



Immunogenic response of *Brucella canis* *virB10* and *virB11* mutants in a murine model

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The *virB* locus, which encodes the type IV secretion system, is a major component of virulence in *Brucella*. A non-polar *virB10* mutant and a *virB11* deletion mutant were constructed in *Brucella canis*. In the mouse model, both mutants were cleared at day 21 post-infection, indicating reduced virulence in mice. After challenging with wild-type *B. canis*, the amounts of CFU recovered at day 15 were significantly lower in the group previously vaccinated with the *virB10* mutant. Levels of IgG1, IgG2a, IgG2b, and IgM, the induction of the cytokines IL-2, IL-4, IL-10, and the production of IFN- γ were measured in lymphocyte cultures. All strains elicited similar levels of different antibody isotype profiles, and no significant differences were detected ($P < 0.05$). The wild-type strain induced a rapid and strong INF- γ response at 24 h, while both mutants induced mild INF- γ responses at 24 h, which remained constant over the course of sampling. Our results suggest that the *virB* mutants elicit a protective immunity and may be considered as candidates for studies to be conducted in dogs against canine brucellosis.

Keywords: *Brucella canis*, *virB* mutants, mouse model

INTRODUCTION

The *Brucella* genus consists of Gram-negative, facultative intracellular microorganisms that cause disease in diverse animal species, including humans (Sieira et al., 2000). *Brucella* species can survive within professional and non-professional phagocytes, thus avoiding the host defense mechanisms. Host resistance to facultative intracellular pathogens such as *Brucella* depends mainly on the induction of cell-mediated immunity (Golding et al., 2001; Baldwin and Parent, 2002; Baldwin and Goenka, 2006). The capacity of antigens to selectively induce a Th1 or Th2 response is an important aspect in the development of vaccines against intracellular *Brucella* pathogens (Zhan et al., 1996). Studies performed in the murine model show that live attenuated vaccines induce a cellular response characterized by the production of IFN- γ and IL-2 (Zhan et al., 1995). In the case of canine brucellosis, no vaccine is available; thus, studies concerning *Brucella canis* virulence factors, as well as interactions between host-pathogen should be performed in order to develop immunogens to prevent disease in dogs.

A virulence factor that has been shown to be essential for *Brucella* is the type IV secretion system (T4SS), which is encoded by the *virB* operon (Hong et al., 2000; Sieira et al., 2000) and is required for the establishment and persistence of infection in the murine model (Sun et al., 2002; Rolan and Tsolis, 2008). It has been shown that *virB* mutants of *B. abortus* and *B. melitensis* have reduced capacities to survive and replicate in professional and non-professional phagocytic cells (Sun et al., 2005; Rolan and Tsolis, 2008).

In this study, a *B. canis* non-polar *virB10* mutant and a *virB11* null mutant were evaluated for their roles in protecting against an experimental challenge. The induction of the cytokines IL-2, IL-4, and IL-10 as well as the production of IFN- γ in spleen cells from mice inoculated with *B. canis* mutants were assessed, as was the production of IgG1, IgG2a, IgG2b, and IgM in the murine model.

MATERIALS AND METHODS

BACTERIA STRAINS, MEDIA, AND CULTURE CONDITIONS

The *B. canis* wild-type strain, the mutants *virB10* and *virB11*, and their complemented mutants were cultured at 37°C in *Brucella* agar or *Brucella* broth with orbital shaking (200 rpm) for 18 h. *Escherichia coli* JM109 and DH5 α strains were cultured at 37°C in Luria-Bertani agar or broth with orbital shaking (200 rpm) for 18 h. When necessary, the following antibiotics were added: gentamycin (2.5 μ g/ml) and ampicillin (50 μ g/ml; SIGMA Aldrich, St. Louis, MO, USA).

CONSTRUCTION OF *virB10* AND *virB11* MUTANTS

To obtain a non-polar mutation in the *virB10* gene, an insertional mutant was constructed. The plasmid pB2A3, which contains the *virB10* gene, was linearized with *Nru*I and ligated with a 0.7-kb *Sma*I fragment encoding a gentamycin resistance cassette. The resulting plasmid, pB2A3::Gm, was electroporated into *B. canis* where it is incapable of autonomous replication, and double-homologous recombinant events (Gm^r Amp^s) were selected using gentamicin resistance (Sieira et al., 2000).

To generate a mutant with a deletion in the *virB11* gene, a 1.2-kb fragment of the plasmid pGEM-T-*virB10* that contained a *virB10* gene was ligated into pBluescript KSII to generate the plasmid pBlue-*virB10*. A 0.7-kb *Bam*HI fragment encoding a gentamycin resistance cassette was ligated into the *Bam*HI site of pBlue-*virB10* to generate the plasmid pBlue-*virB10*-Gm. An 850-bp that contained the *orf13-orf12* region was cloned into the pGEM-T Easy vector and was recovered by *Not*I digestion. This fragment was ligated into pBlue-*virB10* at the *Not*I site to generate the plasmid pBlue-*virB10*::Gm-ORF13-ORF12. Five micrograms of pBlue-*virB10*::Gm-ORF13-ORF12 was electroporated into wild-type *B. canis*. Accordingly, colonies that were Gm^r and Amp^s were selected as possible double-homologous recombinants.

COMPLEMENTATION OF BRUCELLA CANIS virB10 AND virB11 NON-POLAR MUTANTS

The entire *virB10* gene was cloned into the pBBR1MCS-4 plasmid at the *Eco*RI site to generate the pGP10c plasmid. The pGP10c plasmid was electroporated into *B. canis virB10*::Gm, and Amp^r strains were selected. The presence of the plasmid was confirmed by alkaline lysis. To restore the *virB11* gene, the complete gene was introduced into the pBBR1MCS-4 plasmid at the *Eco*RI site to generate the pGP11c plasmid. The pGP11c plasmid was then transformed into *B. canis ΔvirB11*::Gm by electroporation; Amp^r strains were selected, and the presence of the plasmid was confirmed by alkaline lysis.

EXPERIMENTAL INFECTION IN MICE

Female 6- to 7-week-old BALB/c mice were obtained from Harlan, Mexico. The mice were placed in cages with water and feed *ad libitum* and were acclimated for 1 week before beginning the experiments. Three groups of 16 mice each were formed for the *in vivo virB* mutant protection experiments.

SURVIVAL OF virB MUTANTS IN MICE

The survival of *virB* mutants, of the complemented mutants *virB10*::Gm and *ΔvirB11*::Gm and of wild-type *B. canis* was determined by quantitating the CFU of each strain in mouse spleens at different time intervals post-infection. Groups of 15 7-week-old female BALB/c mice were injected i.p. with 1.4×10^8 CFU of the *virB10*::Gm strain or the *ΔvirB11*::Gm strain in 0.1 ml of PBS. At 7, 21, 35, and 45 days post-infection (p.i.), animals were sacrificed, and spleens were removed and homogenized in PBS. Tissue homogenates were serially diluted with PBS and were plated onto tryptic soy broth-agar with the appropriate antibiotics to determine the numbers of CFU per spleen.

For protection experiments, groups of 16 mice were immunized i.p. with 1.4×10^8 CFU of strain *virB10*::Gm or strain *ΔvirB11*::Gm or with PBS (unvaccinated control). Six weeks post-inoculation, both groups were challenged i.p. with 5×10^4 CFU of virulent wild-type *B. canis* per mouse. Two and 4 weeks post-challenge, eight mice per group were sacrificed, and the numbers of CFU recovered from spleens were determined. Protection units were defined as the difference between the mean log of the numbers of CFU from immunized mice and that of the numbers of CFU from mice receiving saline.

LYMPHOCYTE CULTURES AND CYTOKINE INDUCTION

Three groups of five 6- to 7-week-old female BALB/c mice were immunized intraperitoneally with 1.4×10^8 CFU/ml of *B. canis* or the *virB* mutants; each mouse was inoculated at both day 0 and 8. Fifteen days after the first inoculation, the mice were sacrificed, and spleens were extracted for lymphocyte cultures. Spleens from each group of mice were combined and washed three times with Hank's balanced salt solution and were placed in a Petri dish that contained 5 ml of RPMI 1640 medium (Gibco, Laboratories) supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin. The spleens were wrapped with sterile gauze to retain tissue portions and were then macerated. The cellular suspension was transferred into a tube with 5 ml of the same medium and was centrifuged at $400 \times g$ for 3 min. The cells were re-suspended in 0.17 M ammonium chloride for 5 min at 4°C to lyse erythrocytes, and they were then washed three additional times with RPMI. Cells were re-suspended in RPMI medium enriched with 20% bovine fetal serum, 200 mM L-glutamine and 0.1 mM non-essential amino acids. A total of 6.5×10^6 mouse lymphocytes were stimulated with their corresponding mutant or the wild-type strain and were then each distributed into five wells in cell culture plates (Nunclon, Rochester, NY, USA). Wells were inoculated with 10^8 CFU of each one of the strains, and the plates were then incubated at 37°C with 5% CO₂. A positive control was inoculated with concanavalin A. Supernatants were collected at 24, 48, 72, 96, and 120 h after inoculation and were frozen until use; commercial kits were used for the quantification of mouse cytokines IFN-γ, IL-2, IL-4, and IL-10 (Duoset ELISA; R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

DETECTION OF SPECIFIC ANTIBODIES IN SERUM

Antibodies against *virB* mutants were quantified by indirect ELISA using a total extract of wild-type *B. canis* as the antigen. Three groups of 15 mice were immunized intraperitoneally with 1.4×10^8 CFU/ml of wild-type *B. canis* and its mutants, and sera were obtained at 0, 7, 14, 21, and 28 days post-inoculation. IgG1, IgG2a, IgG2b, and IgM isotypes were evaluated using a commercial mouse monoclonal antibody isotyping reagent kit (Sigma Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. Plates were read at a wavelength of 490 nm (VICTOR ELISA reader).

STATISTICAL ANALYSIS

Significance levels were determined using Student's *t*-test to compare between vaccinated and unvaccinated groups and among days. A value of $P < 0.05$ was considered significant.

RESULTS

CHARACTERIZATION AND COMPLEMENTATION OF B. CANIS virB10::Gm AND B. CANIS ΔvirB11::Gm MUTANTS

A total of 5 μg of pB2A3::Gm plasmid was electroporated with a GenePulser (Bio-Rad) into *B. canis* wild-type strain; colonies displaying Gm^r and Amp^s were selected to obtain mutants with a homologous double recombination (Gm^rAmp^s). PCR analysis confirmed that the wild-type *virB10* gene was replaced. The mutated region was amplified to determine the interruption of the *virB10* gene using primers that amplify a 1369-bp fragment

for the parental strain and a 2069-bp fragment for the mutant strain. This procedure generated the non-polar mutant, *B. canis virB10::Gm*.

The plasmid pBlue-*virB10::Gm*-ORF13-ORF12 was electroporated with a GenePulser (Bio-Rad) into *B. canis* wild-type strain to generate the deletion of the *virB11* gene. Gm^r and Amp^s colonies were selected and then analyzed by PCR to confirm that the replacement of *virB11* with *Gm* had occurred. This procedure generated the non-polar *B. canis* mutant, $\Delta virB11::Gm$. To confirm the interruption of the *virB11* gene, an amplification of the mutated region was performed using primers that amplify a 2013-bp fragment for the wild-type strain and a 1900-bp fragment for the mutant strain. This procedure generated the non-polar mutant, *B. canis* $\Delta virB11::Gm$.

To restore the *virB10* gene, a 1.2-kb fragment containing the entire *virB10* gene was amplified and cloned directly into pCR2.1 TOPO; the resulting plasmid was named pGP10c. Plasmid pGP10c was digested with *EcoRI* to recover a 1.2-kb fragment, which was ligated into pBBRMCS-4-*EcoRI* to generate a pBBR4-*virB10*. This plasmid was electroporated into *B. canis virB10::Gm*, and strains resistant to ampicillin were selected. The presence of the plasmid was confirmed using alkaline lysis.

To restore the *virB11* gene, the complete gene was amplified and cloned directly into pCR2.1 TOPO; the resulting plasmid was named pGP11c. This plasmid was digested with *EcoRI* to generate a 1.3-kb product that contained the entire *virB11* gene. The 1.3-kb fragment was ligated into the pBBR1MCS-4 plasmid at the *EcoRI* site to generate the pBBR4-*virB11* plasmid. This plasmid was transformed into *B. canis* $\Delta virB11::Gm$ by electroporation, ampicillin-resistant strains were selected and the presence of the plasmid was confirmed by alkaline lysis.

EXPERIMENTAL INFECTION IN MICE

To evaluate the survival of the *virB10* and *virB11* mutants, the complemented mutants *virB10::Gm* and $\Delta virB11::Gm$ and *B. canis* wild-type strain *in vivo*, groups of mice were inoculated intraperitoneally with 1.4×10^8 CFU of the strains in 0.5 ml of PBS; at different times, the numbers of CFU/ml were determined from spleens. Experimental results revealed that at 7 days post-infection, there were no differences in the recovered CFU of the different strains. By the third week, mutant *virB10* had already been completely eliminated, thus indicating a notable reduction in virulence. Compared to the wild-type strain, mutant *virB11* reduced its bacterial load by 0.2 logs by week 3, and at week 5, the levels were undetectable, suggesting that the loss of some of the *virB* genes resulted in the elimination of the bacteria from the mouse spleens. Mutant *virB11* complemented with plasmid pBBR4-*virB11* recovered its virulence and replicated similarly to the wild-type strain (Figure 1).

To examine the protection induced by *virB* mutants, an immunization experiment was performed. Mice were inoculated i.p. with 1.4×10^8 CFU of *virB10::Gm*, $\Delta virB11::Gm$, or with PBS. Six weeks post-inoculation, animals were challenged with 5×10^4 CFU of *B. canis* wild-type strain. Protection was defined as the difference between the numbers of viable bacteria recovered from spleens of immunized mice and those recovered

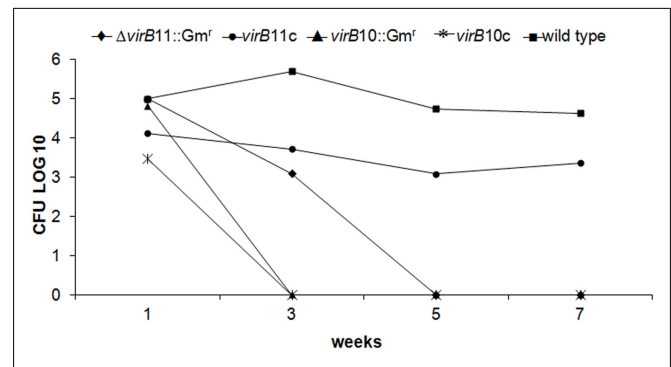


FIGURE 1 | Virulence in mice. Mice were infected i.p. with 1.4×10^8 CFU of *B. canis* wild-type strain, the *virB10* and *virB11* mutants or the *virB*-complemented mutant strains, and the numbers of CFU recovered from spleens at different times post-infection were determined as described in the Section "Materials and Methods": ◆ *B. canis* $\Delta virB11::Gm^r$; ● *B. canis virB11::Gm*; ▲ *B. canis virB10::Gm^r; * *B. canis virB10::Gm*; ■ *B. canis* wild type.*

Table 1 | Protection against *B. canis* wild-type.

Treatment group (n = 8) receiving	Mean log ₁₀ of the no. of brucellae ± SD in spleens on post-challenge day		Log ₁₀ of protection ^a on day	
	15	30	15	30
<i>virB10::Gm</i>	3.84 ± 0.58	3 ± 0.47	1.72 ^b	1.91 ^b
$\Delta virB11::Gm$	4.8 ± 0.68	2.95 ± 0.46	0.76	1.96 ^b
PBS	5.56 ± 0.74	4.91 ± 0.69		

^aLog₁₀ of protection is defined as the difference between the mean log of the numbers of CFU from immunized mice and that of the numbers of CFU from mice receiving saline. ^bP < 0.05 (significant) compared with value for control mice.

from spleens of mice that received saline; results are summarized in Table 1. The *virB10::Gm* inoculation generated significant protection 15 and 30 days post-challenge (1.72 and 2 protection units, respectively). As expected, $\Delta virB11::Gm$ also induced significant protection at day 30 (1.96 protection units). These results emphasize the potential of this mutant for use as a live vaccine for dogs.

MUTANTS INDUCE A HUMORAL RESPONSE SIMILAR TO WILD-TYPE INFECTION

Sera collected at 0, 7, 14, 21, and 28 days post-vaccination were used to detect the presence of specific antibodies against *B. canis* by ELISA. Immunization with *virB* mutants stimulated an antibody response with IgG1, IgG2a, IgG2b, and IgM isotypes. A similar distribution occurred in the sera of mice inoculated with the wild-type strain. Although IgG2a levels increase in mice vaccinated with *virB10*, on day 21 post-infection, there are no significant differences (P < 0.05) compared to the sera of mice challenged with the wild-type strain, in which IgG2a levels increase up to day 28 (data not shown).

CYTOKINE RESPONSES BY *virB* MUTANTS

The immune cellular response was characterized based on the secretion of cytokines at different times in supernatants of lymphocyte cultures obtained from mice previously immunized and later stimulated with the corresponding strains. The wild-type strain induces a rapid and strong INF- γ response with a marked reduction at 96 h, while IL-10 is slightly increased at 72 h. There was no significant difference ($P > 0.05$) in the production of INF- γ between *virB10* and *virB11* mutants. IL-2 and IL-4 levels for both mutants were not statistically significant compared to those produced by the spleen cells of mice inoculated with the wild-type strain (Figure 2).

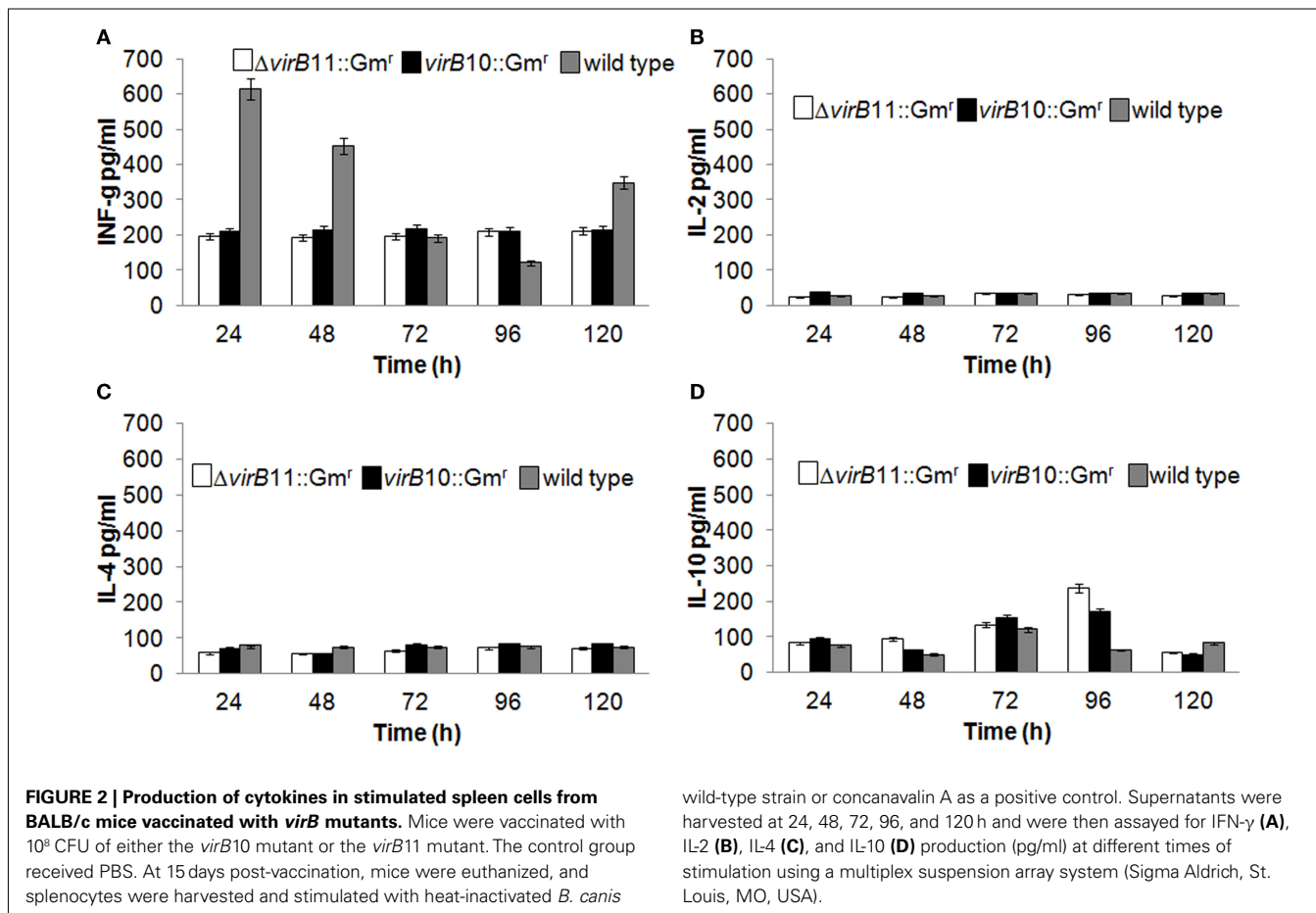
DISCUSSION

The development of a live vaccine for the control and eradication of canine brucellosis is of great importance. Nevertheless, currently, there is no information that describes an appropriate candidate that can be used as a vaccine against *B. canis*.

In this study, it was shown that *virB10* and *virB11* genes perform an essential role in *B. canis* virulence in mice. Studies published by other groups confirm these results and also show that these genes fulfill the same function in other *Brucella* species (Hong et al., 2000; Seira et al., 2000; Comerci et al., 2001; Delrue et al., 2001; Boschirolti et al., 2002; Celli et al., 2003). Through the construction of mutants, it has been shown that the alteration of

either *virB10* or *virB11* with non-polar mutations has a detrimental effect on virulence. Comerci et al. (2001) used non-polar mutations to demonstrate that *virB4*, *virB8*, and *virB10* genes are required for the persistence of *Brucella* infection, while Den Hartigh et al. (2008) demonstrated that non-polar mutations in *virB10* and *virB11* are essential for *B. abortus* virulence in mice.

Brucella virulence mechanisms are not fully understood, but the capacity to cause disease is related to the intracellular survival of the bacteria, which has been associated with the T4SS (Hong et al., 2000; Comerci et al., 2001; Delrue et al., 2001; Boschirolti et al., 2002; Celli et al., 2003). In this study, we conclude that the absence of *B. canis virB10* and *virB11* genes from the *virB* operon is associated with a reduction of virulence in mice. Seira et al. (2000) demonstrated that a *B. abortus virB10* mutant could not be recovered from the spleens of infected mice. In this study, non-polar *virB* mutants had reduced spleen counts compared to the wild-type strain, indicating severe reductions in virulence as estimated by the numbers of viable bacteria from the spleens at all times p.i that were assessed. This phenotype is likely due to a faulty secretion of effector molecules as a result of a T4SS that works deficiently in the absence of some *virB* gene. We also analyzed the protection properties of *virB* mutants in the BALB/c mouse model. Our results show that the mutants protect mice from a challenge with a virulent strain of wild-type *B. canis* with a reduction of approximately 2 log units.



Even though there are detectable levels of anti-*Brucella* IgG, the antibody response to immunization was considered to be low compared to other virulence factors used in immunizations; nevertheless, the IgG1 and IgG2a levels were heterogeneous in both mutants. Our results and numerous examples in the literature indicate that INF- γ levels produced in response to a vaccination candidate do not always correlate with the efficacy of the vaccine during a challenge with *Brucella*. Various *in vitro* studies have demonstrated that murine spleen cells previously inoculated with live vaccines induce a type Th1 response to *Brucella* antigens, while those immunized with inactivated vaccines induce a type Th2 response (Zhan et al., 1995). In mice infected with *B. abortus virB* mutants, serum levels of immunoglobulin IgG2a and cytokines INF- γ and IL-12p40 increase more than in mice infected with *B. abortus* wild-type strain (Rolan

and Tsois, 2008). Our results suggest that the *virB* mutants elicit a strong protective immunity and should be considered as candidates for live vaccines for dogs. The evaluation of the mutants generated by insertion of the gentamycin resistance cassette as a selection marker is permitted in our country (Mexico); however, to be considered as a candidate for a live vaccine, it will be necessary to reconstruct the mutants and remove the gentamycin selection marker using counterselectable *sacB* as a selection marker.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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