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CHARACTERIZATION AND IMMUNOGENICITY OF RLIPL32/1-LIPL21-OMPL1/2 FUSION PROTEIN AS A NOVEL IMMUNOGEN FOR A VACCINE AGAINST LEPTOSPIROSIS

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Abstract – Vaccination is an effective strategy to prevent leptospirosis, a global zoonotic disease caused by infection with pathogenic *Leptospira* species. However, the currently used multiple-valence vaccine, which is prepared with whole cells of several *Leptospira* serovars, has major side effects, while its cross-immunogenicity among different *Leptospira* serovars is weak. LipL32, LipL21 and OmpL1 have been confirmed as surface-exposed antigens in all pathogenic *Leptospira* strains, but their immunoprotective efficiency needs to be improved. In the present study, we generated a fusion gene *lipL32/1-lipL21-ompL1/2* using primer-linking PCR and an engineered *E. coli* strain to express the recombinant fusion protein rLipL32/1-LipL21-OmpL1/2 (rLLO). Subsequently, the expression conditions were optimized using a central composite design that increased the fusion protein yield 2.7-fold. Western blot assays confirmed that rLLO was recognized by anti-rLipL32/1, anti-rLipL21, and anti-rOmpL1/2 sera as well as 98.5% of the sera from leptospirosis patients. The microscopic agglutination test (MAT) demonstrated that rLLO antiserum had a stronger ability to agglutinate the strains of different *Leptospira* serovars than the rLipL32/1, rLipL21, and rOmpL1/2 antisera. More importantly, tests in hamsters showed that rLLO provided higher immunoprotective rates (91.7%) than rLipL32/1, rLipL21 and rOmpL1/2 (50.0-75.0%). All the data indicate that rLLO, a recombinant fusion protein incorporating three antigens, has increased antigenicity and immunoprotective effects, and so can be used as a novel immunogen to develop a universal genetically engineered vaccine against leptospirosis.

Key words: Leptospirosis; triple antigen; genetically engineered vaccine; immunogenicity

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INTRODUCTION

Leptospirosis is a global zoonotic disease in both humans and animals caused by infection with pathogenic *Leptospira* species (Bharti et al., 2003). Human leptospirosis is prevalent in East/Southeast Asia and South America (Ricaldi et al., 2013). However, in recent years, human leptospirosis cases have been frequently reported in Europe, North America, and Africa, where it is considered an emerging or re-emerging infectious disease (Vijayachari et al., 2008; Hartskeerl et al., 2011; Dupouey et al., 2014). Thus, this disease has become a public health problem of global importance (Pappas et al., 2008).

Many animals, including dogs and livestock have been confirmed as the natural hosts of pathogenic *Leptospira* species. Infected animals usually display a mild or symptomless persistent infection but discharge leptospires through their urine for long periods that contaminate soil and natural bodies of water (Adler et al., 2010). Humans suffer from leptospirosis after contact with leptospire-contaminated soil and water (McBride et al., 2005). Pathogenic *Leptospira* species rapidly enter the blood stream after invading the body through the skin and mucosa and then promptly spread to the lungs, liver, and kidneys, where they cause tissue injury (Adler et al., 2010; McBride et al., 2005; Dassanayake et al., 2012). The course of leptospirosis in humans varies from mild to rapidly fatal forms, including influenza-like manifestations such as fever and myalgia, and severe symptoms such as respiratory failure due to diffuse pulmonary hemorrhage and meningitis, and renal failure caused by renal injury and serious jaundice (McBride et al., 2005; Dassanayake et al., 2012; De Brito et al., 2013). Pathogenic *Leptospira* species have been classified into seven genospecies: *L. interrogans*, *L. borgpetersenii*, *L. kirschneri*, *L. noguchii*, *L. santarosai*, *L. meyeri*, and *L. weilii* (Adler et al., 2010; Hu et al., 2014). However, *L. interrogans*, which has been serologically divided into about 230 serovars belonging to 23 serogroups, is the predominant pathogenic genospecies in most countries and areas in the world, including China (Forbes et al., 2012; Miraglia et al., 2013; Sakundarno et al., 2014; Zhang et al., 2012).

Vaccination is an effective strategy to prevent infectious diseases. Since there is a geographical diversity of predominant *L. interrogans* serovars and major animal carriers, and since the cross-immunogenicity among different *L. interrogans* serovars is weak, the currently used leptospirosis vaccine is prepared with killed whole cells of the 3-5 *L. interrogans* serovars prevailing in local areas (Fitzgerald et al., 2009; Verma et al., 2013). However, currently vaccines against leptospirosis are of low efficacy, have an unacceptable side-effect profile, and provide no cross-protection against the different serovars of pathogenic leptospires. (Fitzgerald et al., 2009; Verma et al., 2013; Srikram et al. 2008). More importantly, the vaccine does not provide immunogenicity for individuals infected with *L. interrogans* serovars that are not included in the vaccine (Hu et al., 2014; Zhang et al., 2012). Therefore, a new vaccine with extensive cross-immunogenicity and minimal side effects would greatly contribute to the prevention and control of leptospirosis.

Leptospiral membrane proteins can act as protective antigens in experimental animals given a lethal challenge of *L. interrogans* (Atzingen et al., 2010). Previous studies have revealed that membrane proteins such as OmpL1, LipL21, LipL32, LipL41, LipL46, LenA, Loa22, and LigB are only expressed in pathogenic *Leptospira* species (Haake et al. 1993; Haake et al., 2000; Shang et al., 1996; Cullen et al., 2003; Matsunaga et al., 2006; Verma et al., 2010; Ristow et al., 2007; Matsunaga et al., 2003). However, our previous studies confirmed that the *lipL32*, *lipL41*, and *ompL1* but not the *lipL21* genes in 15 strains belonging to 15 serovars in 15 serogroups of pathogenic *L. interrogans*, *L. borgpetersenii*, and *L. weilii* in China have different genotypes (*lipL32/1* and *lipL32/2*, *lipL41/1* and *lipL41/2*, and *ompL1/1*, *ompL1/2*, and *ompL1/3*), but the major predominant *L. interrogans* serovars in China express OmpL1/2- and LipL32/1-type proteins (Dong et al., 2008; Luo et al., 2009). Compared to recombinant OmpL1, LipL21 and LipL32, recombinant LipL41 has been shown to provide less immunogenicity in guinea-pigs (Dong et al., 2008; Luo et al., 2009). Therefore, the LipL21, OmpL1/2, and LipL32/1 proteins of *L. interrogans* have potential use for developing a new universal genetically engineered vaccine against leptospirosis.

Prokaryotic microbes including *Leptospira* possess a much more complicated antigenic composition than viruses (Nally et al., 2007). In our view, the use of multiple-protein antigens as the immunogen will improve the immunoprotective effect of genetically engineered vaccines against leptospirosis. In the present study, we generated a fusion gene *lipL32/1-lipL21-ompL1/2* and its prokaryotic expression system. The cross-immunoreactivity and immunogenicity in hamsters of the recombinant triple-protein antigen expressed by this fusion

gene were subsequently determined. Moreover, the expression conditions of the triple-protein antigen were optimized.

MATERIALS AND METHODS

Leptospiral strains and culture

Fifteen strains belonging to 15 serovars in 15 serogroups of three pathogenic *Leptospira* genospecies (Table 1), which are prevalent in China and officially used as the standard strains for serological diagnosis of Chinese leptospirosis patients, were provided by the Chinese National Institute for Control of Pharmaceutical and Biological Products (Table 1). The strains were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium at 28°C (Zhao et al., 2013). *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai strain Lai, serogroup Grippotyphosa serovar Grippotyphosa strain Lin-6, and serogroup Pomona serovar Pomona strain Luo used in animal protective test were maintained by serial passage in hamsters to preserve virulence.

Serum samples

Serum samples from 133 leptospirosis patients with microscopic agglutination test (MAT) titers >1:100 collected in the last three years were provided by the Centers of Disease Prevention and Control of Sichuan, Jiangxi and Zhejiang Provinces in China (Table 1). Serum samples from 12 individuals without antibodies against *Leptospira* as determined by MAT collected during routine health examination were provided by the Affiliated Hospitals of Zhejiang University School of Medicine. The collection of serum samples was approved by the Research Ethics Committee of Zhejiang University School of Medicine and informed written consent for sample collection was given by all participants.

Animals

New Zealand rabbits (3.2 to 3.5 kg) and male Syrian hamsters (25 ± 2 g, 4 weeks old) were provided by the Experimental Animal Center of Zhejiang University. The experimental protocols were approved by the Animal Ethics Committee of Zhejiang University School of Medicine.

Amplification and sequencing of target leptospiral genes

Genomic DNA from *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai strain Lai was extracted using a bacterial DNA extraction kit (BioColor, China). In order to decrease the size of the *lipL32/1*, *lipL21*, and *ompL1/2* fusion gene to enhance the recombinant fusion protein expression in the next experiment, the signal peptide sequence-free *lipL21* gene (49-558 bp) and transmembrane sequence-free *lipL32/1* and *ompL1/2* genes (58-816 and 73-960 bp) were amplified by PCR from the leptospiral DNA using the primers P1F/P1R, P2F/P2R, and P3F/P3R (Table 2). The P1R, P2F, P2R, and P3F primers contained a flexible peptide (GGGGSGGGGSGGGGS)-encoding sequence at the 5' terminals and this sequence was not only used to link the three genes but was also beneficial for maintaining the independent immunogenicity of the LipL32/1, LipL21 and OmpL1/2 segments in the fusion protein. The products were examined on 1.5% ethidium bromide pre-stained agarose gel after electrophoresis and then cloned into pUC18-T plasmid to form pUC18-$T^{lipL32/1}$, pUC18-T^{lipL21}, and pUC18-$T^{ompL1/2}$ using a T-A cloning kit (TaKaRa, China) for sequencing by Invitrogen Co.,Shanghai, China.

Generation and identification of lipL32/1-lipL21-ompL1/2 fusion gene

The leptospiral *lipL32/1*, *lipL21*, and *ompL1/2* genes were amplified by PCR as above. The PCR products were extracted using a PCR Product Purification Kit (Axygen, USA), and then quantified by ultraviolet spectrophotometry (Xue et al., 2010). A special PCR was performed to generate the *lipL32/1-lipL21-ompL1/2* fusion gene. The reactive mixture contained all the PCR reagents, except for the primers, using 100 ng DNA from each of the extracts as the template. The reaction was initiated at 94°C for 5 min and then repeated at 94°C for 1 min, 45°C for 1 min, and 72°C

for 3 min for 10 cycles to form a combined template *lipL32/1-lipL21-ompL1/2* (a 2247-bp segment including 759-bp *lipL32/1*, 510-bp *lipL21*, and 888-bp *ompL1/2* plus two 45-bp flexible peptide linker sequences) (Table 2). Subsequently, the primers P1F and P3R (Table 2) were added to the mixture for 30 cycles of amplification at 52°C annealing temperature. The agarose gel electrophoresis, T-A cloning, and sequencing of the amplified *lipL32/1-lipL21-ompL1/2* fusion gene were performed as described above.

Generation of E. coli strain expressing lipL32/1-lipL21-ompL1/2 gene

The pET42a vector (Novagen, USA) and pUC18-T$^{lipL32/1\text{-}lipL21\text{-}ompL1/2}$ were digested with Nde I and Xho I endonucleases (TaKaRa), respectively. The recovered *lipL32/1-lipL21-ompL1/2* segment was linked with the linearized pET42a using T4 DNA ligase (TaKaRa) to form a prokaryotic recombinant expression vector pET42a$^{lipL32/1\text{-}lipL21\text{-}ompL1/2}$ for sequencing. The recombinant vector was transformed into *E. coli* BL21DE3 (Novagen) to form an engineered strain (*E. coli* BL21DE3$^{pET42a\text{-}lipL32/1\text{-}lipL21\text{-}ompL1/2}$). This strain was cultured in kanamycin-containing Luria-Bertani (LB) liquid medium (Oxoid, UK). The pET42a$^{lipL32/1\text{-}lipL21\text{-}ompL1/2}$ was extracted from the culture using a Plasmid MiniPrep Kit (Axygen) for re-sequencing.

Expression and extraction of leptospiral recombinant proteins

The engineered *E. coli* BL21DE3$^{pET42a\text{-}lipL32/1}$, *E. coli* BL21DE3$^{pET42a\text{-}lipL21}$, and *E. coli* BL21DE3$^{pET42a\text{-}ompL1/2}$ were from our laboratory (Dong et al., 2008; Luo et al., 2009). Under induction by 1 mM isopropyl-β-D-thiogalactoside (IPTG) (Sigma, USA), the target recombinant proteins rLipL32/1, rLipL21, rOmpL1/2, and rLipL32/1-LipL21-OmpL1/2 (rLLO) were expressed by the three engineered strains and *E. coli* BL21DE3$^{pET42a\text{-}lipL32/1\text{-}lipL21\text{-}ompL1/2}$, respectively. The expressed recombinant proteins were extracted using an Ni-NTA affinity chromatographic column (BioColor). The yield and purity of the recombinant proteins were estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a Gel Image Analyzer (BioRad, USA).

Optimization of rLLO expression conditions

To increase the yield of rLLO, a central composite design (CCD) from response surface methodology was applied to optimize the expression conditions such as initial pH, dose of IPTG, duration before and after induction, and induction temperature. The details of the experiments are shown in the Supplementary Materials.

Preparation of rLLO antiserum

Rabbits were intradermally immunized on days 1, 10, 20 and 30 with 2 mg per animal of rLLO premixed with Freund's adjuvant. Fifteen days after the last immunization, the serum was collected. Immunodiffusion test was used to assess the titer of antiserum binding to the recombinant fusion protein.

Western blot analysis

The protein concentrations in the leptospiral rLipL32/1, rLipL21, rOmpL1/2, and rLLO extracts were determined using a BCA Protein Quantitative Kit (Beyotime Biotech, China) (Wang et al., 2012). After SDS-PAGE, the recombinant proteins (10 μg each) in gels were electron-transferred onto PVDF membrane (Millipore, USA). Using 1:1000 diluted rabbit anti-rLLO serum as the primary antibody and 1:3000 diluted HRP-labeled goat anti-rabbit IgG (ImmunoResearch, USA) as the secondary antibody, Western blot assays were applied to determine the combination of the recombinant protein antigens with antiserum. In addition, several Western blot assays were performed using rabbit antisera against rLipL32/1, rLipL21, and rOmpL1/2, and against whole cells of *L. interrogans* serogroup Icterohaemorrhagiae serovar Lai strain Lai, serogroup Grippotyphosa serovar Grippotyphosa strain Lin-6, serogroup Autumnalis serovar Autumnalis strain Lin-4, serogroup Pomona serovar Pomona strain Luo, *L. borgpetersenii* serogroup Javanica serovar Javanica strain M-10, and *L. weilii* sero-

group Manhao serovar Manhao 2 strain L-105 as the primary antibody to detect rLLO as above. The antisera against rLipL32/1, rLipL21, rOmpL1/2 and the whole cells of different *Leptospira* strains were made in our laboratory (Dong et al., 2008; Luo et al., 2009).

Microscopic agglutination test

MAT is a typical serological method to diagnose leptospirosis in humans and animals using living leptospires to detect specific agglutination antibodies in serum (Dong et al., 2008; Luo et al., 2009). Briefly, the rLipL32/1, rLipL21, rOmpL1/2, and rLLO antisera were double-diluted with 0.01 M phosphate-buffered saline (PBS, pH 7.4). Each of the dilutions (0.1 ml) was mixed with 0.1 ml of each of the cultures of fifteen standard leptospiral strains (Table 1). The mixtures were incubated at 37°C for 1 h and agglutination was assessed under a dark-field microscope (400×). The highest dilution of antiserum that agglutinated 50% of leptospires was considered the terminal MAT titer (Luo et al., 2009). In the MAT, sera from three normal rabbits were mixed and served as the control.

ELISA

rLipL32/1, rLipL21, rOmpL1/2, and rLLO were diluted with 0.01 M carbonate sodium buffer (pH 9.6). One hundred microliters per well of each of the recombinant protein solutions (20 μg/ml) was loaded in 96-well polystyrene plates for overnight incubation at 4°C. The plates were thoroughly washed with 0.05% Tween 20-PBS and then blocked with 10% BSA-PBS. Using 1:100 diluted sera from 133 leptospirosis patients (Table 1) as the primary antibody and 1:3000 diluted HRP-labeled goat anti-human-IgG (ImmunoResearch) as the secondary antibody, ELISA was performed to determine the optical density at 490 nm (OD_{490}) per well using a microplate reader (BioRad). In the ELISA, the sera from 12 healthy MAT-negative individuals were used as the negative control. If the OD_{490} of a patient's serum sample was higher than the mean plus three-fold standard deviation (SD) of the negative control, the result was regarded as positive (Lewis et al., 2003).

Immunoprotective test in hamsters

Hamsters were intraperitoneally injected with 1×10^5, 5×10^5, 1×10^6, 5×10^6, 1×10^7, 5×10^7, or 1×10^8 of *L. interrogans* serovar Lai strain Lai, serovar Grippotyphosa strain Lin-6, or serovar Pomona strain Luo, and eight animals were used per group (Kassegne et al., 2014). Eight negative control animals were intraperitoneally injected with EMJH medium. The animals were monitored twice daily and their survival was recorded within 14 days after challenge to obtain the minimal lethal dose of all animal deaths in a group (100% MLD). Fifteen groups of hamsters (A1-3, B1-3, C1-3, D1-3, and E1-3) were subcutaneously immunized twice at a weekly interval with 200 μg rLipL32, rLipL21, rOmpL1/2, rLLO, or a mixture of equimolar rLipL32/1, rLipL21, and OmpL1/2 (1:1:1) using 1 mg aluminum hydroxide (Sigma) as the adjuvant. In addition, another three groups of hamsters (E1-3) were immunized with 200 μg BSA (Sigma) as above and served as the control. On the fifteenth day after the last immunization, the immunized hamsters were intraperitoneally injected with double the 100% MLD of strain Lai, Lin-6, or Luo (10^6, 10^7, or 10^7 leptospires) as above. The animals were monitored twice daily and their death/survival was recorded within two weeks after challenge. The χ^2 test was used to determine significant differences among the percentages of immunogenicity. Statistical significance was defined as $p < 0.05$.

Pathological examination and leptospiral isolated culture

Liver, lung and kidney samples from the hamsters that had died or survived in the immunoprotective test were fixed, embedded, sectioned and HE-stained as previously described (Kassegne et al., 2014). Pathological changes in the samples were observed under a light microscope. In addition, 2-3 small pieces (~3×3 mm) of each of the kidney samples was inoculated in EMJH medium for 21-day incubation at 28°C and then examined by dark-field microscopy.

RESULTS

PCR and sequencing results

PCR showed that the *lipL32/1-lipL21-ompL1/2* fusion gene was generated using the flexible peptide sequence-based primers (Fig. 1A). The sequencing data confirmed that the generated *lipL32/1-lipL21-ompL1/2* fusion gene had the same sequences as the three separate *lipL32/1*, *lipL21* and *ompL1/2* genes (GenBank Nos: GI7330696, GI 30171159, and GI 348937).

Optimal expression conditions for rLLO

After induction by IPTG, the rLipL32/1, rLipL21, and rOmpL1/2 were efficiently expressed (Fig. 1B). However, the yield (37.78 mg/L) of rLLO under optimal expression conditions (initial pH 7.9, 0.2 mM IPTG, 2.5 h duration before induction, 5.83 h duration after induction, and 31°C induction temperature) was markedly higher than that of 13.95 mg/L under routine expression conditions (initial pH 7.4, 1 mM IPTG, 2 h duration before induction, 4 h duration after induction, and 37°C induction temperature) (Fig. 1C). Besides, each of the recombinant proteins extracted by Ni-NTA affinity chromatography showed a single band on gels (Fig. 1B and C). The details of the optimal expression of rLLO are shown in the Supplementary Materials.

Effective combination between recombinant proteins and antisera

Western blot assays demonstrated that the rLLO antiserum combined with rLipL32/1, rLipL21, rOmpL1/2 and rLLO (Fig. 2A), while the rLipL32/1, rLipL21 and rOmpL1/2 antisera also recognized rLLO (Fig. 2B). More importantly, rLLO gave positive immunoblotting results with the antisera against four strains from different serogroups or serovars of *L. interrogans* and two strains belonging to *L. borgpetersenii* and *L. weilii* (Fig. 2C). These data suggested that the product expressed by the *lipL32/1-lipL21-ompL1/2* gene possesses the immunogenicity of LipL32/1, LipL21 and OmpL1/2, and representative strains of *L. interrogans*, *L. borgpetersenii* and *L. weilii*, all pathogenic *Leptospira* genospecies found in China (Hu et al., 2014; Zhang et al., 2012),

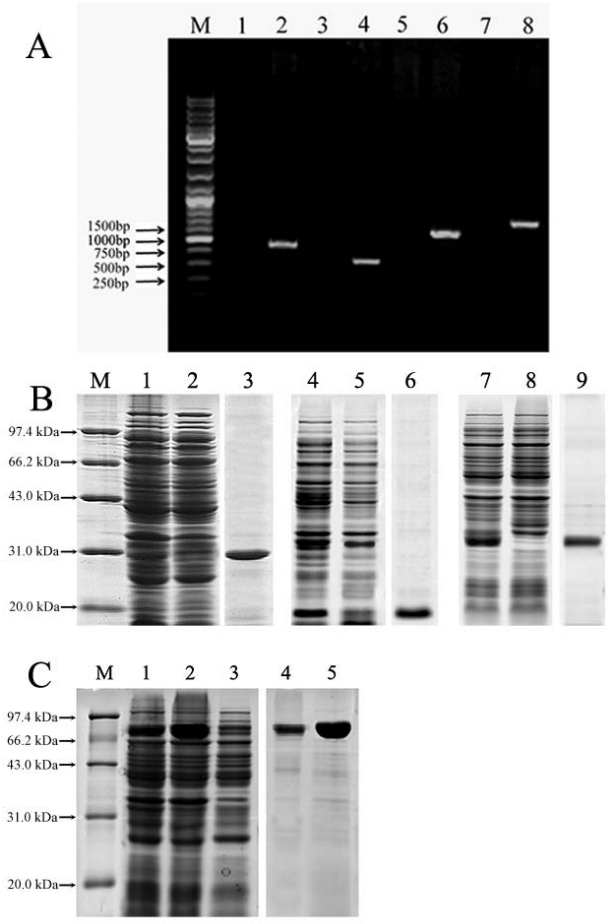

Fig. 1. Expression of single and fusion genes of *L. interrogans*. (A) Amplification fragments of the target genes. Lane M: DNA ladder (TaKaRa); Lanes 2, 4, 6, 8: the amplification fragment of lipL32/1, mlipL21, ompL1/2 and lipL32/1-mlipL21-ompL1/2 fusion gene, respectively. (B) Expressed and extracted rLipL32/1, rLipL21, and rOmpL1/2. Lane M, protein marker (BioColor); lanes 1, 4, and 7 / 3, 6, and 9, expressed / extracted rLipL32/1, rLipL21, and rOmpL1/2 (~29.1, 19.6, and 34.0 kDa), respectively; lanes 2, 5, and 8, wild-type pET42a-transformed *E. coli* strain used as control. (C) Higher yield of rLLO under optimal expression conditions. Lane M, protein marker (BioColor); lanes 1 and 2, rLLO (~86.1 kDa) expressed under the optimal expression conditions (lane 2) was higher than that under the routine expression conditions (lane 1); lane 3, wild-type pET42a-transformed *E. coli* strain used as control; lanes 4 and 5, rLLO extracted from the same volume of culture under routine and optimal expression conditions by Ni-NTA affinity chromatography.

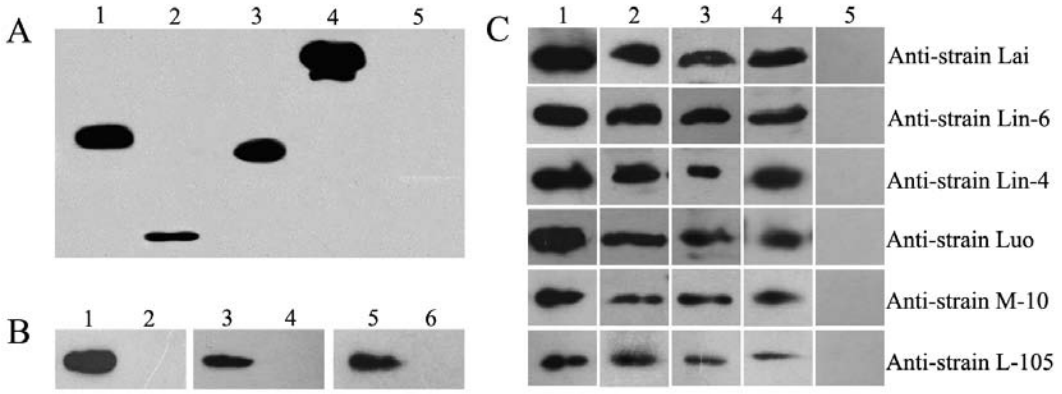

Fig. 2. Western blotting results of leptospiral recombinant proteins with antisera. (A) Combination of rabbit anti-rLLO serum with rLipL32/1, rLipL21, rOmpL1/2, and rLLO. Lanes 1-4, immunoblotting bands of 1:1000 diluted antiserum with rLipL32/1, rLipL21, rOmpL1/2, and rLLO, respectively; lane 5: blank control. (B) Combination of rLLO with rabbit anti-rLipL32/1, anti-rLipL21, and anti-rOmpL1/2 sera. Lanes 1, 3, and 5, immunoblotting bands of rLLO with 1:1000 diluted anti-rLipL32/1, anti-rLipL21, and anti-rOmpL1/2 sera, respectively; lanes 2, 4 and 6, blank controls. (C) Combination of leptospiral recombinant proteins with rabbit antisera against different *Leptospira* serovars. Lanes 1-4, immunoblotting bands of rLLO, rLipL32/1, rLipL21, and rOmpL1/2, respectively, with 1:1000 antiserum against whole cells of *L. interrogans* serovar Lai strain Lai, serovar Grippotyphosa strain Lin-6, serovar Autumnalis strain Lin-4, serovar Pomona strain Luo, *L. borgpetersenii* serovar Javanica strain M-10, and *L. weilii* serovar Manhao 2 strain L-105; lane 5, blank control.

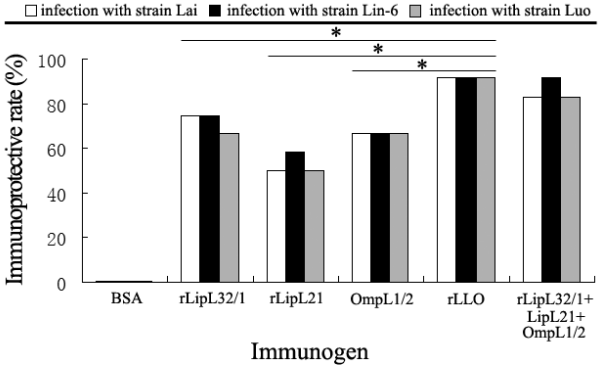

Fig. 3. Comparison of the immunoprotective rates in hamsters immunized with different leptospiral recombinant proteins after challenge. *L. interrogans* serovar Lai strain Lai, serovar Grippotyphosa strain Lin-6, and serovar Pomona strain Luo with double 100% MLD were used as the challenges. Each of the groups challenged with different leptospiral strains contained 12 animals. No significant difference was found between the immunoprotective rates in the hamsters immunized with rLLO and those immunized with the mixture of equimolar rLipL32/1, rLipL21, and rOmpL1/2 ($\chi^2 = 0.56$, $p > 0.05$). BSA was used as the control and all the BSA-immunized hamsters died after challenge. $^*p < 0.05$ vs. immunoprotective rates in hamsters immunized with the same concentrations of single rLipL32/1, rLipL2, or rOmpL1/2; $\chi^2 = 4.60$, 13.57, and 6.70).

probably express the LipL32, LipL21 and OmpL1 proteins.

Cross-agglutination of rLLO antiserum with different leptospiral strains

The MAT confirmed that the rLLO antiserum agglutinated all the 15 standard strains belonging to 15 serovars in 15 serogroups of *L. interrogans*, *L. borgpetersenii* and *L. weilii* with 1:100 to 1:400 titers (Table 3). The MAT titers of rLLO antiserum against 7 strains of *L. interrogans* (1:200-1:400) and 1 strain of *L. borgpetersenii* (1:200) were higher (1:100-1:200 and 1:100) than those of rLipL32/1, rLipL21 and rOmpL1/2 antisera (Table 3). These data suggested that LipL32/1, LipL21, and OmpL1 are common antigens of pathogenic *Leptospira* genospecies, and the rLLO antiserum has a more powerful cross-agglutinative effect, probably due to its multiple binding sites.

High positive rates of anti-rLLO antibody ELISA in sera from leptospirosis patients

The cut-off value (mean OD_{490} + 3×SD) in ELISA from the sera of *Leptospira* antibody-negative healthy individuals was 0.22. Based on the cut-off value, 98.5% (131/133) of the sera from 133 patients infected with *L. interrogans* serogroup Icterohaemorrha-

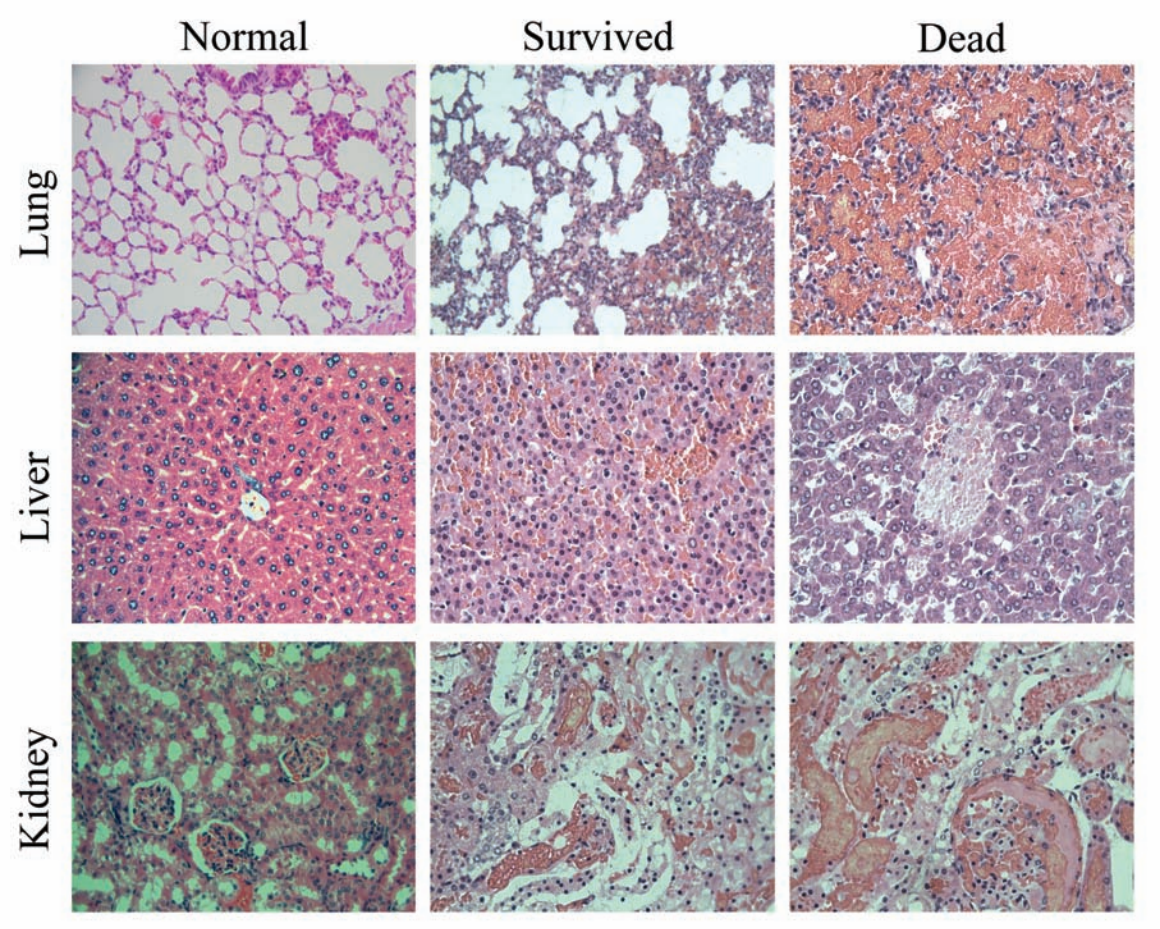

Fig. 4. Diversity of pathological damage in immunized and non-immunized hamsters after infection with *L. interrogans* (HE stain). The hamsters killed by infection with *L. interrogans* displayed serious diffuse hemorrhage in the lung, extensive hepatocyte necrosis in the liver, and serious congestion and focal epithelial cell necrosis in the kidney, but the surviving hamsters immunized with rLLO exhibited slight pulmonary hemorrhage, and mild hepatic and renal congestion.

giae serovar Lai (n = 60), serogroup Grippotyphosa serovar Grippotyphosa (n = 31), serogroup Pomona serovar Pomona (n = 30) and serogroup Autumnalis serovar Autumnalis (n = 12) were positive (Table 4). Only two serum samples, from patients infected with serovar Lai and serovar Pomona, showed negative results. These data suggested that LipL32/1, LipL21 and/or OmpL1/2 are dominant antigens of *L. interrogans* that induce specific antibodies in patients.

Strong immunoprotective effect of rLLO in hamsters

All the BSA-immunized hamsters (control) died after challenge with a lethal dose of *L. interrogans* serovar Lai strain Lai, serovar Grippotyphosa strain Lin-6 or serovar Pomona strain Luo. However, immunization with rLLO provided higher rates of immunogenicity (91.7%) after challenge than immunization with the same concentration of single rLipL32/1, rLipL21 or rOmpL1/2 (66.7-75.0%, 50.0-58.3%, and 66.7%; $\chi^2 = 4.60$, 13.57 and 6.70; $p < 0.05$) (Table 5 and Fig. 3). However, the immunoprotective rate in hamsters immunized by a mixture of equimolar single rLipL32/1, rLipL21 and OmpL1/2 (83.3-91.7%) was similar to that in hamsters immunized with rLLO ($\chi^2 = 0.56$, $p > 0.05$). In addition, the dark-field micro-

scopic examination showed that no leptospires were found in any of the cultures of kidney samples from surviving hamsters (n = 133), while 71.1% (59/83) of the cultures from dead hamsters displayed leptospire growth.

Pathological changes in hamsters after infection with L. interrogans.

Pathological examination showed that the hamsters killed by infection with *L. interrogans* displayed serious diffuse pulmonary hemorrhage, extensive hepatocyte necrosis and serious renal congestion and focal epithelial cell necrosis, while the surviving hamsters immunized with rLLO exhibited mild pathological injury (Fig. 4).

DISCUSSION

Pathogenic *L. interrogans* comprise a large group of heterogenic spirochetes that includes at least 230 serovars belonging to 23 serogroups with a large and diverse distribution in different regions of the world (Pappas et al., 2008; Adler et al., 2010; McBride et al., 2005). For instance, *L. interrogans* serovar Lai or Copenhageni is predominant in China, Europe and Brazil (Forbes et al., 2012; Miraglia et al., 2013; Zhang et al., 2012), while pathogenic *L. santarosai* serovar Shermani is prevalent in Taiwan (Chou et al., 2012). On the other hand, the numerous serovars of *L. interrogans* indicate the diversity of antigens on their surface. This is why the currently used whole-cell leptospirosis vaccine is composed of several *L. interrogans* serovars. However, this vaccine is limited in application due to the LPS-based adverse reaction and the weak cross-immunogenicity among different *L. interrogans* serovars (Verma et al., 2013). For instance, at the turn of the 21st century, *L. interrogans* serogroup Sejroe serovar Medanesis caused several outbreaks in South China, but vaccination with a triple whole-cell vaccine composed of *L. interrogans* serovars Lai, Grippotyphosa and Autumnalis had no immunoprotective effect (Hu et al., 2014). Thus, the development of universal vaccines with minimal side effects is crucial for the further prevention and control of human leptospirosis.

LPS-free, protein antigen-based and genetically engineered vaccines have been anticipated for the prevention of leptospirosis in humans, but at present no commercial products of this kind are available. Protein antigens with high expression, extensive distribution and powerful antigenicity are extremely important for developing genetically engineered vaccines (Lin et al., 2011; Pinne et al., 2013). LipL32, and then LipL21, have been confirmed to be highly expressed in different pathogenic leptospiral strains (Haake et al., 2000; Cullen et al., 2003). A previous study reported that LipL32 is a submembrane lipoprotein of *Leptospira* (Pinne et al., 2013). However, our previous analysis showed that LipL32 has six main surface membrane regions in its molecule (amino acids 75-80, 105-130, 150-180, 185-200, 210-220, and 245-255) (Luo et al., 2009). The MAT is an immunoagglutinative test using living leptospires as the antigens (Dong et al., 2008). The MAT in this study showed that the rLipL32 antiserum agglutinated all the tested *L. interrogans* strains. In addition, a recent study has shown that LipL32 is exposed on the surface of leptospires to induce an inflammatory reaction through binding to Toll-like receptor-2 on HK2 human renal cells (Lo et al., 2013). These data suggested that LipL32 or at least some regions of LipL32 are surface-exposed. Our recent study showed that OmpL1, Loa22 and LigB but not LenA are expressed by all the strains belonging to the 15 *L. interrogans* serovars prevailing in China. However, the low expression level of Loa22 and the high molecular weight of LigB (~220 kDa) limit them as immunogens for developing genetically engineered vaccines. Thus, we selected LipL32, LipL21, and OmpL1 to generate a fusion protein that can be used as a novel immunogen in a genetically engineered leptospirosis vaccine.

High expression of recombinant protein antigens is a key problem in the development of genetically engineered vaccines (Peleg et al., 2012). In this study, we optimized the expression conditions of rLLO, which resulted in a 2.7-fold increase in the fusion protein yield compared to routine expression conditions. The Western blot assays confirmed that rLLO was recognizable by rLipL32/1, rLipL21

and rOmpL1/2 antisera, while rLLO antiserum also bound to rLipL32/1, rLipL21 and rOmpL1/2, indicating that rLLO maintained the immunogenicity of all three single-protein antigens. Furthermore, the rLLO antiserum showed stronger agglutination with all the tested standard strains belonging to *L. interrogans*, *L. borgpetersenii* and *L. weilii* than the rLipL32/1, rLipL21 and rOmpL1/2 antisera. The ELISA demonstrated that 98.5% of the serum samples from patients infected with *L. interrogans* serovars Lai, Grippotyphosa, Pomona and Autumnalis combined with rLLO. These data indicated that rLLO protein has stronger immunogenicity than separate LipL32/1, LipL21 and OmpL1/2.

So far, only three pathogenic *Leptospira* genospecies (*L. interrogans*, *L. borgpetersenii* and *L. weilii*) have been found in China (Zhang et al., 2012). However, *L. interrogans* acts as the causative agent for all Chinese leptospirosis patients, while *L. borgpetersenii* and *L. weilii* have only been isolated from animal hosts (Li et al., 2013). Strains of *L. interrogans* serovar Lai are responsible for ~60% of Chinese leptospirosis infections (Jin et al., 2009; Li et al., 2013; Hu et al 2013). *L. interrogans* serovar Grippotyphosa is also a common pathogen in China, while serovar Pomona is especially prevalent in North China (Zhang et al., 2012; Li et al., 2007). The requirement for high production vaccine against leptospirosis is urgent; therefore, we selected the strains belonging to these three serovars that are most prevalent in China as challenges in the animal immunoprotective test. The results showed that rLLO provided higher immunoprotective rates (91.7%) against lethal infection with the different *L. interrogans* serovars than single rLipL32/1, rLipL21 or rOmpL1/2 (50.0-75.0%), probably due to the higher immunogenicity of a macromolecular fusion protein than a micromolecular single protein. Although the immunoprotective rates in the hamsters immunized by the mixture of rLipL32/1, rLipL21 and OmpL1/2 (83.3-91.7%) approached that by rLLO immunization, multiple expression and extraction of the three single protein antigens is less favorable for industrial production,

Pathogenic *Leptospira* species can invade the lungs, liver and kidneys, but only remain in the animal kidney for a long period of time to discharge leptospires from urine during infection (Zhang et al., 2012). Therefore, the culture of leptospires isolated from animal kidney tissue is a suitable method for assessing the elimination of leptospires achieved by the elicited immune response after vaccination. The results showed that no leptospires could be isolated from the kidneys of the surviving vaccinated hamsters after challenge. Moreover, the surviving rLLO-immunized hamsters also showed milder pulmonary, hepatic and renal injury compared to the dead non-immunized hamsters after challenge. Taking these findings together, rLLO can be used as an antigen for developing a novel universal genetically engineered vaccine that has high cross-protection against serovars of pathogenic leptospires that are prevalent in China.

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SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Variables used in central composite design (CCD)

Our preliminary experiments revealed that the major factors influencing the yields of the target recombinant protein rLipL32/1-LipL21-OmpL1/2 (rLLO) were the initial pH (H), duration before induction (D1), duration after induction (D2), concentration of isopropyl-β-D-thiogalactoside (IPTG) inducer (C) (Sigma, USA) and induction temperature (T). Based on these data and a previous report (Aravindan et al., 2007), a CCD from response surface methodology (RSM) was used to optimize the protein expression conditions (Maldonado et al., 2007). The selected independent variables and variable levels in CCD are listed in Table S1. The CCD experimental scheme contained 52 assays including 42 factorial experiments and 10 duplicate tests at central points. LB medium was used to culture *E. coli BL21DE3*$^{pET42a\text{-}lipL32/1\text{-}lipL21\text{-}ompL1/2}$.

$$Y \text{ (predictive values)} = 36.4099 + 1.1509325 \times H + 1.169631 \times D1 - 0.784195 \times D2 + 0.4976513 \times C + 0.879023 \times T - 3.466545 \times H \times H + 0.2053125 \times H \times D1 - 0.280312 \times H \times D2 - 0.784688 \times H \times C + 0.5409375 \times H \times T - 2.010444 \times D1 \times D1 - 1.135313 \times D1 \times D2 + 0.1740625 \times D1 \times C - 0.239062 \times D1 \times T - 3.068185 \times D2 \times D2 + 0.3346875 \times D2 \times C - 0.280937 \times D2 \times T - 2.635884 \times C \times C + 0.2909375 \times C \times T - 2.195545 \times T \times T$$

Fig. S1. The mathematic model for predicting the optimal conditions for rLLO expression. The data for establishing the model were from the CCD-based expression assays.

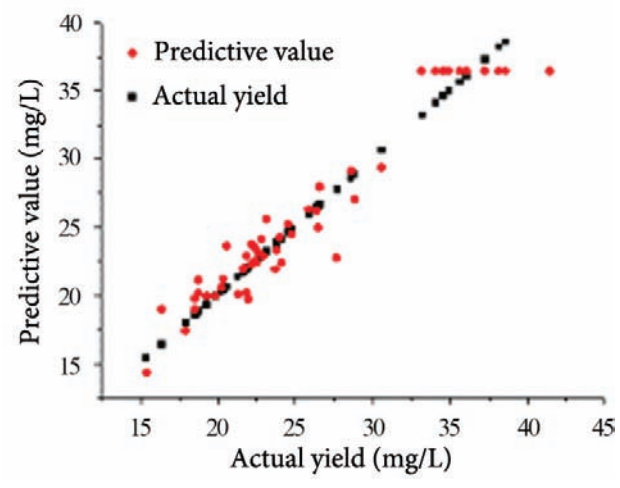

Fig. S2. High fitness of predictive values with actual yields in CCD-based expression assays. The predictive values for rLLO yields (red points) approached the actual yields (black points) in the CCD-based expression assays.

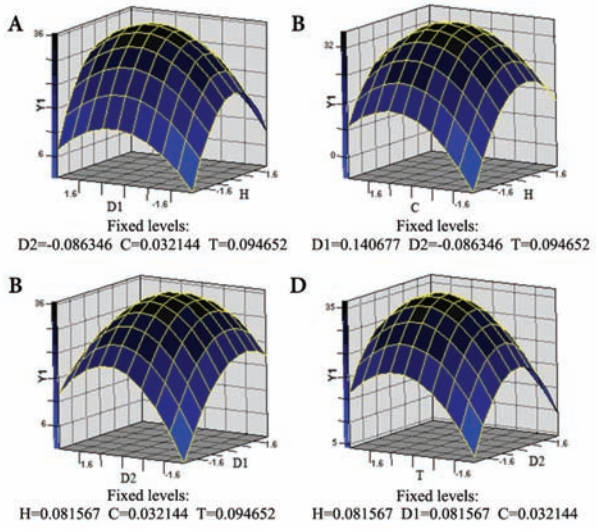

Fig. S3. Optimal expression conditions of rLLO, determined by response surface methodology (RSM). (A) Influence of interaction between the initial pH (H) and duration before induction (D1) on the yield of rLLO. The highest point of the response surface indicates the optimal H and D1 parameters to harvest the highest yield of the fusion protein. (B) Influence of interaction between the initial pH (H) and concentration of IPTG used (C) on the yield of rLLO. The legend is the same as in A but with the interaction between H and C variables. (C) Influence of interaction between the durations before induction (D1) and duration after induction (D2) on the yield of rLLO. The legend is the same as in A but with the interaction between the D1 and D2 variables. (D) Influence of interaction between the duration after induction (D2) and temperature of induction (T) on the yield of rLLO. The legend is the same as in A but with the interaction between D2 and T variables.

Establishment of a mathematic model for predicting optimal expression conditions

The correlation between the actual yields of rLLO and their statistical coding values from the CCD expression assays were described as $X_i = A_i - A_0/\Delta A$ (where X_i is the variable coding value, A_0 is the actual value at the central point A_i, and ΔA is the differential of the variable). Using the reported formula $Y = b_0 + \Sigma b_i x_i + \Sigma b_{ii} x^2_i + \Sigma\Sigma b_{ij} x_i x_j$ (where Y is the response value, X_i is the variable, b_0 is a constant, and b_i, b_{ii}, and b_{ij} are the linear, square, and cross-product coefficients) as a basis (Aravindan et al., 2007), a mathematic model was established to predict the optimal parameters during rLLO expression.

Statistical evaluation of the mathematical model

The validity of the mathematical model established to predict the optimal conditions for rLLO expression was determined by the change of determination coefficient (R^2) values and analysis of variance (ANOVA). A higher R^2 value indicates that the variation in the model is probably due to the change of

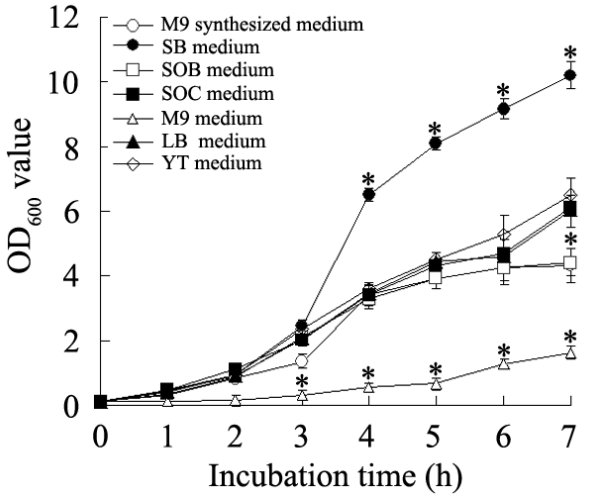

Fig. S4. Growth of *E. coli BL21DE3*$^{pET42a\text{-}lipL32/1\text{-}lipL21\text{-}ompL1/2}$ in different media for the indicated times as determined by spectrophotometry. The values at OD_{600} reflect the numbers of bacteria in the cultures. The number in SB medium was significantly greater than that in the other six types of media, while the number in YT medium the least. *p <0.05 *vs* the bacterial number in SOB, SOC, LB, M9, or M9 synthetic medium.

variables, reflecting its feasibility and applicability (Wang et al., 2005).

Measurement of rLLO yield

The rLLO expressed in *E. coli* was extracted using an Ni-NTA affinity chromatographic column (BioColor, China). The extracted rLLO was quantified using a BCA Protein Quantitative Kit (Beyotime Biotech, China) and then diluted with 0.01 M phosphate-buffered saline (pH 7.4). The dilutions with different rLLO concentrations were electrophoresed in sodium dodecyl sulfate polyacrylamide gel and then electron-transferred onto PVDF membrane (Millipore, USA). Using 1:1000 diluted rabbit anti-rLLO serum as the primary antibody and 1:3000 HRP-labeled goat anti-rabbit IgG (ImmunoResearch, USA) as the secondary antibody, several Western blot assays were performed and the immunoblotting bands were quantified by densitometry (gray-scale determination) using a Gel Image Analyzer (BioRad, USA). Based on the gray-scale values, a standard curve was constructed for the rLLO concentrations. The yields of rLLO in CCD expression assays were determined by Western blot assay and gray-scale values as above as well as calculations based on the rLLO concentration standard curve.

Medium selection

The type of medium can affect the growth of bacteria and cause changes in the rLLO yields. Therefore, SB, SOB, SOC, LB, YT, M9 and synthetic M9 media were selected to culture *E. coli BL21DE3*$^{pET42a\text{-}lipL32/1\text{-}lipL21\text{-}ompL1/2}$ under the optimal expression conditions, and the growth of the *E. coli* strain in different media was assessed by spectrophotometry at OD_{600}. In addition, the effects of the glucose and sodium acetate concentrations in media and the amount of bacterial inoculation on the growth of the *E. coli* strain were also determined as above. One-way analysis of variance (ANOVA) was used to determine significant differences of the bacterial number in different media and culture conditions. Statistical significance was defined as p <0.05.

RESULTS

Major variables affecting the expression of rLLO

The yields of rLLO in the 10 duplicate tests at central points (43-52) were higher (33.20-41.43 mg/L) than in the 42 factorial experiments (1-42) (15.39-30.61 mg/L) (Table S2). ANOVA confirmed that the independent variables H, D1, T and D2 significantly influenced the yields (p <0.01 for H, D1, and T; p >0.05 for D2), but the independent variable C had no influence (Table S3). However, the yields were significantly affected by different D1 and D2 values (p <0.01), indicating an interaction between the durations before and after induction (Table S3).

High efficiency and reliability of the mathematical model

Based on the regression coefficients of the variables, a mathematical model was established to predict the optimal expression conditions of rLLO (FIG. S1). The R^2 value of this model reached 87.83%, indicat-

ing that it accurately predicted most of the protein yield variations. A high coincidence with a 96.34% degree of fitness between the predictive expression values and actual yields of rLLO was also found in the CCD-based expression assays (Table S4 and Fig. S2).

Optimal expression conditions for rLLO

The results from RSM indicated that the highest yield (37.78 mg/L) of rLLO was obtained under the optimal expression conditions H = 7.9, D1 = 2.5 h, C = 0.2 mM, D2 = 5.83 h, and T = 31°C (Fig. S3). However, the yield was low (13.95 mg/L) under routine expression conditions (H = 7.4, C = 1 mM, D1 = 2 h, D2 = 4 h, and T = 37°C).

Maximal bacterial growth in SB medium

Among the seven media tested, SB medium enabled *E. coli BL21DE3*$^{pET42a\text{-}lipL32/1\text{-}lipL21\text{-}ompL1/2}$ to reach maximal growth (FIG. S4). However, 2-20% glucose, 0.05-0.25 mM sodium acetate, and 1-6% bacterial inoculation had no influence on bacterial growth (data not shown).

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