INFECTION AND IMMUNITY, Aug. 2009, p. 3244–3248 Vol. 77, No. 8
0019-9567/09/$08.00+0 doi:10.1128/IAI.00395-09

Directed Mutagenesis of the *Rickettsia prowazekii pld* Gene Encoding Phospholipase D▽§

Lonnie O. Driskell,[1] Xue-jie Yu,[2] Lihong Zhang,[2] Yan Liu,[2,3] Vsevolod L. Popov,[2] David H. Walker,[2] Aimee M. Tucker,[1] and David O. Wood[1]*

Department of Microbiology and Immunology, Laboratory of Molecular Biology, University of South Alabama College of Medicine, Mobile, Alabama 36688[1]; Department of Pathology, Center for Biodefense and Emerging Infectious Diseases, University of Texas Medical Branch, Galveston, Texas 77555-0609[2]; and Anhui Province Center for Disease Control and Prevention, Hefei, Anhui Province, People's Republic of China[3]

Received 8 April 2009/Returned for modification 21 April 2009/Accepted 26 May 2009

***Rickettsia prowazekii*, the causative agent of epidemic typhus, is an obligately intracytoplasmic bacterium, a lifestyle that imposes significant barriers to genetic manipulation. The key to understanding how this unique bacterium evades host immunity is the mutagenesis of selected genes hypothesized to be involved in virulence. The *R. prowazekii pld* gene, encoding a protein with phospholipase D activity, has been associated with phagosomal escape. To demonstrate the feasibility of site-directed knockout mutagenesis of rickettsial genes and to generate a nonrevertible vaccine strain, we utilized homologous recombination to generate a *pld* mutant of the virulent *R. prowazekii* strain Madrid Evir. Using linear DNA for transformation, a double-crossover event resulted in the replacement of the rickettsial wild-type gene with a partially deleted *pld* gene. Linear DNA was used to prevent potentially revertible single-crossover events resulting in plasmid insertion. Southern blot and PCR analyses were used to confirm the presence of the desired mutation and to demonstrate clonality. While no phenotypic differences were observed between the mutant and wild-type strains when grown in tissue culture, the *pld* mutant exhibited attenuated virulence in the guinea pig model. In addition, animals immunized with the mutant strain were protected against subsequent challenge with the virulent Breinl strain, suggesting that this transformant could serve as a nonrevertible, attenuated vaccine strain. This study demonstrates the feasibility of generating site-directed rickettsial gene mutants, providing a new tool for understanding rickettsial biology and furthering advances in the prevention of epidemic typhus.**

Epidemic typhus, caused by *Rickettsia prowazekii*, is an infamous disease in human history that continues to affect populations subjected to nonhygienic, louse vector-infested conditions that unfortunately continue to threaten much of the world's population. Recent outbreaks of epidemic typhus underscore the continued menace that this disease maintains (11, 12, 18). In the United States, *R. prowazekii* infection of humans has been associated with the existence of a zoonotic reservoir, the flying squirrel (4, 16), and serological surveys have identified individuals with antibodies reactive with *R. prowazekii* in the homeless population in Houston, Texas (14). In addition, *R. prowazekii* has been designated a category B select agent, raising additional concerns regarding its use as an agent of bioterrorism. Regrettably, a safe and effective vaccine is not currently available.

Although the attenuated Madrid E strain of *R. prowazekii* has been used as a live vaccine and was shown to provide good protection (6), it unfortunately also demonstrated the ability to revert to virulence (2). However, the protection that was afforded by the Madrid E strain suggests that a nonrevertible, avirulent strain of *R. prowazekii* would be a safe and effective vaccine against this deadly infectious agent. Several genes have been associated with virulence either by a gene sequence difference observed between the avirulent and virulent strains or by analysis of gene activities in a surrogate bacterial model (21, 24). One such rickettsial gene, the *R. prowazekii pld* (Rp819) gene, encodes a protein with homology to the phospholipase D (PLD) family of proteins. This protein has been shown to exhibit PLD activity and has a conserved secretion signal, suggesting that the encoded protein could be exposed at the host-pathogen interface (15). Interestingly, Whitworth et al. demonstrated that expression of the rickettsial *pld* gene in *Salmonella enterica* serovar Typhimurium allowed the salmonellae to escape from the phagosome (21). This activity suggests an important role for PLD in rickettsial intracellular growth and identifies this protein as a possible virulence factor for *R. prowazekii*.

R. prowazekii is a bacterial pathogen that grows only within the cytosol of eukaryotic host cells. The obligately intracellular nature of *R. prowazekii* greatly diminishes the ability to genetically manipulate the rickettsiae and obtain cloned mutants that can be exploited as probes of rickettsial physiology and metabolism or, in the case of virulence genes, as potential live vaccines. While random transposon mutagenesis has been successfully performed in several rickettsial species (3, 7, 8) and homologous recombination has been used to insert a specific antibiotic resistance mutation as well as an entire plasmid into the *R. prowazekii* genome (9, 10), to the best of our knowledge, a directed knockout of a rickettsial gene has not been reported. In this study, we targeted the *pld* gene for knockout in order to

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Laboratory of Molecular Biology, University of South Alabama, Mobile, AL 36688-0002. Phone: (251) 460-6324. Fax: (251) 460-7931. E-mail: dowood@jaguar1.usouthal.edu.

§ Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 8 June 2009.

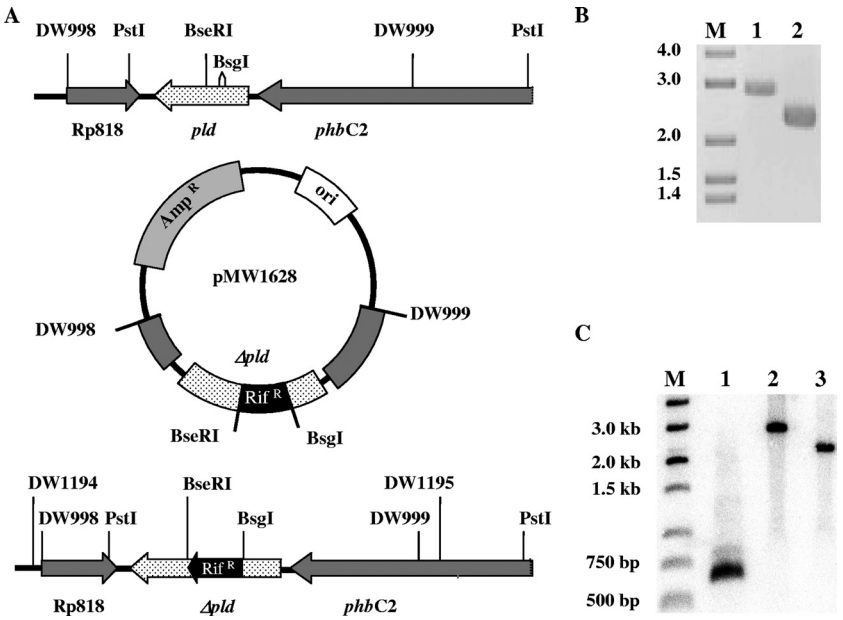

FIG. 1. (A) Physical map of pMW1628 and gene maps of the *pld* locus of the wild-type Evir strain (top) and the Δ*pld* mutant (bottom). Restriction sites and primer locations are indicated. (B) PCR analysis of the Δ*pld* mutant. Chromosomal primers DW1194 and DW1195 were used with template DNAs isolated from *R. prowazekii*. Lane 1, Δ*pld* template DNA product (2,860 bp); lane 2, wild-type template DNA product (2,326 bp). Size markers (M) are indicated in kilobase pairs. (C) Hybridization of a specific *pld* gene probe (lane 1, 622 bp covering the entire open reading frame of the gene) to *R. prowazekii* chromosomal DNA digested with PstI. Lane 2, DNA isolated from the Δ*pld* mutant strain; lane 3, DNA isolated from the wild-type Evir strain. Molecular size markers (M) are indicated.

evaluate its role in rickettsial intracellular growth and virulence as well as to assess the potential role of a *pld* mutant as a live vaccine strain.

MATERIALS AND METHODS

Bacterial strains, host cell lines, and culture conditions. *R. prowazekii* strain Madrid Evir, a virulent revertant of the attenuated Madrid E strain (2), was propagated in and purified from the yolk sacs of embryonated chicken eggs, as previously described (22). Purified rickettsiae were stored frozen in a sucrose-phosphate-glutamate-magnesium buffer solution (0.218 M sucrose, 3.76 mM KH_2PO_4, 7.1 mM K_2HPO_4, 4.9 mM potassium glutamate, and 10 mM $MgCl_2$). Murine fibroblast L929 cells were cultured at 34°C with 5% CO_2 in modified Eagle's medium (Mediatech, Inc., Herndon, VA) supplemented with 10% heat-inactivated newborn calf serum (HyClone Laboratories, Logan, UT) and 2 mM glutamine (Mediatech, Inc.). For selection of *R. prowazekii* rifampin (rifampicin)-resistant mutants, rifampin was added to a final concentration of 200 ng/ml. *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA) (5) was grown in Luria-Bertani medium (1). For the selection of antibiotic-resistant *E. coli* transformants, ampicillin and rifampin both were used at a concentration of 50 μg/ml. A murine macrophagelike cell line (RAW264.7) (13), obtained from the American Type Culture Collection (ATCC TIB-71), was cultured at 34°C with 5% CO_2 in Dulbecco's modification of Eagle's medium (Mediatech, Inc.) supplemented with 10% newborn calf serum (HyClone Laboratories).

Plasmid construction. The *pld* gene, along with flanking regions (Fig. 1A), was PCR amplified from an *R. prowazekii* chromosomal DNA template using primers DW998 (5′-AATGTTAGCATGAATTGAGAG-3′) and DW999 (5′-TGGGTG GTATGTTTCTTGC-3′). The resulting 2,164-bp fragment was inserted into the EcoRV site of pBluescript SKII+ (Stratagene), generating plasmid pMW1625. A 93-bp internal fragment of *pld* contained in pMW1625 was deleted by digestion with restriction enzymes BsgI and BseRI, followed by treatment with the End-It repair kit (Epicentre, Madison, WI) to generate blunt ends. A PCR-generated fragment, containing the *rpsL* promoter-*R. prowazekii arr-2* ($rpsL^P$-$arr\text{-}2_{Rp}$) rifampin resistance cassette (8), was ligated to the linear plasmid, generating plasmid pMW1628 (Fig. 1A). For transforming DNA, a 2,698-bp PCR fragment was generated using primer pair DW998/DW999 and designated Δ*pld* (Fig. 1A). The PCR fragment was gel purified using a Geneclean II kit (MP Biomedicals, Solon, OH), and 10 to 20 μg was used for transformations.

***R. prowazekii* transformation.** Purified rickettsiae were prepared for transformation and electroporated, as previously described (7, 8, 10). Following electroporation in the presence of the Δ*pld* PCR fragment, the rickettsiae were allowed to infect L929 cells (~4×10^7), and the cells were planted into three T185 cm^2 flasks. Twenty-four hours postinfection, the medium was replaced with medium containing the antibiotic rifampin (200 ng/ml). Medium was replaced with fresh antibiotic-containing medium every 4 to 7 days to ensure continued antibiotic selection. Culture expansion resulted in six T185 cm^2 flasks at day 9, following electroporation. Cells were harvested, and an aliquot was planted into five 96-well plates at approximately 3,000 cells per well. At day 13, each well was duplicated, and fresh rifampin-containing medium was added. DNA was harvested from the duplicate plates at day 15 using the Wizard SV 96 genomic DNA purification system (Promega, Madison, WI), and samples were assayed for the presence of the $rpsL^P$-$arr\text{-}2_{Rp}$ cassette by PCR analysis using primers DW316 and 3′arr2 (8). Wells containing $rpsL^P$-$arr\text{-}2_{Rp}$ cells were expanded to 12-well plates and cultured until sufficient numbers of rickettsiae could be detected microscopically. Uninfected L929 cells were added to the cultures as needed to provide host cells for the growing rickettsial population. Chromosomal primers, located outside of the recombination site and therefore not found within the transforming DNA, were designated DW1194 (5′-TCTTAATACTAACAATTATTGAC-3′) and DW1195 (5′-GGGTTAATCCTGATACCTC-3′). These primers amplify a 2,326-base-pair fragment when wild-type *R. prowazekii* genomic DNA is used as template (Fig. 1A). In contrast, a *pld* mutant resulting from recombination would be predicted to generate a 2,860-base-pair fragment (Fig. 1A).

Electron microscopy. RAW264.7 cells, cultured as monolayers in 6-well plates, were infected with rickettsiae harvested from L929 mouse fibroblast cells by ballistic shearing. L929 cell lysates were centrifuged at 200 × *g* for 5 min. The supernatant (1 ml) was added to each well of the plate, with the plate being maintained at 4°C on ice. The plates were then centrifuged at 200 × *g* for 1 h at 4°C. The medium was aspirated from the plates, and the monolayers were washed three times with ice-cold phosphate-buffered saline. Medium prewarmed to 37°C was added to the wells, and the plates were incubated at 37°C. At selected time points (10, 30, 45, and 60 min), the samples were fixed for electron microscopy in Ito's fixation solution. The samples were thin sectioned and observed by electron microscopy. Twenty electron micrographs were obtained for each sample, and the number of rickettsiae inside a vacuole rather than in the cytosol was determined by two of the investigators, without knowledge of which strain was being analyzed.

Animal experiments. The *R. prowazekii* Δ*pld* mutant was evaluated for virulence in guinea pigs. Male Hartley strain guinea pigs (200 g) were purchased from Charles River Laboratories (Wilmington, MA) and housed in the animal biosafety level 3 laboratory at the University of Texas Medical Branch. All animal studies were conducted in accordance with the Institutional Animal Care and Use Committee Guidelines. Rickettsial strains were cultured in L929 cells and stored at −80°C. The 50% tissue culture infective dose of the rickettsial samples was determined using the frozen stocks. Rickettsial strains were inoculated intraperitoneally into three animals per time point, and temperature and body weight were measured daily for 10 to 13 days postinoculation. To determine significance, an unpaired, one-tailed Student's *t* test was performed for pairwise comparisons between the Δ*pld* mutant and the control and between the wild type and the control. *P* values less than 0.05 were considered significant. Guinea pigs were bled via the toenail on days 4 and 7 and by heart puncture on the day of sacrifice. DNA was extracted from blood samples using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) and used as a template in nested PCR with 17-kDa gene-specific primers (20).

RESULTS

***pld* mutant isolation.** In initial transformation experiments with circular plasmid DNA, we often obtained single crossovers that resulted in insertion of the entire plasmid into the genome (data not shown). These single-crossover events resulted in transformants that were rifampin resistant and positive for our screening target gene $rpsL^P$-$arr\text{-}2_{Rp}$, complicating the search for the desired double-crossover mutants. To force double crossovers and obtain replacement of the wild-type *pld* gene with a partially deleted *pld* mutant, a PCR-amplified linear fragment was used in the transformation. In addition to restricting recombination to events that generate the desired mutant, linear DNA may provide a more efficient substrate for rickettsial recombination. Identification and isolation of an *R. prowazekii pld* mutant employed a PCR-based screening approach coupled with limiting dilution. To demonstrate the presence of the desired mutant, populations were screened by PCR to detect size differences at the *pld* gene locus. Based on genome sequence information, primer pair DW1194/DW1195 amplifies a 2,326-bp fragment from the wild-type Madrid Evir strain. In contrast, the desired mutant containing a partial deletion of the *pld* gene and the insertion of the rifampin-resistant cassette would generate a 2,860-bp fragment. As seen in Fig. 1B, lane 1, DNA isolated from the clonal transformant population generated a DNA fragment of the predicted size (2,860 bp) for a *pld* replacement mutant. A 2,326-bp product indicating the presence of wild-type rickettsiae was not detected, supporting the clonality of the transformant population. The *R. prowazekii* Madrid Evir *pld* mutant was also analyzed for the presence of Δ*pld* by Southern blotting (17). A 622-bp *pld*-specific probe generated using primers DW1266A (5′-ATGAAGAGCAAAAATAATAAATTTATAGC-3′) and DW1267A (5′-TATACTAAAAATGTACTGCATTACTCG-3′) that recognizes both wild-type and mutant *pld* sequences was used for hybridization. This probe hybridized to an expected 2,222-bp PstI fragment of wild-type chromosomal DNA (Fig. 1C, lane 3). However, only the single predicted 2,756-bp PstI fragment representing a partial deletion and insertion of the rifampin resistance cassette was detected in the cloned transformant (Fig. 1C, lane 2), confirming the clonality of the *pld* mutant. The sequence of the *R. prowazekii pld* region of the Δ*pld* strain, showing the location of the deletion and $rpsL^P$-$arr\text{-}2_{Rp}$ insertion, is shown in Fig. S1 in the supplemental material.

To assess virulence of the *pld* mutant, two model systems were employed. First, growth of the mutant in the macrophagelike RAW264.7 cell line was examined. Studies have demonstrated that the virulent Breinl strain of *R. prowazekii* can persist and grow in this phagocytic cell line, while the avirulent Madrid E strain cannot (19). Interestingly, the *pld* mutant strain was able to grow in the RAW264.7 cell line. Dramatic growth differences between the mutant and the wild-type virulent strain were not detected using either direct visual counting or quantitative PCR to determine rickettsial load (data not shown), indicating that the genetic mutations responsible for the attenuated phenotype in the Madrid E strain and Δ*pld* are different. We also examined whether the rickettsial strains could be distinguished by temporal differences in their ability to escape the phagosome. Analysis of electron micrographs (data not shown) at 10, 30, 45, and 60 min following infection revealed no significant differences between the Madrid Evir, Madrid E, or the *pld* mutant strains in their ability to escape the phagosome. All of the rickettsiae were found in the cytosol at the later time points. Only at 10 min could some of the rickettsiae be found in vacuoles, and the numbers of those inside vacuoles versus those released into the cytosol were indistinguishable among the three strains. However, distinct phenotypic differences were observed using the guinea pig model for assessment of rickettsial virulence.

Initially, guinea pigs were inoculated with high doses (1×10^9 rickettsiae/guinea pig) of either *R. prowazekii* strain Madrid Evir or the Δ*pld* mutant. Sham-infected animals were included as controls. At these high doses of rickettsiae, both the wild type and Δ*pld* mutant caused fever in guinea pigs (data not shown). However, body weight loss was more severe and prolonged in guinea pigs infected with the wild-type strain, suggesting that the Δ*pld* mutant may be less virulent. The increase in body temperature in these initial experiments was likely due to a host response to such a large rickettsial inoculum. To test this hypothesis, inoculations were performed using a lower dose of rickettsiae (1×10^6 rickettsiae per guinea pig). The wild-type Madrid Evir strain caused fever beginning at day 5 and peaking at day 9 to 10, while animals inoculated with the Δ*pld* mutant remained afebrile (Fig. 2A). Analysis of the body weight change also revealed a difference between the disease caused by wild-type *R. prowazekii* and the Δ*pld* mutant (Fig. 2B). Animals infected with the wild-type strain ceased to gain weight after day 5, while the animals infected with the Δ*pld* mutant continued to gain weight at a rate similar to that of the control animals. Rickettsial DNA was detected in the blood of guinea pigs obtained on day 7 after inoculation with both *R. prowazekii* strain Madrid Evir and the Δ*pld* mutant, confirming that the guinea pigs were infected.

To assess whether exposure to the Δ*pld* mutant strain of *R. prowazekii* could confer protective immunity, guinea pigs previously inoculated with the high dose of mutant rickettsiae were challenged with 1×10^9 rickettsiae of the virulent Breinl strain of *R. prowazekii* 5 weeks after the initial inoculation. Guinea pigs immunized with the Δ*pld* mutant strain did not develop fever or undergo a weight change when challenged (Fig. 3). The level of protection was similar to that seen when the animals were immunized with the wild-type Madrid Evir strain. In contrast, animals inoculated with saline exhibited increased body temperature and failed to gain weight following challenge. These results indicate that inoculation with the Δ*pld*

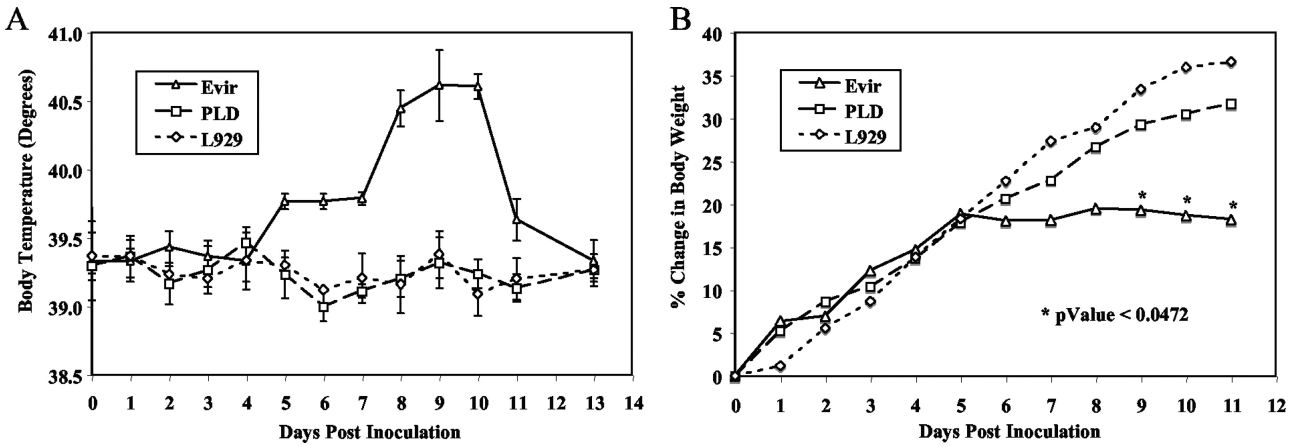

FIG. 2. Body temperature (A) and body weight (B) changes for guinea pigs inoculated intraperitoneally with 1×10^6 rickettsiae. Since L929 cells were used to propagate the rickettsiae, one set of animals was inoculated only with host cells as a control (◇). Animals were inoculated with either the Evir strain (△) or the Δ*pld* mutant (□). The asterisks denote significance at *P* values of <0.05.

mutant conferred effective immune protection against virulent *R. prowazekii* in the guinea pig model.

DISCUSSION

Genetic manipulation of *R. prowazekii* via transformation has been reported previously (7–10). However, due to the obligately intracellular nature of rickettsial growth, genetic manipulation of this pathogen remains a laborious and time-consuming undertaking. In this study, we demonstrate that, despite the challenges involved, it is feasible to target specific rickettsial genes for mutagenesis. An important variation to the transformation protocol that leads to successful mutant isolation was the use of linear DNA in transformations. When intact plasmids were used as transforming DNA, single-crossover events led to the insertion of the entire plasmid into the rickettsial genome. While this can be an effective mechanism for generating useful rickettsial mutants or for inserting genes into specific locations for complementation studies, the objective of our experiments was to generate a nonrevertible gene knockout via gene replacement. Thus, we employed a PCR-generated linear-transforming DNA in order to force a double-crossover event, resulting in the desired knockout. As reported previously (7), one of the more difficult challenges in rickettsial genetics is the isolation of a pure clone owing to spontaneous mutations and conferring resistance to rifampin in the population under selective pressure. Once again, in the current study, this was accomplished using limiting dilution.

The *pld* gene was chosen as the target for this study because of its potential association with rickettsial virulence. Somewhat surprising was the fact that no difference in growth between the Δ*pld* mutant and the wild-type virulent strain was detected within RAW264.7 cells. The ability to grow in this macrophagelike cell line has traditionally been used to differentiate the virulent Breinl strain and the avirulent Madrid E strain (19). Interestingly, electron microscopic analyses comparing

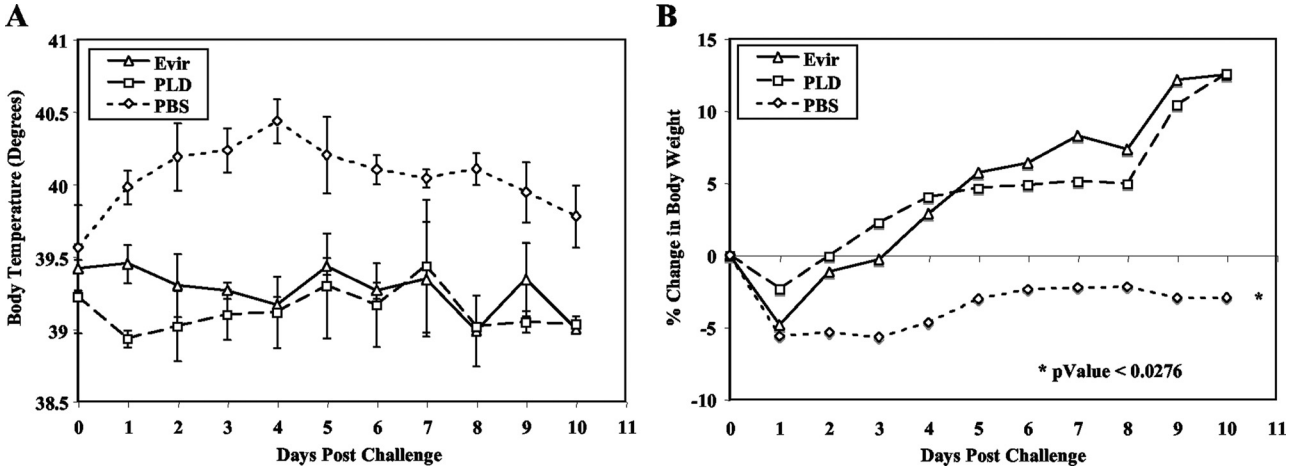

FIG. 3. Body temperature (A) and body weight (B) changes for guinea pigs previously inoculated with 1×10^9 rickettsiae and challenged at 5 weeks, following inoculation with 1×10^9 rickettsiae of the virulent Breinl strain of *R. prowazekii*. The guinea pigs were immunized with the Evir strain (△) and the Δ*pld* mutant (□) or sham inoculated with saline (◇). The asterisk denotes significance at *P* values of <0.05 for all data points. PBS, phosphate-buffered saline.

escape from the phagosome during infection revealed no differences among the Madrid Evir strain, the Δ*pld* mutant, and the Madrid E strain. In addition to demonstrating that a mutation in the *pld* gene does not affect phagosomal escape, these observations also provide evidence that the impairment of the Madrid E strain to grow in the macrophagelike cell line is not caused by a failure or delay in escape from the phagosome. These results support a previous study by Winkler and Daugherty that demonstrated the ability of RAW264.7 cytoplasm to differentiate between the virulent Breinl strain and the avirulent Madrid E strain (23). While growth of the Δ*pld* mutant in this cell line was not noticeably impaired, it was clearly attenuated in the guinea pig model used for testing rickettsial virulence. Obviously, this suggests that additional virulence factors are involved. Continued studies to characterize the mechanisms involved in this attenuation are required. Since the guinea pig is not an ideal animal model for *R. prowazekii*, we plan to use the nonhuman primate animal model to test the virulence of the Δ*pld* mutant and its ability to provide protection.

Enzymatic assays to compare PLD activity between the wild type and the Δ*pld* mutant have been complicated by assay sensitivity and possible contamination with host-derived phospholipases. To confirm that the Δ*pld* mutation is responsible for the observed phenotype, a complementation experiment is appropriate. However, the lack of established rickettsial plasmid vector systems precludes a simple plasmid transformation with a wild-type *pld* gene. Another option is the single-crossover insertion of a plasmid containing a wild-type gene or the introduction of the wild-type gene via a transposon that expresses a functional PLD. The feasibility of this approach has been shown with the *gltA* gene, encoding citrate synthase where a wild-type copy of the gene was inserted at the *gltA* site (9). However, although feasible, this experiment faces the same isolation, cloning, and interpretation challenges that were faced in isolating the original clone. This prevents a timely analysis of complementation. Despite the challenges involved, this study describes the first site-directed, nonrevertible knockout, generating a potential live vaccine candidate for epidemic typhus. In addition, the ability to target selected rickettsial genes for knockout provides a new tool for dissecting rickettsial biology.

ACKNOWLEDGMENTS

We thank Zhikai Zhang for performing the PCR guinea pig assays. We also thank Andria Hines and Andrew Woodard for excellent technical assistance with the rickettsial cloning experiments and quantitative PCR analysis, respectively.

This study was supported by National Institute of Allergy and Infectious Disease grants UO1 AI071283 (to X.J.Y.) and RO1 AI20384 (to D.O.W.).

REFERENCES

1. **Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1997. Current protocols in molecular biology, vol. 1, 2, and 3. John Wiley & Sons, Inc., New York, NY.
2. **Balaeva, N. M., and V. N. Nikolskaya.** 1973. Increased virulence of the E vaccine strain of *Rickettsia prowazeki* passaged in the lungs of white mice and guinea pigs. J. Hyg. Epidemiol. Microbiol. Immunol. **17:**11–20.
3. **Baldridge, G. D., N. Burkhardt, M. J. Herron, T. J. Kurtti, and U. G. Munderloh.** 2005. Analysis of fluorescent protein expression in transformants of *Rickettsia monacensis*, an obligate intracellular tick symbiont. Appl. Environ. Microbiol. **71:**2095–2105.
4. **Bozeman, F. M., S. A. Masiello, M. S. Williams, and B. L. Elisberg.** 1975. Epidemic typhus rickettsiae isolated from flying squirrels. Nature **255:**545–547.
5. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. A high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–379.
6. **Fox, J. P.** 1956. Immunization against epidemic typhus; a brief general review and a description of the status of living, avirulent R. prowazeki (strain E) as an immunizing agent. Amer. J. Trop. Med. Hyg. **5:**464–479.
7. **Liu, Z.-M., A. M. Tucker, L. O. Driskell, and D. O. Wood.** 2007. *mariner*-based transposon mutagenesis of *Rickettsia prowazekii*. Appl. Environ. Microbiol. **73:**6644–6649.
8. **Qin, A., A. M. Tucker, A. Hines, and D. O. Wood.** 2004. Transposon mutagenesis of the obligate intracellular pathogen *Rickettsia prowazekii*. Appl. Environ. Microbiol. **70:**2816–2822.
9. **Rachek, L. I., A. Hines, A. M. Tucker, H. H. Winkler, and D. O. Wood.** 2000. Transformation of *Rickettsia prowazekii* to erythromycin resistance encoded by the *Escherichia coli ereB* gene. J. Bacteriol. **182:**3289–3291.
10. **Rachek, L. I., A. M. Tucker, H. H. Winkler, and D. O. Wood.** 1998. Transformation of *Rickettsia prowazekii* to rifampin resistance. J. Bacteriol. **180:**2118–2124.
11. **Raoult, D., J. B. Ndihokubwayo, H. Tissot-Dupont, V. Roux, B. Faugere, R. Abegbinni, and R. J. Birtles.** 1998. Outbreak of epidemic typhus associated with trench fever in Burundi. Lancet **352:**353–358.
12. **Raoult, D., T. Woodward, and J. S. Dumler.** 2004. The history of epidemic typhus. Infect. Dis. Clin. N. Am. **18:**127–140.
13. **Raschke, W. C., S. Baird, P. Ralph, and I. Nakoinz.** 1978. Functional macrophage cell lines transformed by Abelson leukemia virus. Cell **15:**261–267.
14. **Reeves, W. K., K. O. Murray, T. E. Meyer, L. M. Bull, T. F. Pascua, K. C. Holmes, and A. D. Loftis.** 2008. Serological evidence of typhus group rickettsia in a homeless population in Houston, Texas. J. Vector Ecol. **33:**205–207.
15. **Renesto, P., P. Dehoux, E. Gouin, L. Touqui, P. Cossart, and D. Raoult.** 2003. Identification and characterization of a phospholipase D-superfamily gene in rickettsiae. J. Infect. Dis. **188:**1276–1283.
16. **Reynolds, M. G., J. W. Krebs, J. A. Comer, J. W. Sumner, T. C. Rushton, C. E. Lopez, W. L. Nicholson, J. A. Rooney, S. E. Lance-Parker, J. H. McQuiston, C. D. Paddock, and J. E. Childs.** 2003. Flying squirrel-associated typhus, United States. Emerg. Infect. Dis. **9:**1341–1343.
17. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503.
18. **Tarasevich, I., E. Rydkina, and D. Raoult.** 1998. Outbreak of epidemic typhus in Russia. Lancet **352:**1151.
19. **Turco, J., and H. H. Winkler.** 1991. Comparison of properties of virulent, avirulent, and interferon-resistant *Rickettsia prowazekii* strains. Infect. Immun. **59:**1647–1655.
20. **Webb, L., M. Carl, D. C. Malloy, G. A. Dasch, and A. F. Azad.** 1990. Detection of murine typhus infection in fleas by using the polymerase chain reaction. J. Clin. Microbiol. **28:**530–534.
21. **Whitworth, T., V. L. Popov, X. J. Yu, D. H. Walker, and D. H. Bouyer.** 2005. Expression of the *Rickettsia prowazekii pld* or *tlyC* gene in *Salmonella enterica* serovar Typhimurium mediates phagosomal escape. Infect. Immun. **73:**6668–6673.
22. **Winkler, H. H.** 1976. Rickettsial permeability: an ADP-ATP transport system. J. Biol. Chem. **251:**389–396.
23. **Winkler, H. H., and R. M. Daugherty.** 1983. Cytoplasmic distinction of avirulent and virulent *Rickettsia prowazekii*: fusion of infected fibroblasts with macrophage-like cells. Infect. Immun. **40:**1245–1247.
24. **Zhang, J. Z., J. F. Hao, D. H. Walker, and X. J. Yu.** 2006. A mutation inactivating the methyltransferase gene in avirulent Madrid E strain of *Rickettsia prowazekii* reverted to wild type in the virulent revertant strain Evir. Vaccine **24:**2317–2323.

Editor: R. P. Morrison