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Identification and Cloning of a Fraction 1 Protein of *Yersinia pestis* that Produces Protective Immune Responses

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Abstract The capsule that surrounds *Yersinia pestis* cells is composed of a protein-polysacchride complex; the purified protein component is fraction 1 (F1) antigen. We report the cloning of the *caf*1 gene and its expression in *Escherichia coli* using the vector pET102/D-TOPO and the F1-specific monoclonal antibody. The recombinant F1 (rF1) antigen had a molecular size of 17.5 kDa, which was identical to that of the F1 antigen produced by *Y. pestis*. Recombinant F1 protein was found to react to polyclonal antiserum to *Y. pestis* F1. Recombinant F1 was purified by ProBond purification system and induced a protective immune response in BALB/c mice challenged with up to 10^5 virulent *Y. pestis*. Purified rF1 protein was used in an ELISA to evaluate the ability of a method to detect antibodies to *Y. pestis* in animal sera. These results strongly indicated that the rF1 protein is a suitable species-specific immunodiagnostic antigen and vaccine candidate.

Key words: *Y. pestis*, fraction 1 antigen

Although *Y. pestis* no longer causes pandemics, the bacterium is still a public health problem in the Asian subcontinent and Far East [5]. Most diseases caused by *Y. pestis* are of bubonic form, arising from a bite of a flea that has fed on blood of an infected rodent. Some cases of bubonic plague progress to a pneumonic form, which can be transmitted human-to-human, leading to epidemics [6].

Y. pestis, the causative agent of plague, still represents a serious public health threat in various regions of the world and, at the same time, it is gaining attention as a potential agent in bioterrorism. Even though live and killed whole-cell vaccines are available for human use, serious drawbacks limit their use for prevention of natural or human-inflicted outbreaks [13]. Two *Y. pestis* proteins, F1 and V, are known to be effective immunogens and have been proposed as candidates for a combined subunit vaccine against plague [1].

The fraction 1 capsular protein (F1), which is encoded by a 100-kb pFra plasmid, forms a large gel-like capsule containing multimeric F1 aggregates [4]. The F1 gene was found to code for a 17.5-kDa polypeptide carrying a putative secretion signal [9]. F1 protein is considered to be an important but not essential virulence factor unique to *Y. pestis* [7, 18]. Deletion of the F1 gene does not abolish virulence, but leads to a delay of disease onset in animal models. The detection of antibodies against F1 is the basis of standard serological tests for the surveillance and diagnosis of plague, as infected animals and patients produce a strong antibody response to this antigen [20].

We report the molecular cloning and expression of the F1 protein gene of *Y. pestis* and the antigenicity and immunogenicity of recombinant protein. In addition, we produced monoclonal antibodies against *Y. pestis* F1 antigen.

Materials and Methods

Bacterial Strains and Animals

Y. pestis was cultured aerobically at 28°C in BHI medium or CIN agar, supplemented with *Yersinia* antimicrobic supplement CN (Difco). *E. coli* BL21 was cultured and stored, as described by Sambrook and Russel [16]. Six-week-old BALB/c female mice (Japan SLC) were used throughout this study.

Manipulation of DNA

The gene encoding F1 antigen (*caf1*) was amplified from *Y. pestis* DNA by PCR with 100 pmol of primers that are identical to sequences from the 5' and 3' ends of the gene: The sequences were 5' primer (F1/F:CACCATGGACAA-TGGAAACATC GATAC) and 3' primer (F1/R:TTATTG-

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GTTAGATACGGTTACG). To make directional cloning, the forward PCR primer contains the sequence CACC at the 5'-end of the primer. A DNA fragment was obtained after 30 cycles of amplification [95°C, 20 s; 58°C, 20 s; 72°C, 20 s (MJ research)]. The PCR product was then cloned into the Invitrogen pET102/D-TOPO vector and transformed into chemically competent *E. coli* TOP10F' cells. Plasmid DNA from these clones was sequenced by MACROGEN using primers included in the Invitrogen TA cloning kit. The primers were T7 forward (5'-TAATACGACTCACTATAGGG-3') and V5 C-terminal reverse (5'-ACCGAGGAGAGGGTTAGGGAT-3'). Plasmid DNA from a clone showing the correct construct with a thrombin cleavage site was transformed into chemically competent *E. coli* BL21 Star (DE3)pLysS cells for expression of the six-histidine-tagged fusion protein.

Expression of Recombinant F1 Antigen and Purification of rF1

The expression strain was cultivated overnight at 37°C in Lennox Broth (LB) medium supplemented with 100 mg/l ampicillin. A 0.5-ml aliquot of an overnight culture was mixed with 3 ml of medium and incubated under shaking for 60 min at 37°C to an optical density of 0.5 at 600 nm, induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cultivated for 4 h at 37°C. The recombinant F1 protein was isolated and purified using ProBond™ columns (Invitrogen Corporation, U.S.A.).

SDS-PAGE and Immunoblot Analysis

The protein sample was suspended in equal volume of sample buffer, boiled for 5 min, and electrophoresed on 12.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) followed by staining with Coomassie blue R or transferred to polyvinylidene fluoride (PDVF) membranes (Immobilon-Millipore, MA, U.S.A.). Molecular weight markers were (LMW): phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and lactalbumin (14.4 kDa) (BioRad, Hercules, CA, U.S.A.). For the immunoblot, protein bands were transferred to PDVF membranes at 80 V for 50 min. Membrane proteins were successively incubated with mouse anti-F1 antigen polyclonal serum and alkaline phosphate-conjugated goat anti-mouse IgG (H&L. Promega, Madison, WI, U.S.A.) [11]. Bound antibodies were detected by nitroblue tetrazolium/5-bromo-4-chloro-3-indolyphosphate (Sigma, MO, U.S.A.).

Analysis of Blood Sample

Isotype and titer of the antibody response in serum samples were measured by ELISA. For the soluble rF1 antigens, 96-well plates (Dynatech, Chantilly, VA, U.S.A.) were coated with 100 µl of antigen [5.0 µg/ml in PBS (pH 7.4)] per well and incubated overnight at 4°C. Plates were washed three times in PBS (pH 7.4) with 0.1% Tween 20. Serum samples were applied at a starting dilution of 1:100 in PBS (pH 7.4)–0.1% Tween 20–5% (wt/vol) skim milk, and serially diluted twofold to 1:12,800 in a final volume of 100 µl/well. The plates were incubated for 1 h at 37°C, washed, and then 100 µl of 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Kirkegaard & Perry) was added per well. After 1 h of incubation at 37°C, plates were washed, and 100 µl of 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) two-component substrate system (Kirkegaard & Perry) was added. Following incubation at 37°C for 30 min, plates were read at 405 nm on a Bio-Rad plate reader. The endpoint titer was determined by the highest test serum dilution with optical density (OD_{405}) of ≥0.20 at 405 nm after subtraction of OD_{405} for the blank wells with no antigen added. The results are expressed as the geometric mean titer of the reciprocal endpoint dilution for three separate experiments.

The measurement of IgG subclass titer was carried out on a pooled sample representative of the immunization group. Each of the peroxidase-labeled secondary antibodies against mouse, IgG1, IgG2a, IgG2b, and IgG3 (HarlanSeraLab, Crawley Down, U.K.), at dilution 1:2,000 was used, and they were found to be specific and to have equivalent sensitivity of detection (1 ng/ml) for their homologous IgG subclass proteins, by using standard curves with the respective IgG subclass proteins.

Immunization Protocol

Groups of 18 mice were allocated to one of three immunization regimens, described below. All groups received a single immunization on day 1, delivered by the intraperitoneal (i.p.) route. All animals were dosed with a total volume of 100 µl divided equally in the abdomen. Group 1 animals were dosed with 50 µg of rF1 antigen in phosphate-buffered saline (PBS). Group 2 animals were dosed with *E. coli* pET/F1 in PBS. Groups 3 animals received 100 µl of PBS alone. After thirty days post-inoculation, mice were bled and challenged intraperitoneally with log dilution of *Y. pestis* (ATCC19428) suspended in 100 µl of PBS. The lethal dose required to kill 50% of the animals (LD_{50}) within 21 days was calculated by the method of Reed and Muench [14].

RESULT

PCR and Cloning of rF1

The *caf*1 gene size was determined after amplification by PCR. A PCR product was obtained, and the *caf*1 gene size was about 370 bp (Fig. 1).

Expression of rF1 Antigen and Immunoblot Analysis

The product gene was cloned downstream of the N-terminal hexa-histidine gene encoded by the plasmid pET102/

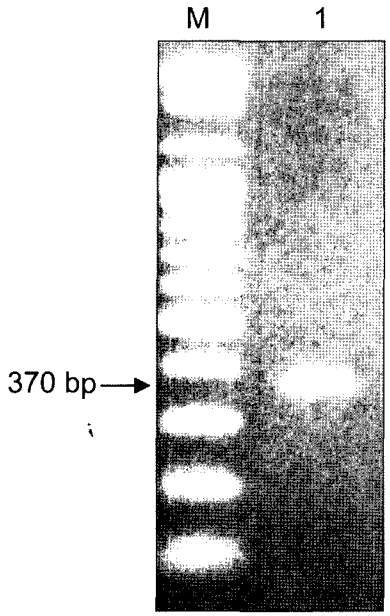

Fig. 1. Amplified products of the *caf*1 gene from DNA of *Y. pestis* ATCC23053 by PCR.
Lane M, 100-bp ladder; lane 1, product of the *caf*1 gene amplified by PCR.

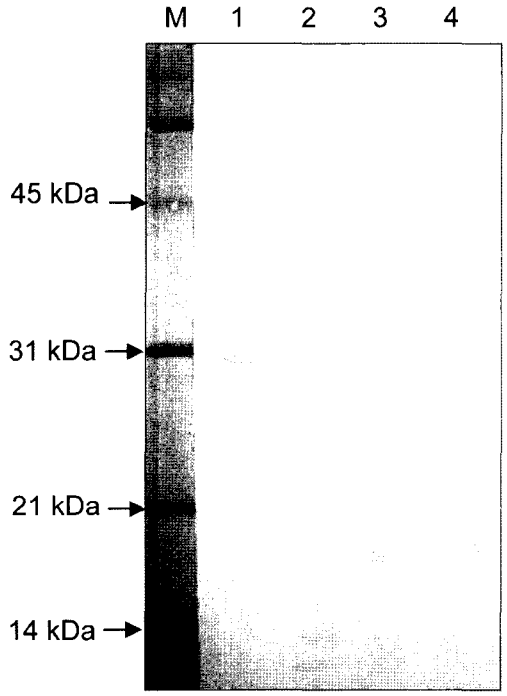

Fig. 2. Immunoblot analysis of purified recombinant F1 protein produced by the pET/F1. The purified recombinant F1 proteins were resolved by 12.5% SDS-PAGE, transferred to PVDF membranes, and reacted with monospecific polyclonal anti-mouse sera against recombinant F1 protein.
Lane M, protein molecular weight marker; lane 1, not induced by IPTG; lane 2, purified 6×His-F1 fusion protein induced with 0.5 mM IPTG, and grown further for 4 h; lane 3, purified recombinant F1 protein; lane 4, whole-cell protein of *Y. pestis*.

D-TOPO vector to form an in-frame fusion. An IPTG-induced culture of *E. coli* BL21 Star (DE3)pLysS, containing the recombinant plasmid pET102/F1, produced a unique protein that was detected by Western blotting with mouse anti-F1 serum (Fig. 2). The molecular size of this protein was determined by SDS-PAGE and found to be similar to the predicted value of 30 kDa for the 6×His-F1 fusion. The recombinant F1 proteins were purified using ProBond™ Columns, and the elution fractions were detected by SDS-PAGE and immunoblot assay (Fig. 2).

Antibody Titer and Immunoglobulin Subtype in Serum

Animals given a single dose of antigens responded by day 13 with high serum IgG titers to F1. By comparison, however, animals given a single dose of *E. coli* pET/F1 vaccine had a significantly lower anti-F1 IgG titer. All mice immunized with rF1 and *E. coli* pET/F1 had detectable serum IgG responses to F1 antigen (Fig. 3), although the levels of response differed between animals. This pattern of response to F1 antigen was highly reproducible between experiments. The predominant IgG antibody subclass to F1 antigen in the serum was found to be IgG2a (Fig. 3). No anti-F1 antigen antibodies were detected, when immunized with PBS.

Protective Properties of rF1 Antigen

BALB/c mice immunized intraperitoneally with rF1 (~10 μg) or *E. coli* pET/F1 (10^5 CFU) were challenged 13 days after the immunization with 1×10^5 to 1×10^7 CFU of virulent *Y. pestis* via the intraperitoneal route. All control mice and *E. coli* pET/F1-immunized mice succumbed to infection with 1×10^7 CFU of virulent *Y. pestis* by day 3 post challenge (Table 1). On the other hand, immunization with rF1 (~10 μg) resulted in a 33% survival rate (2 out of 6). All the animals immunized with 1×10^5 CFU were fully protected and survived without development of externally visible symptoms during the 21-day observation period. In contrast, all six mice in the control group, which received a 1×

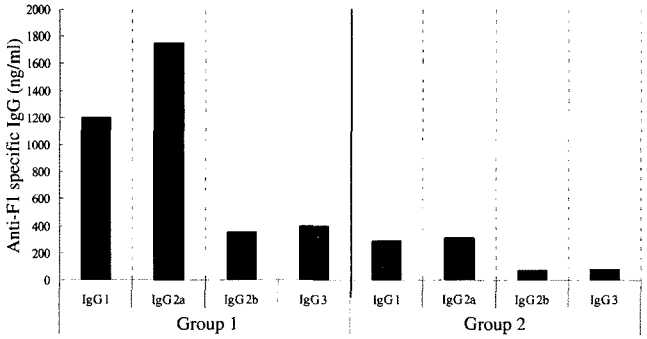

Fig. 3. F1 antigen-specific IgG isotype response induced following immunization with rF1 (Group 1) and *E. coli* pET/F1 (Group 2). Antibodies were measured on day 13 after challenge with *Y. pestis* ATCC19428.

Table 1. Mortality data for vaccinated BALB/c mice challenged with *Y. pestis*.

Immunization	Challenge dose (CFU of *Y. pestis*)	No. of mice surviving*	Time to death
PBS	1×10^5	0/6	56 h
	1×10^6	1/6	56 h
	1×10^7	0/6	56 h
Heat-killed *E. coli* BL21 (pET/F1)	1×10^5	1/6	117 h
	1×10^6	0/6	106 h
	1×10^7	0/6	68 h
Recombinant F1 antigen (~10 μg)	1×10^5	6/6	
	1×10^6	5/6	62 h
	1×10^7	2/6	69 h

*Each group had six mice. Deaths were recorded up to 21 days post challenge.

10^5 CFU dose of *Y. pestis*, succumbed a mean time to death of 56 h.

DISCUSSION

The capsule of *Y. pestis* is composed of high molecular size polymer of a single 17.5 kDa polypeptide (the capsular F1 subunit, caf1 subunit). It is highly immunogenic. Active immunization with purified F1 and passive immunization with anti-F1 antibodies have both been shown to protect mice from experimental bubonic and pneumonic plague [20]. The capsule that surrounds *Y. pestis* cells is composed of a protein-polysaccharide complex; the purified protein component is F1 antigen [15] with a molecular mass of 17.5 kDa [17]. F1 is fully expressed only at 37°C and believed to confer resistance to phagocytosis, possibly by forming aqueous pores in the membranes of phagocytic cells [18]. In this study, the pET102/D-TOPO expression vector was chosen to avoid accumulation of recombinant protein in the form of inclusion body. The *caf1* gene (370 bp) was amplified from the pFra plasmid of the reference ATCC19428 of *Y. pestis*, and cloned and expressed in *E. coli*, yielding relatively large amounts of recombinant fusion protein that could easily be purified by a ProBond system. The molecular weight of rF1 was estimated to be 17 kDa by SDS-PAGE, which is almost similar to the values of 17.5 kDa reported for native F1 antigen [20] and 17,664 predicted from the amino acid sequence. Recombinant F1 antigen was recognized by Western blots, using monospecific polyclonal antiserum (Fig. 2). It was not cross-reactive with *Y. enterocolitica* and *Y. pseudotuberculosis* (data not shown).

In this study, i.p.-administered rF1 vaccine has been demonstrated to protect animals from exposure to *Y. pestis* in excess of 1×10^5 CFU. In contrast, an *E. coli* pET/F1 vaccine failed to protect animals against this dosage of *Y. pestis*. Thus, the rF1 vaccine even after a single dose provided six-fold protection against *Y. pestis* (1×10^5 challenge dose of *Y. pestis*), compared with the IPTG-inducted *E. coli* pET/F1 vaccine (Table 1). This level of protection in a mouse model against a wild-type strain of *Y. pestis* is greater than that published previously for a single-dose vaccine [1].

A number of previous studies have indicated that antibody-mediated immunity is important in protection against plague [17]. This may be expected, since *Y. pestis* is a predominantly extracellular infection. Immunization of mice with the plague subunit vaccine resulted in a strong total IgG1 response, which correlated with protection [19, 10]. Furthermore, transfer of mouse hyperimmune serum into naïve mice protected the latter against plague challenge [19]. In this study, we assessed whether the protection afforded by rF1 was mediated by antibody, by investigating F1 antigen-specific IgG responses in individual mice following immunization. However, there was no clear correlation between anti-F1 antigen and specific IgG protection. Nevertheless, the serum IgG concentration of 5 survivors within the rF1 vaccinated group showed 600 ng/ml. The IgG concentration of 8 remaining mice showed below this level (on the average of 380 ng/ml). Furthermore, the mean total F1 antigen-specific IgG response was greater in the rF1 immunized mice that survived plague challenge than with the non-survivors (Table 1). These data suggest that antibody against F1 antigen may provide an indicator of protection against *Y. pestis* challenge.

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