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Identification of a unique gene cluster of *Brucella* spp. that mediates adhesion to host cells

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Abstract

Brucella, the causative agent of brucellosis, a major zoonotic disease affecting a broad range of mammals, is a gram negative bacterium whose virulence is dependent on the capacity to attach and invade different cells of the host. The bacterium is able to infect through a diverse repertoire of epitheliums: skin, airways or gastric. Although much has been studied on the mechanisms *Brucella* uses to establish an intracellular replication niche, almost none is known on how the bacterium adheres and invades host cells. We report here the identification of a pathogenicity island that harbors a gene homologous to proteins with bacterial immunoglobulin-like domains present in other pathogens that play a role in attachment and invasion. Deletion of the entire island results in a mutant with a reduced attachment capacity measured by intracellular replication and adhesion assays. Intraperitoneal and oral experimental infection of mice strongly suggests that this island plays a role during the oral infection probably mediating attachment and trespassing of the gastric epithelium to establish a systemic infection.

Keywords

BRUCELLA; ADHESION; VIRULENCE

1. Introduction

Adhesion of pathogens to host tissues is one of the initial and crucial steps in the process of infection and, as such, an important and complex virulence trait. Adherence determines host specificity, point of entry into the host, tissue and cellular tropism and even establishment of the replicative niche. Bacteria have the ability to selectively adhere and invade specific host cells through the presence of surface adhesins that specifically recognize host cell receptors and mediate entrance. The immense variety and redundancy of bacterial receptors that facilitate attachment and invasion in different pathogens highlights the importance of this step during the infectious process. Pathogens have evolved different strategies to penetrate the host depending on the life cycle or, more specifically, the point and mechanism of entry. This repertoire is extensive and includes different types of adhesins that recognize specific host cell receptors, secretion systems that mediate internalization and bacterial surface structures (lipids or polysaccharides) that mediate attachment [1]. *Brucella* spp. are Gram-

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negative facultative intracellular bacteria that cause brucellosis, a worldwide-distributed zoonosis affecting a broad range of mammals, ranging from dolphins and domestic animals to humans. Brucellosis remains endemic in many developing countries, where it causes important economic losses [2]. In humans, brucellosis is a serious debilitating disease that can have a fatal outcome. The disease is characterized by diverse pathological manifestations such as oscillating fever, osteoarticular complications, endocarditis and several neurological disorders [3]. In domestic animals such as cattle, goats and sheep, the outstanding manifestation of the pathology is miscarriage in pregnant females and sterility in males, as a result of bacterial colonization of the placenta, fetal tissues and sexual organs [2]. Infection depends on contact with infected animals or their products. After invasion of the host, the bacteria survive within mononuclear phagocytes and the infected monocytes (or macrophages) play a crucial role in the dissemination of the bacteria to specific locations of the body (spleen, brain, heart, and bones) leading in many cases to a chronic infection.

Although a great load of knowledge has been accumulated over the past years on how *Brucella* evades the host response and establishes a secure niche for bacterial replication [4, 5], very little is known about the microbial determinants that participate in the early steps of infection (adhesion to tissues and penetration of epithelial barriers for example). Even though it is well established that *Brucella* is able to infect hosts through the mucosa such as the respiratory airways, abrasions in the skin or the gastrointestinal tract; no virulence factors directly involved in the penetration of these barriers have been identified to date. Additionally, few virulence factors involved in adhesion and invasion of host cells have been reported in *Brucella*. Lipopolysaccharide (LPS) is one of the most studied virulence factors and its absence significantly affects adhesion and invasion of host cells mainly because LPS deficient strains (rough strains) have an altered bacterial surface [6, 7]. The mutations in these cases are highly pleiotropic and impact a broad range of surface molecules, thus turning complicated to determine the exact nature of the adhesion/invasion defect. Mutants in the BvrR/BvrS double component system also display an invasion defect. As with the LPS, mutants in this system are highly pleiotropic and affect a broad range of surface proteins [8]. To date only one protein has been identified as an adhesin involved in the adherence to epithelial cells in *Brucella* [9]. SP41 was demonstrated to be involved in adhesion and invasion of epithelial cells by antibody inhibition and infection assays using null mutants [9]. The gene coding for SP41 is the *ugpB*, and codes for a periplasmic glycerol-3-phosphate binding ATP-binding cassette (ABC) transporter present in several pathogenic and non-pathogenic bacteria and localized in the outer membrane. In this report the authors do not rule out if this protein is necessary in an animal model of infection and, thus, the importance of it in virulence is not discussed. Recently a gene present in all *Brucella* species was informed to be critical for internalization in HeLa cells [10]. This open reading frame (BMEI0216 in *B. melitensis*) is also present in several other bacteria and is an 84 amino acid protein annotated as a transglycosylase associated protein. The results demonstrate that the mutant displays a severe invasion deficiency at 1 and 2 hours post-infection [10] although the paper does not rule out if the defect is at the level of adhesion or invasion. As with the other report, these authors do not determine the role of this gene in virulence in an animal model.

The present report identifies a chromosomal region present in all *Brucella* species that has a G+C content significantly reduced compared to the rest of the chromosome. This characteristic is one of the signature tags in horizontally transmitted genomic regions and, thus, may harbor putative virulence genes. The data demonstrate that this region encodes an adhesin important for attachment and invasion of host cells and that it plays a role in infection through the oral route.

2. Material and methods

2.1. Bacterial strains, media and culture conditions

Brucella strains used in this report were derived from the wild type 2308 biovar. All strains were grown at 37°C in Tryptic Soy Agar (TSA; Difco/Becton-Dickinson, Sparks, MD) or in Tryptic Soy Broth (TSB) in an orbital shaker at 250 rpm. *Escherichia coli* strains were grown at 37°C in Luria-Bertani broth.

When necessary, media were supplemented with the appropriate antibiotics at the following concentrations: Ampicillin, 100 g/ml; Kanamycin, 50 g/ml; Nalidixic, 5g/ml. Manipulation of *B. abortus* was performed in a Biosafety Level 3 facility at the University of San Martín, Buenos Aires.

2.2. Cloning, gene disruption, and generation of mutant strains

2.2.1. Construction of plasmid pSM12—A DNA fragment of 5200 bp containing the genes of *B. abortus* 2308 (Bab1_2009/2010/2011/2012) was amplified from genomic DNA using primers MDG06 (5'-CGAGCTCTACCGGAGGAGATGGTG-3') and MDG07 (5'-CCGCTCGAGCACTCGGCAGTGGGTCTGTT 3') digested with *SacI* and *XhoI* and ligated into pBBR1-MCS4 [11].

2.2.2. Construction of plasmid pSM14—In order to delete region Bab1_2009–2012, two PCR fragments were generated from regions flanking the island. Oligonucleotides CC19 (5'-CCGGAATTC CACTCGGCAGTGGGTCTGTT-3') and CC20 (5'-TTTACCTCTAATATAATTA-3') were used to amplify 500 bp of the upstream region and CC21 (5'-ATATTAGAGGGTAAAGGCCGTCTTATACTATTTCAGA -3') and CC22 were used to amplify 500 bp of the downstream region. Both fragments, containing complementary regions, were ligated by overlapping PCR using oligonucleotides CC19 and CC22. The resulting fragment was digested with *EcoRI* and *BamHI* and cloned into the pK18mobSacB plasmid [12].

2.2.3. Construction of strain SM001—Plasmid pSM14 was transferred to *B. abortus* 2308 by biparental mating and Kanamycin resistant colonies were selected. These clones are the result of a single-homologous recombination and thus harbor the *sacB* gene. Selection with sucrose, excision of plasmids, and generation of deletion mutants were performed as described previously [13]. Double recombination events were identified by sensitivity to kanamycin, and the clean deletion clones were identified by colony PCR using primers CC19 and CC22.

2.3. Cell infection assays

Intracellular replication was evaluated in HeLa and J774 cells using the antibiotic protection assay [7]. Briefly, cells were seeded in 24-well plates in minimal essential medium (Gibco) for HeLa and RPMI 1640 (Gibco) for J774, supplemented with 10% fetal bovine serum and 2 mM L-glutamine without antibiotics (complete culture medium) at 10⁵ cells per ml. *Brucella* strains and mutants were grown in liquid medium for 24 h and resuspended in cell media. The suspension was added to the cells at a multiplicity of infection ranging from 100 to 500 depending on the experiment, and centrifuged at 1000 rpm for 10 min. After 1 h of incubation at 37°C 5% CO₂, fresh complete culture medium with 50 µg of gentamicin per ml and 100 µg of streptomycin per ml was added to the monolayer to eliminate extracellular bacteria. At 4, 24, and 48 hours post-infection, the monolayers were washed three times with phosphate-buffered saline (pH 7.4) and lysed with 0.1% Triton X-100 in deionized water. The Triton lysates were then diluted serially and plated on TSB agar with the appropriate antibiotics to determine the number of CFU recovered per milliliter.

2.4. Immunofluorescence microscopy

For immunofluorescence, the primary antibodies used were a rabbit anti-*Brucella* polyclonal antibody (dilution 1:1,500) or mouse anti-M84 monoclonal antibody (dilution 1:1,000) [14]. The secondary antibodies used were goat anti-mouse or goat anti-rabbit Alexa Fluor 568 or 488 (Molecular Probes, Invitrogen Co.) at a 1:4,000 dilution. For DNA staining, 40,6-diamidino-2-phenylindole DAPI dye at 0.5 mg/ml (final concentration) was used.

HeLa or J774 cells were plated on glass coverslips (5×10^4 cells per coverslip) and infected as described above. At different time points after infection, the cells were washed five times with PBS to remove non-adhered bacteria and fixed for 15 min in 4% paraformaldehyde (pH 7.4) at room temperature and then processed for immunofluorescence labeling. Coverslips were washed three times with PBS, incubated for 10 min with PBS added with 50 mM NH_4Cl in order to quench free aldehyde groups. Coverslips were then incubated with primary antibodies in a PBS 10% horse serum 0.1% saponin solution for 40 min at room temperature, washed in PBS containing 0.1% saponin and then incubated with secondary antibodies in a PBS 10% horse serum 0.1% saponin solution. The coverslips were mounted onto glass slides using FluorSave Reagent (Calbiochem). Cells were observed on the microscope (Nikon-Eclipse T2000) using a 100x oil immersion objective. Projections were saved in TIFF format and imported to ADOBE PHOTOSHOP CS where images were merged using RGB format.

2.5. Adhesion and invasion assays

To determine adhesion and invasion infected HeLa cells were fixed for 15 min in 4% paraformaldehyde (pH 7.4) at room temperature at different time points post-infection. Coverslips were washed three times with PBS, incubated for 10 min with PBS added with 50 mM NH_4Cl and before permeabilization incubated with the primary antibody rabbit anti-*Brucella* polyclonal antibody (dilution 1:1,500) in a PBS 10% horse serum solution. Then cells were washed and incubated with the other primary antibody mouse anti-M84 (anti O antigen) monoclonal antibody (dilution 1:1,000) in a PBS 10% horse serum 0.1% saponin solution and then incubated with secondary antibodies (Alexa Fluor 568 or 488, Molecular Probes, Invitrogen Co) in a PBS, 10% horse serum, 0.1% saponin solution. The coverslips were mounted as described before. Invasion was determined as the number of bacteria positive for both labels versus the ones positive for the anti-rabbit labeling. Adhesion was determined counting the number of bacteria associated per 100 HeLa cells.

2.6. Virulence in mice

Virulence was determined quantifying the survival of the strains in the spleens after 7, 15 or 21 days post-infection as previously described [7, 15]. Groups of five or ten nine-week-old female BALB/c mice were injected intraperitoneally or orally with 5×10^4 or 10^9 CFU respectively of *B. abortus* wild type or mutant strains in 0.2 ml of sterile PBS (in the case of the oral infection by gavage). At each time point analyzed animals were euthanized, the spleens removed, and homogenized in 2ml of PBS. Tissue homogenates were serially diluted with phosphate-buffered saline and plated on TSB agar with the appropriate antibiotics to determine the number of CFU per spleen.

3. Results

3.1. Identification of a chromosomal region with an extremely low G+C content

In order to identify novel virulence factors in *Brucella abortus* we searched the genome for regions that deviate from the average G+C content with the premise that the acquisition of horizontally transmitted regions that have occurred recently in evolution have not homogenized with the rest of the genome and, hence, have a significant difference in the G

+C content compared with the rest of the genome. Additionally, in many cases, horizontally transmitted genetic information is involved in virulence processes. Using the program ARTEMIS [16] we identified 12 regions that deviate from the average G+C content plus or minus 2.5 standard deviation errors in the *Brucella abortus* 2308 genome [17]. Once these regions were identified we analyzed each one of them individually identifying the open reading frames and determining for each, conservation among *Brucella* species, presence in other bacteria and domain structure. Using these parameters we identified a cluster of genes, designated Bab1_2009–2012 that harbors four open reading frames only present in the *Brucella* genus and that has a G+C content of 40%, significantly lower than the average 57% of the rest of the genome (Fig. 1). None of the four open reading frames located in this region, have a significant homology to any protein in the database and are conserved in all the *Brucella* species sequenced to date. One of these genes, Bab1_2009, has a bacterial immunoglobulin like domain (BID_1 domain) present in several phage and bacterial adhesins including the intimin of the enterohemorrhagic *Escherichia coli* [18] that mediates the intimate bacterial-host cell interaction. Additionally, during a genetic screen that we performed to identify N-terminal sequences that mediate secretion, we isolated this gene as a potential surface exposed or secreted polypeptide [19]. Bab1_2010 codes for an open reading frame of 359 amino acids, is immediately upstream of Bab1_2009 probably forming part of the same cistron, is present only in the *Brucella* genus and has no detectable homology to any protein in database. Analysis of Bab1_2010 using several domain identification and structural databases showed no detectable homology to any known protein even using low threshold parameters. Bab1_2011 and Bab1_2012 code for 129 and 518 amino acid proteins respectively, are also only present in the genus *Brucella*, and have no homology to any proteins known so far. Like Bab1_2010, analyses using several domain identification and structural databases showed that, neither Bab1_2011 nor Bab1_2012, have conserved domains or structural features that could allow speculation on their function. Bab1_2011 has a predicted signal peptide indicative of a putative periplasmic localization.

3.2. The genomic region Bab1_2009–2012 is involved in the internalization into host cells

Because the putative protein product of Bab1_2009 has a domain homologous to the immunoglobulin-like domain of the attaching protein intimin of enterohemorrhagic *E. coli*, we hypothesized that this genomic region could be involved in adhesion and invasion of host cells in *B. abortus*. To test this we constructed a deletion mutant lacking the whole genomic region, which we named SM001, and analyzed intracellular replication in HeLa, a non-professional phagocytic cell line and in J774, a mouse macrophagic cell line. As can be observed in Fig. 2 panel A, deletion of the entire genomic region, significantly reduced the bacterial load at 4 hrs post-infection (more than 10 times). Even though there was a significant reduction during the initial hours of infection, the bacteria that entered the cells were able to replicate efficiently and the bacterial load at 48 hrs post-infection was not significantly less than that of the parental strain. This indicates that the mutant strain, once inside, is able to multiply intracellularly at the same rate or faster than the wild type. To test if this phenotype was restricted to HeLa cells we performed the same experiment with the macrophagic cell line J774. As can be observed in Fig. 2 panel B, the mutant strain showed the same phenotype that in HeLa cells. These findings indicate that this genomic region encodes a protein involved in the adhesion or the internalization process.

3.3. The genomic region Bab1_2009–2012 is involved in the attachment to host cells

The defect observed at 4 hrs post-infection in the intracellular replication assays could be due to a diminished adhesion or a reduced invasion. These two properties are completely different; adhesion is the initial process in which the bacteria recognize surface receptors and attach to the cell, and invasion is the active process by which the bacteria are able to reach an intracellular location. To distinguish between these two possibilities we infected

HeLa cells with a multiplicity of infection of 500:1 and, at 4 hrs post-infection, fixed the samples and performed an in/out staining with a rabbit polyclonal anti-*Brucella* and a monoclonal anti-O-antigen (M84). For this experiment we scored the number of bacteria associated with a cell and the percent of internalized bacteria. Fig. 3, panels A and B shows the results of these assays. As can be observed in panel A, deletion of the island significantly reduced the number of internalized bacteria and this defect is probably the consequence of a reduced adhesion capacity of the mutant since, when we scored the amount of bacteria associated per cell (attachment), it also showed a significant reduction (panel B). Interestingly, although plasmid pSM12 complemented the invasion phenotype to levels similar to the wild type ones, it significantly increased attachment compared to the parental strain. These findings indicate that this genomic region encodes an adhesin important for attachment to host cells rather than a factor involved in the internalization process (invasion).

3.4. The genomic region Bab1_2009–2012 participates in the oral route of infection

The fact that this genomic region participates in the adhesion to host cells, prompted us to evaluate the role it might play in the virulence process. To test this, we intraperitoneally infected groups of five mice with 5×10^4 CFU of *B. abortus* wild type 2308, the SM001 strain or the SM001 strain complemented with a plasmid coding for the entire genomic region. 21 days post-infection, mice were euthanized and the bacterial load in spleens determined by direct CFU count. As shown in Fig. 4 panel A, no significant differences were observed between the parental and the mutant strain by this route of infection. Because this genomic region encodes an adhesin important for attachment to host cells we speculated that it might play a role in the adhesion and penetration of epithelial barriers during the infectious cycle. One of such barriers is the gastric epithelium that plays an important role in the oral route of infection. To test if this region is involved in this route of infection we inoculated, orally, groups of 8 or 10 mice with 10^9 CFUs with the wild type 2308, the SM001 or the complemented strain. 7 and 21 days post-infection the bacterial load in spleens was determined as indicated in materials and methods. Comparison of the bacterial colonization capacity with this type of infection measures the capacity of the strain to trespass the gastric barrier and to establish a systemic infection. As shown in Fig. 4 panel B, the mutant strain displayed a significant reduction in the capacity to colonize the spleen when orally administered. The complemented strain did not show a significant increase compared to the mutant although there was a clear tendency that suggested a partial complementation (not shown). This is not surprising as it has been reported that complementation of virulence phenotypes in the animal model are often difficult to achieve due to the lack of selective pressure (i.e. antibiotic presence) in the mouse. These results demonstrate that the genomic region Bab1_2009–2012 is involved in the infection via the oral route, probably mediating bacterial attachment to the gastric epithelium and facilitating its trespassing.

4. Discussion

Virulence is the consequence of a complex series of phases that pathogens must overcome in order to establish a successful infection. One of the early stages of the infectious process is penetration of the host and attachment to tissues that will, ultimately, lead to invasion of specific cell types that varies depending on the biology of the pathogen. Because of this, adhesion or attachment to host cells is one of the bottleneck stages in an infectious process and most pathogens have evolved redundant strategies to achieve it. Many bacterial pathogens have adhesive pillus that mediate an intimate interaction with the host cell and, in many cases, are important for an efficient invasion [1] and mediating attachment to specific tissues where these bacteria proliferate and reside. Regardless of the pathogens lifestyle the molecules that mediate attachment to cells and tissues play central roles in the virulence

process and their identification and characterization are essential to develop preventive and/or therapeutic strategies.

Brucella abortus, the causative agent of bovine brucellosis, is a pathogenic bacterium whose virulence depends on the capacity to establish an intracellular replication niche [20]. To date, very little is known on how *Brucella* enters the hosts and reaches the tissues where it will actively replicate. It is accepted that bacteria penetrate the host through abrasions in the skin or by trespassing the mucosal barriers such as the respiratory or gastric ones. Even though this fact has been widely known for some time, there is no virulence factor identified, to date, that participate in this process. Attachment of the bacterium to the tissues through which it enters is an essential step in the process of infection and the pathogen must have surface molecules involved in these early stages.

To date absolutely nothing is known on how *Brucella* crosses epithelial barriers in order to reach dendritic cells or macrophages where it will, ultimately, establish an infectious niche. The normal routes of infection of *Brucella* necessarily implicate that the pathogen must overcome one or more epitheliums to reach its final destination and, to achieve this; it must be able to attach to these tissues. Due to the fact that the pathogen is able to access the host through a wide range of points of entry, it is valid to speculate that it must code for a plethora of virulence factors devoted to mediate invasion depending on what tissue it will encounter. To date only two reports have identified *Brucella* genes directly involved in the adhesion and/or invasion of host cells [9, 10] and one has described that sialylated residues present in these cells are important in the attachment process [21].

In this report we identify in *Brucella abortus* a pathogenicity island only present in the *Brucella* genus, which codes for an adhesin that mediates attachment and invasion to the host cell and, as a consequence, enhances invasion. This pathogenicity island encodes four open reading frames with no homology to any known proteins and has a G+C content significantly lower than the rest of the genome, indicative of probable horizontal transfer. One of these genes, Bab1_2009, codes for a protein with a bacterial immunoglobulin-like domain also present in the intimin of enterohemorrhagic *E. coli* and other invasins, that mediate adhesion to host cells. Deletion of the entire region resulted in a mutant with a significant reduction in the invasion and adhesive capacity indicating that the island plays a role during the attachment and invasion phases. The fact that a mutant shows a phenotype in an *in vitro* model (cell culture) does not necessarily mean that it will have a virulence defect in an animal model. To evaluate if the pathogenicity island plays a role in virulence, we initially evaluated the performance of the mutant strain when administered intraperitoneally in mice and observed no differences compared to the wild type. Intraperitoneal inoculation of a pathogen represents an acute model of infection where the bacteria do not have to trespass any epithelial barriers in order to reach the target cells where they will establish an infectious niche (in the case of *Brucella* immune cells of the reticuloendothelial system). For this reason, and the fact that the region is involved in invasion/attachment, we reasoned that maybe they help in the adhesion to the gastric epithelial and participate in crossing it. To evaluate this, we orally infected mice and determined the bacterial load in the spleen as a way of measuring the capacity of the mutant to establish a systemic infection. Our results demonstrate that the mutant is less efficient in establishing a successful systemic infection when orally administered thus strongly suggesting that the island plays an important role in crossing the gastric epithelial barrier. The results presented here cannot rule out if the defect is due to a diminished capacity to trespass this barrier or if the mutant persists less in the gut (because of a reduced tissue adhesion) and, thus, the efficiency is reduced due to a lower bacterial load over time. Future research, including the identification of the receptor of this adhesin, will be needed to discern between these two possibilities.

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A

% G+C

57% →

38% →

B

<i>B. abortus</i>	2	VLKLVKVDKDNALSNGTDFDLVTATLTRDGGQKFVGGVTFSLPSNDAAVFETEQTKKLSA	61
		+ ++K DK A++NG D T + ++G Q Q VTFS +N QT+ A	
<i>E. coli</i>	4	ITEIKADKTTAVANGKDAIKYTVKVMKNG-QPVNNQSVTFSS--TNFGMFNGKSQTQ---A	57
<i>B. abortus</i>	62	TTGNDGTATAIIIRNAAGGGTVTVTCVLDADGKVSD	97
		TTGNDG AT + ++AG TV+ T A+ K ++	
<i>E. coli</i>	58	TTGNDGRATITLTSSSAGKATVSATVSDGAEVKATE	93

Fig. 1.

A. Schematic representation of the chromosomal region encoding genes Bab1_2009 to Bab1_2012 only present in the *Brucella* genus as shown with the Artemis free genome viewer (<http://www.sanger.ac.uk/resources/software/artemis/>). The G+C content of the island compared to the neighboring regions is shown on the top line. **B.** Protein alignment of the bacterial immunoglobulin-like domains of *B. abortus* Bab1_2009 with the intimin of *E. coli*.

Fig. 2.

Deletion of the Bab1_2009–2012 genomic region reduces invasion of non-professional and professional phagocytes. **A.** Intracellular replication of wild type, deletion of the genomic island (SM001) and complemented strains in HeLa cells determined by the antibiotic protection assay (see material and methods). **B.** Intracellular replication of wild type, deletion of the genomic island (SM001) and complemented strains in J774 cells determined by the antibiotic protection assay (see material and methods).

Fig. 3.

Deletion of the Bab1_2009–2012 genomic region significantly reduces attachment and invasion of non-professional phagocytes. **A.** Internalization capacity of the wild type, SM001, and complemented strains in HeLa cells. The number of internalized bacteria was determined by an in/out staining at 4 hours post-infection as described in materials and methods. **B.** Attachment of the wild type, the deletion and the complemented strains to HeLa cells. The number of bacteria associated per 100 cells was determined by staining with a monoclonal anti O-antigen at 4 hours post-infection as described in materials and methods. All assays were performed at a multiplicity of infection of 1:500. * $P < 0.005$ and ** $P < 0.02$, significantly different compared to the wild type strain.

Fig. 4. Virulence in the mouse model. **A.** Intraperitoneal infection of groups of five nine-weeks old Balb/c female mice with 105 CFU of wild type and island deleted strains. Bacterial load in the spleens at 21 days post-infection was determined as described in materials and methods. **B.** Bacterial load in the spleens of orally infected nine-weeks old Balb/c female mice with 109 CFU of wild type and island-deleted strains at 7 and 21 days post-infection. * and ** Significantly different: * $P=0.056$ and ** $P=0.023$.