an *icmF*-containing gene form the core set of genes that suggest the presence of a type VI secretion system. We also found through BLASTP analysis that the deduced product of an ORF 380 bp downstream of *pdpB* (shown as *vgr* in Fig. 1) shows a weak similarity (E-value 0.15) to the family of *vgr*-encoded proteins, such as VgrG [24] which is secreted by a T6SS in *V. cholerae*. Vgr proteins are hydrophilic proteins that contain yline-glycine repeats, and are found in a number of gram negative pathogens. Another ORF, 4587 bp downstream of *pdpB* show similarity (E-value, 0.0005) to proteins in COG3455 that includes the IAHP-associated protein DotU. The clustering of *iglAB* and the *icmF*-containing *pdpB* gene, together with two other IAHP-associated genes strongly suggests that the FPI carries a type VI secretion system.

**IglA expression in an mglAB background**

Previously RT-PCR analysis of the level of *iglA*, *iglC* and *iglD* transcripts revealed a role of MglA in regulating expression of the *iglABCD* operon mRNA production [13]. We wished to test if IglA protein expression levels are depressed in mutant *mglA* and *mglB* backgrounds. Western immunoblot analysis of IglA in an *mglA* mutant and an *mglB* background revealed that IglA is not expressed at detectable levels in these strains (Fig. 2).

**IglA expression during intramacrophage growth**

Previous studies provide evidence that MglA expression peaks at about 5 hours after infection of macrophages

We recently described a *Francisella* pathogenicity island (FPI) harboring several genes necessary for intracellular growth. Four FPI genes, *iglABCD*, are organized in an apparent operon [12]. The production of IglC mRNA is in part dependent on MglA [13] which is thought to be a global regulator of virulence factors in *F. tularensis*. By analogy with its *Escherichia coli* homologue, SspA, MglA likely interacts with RNA polymerase to directly or indirectly alter transcription of several genes [14]. Disruption of *mglA* or *mglB* results in mutants that are severely attenuated for virulence [15]. IglC has been shown to be induced about four-fold during intracellular growth relative to broth growth and necessary for virulence [16-18], and it was recently demonstrated that inactivation of *iglC* and *mglA* result in mutants that remain in phagosomes that fuse with lysosomes [19,20]. Although an *iglA* transposon insertion mutant has been shown to be defective for intracellular growth, it could not be ruled out that the observed phenotype was due to interruption of transcription of downstream genes, including *iglC* [17].

In this study, we use *F. novicida* to investigate the properties of IglA and its role in *F. novicida* intracellular growth. *F. novicida* is particularly suited for these studies since, unlike *F. tularensis*, it contains only one copy of the FPI, and this simplifies the construction of mutants. Further, the biology of *F. novicida* growth in human macrophages is indistinguishable from that of *F. tularensis* strains [9,11], and thus *F. novicida* serves as a valid surrogate for virulent strains when studying basic aspects of *Francisella* intracellular growth. In this work we supply evidence that IglA is a cytoplasmic protein that interacts with IglB, and is required for intramacrophage growth.

**Results**

**IglAB homologues in diverse bacteria are organized in a conserved gene cluster**

Homologues of *iglA* and *iglB* exist in several bacterial species that are either animal or plant pathogens or plant symbionts [12] but there are no known homologues of *iglC* or *iglD*. IglAB homologues in *Vibrio cholerae*, *Salmonella enterica*, *Rhizobium leguminosarum*, and other bacteria are found in a cluster of genes encoding proteins known as IcmF-associated homologous proteins (IAHPs) [21-23]. Recently, it was demonstrated that this gene cluster encodes components of a proposed type VI secretion system (T6SS) in *Vibrio cholerae* [24].

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[25], and that IglC expression is maximal at between 6 and 24 hours after infection [16]. To access the pattern of IglA expression during *F. novicida* infection of macrophages, we lysed J774 macrophages at various time points after infection with the wild type strain U112 and examined the lysates for IglA using immunoblotting. In our assays IglA was first detectable at 8 hours post-infection, peaked at 10 hours, and showed a decline by 12 hours (Fig. 3). In broth grown cultures IglA appeared to be maximally expressed at the late logarithmic phase of growth. IglA is cytoplasmically located Knowing the cellular localization of a protein can help lead to a hypothesis as to its biological role. To investigate the subcellular localization of IglA, we fractioned *F. novicida* U112 into soluble and membrane-associated fractions and determined the amount of IglA in each fraction by immunoblot analysis. The data from this experiment revealed that IglA is exclusively a soluble protein (Fig 4). Although IglA lacks a signal peptide sequence, it could not be ruled out that IglA localizes to the periplasm by a novel mechanism. Therefore, we isolated the periplasmic contents from *F. novicida* and determined by immunoblotting that IglA does not localize to this compartment. We also failed to detect IglA in culture supernatant (data not shown). The data from these experiments strongly suggest that IglA is a cytoplasmic protein. In agreement with this, the IglA homologue in *Salmonella enterica* has been predicted to be localized to the cytoplasm [23].

**IglA interacts with IglB in vivo**

To investigate interactions of IglA with other *F. novicida* proteins we performed immunoprecipitations with anti-IglA antibody on soluble proteins. A co-precipitating protein with a relative molecular mass of approximately 60 kDa was detected (Fig 5A). This protein band was excised and subjected to MALDI-TOF analysis, and the resulting peptide fragment masses were submitted to searches against predicted peptide fragments of prokaryotes in the MASCOT data bank. This analysis revealed that the only significant match was IglB from *F. novicida* (Fig. 5B). The relative molecular mass of the co-precipitated protein is consistent with this result as IglB is predicted to be 58 kDa. Immunoprecipitations performed with an i**glA** null strain did not result in the appearance of the 60 kDa band, nor did immunoprecipitations of U112 done with pre-immune serum. These results strongly suggest that IglA and IglB interact in the cytoplasm of *F. novicida*.

Supporting the hypothesis that IglA interacts with IglB is the finding that IglB mutants but not IglC mutants lack detectable IglA (see below, Fig. 8). Presumably a lack of
association of IglA with IglB makes the former susceptible to degradation.

Deletion mutagenesis of iglA and complementation of the mutant strain

An iglA deletion mutant, ODB2, was constructed using a two-step integration-excision method (Fig. 6A). First, the PCR-amplified 1.5 kbp regions flanking iglA were joined so as to leave iglB intact, including its ribosome binding region. This recombinant construct was ligated to an erythromycin resistance-sacB cassette and the ligation mixture was used to chemically transform F. novicida JL0 to erythromycin resistance. The JL0 strain is a derivative of U112 that has a deletion in one of its putative sucrose hydrolase genes, and is thus sensitive to sucrose when sacB is expressed. This strain behaves like wild type in our virulence assays (data not shown). An erythromycin resistant colony was grown and plated on agar containing 10% sucrose which acts as a counter selective marker for the sacB gene. Sucrose sensitive strains were examined for loss of iglA by PCR (Fig. 6B). Attempts to genetically complement the ΔiglA strain by incorporating iglA into a F. tularensis plasmid pFNLT1 [26] failed, presumably because the over-expression of IgLA was lethal to F. novicida.

Figure 3

IglA expression in J774 macrophages. Western blot showing expression of IglA during infection of macrophages. J774 macrophages were infected with parent strain U112 (m.o.i 300:1) and lysed at the indicated time post infection. Loading was normalized according to the number of viable bacteria (CFU) in each sample as determined by plating on TSA-C plates. Lane J774, uninfected macrophages. TSB, broth grown U112 grown to indicated optical density (600 nm). All samples were normalized to 10^7 CFU by viable counts. The macrophage cell lysates altered the appearance of the IglA bands, but control experiments showed that the cell lysates did not mask IglA reactivity with antibody.

Figure 4

Subcellular localization of IglA. Anti-IglA was used to probe Western immunoblot of subcellular fractions of F. novicida. The sarkosyl insoluble fraction represents an enrichment of outer membrane protein and the sarkosyl soluble fraction contains largely inner membrane protein. Samples were prepared as outlined in Methods and normalized to 10 µg protein per lane before separation on a 12% SDS-PAGE gel. Results are representative of three independent experiments.

Figure 5

Co-immunoprecipitation of a 60 kDa protein with IglA. Panel A. Anti-IglA serum co-immunoprecipitates a circa 60 kDa soluble protein (arrow, lanes 1 and 4). The band is absent in control reactions with non-specific antibody (lane 3) and in immunoprecipitations with an iglA mutant (lane 2). Numbers shown indicate molecular mass standards. Results are representative of those of three experiments. Panel B. MALDI-TOF identified the 60 kDa protein as IglB. Underlined sequences indicate peptides identified by MALDI-TOF. The second and third regions each represent two peptides (break after the "R"). Of 25 queries submitted, 9 showed significant identity with rabbit heavy chain and 9 showed significant identity with IgB of F. novicida. No other significant hits were found in the MSDB 20060224 databank.
ida. Hence, an in cis complementation approach was devised, allowing iglA to be incorporated into the chromosome linked to a kanamycin resistance marker (Fig. 7A and 7B). The iglA deletion strain failed to produce IglA as determined by Western immunoblotting (Fig. 8). However, the ΔiglA strain retained expression of IglC at parental strain levels. In cis complementation of the ΔiglA strain resulted in a strain that regained partial expression of IglA. An insertion mutant of iglB gave a reduction in the amount of IglC that was made, and this is not surprising since many insertion mutation decrease the expression of downstream genes. Surprisingly, this same mutant lacked expression of IglA, suggesting that the co-expression of IglB is needed for expression of IglA or to prevent degradation of IglA. Disruption of iglC however, does not affect the amount of IglA detected (Fig. 8).

IglA is required for growth in the J774 macrophage cell line
Previous work has suggested that IglA is required for F. novicida intramacrophage growth and virulence; however, its role has never been unequivocally demonstrated. In order to assess the requirement for IglA expression in intramacrophage growth we used our defined deletion and complemented strains to infect a culture of the J774 macrophage cell line. The data shown in figure 9 illustrates that the ΔiglA strain is incapable of intramacrophage growth, as is the iglC negative strain, CG62. The ΔiglA strain that was complemented for IglA production partially regained its ability to grow in macrophages. The residual defect in intracellular growth is not unexpected since we showed that the expression of IglA was not at wild type levels. ΔiglA replicated as the parental strain in broth (data not shown).

The ΔiglA strain has lowered virulence in chicken embryos
When the ΔiglA strain was used to infect chicken embryos it caused low mortality when compared to wild type F. novicida (Fig. 10). The wild type strain of F. novicida caused 100% mortality at day 5 post infection at an infecting dose of 600 CFU, whereas the ΔiglA strain caused only 14% mortality at day 6 with an infecting dose of 4,500 CFU (Fig. 10) or 50% mortality at day 6 with an infecting dose of 45,000 CFU (data not shown).

Discussion
There is growing evidence that the iglABCD operon is needed for F. tularensis intracellular growth and virulence and that the MglAB proteins are involved in regulating the expression of iglABCD. However, there is very little genetic and corresponding biochemical data demonstrating the roles of MglAB and IglAB and their corresponding homologues in other bacteria. For example, while it is clear that MglA plays a role in regulating the amount of iglABCD transcript it is unclear if the role precisely corresponds to that of the E. coli SspA protein. The data that exists for the functioning of SspA suggest that much of the regulation of stationary phase proteins occurs indirectly via the repression of H-NS, and that some of the effect of SspA is post-transcriptional [14].

There is also growing evidence that proteins encoded by IAHP clusters, of which IglAB homologues are important
components, are involved in secretion of proteins from gram-negative bacteria [24,27]. There are approximately 30 homologues of *iglAB* and in every case the two genes are adjacent to each other and arranged in the same gene order. In this work we provided biochemical evidence that the IgLAB proteins physically associate with each other and are localized to the cytoplasm. The surprising finding that inactivation of the *iglB* gene results in the disappearance of the IgLA protein suggest that the presence of IgLB is required for IgLA to be stable.

IgLA was first identified as a locus that when inactivated by a transposon insertion rendered *F. novicida* defective for growth in macrophages [17]. However, it could not be ruled out that the effect was due to interruption of transcription of downstream genes. In this report, we provide strong evidence that IgLA is necessary for intracellular growth as a non-polar *iglA* deletion mutant was defective for growth in a mouse macrophage-like cell line. In *cis* complementation of the ΔiglA strain restored intramacrophage growth although the growth was slower than in the wild type strain. The *in cis* complementation strategy created two *iglA* promoter regions on the chromosome, one on either side of a kanamycin resistance cassette. It is conceivable that this results in aberrant regulation of *iglA* expression, which could explain why the growth of the complementation strain lags early during infection. We were unable to complement the *iglA* deletion mutant *in trans* with pFNLTP1::*iglA*, a high copy-derivative of an endogenous *Francisella* plasmid. Presumably, over-expression of IgLA was lethal to *F. novicida*.

We hypothesize that IgLA and IgLB are cytoplasmic, chaperone-like proteins that are involved in secretion of viru-
lence factors. Therefore, the biological significance of IglAB interaction may be to secrete *Francisella* effector molecules. In other pathogens, secretion of virulence proteins often requires interaction between two cytoplasmic proteins. For example, in *Yersinia pestis*, a complex composed of SycN and YscB function as chaperones for YopN [28], which is secreted to the cell surface [29]. Also, interaction of IcmS and IcmW is required for translocation of effector proteins via the Dot/Icm complex during *Legionella pneumophila* intracellular growth [30,31]. Hager et al. recently demonstrated protein secretion by *F. novicida* [32]. We did not observe any difference in secreted peptides between broth-grown wild type *F. novicida* and the ΔiglA strain by SDS-PAGE electrophoresis (data not shown). This observation is not surprising given the fact it has been demonstrated that secretion involving IAHPs is a highly regulated or an in vivo-induced process [27].

In summary, our results suggest that IgLA and IgLB are interacting cytoplasmic proteins that are required for intramacrophage growth. The significance of the interaction may be to secrete effector molecules that affect host cell processes.

**Conclusion**

The *Francisella* Pathogenicity Island harbors uncharacterized genes implicated in virulence. By constructing an in-frame deletion mutant we have shown that the FPI gene *iglA* is needed for intramacrophage growth. Biochemical characterization of IgLA strongly suggests that it is a cytoplasmic protein that interacts physically with IgLB. In addition, we provide data that show IgLA is induced during infection of macrophages. Bioinformatics analysis of the FPI suggests that it is similar to virulence loci that encode a protein secretion apparatus. We propose that IgLA and IgLB are chaperone-like proteins that are part of a secretion system in *F. novicida*.

**Methods**

**Bacterial strains and culture conditions**

All strains used in this work are listed in Table 1. *F. novicida* strains were grown in trypticase soy broth supplemented with 0.1% cysteine (TSBC) or on trypticase soy agar supplemented with 0.1% cysteine (TSAC) unless stated otherwise. Kanamycin (45 μg/ml) or erythromycin (30 μg/ml) or 10% sucrose were added as needed.

**Subcellular fractionation**

1000 ml of overnight *F. novicida* U112 culture was harvested and resuspended in 50 ml of cold phosphate buff-
Table 1: Strains and plasmids used in study.

| Name     | Phenotype/Relevant Characteristics                           | Reference or Source       |
|----------|--------------------------------------------------------------|---------------------------|
| U112     | Francisella novicida prototype strain.                      | ATCC                      |
| JL0      | U112, α-sucrase hydrolase strain used to make deletion mutants. | Laboratory strain         |
| ODB2     | JL0, ΔiglA                                                   | This study                |
| ODB7     | U112, iglA::EmR                                              | This study                |
| ODB1     | U112ΔiglA                                                   | This study                |
| ODB5     | ΔiglA::KmR, in cis complementation of iglA in strain ODB2   | Gray et al. (2002)        |
| CG62     | U112, iglC::TrmMax2                                          | Baron et al. (1998)       |
| GB2      | U112, mglA                                                  | Invitrogen                |
| GB6      | U112, mglA::mTn 10Km                                         | Invitrogen                |
| DH5α     | F-Φ80dlocZΔM15ΔiglZYA-argF)U169 recA1 endA1 hsdR17(rK -, mK +) phoA supE44 thi-1 gyr A96 relA1 λ- |                     |
| pCR2.1   | Cloning vector, AmpK KmR                                    |                           |

NaCl, 10 mM Na₂H₃PO₄, pH 7.2. After the final wash, complexes were resuspended in 30 μl SDS-PAGE loading buffer and the sample was boiled for 5 min. Beads were removed by centrifugation and released proteins were separated on a 12% Sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) gel. The immunoprecipitated material was examined by immunoblotting with anti-IglA to confirm that IglA was present (data not shown).

**SDS-PAGE and Western blotting**

To normalize the amount of protein added to each lane, the concentration of protein samples were determined by use of the BCA assay (Pierce). SDS-PAGE was performed according to standard techniques. Separated proteins were transferred onto a Trans Blot nitrocellulose (BioRad) or Immobilon-FL (Millipore) membrane and blocked with 5% skim milk (Difco) in PBS. Anti-IglA, and anti-IglC antibody were used at dilutions of 1:4,000 and 1:500 respectively. To detect bound antibody blots were incubated with IRDye800DX-conjugated goat anti-rabbit or IRDye700DX-conjugated goat anti-rat immunoglobulin G (Rockland, Gilbertsville, Pa.) and visualized in a LiCor Odyssey imaging system.

**MALDI-TOF**

Following SDS-PAGE separation of proteins in-gel digestion with trypsin was carried out, and peptides extracted. 10 μl of the peptide sample was loaded on to a C18 zip tip and washed three times in 10 μl of 0.1% TFA and eluted with 2 μl of 50% ACN and 0.1% TFA containing 10 mg/ml 4-hydroxy alpha cyanocinnamic acid. MALDI-TOF MS analysis of the peptides was carried out using a Voyager-DE STR (Applied Biosystems, Foster City, CA). Mass fingerprint analysis was done using Mascot (Matrix Science, UK).

**Construction of iglA deletion mutant**

IglA deletion mutant, ODB2, was constructed using a two-step integration-excision method. 1.5 kilobasepair (kbp) regions flanking iglA were amplified with primers iglA L-F.
5’ cgcggccgaagagaattcttgtggtt, iglA L-R 5’ cctcagacag-
cacccagatgtgggtggtt, for the left-hand flanking region, and
with primers iglA R-F 5’ cctcagatctgtggttctggtt, iglA
R-R 5’ cgcggccgaagagaattcttgtggtt, for the right-hand
flanking regions. These were cloned into plasmid pCR2.1
(Invitrogen) and verified by sequencing. The flanking
regions were then joined by ligation. The flanking region
construct was ligated to an erythromycin resistance-sacB
cassette and the ligation mixture was used to chemically
transform F. novicida JL0 to erythromycin resistance as
previously described [35]. The JL0 strain (Ludu et al.,
unpublished data) is a derivative of the F. novicida U112
prototype strain that has a deletion in a sucrose hydrolase
gene, and thus is sensitive to sacB expression in the pre-
esence of sucrose. An erythromycin resistant colony was
grown and plated on TSAC containing 10% sucrose which
acts as a counter selective marker for the sacB gene.
Sucrose sensitive strains were examined for loss of iglA by
PCR.

The iglA and iglB allelic replacement mutants, ODB7 and
OBD1, were constructed as previously described [12].
Briefly, 1.5 kbp regions flanking iglB were PCR amplified
with primers iglB L-F 5’ cgcggccgaagagaattcttgtggtt,
iglB L-R 5’ cctcagagattctgtggtt, iglB R-F 5’ cctcagatctgtggttctggtt,
iglB R-R 5’ cgcggccgaagagaattcttgtggtt, iglB
regions were then joined by ligation. The flanking
region of the sucrose hydrolase gene in
F. novicida
was cloned into a phagolysosome upon activation by IFN-gamma.

In cis complementation
IglA and its promoter region were amplified with primers
iglA int-L 5’ CCCCTCGAGACGGCTTTTCAATATTGGTTT
and iglA int-R 5’ CCCCTCGAGACCTCTGAGATGATC-
CCCCAAA incorporating added XhoI sites and ligated to a
kanamycin resistance cassette carrying a F. novicida
promoter (Ludu et al., unpublished data). The construct was
used to transform ODB2 as previously described [35]. The same primers
used for construction of ODB2 were used for ODB7.

Macrophage infection assay
Macrophage infection assays were performed essentially
as described previously [2]. Briefly, 1774.1 mouse macro-
phage-like cells were infected with F. novicida strains at
a multiplicity of infection of 50:1 (bacterium-to-macro-
phage), and monolayers were incubated for 2 h in Dul-
becco’s Modified Eagle Medium containing 10% fetal
bovine serum (DMEM), washed five times in Dulbecco’s
Phosphate Buffered Saline (DPBS), and incubated at
37 °C in 5% CO2. Macrophages were lysed in 0.1% deox-
ycholate at 0, 24, 48 and 72 h post infection. To determine
bacterial growth, lysed macrophages and culture superna-
tants were serially diluted in DPBS and plated on TSAC. As
F. novicida does not grow in DMEM, this allows for an ade-
quate determination of intracellular growth [2].

Chicken embryo infections
Fertilized White Leghorn eggs were obtained from the
University of Alberta Poultry Research Station. Seven-day
old embryos were injected under the chorioallantoic
membrane with various doses of 100 μl of F. novicida
diluted in PBS as previously described [36]. The embryos
were monitored for death for 6 days.

Authors’ contributions
OMB performed all experiments, constructed iglA and iglB
mutants and drafted the manuscript. JSL constructed the
deletion of the sucrose hydrolase gene in F. novicida. FEN
was the principal investigator and supervised the project.
All authors read and approved the final manuscript.

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