RESEARCH LETTER

Attempts at validating a recombinant *Flavobacterium psychrophilum* gliding motility protein N as a vaccine candidate in rainbow trout, *Oncorhynchus mykiss* (Walbaum) against bacterial cold-water disease

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Abstract

The *Flavobacterium psychrophilum* gliding motility N (GldN) protein was investigated to determine its ability to elicit antibody responses and provide protective immunity in rainbow trout *Oncorhynchus mykiss* (Walbaum). GldN was PCR-amplified, cloned into pET102/D-TOPO, and expressed in *Escherichia coli*. Bacteria expressing recombinant GldN (rGldN) were formalin-inactivated and injected intraperitoneally (i.p.) into rainbow trout with Freund's complete adjuvant (FCA) in four separate studies that used two different immunization protocols followed by challenge evaluations. Fish injected with *E. coli* only in FCA served as the control. Antibody responses to *F. psychrophilum* whole-cell lysates measured by ELISA were low in all four studies. Protection against *F. psychrophilum* challenge was observed in the first study, but not in the three following studies. The discrepancies in results obtained in the later studies are unclear but may relate to formalin treatment of the antigen preparations. Overall, it appeared that rGldN delivered i.p. as a crude formalin-killed preparation is not a consistent vaccine candidate, and more work is required. Additionally, this study illustrates the importance of conducting multiple *in vivo* evaluations on potential vaccine(s) before any conclusions are drawn.

Introduction

Bacterial cold-water disease (BCWD) caused by *Flavobacterium psychrophilum* is a major contributor to losses in the rainbow trout, *Oncorhynchus mykiss* (Walbaum), and salmonid farming around the world. Outbreaks of BCWD can result in 50–60% mortality (Holt, 1987), but further losses due to deformities and reduced growth in survivors contribute to additional economic impacts for the farmer. Despite a number of publications addressing vaccination against BCWD, there is still no commercially available vaccine. Proteins of *F. psychrophilum* have been identified including those in the 70–100 and 41–49 kDa range that can provide protection against BCWD in laboratory evaluations (LaFrentz *et al.*, 2004). A number of proteins within these fractions were subsequently identified as being immunogenic (LaFrentz *et al.*, 2011) along with other proteins of the bacteria (Sudheesh *et al.*, 2007). We have previously evaluated five of these proteins for their efficacy as recombinant protein vaccines (Plant *et al.*, 2009, 2011). This study continues our previous work that failed to identify any potential recombinant protein vaccine candidates and examines the potential of recombinant gliding motility protein N (rGldN) as a vaccine against BCWD.

Gliding motility is a common characteristic of members of the *Bacteroidetes* phylum of which *F. psychrophilum* is a member. Gliding occurs despite a lack of flagella or pili and the mechanism responsible in *F. psychrophilum* is unknown. However, in *Flavobacterium johnsoniae,* gliding motility is thought to involve cell surface complexes consisting of the Gld proteins which facilitate the movement of adhesive proteins from one to another (Nelson *et al.*, 2008). A substantial amount of research has been carried

out on gliding motility in *F. johnsoniae* that has identified multiple genes involved in gliding (Agarwal *et al.*, 1997; Hunnicutt & McBride, 2000, 2001; Hunnicutt *et al.*, 2002; McBride *et al.*, 2003; McBride & Braun, 2004; Braun & McBride, 2005; Braun *et al.*, 2005). As a result of the sequencing of the complete *F. psychrophilum* genome (Duchaud *et al.*, 2007), comparison of the gliding motility genes between *F. psychrophilum* and *F. johnsoniae* revealed extensive similarities. In *F. johnsoniae,* GldN is essential for motility, and it is thought to be involved in the secretion of motility proteins to the cell surface in concert with a number of other Gld proteins (Rhodes *et al.*, 2010). Recently, a link was found between protein translocation and the motility apparatus in members of the *Bacteroidetes* phylum. A protein secretion system was identified and termed the Por secretion system (PorSS) (Sato *et al.*, 2010), of which GldN is thought to be one component. The PorSS is thought to have a similar role to the type III secretion system present in other bacterial species and therefore could be exploited for disease control.

Because our previous work on potential candidate antigen(s) was unsuccessful at identifying one for a recombinant vaccine, the *F. psychrophilum* GldN protein was examined. This choice was supported by the following: (1) identification of GldN as an immunogenic protein within the protective high- and mid-molecular mass fractions (LaFrentz *et al.*, 2011); (2) GldN is upregulated *in vivo* and *in vitro* under iron-restricted conditions (LaFrentz *et al.*, 2009); (3) GldN is probably exposed on the cell membrane (Braun *et al.*, 2005); and (4) GldN has a potential role in the Por secretion system in *F. johnsoniae* (Sato *et al.*, 2010).

Materials and methods

Fish and rearing conditions

Rainbow trout (mean weight 2 g) were used for the immunization trials at Clear Springs Foods, Buhl, Idaho, USA. Fish were maintained in 378-L tanks supplied with ultraviolet light treated specific pathogen-free spring water at 15 °C. Fish were fed pelleted trout food (Clear Springs Foods) at 1% body weight day^{-1}.

Bacterial culture

Tryptone yeast extract salts (TYES) broth or agar was used to culture *F. psychrophilum* (strain CSF-259-93) (Holt *et al.*, 1993) at 15 °C for 72 h. Bacteria were harvested from broth by centrifugation for 15 min at 4300 ***g***. The pellet was resuspended in phosphate-buffered saline (PBS) and the optical density (OD) set at 0.2, 0.4, or 0.6 at 525 nm.

Plasmid construction

The gene encoding gliding motility protein N (*gldN*) was amplified by polymerase chain reaction (PCR) directly from glycerol frozen stocks of *F. psychrophilum* CSF-259-93. Primer sequences for *gldN* were forward 5′-CACCATGAATTGGAGAAATT-3′ and reverse 5′-GTA ATTCCACATATCTTGCT-3′. Methods used for PCR and cloning have been described in detail previously (Plant *et al.*, 2009). Cycle parameters for *gldN* were as follows: 94 °C for 2 min, 35 cycles of 94 °C for 15 s, 45 °C for 30 s, 68 °C for 1 min, concluding with an extension at 68 °C for 7 min. The purified PCR product was cloned into pET102/D-TOPO (Invitrogen, Carlsbad, CA), stored in *Escherichia coli* TOP10 cells (Invitrogen) at −80 °C, and transformed into *E. coli* BL21 Star (DE3) cells (Invitrogen) for protein expression.

Recombinant GldN expression

Expression of rGldN was achieved with culture in Luria–Bertani (LB) broth containing 50 μg mL^{-1} carbenicillin and 1% glucose at 37 °C overnight. The next day, 1 mL of the overnight culture was transferred into 50 mL of LB with 50 μg mL^{-1} carbenicillin at 37 °C. Expression of rGldN was induced 2 h later with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h (Marbach & Bettenbrock, 2012). The culture was centrifuged at 3000 ***g*** for 10 min to pellet the cells and resuspended in 5 mL PBS. A culture of *E. coli* containing the empty vector pET102/D-TOPO was treated in the same manner as *E. coli* expressing rGldN and served as a control. Whole bacteria were killed using 0.5% formalin (v/v) overnight at 4 °C, subsequently washed twice with PBS, and finally resuspended to an OD at 600 nm (see Table 1).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and immunodetection

Detailed methods for SDS-PAGE and Western blotting have been described previously (Plant *et al.*, 2009). Briefly, proteins were separated on a 10–20% Tris-glycine gel (Invitrogen) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The molecular weights of proteins were compared to Precision Plus prestained protein standards (Bio-Rad), and gels were stained with SimplyBlue™ SafeStain (Invitrogen). Blots were immunodetected with mouse anti-His monoclonal (1 : 5000, Invitrogen) or serum from fish injected with *E. coli* expressing rGldN (*E. coli* rGldN, 1 : 200) as primary antibody. Whichever primary antibody was used, the immunodetection protocol followed the method described

Table 1. Experimental parameters used in four different studies that examined the vaccine potential of *Escherichia coli* produced *Flavobacterium psychrophilum* gliding motility protein N (rGldN) in Freund's complete adjuvant (FCA) injected intraperitoneally into rainbow trout (*Oncorhynchus mykiss*) held at a constant 15 °C. Each study also included a mock-infected negative control for each treatment group

Study	Treatments	Immunization dose (OD600)	4 weeks postbooster	Challenge parameters	
				Weeks postprimary immunization	Injection dose (OD525)
1	*E. coli*/FCA	2.5*	–	8	0.2†, 0.4‡
	E. coli rGldN/FCA	2.5			
2	*E. coli*/FCA	5.0§	–	8	0.4, 0.6¶
	E. coli rGldN/FCA	2.5			
	E. coli rGldN/FCA	5.0			
3	*E. coli*/FCA	5.0	–	6	0.2, 0.4
	E. coli rGldN/FCA	2.5			
	E. coli rGldN/FCA	5.0			
4	*E. coli*/FCA	5.0	+	8	0.2, 0.4
	E. coli rGldN/FCA	2.5			
	E. coli rGldN/FCA	5.0			

*Equivalent to 200 μg of total bacterial protein.
†Equivalent to 2×10^8 colony-forming units (CFU) mL^{-1}.
‡Equivalent to 5×10^8 CFU mL^{-1}.
§Equivalent to 400 μg of total bacterial protein.
¶Equivalent to 1×10^9 CFU mL^{-1}.

previously (LaFrentz *et al.*, 2004; Plant *et al.*, 2009) with one exception: 1 : 5000 of the final antibody–goat anti-mouse immunoglobulin alkaline phosphatase conjugate (Bio-Rad) was used. Formalin-killed *E. coli* rGldN alongside live *E. coli* rGldN was also stained with InVision™ His-tag in-gel stain (Invitrogen) as a quick method to confirm expression of a single protein.

Immunization trials

The experimental regimens for the *in vivo* trials with rGldN are shown in Table 1. Common to all studies were the following methods. Formalin-killed *E. coli* or formalin-killed *E. coli* rGldN was adjusted to either 2.5 or 5.0 OD600, which resulted in the fish receiving either 200 or 400 μg of total protein, respectively. The inactivated bacteria were mixed with equal volumes of Freund's complete adjuvant (FCA, Sigma) and emulsified by vortexing. In the first study, fish were immunized with 200 μg of total protein and challenged 8 weeks postvaccination (800 degree days). In the second and third studies, fish were immunized with 200 or 400 μg of protein and challenged 6 or 8 weeks postvaccination (600 or 800 degree days). In the final study (#4), fish were again immunized with 200 or 400 μg of protein but were booster vaccinated with the same dose 4 weeks (400 degree days) later. Challenges were conducted 4 weeks postbooster.

Prior to injection, rainbow trout were anesthetized by immersion in 50 mg L^{-1} tricaine methane sulfonate (MS-222, Argent). Groups of 150 fish were intraperitoneally (i.p.) injected with 50 μL of each treatment. Prior to immunization, blood samples were taken from 10 fish by severing the caudal peduncle and collecting blood in heparinized hematocrit tubes. Samples were pooled into 2 five fish pools. Serum was collected by centrifuging at 15 000 ***g*** for 5 min at room temperature and stored at −80 °C. In the fourth study, the fish were booster immunized after 4 weeks similar to the primary immunization described above.

Antibody detection and bacterial challenge

Prior to challenge, 15 rainbow trout from each group were bled and pooled into five pools of three fish. Pooled serum was used to detect antibodies to *F. psychrophilum* whole-cell lysate by ELISA (LaFrentz *et al.*, 2002). The ELISA titer was defined as the reciprocal of the dilution with an OD at least twice the negative control.

Fish were challenged either 6 or 8 weeks postprimary immunization with 25 μL of *F. psychrophilum* CSF-259-93 by subcutaneous injection with a 30-gauge needle at the dorsal midline, posterior to the dorsal fin. Duplicate groups of 25 fish from each treatment were injected with *F. psychrophilum* at an OD525 of 0.2, 0.4, 0.6, or PBS as a mock-infected control group. These ODs correlate with 2×10^8 colony-forming units (CFU) mL^{-1}, 5×10^8 CFU mL^{-1}, 1×10^9 CFU mL^{-1}, respectively (Holt, 1987). Mortalities were recorded daily for 28 days. Spleen tissue of at least 20% of the mortalities was streaked onto TYES agar to recover *F. psychrophilum*.

Statistics

Statistical analyses were performed using STATISTICA version 6. Student's *t*-tests were used to determine significant ($P < 0.05$) differences in mortality and antibody levels between treatments.

Results

Study 1

In both the low- and high-dose challenges, mortality was significantly ($P < 0.05$) reduced in the rGldN treatment groups (Table 2) and relative percent survival (RPS) was 46 and 42, respectively. *Flavobacterium psychrophilum* was isolated from all of the sampled mortalities. Antibody titers as measured by ELISA were low in both groups and not statistically different (Table 2).

Study 2

Given the positive evidence of protection in study 1, study 2 was designed using a higher dose of the formalin-killed *E. coli* rGldN along with the original antigen dose of the first study. *Flavobacterium psychrophilum* was isolated from all of the mortalities sampled, but in contrast to study 1, study 2 showed only minor protection at either antigen dose (Table 2). At the highest challenge dose tested, the RPS was 12 and 23 at the low and high antigen dose, respectively. In this study, there were no statistical differences between treatments. Similar to study 1, ELISA antibody titers were low in all treatments with no statistical differences (Table 2).

Studies 3 and 4

To resolve the disparity between studies 1 and 2, two further studies were carried out simultaneously. Study 3 included a challenge evaluation at 6 weeks rather than 8 weeks and study 4 included a booster immunization at 4 weeks and a challenge at 8 weeks postprimary immunization. The cumulative percent mortality obtained from the challenges for both studies was lower, and while there appeared to be minor protection in some of the groups, again there were no statistical differences in mortality between the different treatment groups (Table 2). *Flavobacterium psychrophilum* was isolated from all of the mortalities sampled. Antibody titers measured by ELISA in fish from study 3 were low (Table 2). In study 4, antibody titers from the lowest immunization dose (2.5 OD) were very low, but at the higher immunization dose (5.0 OD), titers were higher and significantly ($P < 0.05$) different from both the control group and the low dose group (2.5 OD) (Table 2).

Protein visualization and immunodetection

Overexpression of rGldN was observed in live *E.coli,* but after formalin inactivation, the protein was no longer visible on SDS-PAGE gels (Fig. 1a). An in-gel His-tag stain also did not detect rGldN at its predicted size (Fig. 1b). Immunodetection was employed as a more sensitive method to detect rGldN in formalin-killed preparations (Fig. 1c and d). The immunodetection did show that serum from fish injected with formalin-killed whole-cell *E. coli* expressing rGldN recognized the GldN protein in

Table 2. Mean percent mortality of rainbow trout immunized with formalin-killed *Escherichia coli* expressing recombinant *Flavobacterium psychrophilum* gliding motility protein N (rGldN) and antibody titers against *F. psychrophilum* lysate measured by ELISA

Study	Treatments	Immunization dose (OD600)	Mean percent mortality ±SE[†]		Mean antibody titer
			Low	High	
1	*E.coli*/FCA	2.5[‡]	82 ± 6	82 ± 14	300
	E. coli rGldN/FCA	2.5	44* ± 4	47* ± 13	100
2	*E.coli*/FCA	5.0[§]	80 ± 0	86 ± 10	520
	E. coli rGldN/FCA	2.5	92 ± 4	76 ± 4	880
	E. coli rGldN/FCA	5.0	78 ± 2	66 ± 1	480
3	*E.coli*/FCA	5.0	42 ± 18	43 ± 3	840
	E. coli rGldN/FCA	2.5	48 ± 4	25 ± 2	140
	E. coli rGldN/FCA	5.0	40 ± 12	37 ± 1	280
4	*E.coli*/FCA	5.0	31 ± 7	29 ± 4	20
	E. coli rGldN/FCA	2.5	21 ± 5	34 ± 2	80
	E. coli rGldN/FCA	5.0	34 ± 8	38 ± 1	1280*

There was no mortality in any mock-infected negative control for each treatment group in each study.

*Significant differences ($P < 0.05$) within a study.

[†]In each study, 'low' refers to a challenge dose of either 2×10^8 colony-forming units (CFU) mL^{-1} or 5×10^8 CFU mL^{-1} and 'high' refers to a challenge dose of 5×10^8 CFU mL^{-1} or 1×10^9 CFU mL^{-1}.

[‡]Equivalent to 200 μg of total bacterial protein.

[§]Equivalent to 400 μg of total bacterial protein.

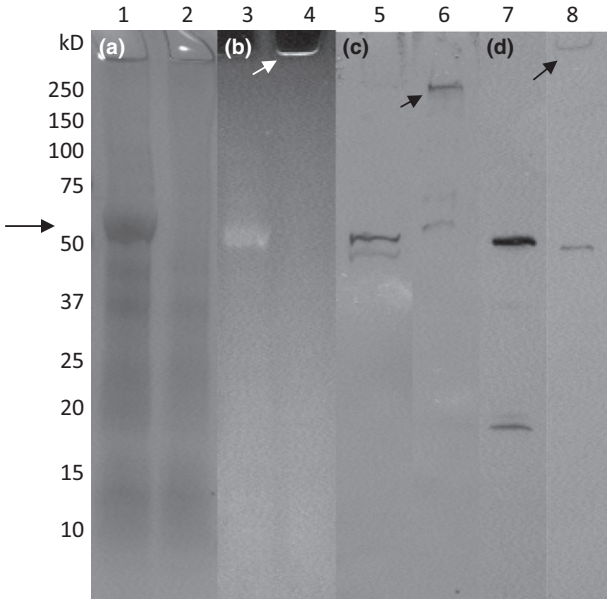

Fig. 1. (a) SDS-PAGE showing *Escherichia coli* lysate expressing recombinant gliding motility protein N (rGldN, horizontal arrow), lane 1: live *E. coli*, lane 2: formalin-killed *E. coli*. (b) SDS-PAGE showing *E. coli* lysate expressing rGldN stained with InVision™ His-tag in-gel stain (Invitrogen), lane 3: live *E. coli*, lane 4: formalin-killed *E. coli*. (c) Immunodetection of *E.coli* expressing rGldN with sera from fish previously injected with formalin-killed *E.coli* rGldN, lane 5: live *E.coli*, lane 6: formalin-killed *E.coli*. (d) Immunodetection of *E. coli* expressing rGldN with anti-His-tag antibody, lane 7: live *E. coli*, lane 8: formalin-killed *E.coli*. Diagonal arrows indicate a large molecular mass at the very top of the gel in all formalin-treated lanes.

live *E. coli* rGldN (Fig. 1c) and *F. psychrophilum* (data not shown). The same sera also produced a faint band at the approximate size of rGldN in formalin-killed *E.coli* rGldN preparations. These observations indicate the fish vaccinated with formalin-killed *E. coli* expressing rGldN produced antibody that was directed toward rGldN. Detection using the anti-His antibody as the primary antibody detected a band at rGldN's predicted size in formalin-killed preparations and in live *E. coli* cells (Fig. 1d). Interestingly, regardless of the primary antibody or detection method, a band was observed at the very top of the gel in formalin-killed *E. coli* rGldN, suggesting rGldN was cross-linked into an extremely large protein complex that was largely unable to migrate into the gel (Fig. 1b lane 4, C lane 6, and D lane 8). However, it appeared that a portion of the cross-links were reversed by the SDS loading buffer and heating, prior to running on the gel, allowing some rGldN to be observed at its correct size when detected with anti-His antibody and immune fish sera.

Discussion

Study 1 used a statistically reliable number of fish, and the results suggested that rGldN expressed and delivered using whole-cell *E.coli* was a potential vaccine candidate. However, the overall results of the entire study illustrate the problems with demonstrating such results consistently. Expression of rGldN in *E. coli* was relatively simple, and overexpression of rGldN was clearly observed by staining. Nevertheless, we were unable to purify the protein despite attempting many different methods. It is likely that this is related to the probable membrane location of the protein (Braun *et al.*, 2005), and as a consequence, the protein was delivered in and on formalin-killed whole-cell *E. coli*.

The disappearance of rGldN at its predicted molecular weight after formalin treatment suggested the protein was cross-linked by the formalin and the positive results of the first study led us to believe it was a suitable inactivation method. Formaldehyde is commonly used to inactivate pathogens and toxins for vaccine use and it is quick to penetrate cells, inactivate enzymes, and cross link proteins to proteins, DNA, and RNA (Orlando *et al.*, 1997; Vasilescu *et al.*, 2004). Cross-linking is dependent on the lysine residues on the surface of proteins and also the physical proximity of the proteins (Vasilescu *et al.*, 2004). The conditions used for formalin inactivation in the current study (0.5% formalin overnight at 4 °C) are not dissimilar from those used by Shimmoto *et al.* (2010) (0.3% formalin for 24 h at 37 °C) who observed protection. Nencioni *et al.* (1991) observed that treatment of antigens with higher concentrations of formalin, such as 0.42%, altered the quality of the immune response. Antigens treated with lower formalin concentrations, such as 0.035%, were still inactivated, but the quality of the immune response was unaffected. Additionally, masked or missing epitopes may result from exposure to formalin for longer periods of time than is necessary, which can increase cross-linking and denaturation of proteins (Orlando, 2000). It is possible that this may have occurred with particular immunogenic epitopes of rGldN. Formalin treatment of an *F. columnare* bacterin altered one particular epitope so that it was no longer recognized by serum from naturally infected fish (Bader *et al.*, 1997). With this in mind, it would seem prudent to optimize formalin concentration and exposure time and to keep it to a minimum in future studies. However, formalin-killed vaccine preparations have been quite extensively evaluated in fish and are often successful (Azad *et al.*, 2008; Ji *et al.*, 2008; Kubilay *et al.*, 2008; Leal *et al.*, 2010; Locke *et al.*, 2010). Commonly, these are formalin-killed bacterins of fish pathogens rather than bacterins made from

heterologous expression hosts. Publications concerning recombinant protein expression in *E. coli* delivered as a formalin-killed bacterin are less common, but this can still be an effective method of immunization. Shimmoto *et al.* (2010) demonstrated significant protection in red sea bream, *Pagrus major* (Temminck & Schlegel), vaccinated with a major capsid protein of red sea bream iridovirus delivered as a crude preparation of recombinant protein expressed in formalin-inactivated *E. coli*.

Nevertheless, in the studies presented here, the formalin-killed *E. coli* expressing rGldN conferred good protection in the first study but did not confer significant protection in the subsequent studies. Although GldN is considered to be a membrane protein (Braun *et al.*, 2005), it may not be completely surface exposed and it is possible this may account for the lack of protection observed in the latter studies. Another possibility is that a single protein may not be able to adequately stimulate the immune response to confer protection against BCWD. There are many other proteins involved in gliding motility and the putative PorSS in *F. psychrophilum*. It is possible that a combination of these or other proteins may be required to elicit a significant, reproducible protective response and should be examined in future studies.

Antibody titers measured by ELISA to a *F. psychrophilum* lysate were consistently low in all four studies, aside from the 5.0 OD immunized group in study 4. Antibody levels in the negative control *E. coli*/FCA were often very similar to the rGldN treatment groups. It is possible that specific anti-rGldN antibody was limited and that inclusion of FCA in immunization doses resulted in a heightened overall antibody response among all groups (i.e. an adjuvant effect). LaFrentz *et al.* (2002) showed that saline/FCA injection stimulated enhanced antibody levels and was able to confer a level of protection against *F. psychrophilum*. The amount of rGldN present within each preparation may not have been enough to stimulate an adequate antibody response, and it is interesting to see the highest ELISA titers were in the final study using a booster immunization, at the highest antigen dose. It is possible that antibody titers in that group were still too low to provide protection or that the antibody was directed against nonprotective epitopes of GldN. Different results may have been obtained had it been possible to purify the protein and use this as the coating antigen for the ELISA assays. Evidence from the various immunodetection methods suggests that formalin-treated rGldN is complexed into an extremely large protein that is unable to fully migrate into the SDS-PAGE gel. The response of fish sera to rGldN expressed in live *E. coli* indicated that fish immunized with formalin-inactivated preparations were producing antibody specific for rGldN, despite the cross-linking. However, previous studies have also shown that antibody-mediated protection of rainbow trout against *F. psychrophilum* only provides partial protection and stimulation of various innate and/or adaptive responses may be required for complete protection (LaFrentz *et al.*, 2002). This emphasizes the critical need to use *in vivo* evaluations for this type of research rather than relying only on other potential measurements such as antibody titer.

In summary, *F. psychrophilum* rGldN expressed and delivered using whole-cell *E. coli* delivered i.p. as a vaccine has given variable results. However, potentially alternative formalin concentrations and/or other inactivation methods could improve the consistency of this antigen along with including other proteins involved with gliding motility. This work also illustrates the importance of validating the results of potential vaccine candidates. If only the first challenge evaluation had been carried out, the conclusion would have been that rGldN expressed and delivered via whole-cell *E. coli* was a viable vaccine candidate. Variation is inherent with *in vivo* evaluations and demonstration of reproducible results can only be accomplished through multiple challenge evaluations.

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