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(71) Demandeur/Applicant:
INSTITUT PASTEUR, FR

(72) Inventeurs/Inventors:
PICARDEAU, MATHIEU, FR;
RISTOW, PAULA, FR;
BOURHY, PASCALE, FR

(74) Agent: ROBIC

(54) Titre : SOUCHE ATTENUÉE DE LEPTOSPIRA N'EXPRIMANT PAS LA PROTEINE DU GENE LOA22 ET SON
UTILISATION CONTRE LA LEPTOSPIROSE

(54) Title: AN ATTENUATED STRAIN OF LEPTOSPIRA NOT EXPRESSING THE LOA22 PROTEIN AND ITS USE
THEREOF AGAINST A LEPTOSPIRAL DISEASE

(57) **Abrégé/Abstract:**

The present invention related to an attenuated strain of *Leptospira* species which does not allow expression of the Loa22 protein. The present invention also related to the use of the mutant *Leptospira* strain in compositions and methods for the treatment and/or prevention of a leptospiral disease.



ABSTRACT

The present invention related to an attenuated strain of *Leptospira* species which does not allow expression of the Loa22 protein. The present invention also related to the use of the mutant *Leptospira* strain in compositions and methods for the treatment and/or prevention of a leptospiral disease.

AN ATTENUATED STRAIN OF LEPTOSPIRA NOT EXPRESSING THE LOA22 PROTEIN AND ITS USE THEREOF AGAINST A LEPTOSPIRAL DISEASE

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BACKGROUND OF THE INVENTION

Leptospira interrogans is a spirochete responsible for leptospirosis. This disease, considered the most geographically widespread zoonosis, has emerged to become a major public health problem in developing countries [1,2,3]. A large spectrum of mammalian species, including rodents, excretes the pathogen in their urine and serves as reservoirs for transmission. Humans are usually infected through contact with contaminated water or soil. Leptospirosis imparts its greatest burden on poor rural farming and urban slum populations in developing countries [1,2,3]. More than 500,000 cases of severe leptospirosis occur each year, with a mortality rate of 5 to 20% [4]. All control methods implemented to date have been ineffective. Furthermore, little is understood on Leptospira pathogenesis which in turn has hampered the identification of new intervention strategies. Leptospire are highly motile bacteria that are able to penetrate skin and mucous membranes and to rapidly disseminate to other tissues shortly after infection. In susceptible hosts, such as humans, systemic infection produces severe multi-organ manifestations, including jaundice, acute renal failure, and severe hemorrhage in the lungs and other organs. However, in animal reservoirs such as the domestic rat, infection produces chronic and persistent asymptomatic carriage in the renal tubules [1,2,3].

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The virulence mechanisms and more generally the fundamental understanding of the biology of the causative agents of leptospirosis remain largely unknown. To date, only a few proteins have been identified as putative virulence factors. Pathogenic leptospire have been shown to express adhesins [5,6], hemolysins [7], and many lipoproteins, prominent in leptospire and other spirochetes, that could play a role in host-cell interactions [8]. The recent completion of the genome sequence of pathogenic Leptospira strains [9,10,11] has provided a basis for understanding the

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pathogenesis of leptospirosis. However, to date the role of putative virulence factors identified in the genome sequence remains speculative. The lack of genetic tools to manipulate pathogenic *Leptospira* spp. has prevented approaches to test Koch's molecular postulates [12] and directly elucidate the role of these determinants in virulence.

We recently provided the first evidence of gene transfer in *L. interrogans*, involving the transposition of a transposon of eukaryotic origin [13]. This advance has now made it possible to apply genetic approaches to the identification of virulence determinants and vaccine candidates in pathogenic *Leptospira* spp. In this study, we characterized a mutant of the pathogen *L. interrogans* which we obtained by random transposon mutagenesis. This mutant exhibited transposon insertion in a gene, *loa22*, described by Koizumi et al [14] to encode for a lipoprotein of 22-kDa with a C-terminal OmpA domain. Previous studies suggested that this protein may play an important role in infection [14,15,16,17]. Herein, we show that the mutant *loa22*-strain is avirulent in animal models, therefore defining the first virulence factor in *Leptospira* species.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Disruption and complementation of *loa22* in *L. interrogans*.

Analysis of chromosomal DNA from the parental (lane 1), mutant *loa22*- (lane 2), and complemented TK2 strains (lanes 3) by PCR with primers S1a and S1b (A) and Southern blot of *Eco*RI-digested DNA probed for hybridization with the spectinomycin (SpcR) – and kanamycin (KmR)-resistant cassettes (B). Primers S1a and S1b are located in the flanking sequences of the insertion site of the spectinomycin-resistant transposon, into *loa22*. This analysis revealed that there was an insertion of 1.3 kb in the mutant *loa22*- strain and that an additional copy of *loa22* was present in the complemented strain. C. Schematic representation of the genotype of the parental (wt), mutant, and complemented strains. D. ELISA of plates with total bacterial antigens and *Loa22* antiserum (serum dilution 1:800).

Figure 2: Surface localization of Loa22.

Surface immunofluorescence assay was performed with *L. interrogans* wild-type strain (wt), mutant loa22- (loa22-) and mutant loa22- complemented with wild-type loa22 (TK2). Strains were labeled with antibodies against Loa22 and lipoproteins; LipL32, a surface-exposed lipoprotein [19]; and LipL31, a lipoprotein which is associated with the inner membrane and not surface-exposed [19]. Alexa and fluoroscein isothiocyanate (FITC)-conjugated secondary antibodies were used to detect surface-bound antibodies to Loa22 and LipL32 and LipL31, respectively. A DAPI counterstain was used to document the presence of leptospires. A photomicrograph is shown from one of three representative experiments.

Figure 3: Gross examination of infected guinea pigs.

Left panel: Guinea pigs infected with the wild-type (A) and complemented strains (C) with clinical findings of jaundice and haemorrhages which are absent in guinea pigs infected with the mutant loa22- strain (B). Right panel: Lungs of a guinea pig infected with mutant loa22- did not exhibit macroscopic haemorrhage (B) in contrast to lungs of guinea pigs infected with the wild-type (A) and complemented strains (C). Tissues were observed 6 days post-inoculation.

Figure 4: Livers (A to C) and kidneys (D and E) from guinea pigs infected with the wild-type strain (left panel) and mutant loa22- strain (middle panel). All images are from guinea pigs 6 days postinoculation. Right panels show normal livers (A to C) and kidneys (D and E). Tissues were stained by hematoxylin and eosin (x200, A and D), Warthin-Starry (x100, B and E) and immunohistochemistry with antiserum specific to LipL32 (x200, C). Liver of wt -infected animals exhibits important periportal lymphoplasmocitary inflammatory infiltration, loss of parenchymal architecture and increase of biliary canalicules, in comparison to a normal aspect of mutant loa22-. Distortion of liver cords is related to numerous leptospires along cell membranes of hepatocytes in wt-infected animals (A to C). Kidneys of wt-infected animals present haemorrhages in Bowman's spaces, lumen of renal tubules and interstitium (D). Large number of leptospires is seen in Bowman's space, sometimes forming a cap

(E). Histology of kidneys infected with mutant loa22- was considered as normal (D and E).

Figure 5: Histopathologic sections on liver (A), kidney (B) and lung (C) of guinea pigs infected by complemented strain TK2 6 days post-inoculation. Left panel: Hematoxylin and eosin staining (x200) of infected guinea pigs. Right panel: Immunohistochemistry with antiserum specific for LipL32 (x200, except C: x1,000). The liver has a great loss of architecture, areas of necrosis and inflammatory infiltration, associated with numerous leptospire in biliary canaliculi (A). Kidneys present hemorrhages, tubular necrosis, and inflammatory infiltration, with leptospire mainly located in Bowman's Spaces and proximal tubules (B). Lungs have marked intra-alveolar haemorrhages with inflammatory infiltrates and few leptospire are present within septal membranes and, sometimes, into macrophages (C, right panel). Pictures of histopathology were similar between animals infected with wild-type- and complemented strains.

Figure 6: In vivo expression of Loa22 in liver (A) and kidney (B) of guinea pigs infected with *L. interrogans* serovar Lai. Histopathologic sections were stained by immunohistochemistry using Loa22 antiserum (x1,000). Intact organisms were found in biliary ducts (A) and in large number in Bowman's spaces and proximal tubules (B).

Example: Virulence attenuation in *Leptospira interrogans* by disruption of an ompA-like gene

Introduction

Pathogenic mechanisms of *L. interrogans*, the causal agent of leptospirosis, remain largely unknown. This is mainly due to the lack of tools for genetic manipulations of pathogenic species. In this study, we characterized a mutant obtained by insertion of the transposon Himar1 into a gene encoding a putative lipoprotein, Loa22, which has a predicted OmpA-domain based on sequence identify. The resulting mutant did not express Loa22 and was attenuated in virulence in the guinea pig and hamster

models of leptospirosis, whereas the genetically complemented strain was restored in Loa22 expression and virulence. Our results showed that Loa22 was expressed during host infection and exposed on the cell surface. Loa22 is therefore necessary for virulence of *L. interrogans* in the animal model and represents the first genetically defined virulence factor in *Leptospira* species.

Materials & Methods

Bacterial Strains and growth conditions. *Leptospira interrogans* serovar Lai strain Lai 56601 (gift from the National Institute for Communicable Disease Control and Prevention, ICDC China CDC) and other *Leptospira* strains were grown at 30 °C in EMJH [31,32] liquid medium or on 1% agar plates. *E. coli* was grown in Luria-Bertani (LB) medium. When necessary spectinomycin or kanamycin were added to culture media at 50 ug /ml.

Construction of mutant and complemented strains. Random insertion mutagenesis was carried out in low passage *L. interrogans* serovar Lai strain Lai 56601 with plasmid pMSL as previously described [13]. After 4 to 6 weeks of incubation, spectinomycin-resistant transformants were inoculated in liquid medium. Genomic DNA was then extracted and the transposon insertion site of each transformant was identified by LM-PCR as previously described [33]. Among the transformants, we selected a mutant with an insertion into loa22, also called LA0222, at position 220548 in the large chromosome (CI) of *L. interrogans* for further characterization. For complementation, loa22 was amplified with primers OMIA (5'-AGTCGACGGTTTTGGTGGGATGGATAG-3') and OMIB (5'-AGTCGACAGACGTTGAGTTGCCACAGC-3') and cloned into the Sall restriction site of the kanamycin-resistant transposon carried by plasmid pMKL, then resulting in plasmid pMKLoa22. The mutant loa22- strain was then electrotransformed by pMKLoa22 and the transposon insertion site of some transformants was identified as described above. Two transformants, strains TK1 and TK2, were further studied, one, strain TK1, exhibited the kanamycin-resistant transposon at position 1079614

(between LA1074 and LA1075), the other, strain TK2, at position 84051 (into LA0071) of the large chromosome of *L. interrogans*. Confirmation of genotypes was performed by PCR using primers S1a (5'-TTGTTGTGGTGCGGAAGTCG-3') and S1b (5'-GGTCCCGAACAAGCAGAAGG-3'), which are located in the flanking sequences of the transposon inserted into *loa22*, and Southern blots.

Enzyme-linked immunosorbent assay (ELISA). *L. interrogans* strains were grown in EMJH until the culture reaches an OD₄₂₀ of 0.4. *L. biflexa* was also used a control. Concentrations were adjusted to 10⁹ bacteria/ ml in a volume of 20 ml of EMJH and 40 μ l of 37% formaldehyde was added, then incubated 2 h at room temperature and boiled for 30 min. After adjusting pH at 9.6, cultures were centrifuged at 8,000 g for 20 min and pellets resuspended in 10 ml of 0.05 M bicarbonate buffer. Ninety six-well flat-bottom polystyrene assay plates (Immulon) were coated overnight at 4 °C with 50 μ l of total bacterial antigen. Plates were washed three times with phosphate buffered saline (PBS) pH 7.2 and wells blocked with 50 μ l of 5% non fat milk PBS for 45 min at 37 °C. Plates were incubated 45 min at 37 °C with 50 μ l of a 800-fold dilution of *Loa22* antiserum [14] diluted in milk PBS, washed and incubated 1 h at 37 °C with 50 μ l of a 2,500-fold dilution of horseradishperoxidase conjugated sheep affinity purified antibody specific to mouse IgG (Promega). After washing of the plates, 50 μ L of ABTS peroxidase substrate (Roche) was added, and the plates were incubated in the dark at room temperature for 25 min. Optical density was measured using an ELISA reader (Labsystems Multiskan MS) at 405 nm.

Localization of *Loa22* by immunofluorescence. Surface immunofluorescence labelling was performed according to a modified protocol of Cullen et al [34]. Suspensions of 10⁷ live leptospire in 10 μ l of PBS were placed onto poly-L-lysine (Sigma Chem Co.) coated slides for 1 h in a humidified chamber. The slides were washed twice with PBS with 2% bovine serum albumin (PBS-BSA) and were incubated for 1 h with antisera (diluted 1:100 in PBSBSA) to recombinant *Leptospira* proteins. After incubation with mouse antiserum to *Loa22* [14] and rat antisera to LipL32, LipL41, LipL31 and GroEL, the slides were washed gently with PBS-BSA.

Leptospire were fixed by applying cold methanol and incubating the slides for 10 min at -20 °C. The slides were then washed and incubated with donkey anti-mouse IgG antibodies conjugated to Alexa (Molecular Probes) or goat anti-rat IgG antibodies conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories, Inc.) for 1 h at 37 °C. The slides were washed twice with PBS-BSA and incubated with 1 µg/ml of DAPI (Molecular Probes) for 1 h at room temperature. The slides were mounted in anti-fading solution after washing and prior to visualization of stained organisms with fluorescence microscopy.

Animal infections. Golden Syrian male hamsters, five to eight week old, and Hartley male guinea pigs (Charles River Laboratories), two to three week old, were used for this study. Animals were maintained under standard conditions according to institutional guidelines. Water and food were given ad libitum. All animal infections were performed with intraperitoneal injection of low passage strains in 1 ml EMJH medium. Negative control animals were injected intraperitoneally with 1 ml of EMJH medium. Animals were monitored daily for characteristic signs of leptospirosis (i.e. prostration and jaundice) and survival. Surviving animals were sacrificed after a 21 day post-challenge follow-up period.

Histopathology. Guinea pigs were inoculated with 2×10^8 bacteria/ml of wild-type, mutant loa22- and complemented strains of *L. interrogans* serovar Lai strain Lai or EMJH alone. For mutant loa22- strain and EMJH control group infections, 3 guinea pigs were sacrificed 6 and 21 days post inoculation. For wild-type and TK2 strain group infections, tissues were collected at the day of death (5 or 6 days post inoculation). Tissues (liver, kidneys, spleens, and lungs) were fixed in 10% buffered formaldehyde, embedded in paraffin and sectioned according to routine histological procedures to produce 5 µm sections that were then stained with haematoxylin and eosin and Warthin-Starry silver impregnation [35]. For immunohistochemistry, paraffin was removed from the sections with xylene and ethanol. Tissues were then treated in citrate buffer pH 6 at 98 °C for one hour and non-specific sites were blocked by incubation of sections with 1.5% BSA at room temperature for 20 min. Tissues were incubated with 6,000- and 1,000- fold dilution of LipL32 [36] and Loa22

[14] antisera, respectively, overnight at 4 °C. Samples were treated with 0.3% hydrogen peroxide for 30 min at room temperature, then incubated at room temperature for 30 min with goat anti-mouse or anti-rabbit antibodies conjugated to peroxidase (Dako Cytomation). Enzyme reactions were developed using AEC (3-Amino-9-ethylcarbazole) staining Kit (Sigma).

Results

Disruption and complementation of loa22 in *L. interrogans* serovar Lai

Plasmid pMSL [13] was used to deliver the spectinomycin-resistant Himar1 transposon in *L. interrogans* serovar Lai strain Lai. One of the transposon mutants exhibited an insertion in a putative gene, LA0222, encoding a protein (195 amino acids in length) that was reported by Koizumi et al. [14] to be Loa22, a 22-kDa *Leptospira* lipoprotein with OmpA domain; we will therefore further refer to this protein as Loa22. The *L. interrogans* serovar Lai protein (LA0222) exhibits 99 and 96% similarity with orthologs in the pathogens *L. interrogans* serovar Copenhageni (LIC_10191) and *L. borgpetersenii* serovar Hardjobovis (LBL_2925 / LBJ_0158), respectively.

The protein Loa22 exhibits a bipartite structure, which includes an N-terminal domain (residues 1-77), which is unrelated to other eukaryotic or prokaryotic protein domains, followed by an OmpA domain (residues 78-186). Loa22 is a probable lipoprotein with a cleavage site between residues 20 and 21 [18]. C-terminal amino acid sequence analysis of Loa22 revealed that other proteins of *L. interrogans* (LA4337, LA3685, LA0056, LA3615, and LB328) have sequence homology with members of the OmpA family. These *L. interrogans* putative proteins, including Loa22, share between 46 to 59% sequence similarity in their C-terminal domain, but have significant amino acid sequence heterogeneity in their Nterminal domains.

Since there is no replicative plasmid vector available for pathogenic *Leptospira*, we reintroduced the wild-type copy of the gene encoding Loa22 into the spectinomycin-resistant mutant strain by using a kanamycin-resistant transposon carrying loa22 (Figure 1C). As transposition within the chromosome is random, we identified the

transposon insertion sites in several transformants and selected one strain, TK2, for further studies (Figure 1A and B). Enzyme-linked immunosorbent assay (ELISA) (Figure 1D) and immunoblot analysis (data not shown) confirmed the absence of detectable Loa22 in the mutant loa22- strain, whereas the protein was expressed in the wild-type and complemented strains (Figure 1D). Inactivation of *L. interrogans* loa22 did not affect cell morphology and motility. The wildtype, loa22- and TK2 strains had similar cell growth kinetics in liquid EMJH media, indicating that genetic manipulation did not alter growth in vitro.

Loa22 is a surface-exposed protein

Immunofluorescence studies found that Loa22 is a surface-exposed moiety (Figure 2). Antisera to Loa22 labeled the surface of live wild-type and complemented TK2 strains but did not label the surface of the mutant loa22- strain. In control experiments, antisera to LipL32 (Figure 2) and LipL41 (data not shown) labeled the surface of wild-type, mutant loa22- and TK2 strains, indicating that the labeling method was able to detect previously-described surface-exposed LipL32 and LipL41 [19] but not Loa22 in mutant loa22- strain. The procedure specifically detected antibodies bound to the leptospiral surface: antisera to LipL31, a previously-described lipoprotein associated with the inner membrane [19] (Figure 2) and cytoplasmic heat shock protein GroEL (data not shown) did not label live leptospires in this procedure although the antisera strongly labeled fixed, permeabilized leptospires (data not shown). These results are consistent with previous Triton X-114 extraction that showed partitioning of Loa22 into the hydrophobic, detergent phase, indicating that this moiety is a surface-exposed component of the leptospiral outer membrane [14].

Virulence of the mutant loa22- strain is attenuated in experimental animal models

The guinea pig and hamster, the standard experimental models for leptospirosis [1,2], were used to evaluate the virulence of the wild-type, mutant loa22- and complemented strains (Table 1). In two experiments, 10 of 14 and 8 of 8 of the guinea pigs died when inoculated with intraperitoneal challenges of 2×10^8 and 4×10^8 wild-type bacteria, respectively. Infected guinea pigs developed leptospirosis with

characteristic signs such as prostration and jaundice (Figure 3), and died within 4 to 6 days after the infection (Table 1).

In contrast, the mutant loa22- strain demonstrated loss of virulence as reflected by the inability of challenge doses of 2×10^8 and 4×10^8 bacteria to produce death in guinea pigs (14 and 8 animals, respectively) (Table 1). The difference in mortality was significantly lower for animals challenged with the loa22- than with the wild-type strains (0 vs 71% and 0 vs. 100% in experiments 1 and 2, respectively, $P < 0.05$). Guinea pigs infected with the loa22- strain did not demonstrate clinical signs of leptospirosis during the 21 day follow-up period. The mutant loa22- strain was isolated from blood at post-challenge day 3 in 4 of 4 infected guinea pigs which were infected with 2×10^8 bacteria in a separate experiment. In addition, the loa22- strain was isolated from the kidneys of 5 of 7 guinea pigs sacrificed at post-challenge day 21 (experiment 1, Table 1). However, cultures of kidneys from animals infected with the loa22- mutant required an incubation period of more than two weeks to become positive, suggesting that the number of viable leptospire in these tissues was low. These findings indicated that although the mutant did not induce disease, it was able to cause bacteremia and colonization following infection. Sequential in vivo passaging and re-isolation of the loa22- strain from blood or tissues of infected guinea pigs (7 cycles in total) failed to recover a virulent isolate that could induce clinical disease or death in guinea pigs.

Complementation of loa22- restored the virulence phenotype of the mutant loa22- strain in the guinea pig infection model. Challenge doses of 2×10^8 and 4×10^8 of TK2 bacteria caused death in 43 and 75%, respectively, of the inoculated animals (Table 1). Deaths occurred 5 to 9 days after challenge. There were no significant differences between the death rates among guinea pig groups challenged with the wild-type and TK2 strains. DNA was extracted from TK2 strains that were used to challenge guinea pigs and TK2 strains that were re-isolated from guinea pigs during autopsy. Southern blot and PCR analyses demonstrated that these isolates had the complemented loa22 genotype and the spectinomycin and kanamycin cassettes (data not shown),

indicating that the observed restoration in virulence was not due to contamination of inoculating cultures with the wild-type strain.

Hamsters were challenged with wild-type, mutant loa22- and TK2 strains to confirm the findings observed in the guinea pig model. Inoculation with 10⁸ wild-type bacteria induced death in all animals (Table 1). In contrast to what was observed in the guinea pig model, challenge with mutant loa22- bacteria caused death in 1 of 10 hamsters. However, death rates (10% vs. 100%, $p=0.00011$) were significantly lower for hamsters challenged with loa22- than those challenged with wild-type strains. Challenge with the TK2 strain produced death in 60% (6 of 10) of the hamsters, indicating that as in the guinea pig model, complementation of loa22 in the mutant strain partially restored virulence.

Mutant loa22- strain does not produce tissue pathology in the guinea pig model

Necropsy evaluation of guinea pigs infected with wild-type strain at post-challenge days 5 and 6 found macroscopic lesions associated with leptospirosis (Figure 3A). Diffuse hemorrhage was observed in kidneys, as well as multi-focal hemorrhage in lungs, stomach and intestines (data not shown). Splenomegaly was observed, as well as jaundice of the liver and subcutaneous, ascites, and haemothorax. None of these findings were observed, except for splenomegaly, in necropsies of guinea pigs infected with the mutant loa22- strain (Figure 3B). Infection with the TK2 strain, in which loa22- was complemented, produced the complete spectrum of gross lesions observed in infections with wild-type strain (Figure 3C).

Haematoxinilin-eosin staining of sectioned lung, kidney, spleen, and liver from guinea pigs infected with the wild-type strain demonstrated characteristic histopathologic findings for leptospirosis (Figure 4). In liver tissues, there was prominent loss of parenchymal architecture, intense periportal infiltration with lymphocytes and plasma cells, focal necrosis and hemorrhage, mostly in the periportal and perivascular areas (Figures 4A, left panel). Kidneys were characterized by cortical and medullary haemorrhage, interstitial nephritis and tubular necrosis (Figures 4D, left panel). Spleens were haemorrhagic, with focal necrosis in the red pulp (data not shown).

Intraalveolar haemorrhage associated with interstitial infiltration with polymorphonuclear and mononuclear cells was a prominent finding in lung sections (data not shown).

5 However, infection with mutant loa22- strain produced markedly reduced or absent inflammatory responses and tissue pathology in guinea pigs on post-challenge day 6 (Figure 4). Liver tissues demonstrated mild parenchymal dystrobeculation and periportal infiltrates without focal necrosis or haemorrhage (Figure 4A, middle panel). Kidneys, spleens and lungs from mutant-infected animals exhibited sparse or absent
10 inflammatory infiltrates. Infection with the TK2 strain, in which loa22- was complemented, produced similar pathological findings as observed for the wild-type strain (Figure 5).

Silver staining and immunohistochemistry demonstrated the abundant numbers of
15 leptospire in the livers (Figures 4B and C, left panel) and kidneys (Figure 4E, left panel) of guinea pigs infected with the wild-type strain at post-challenge day 6. Sparse numbers of leptospire were found in the interstitial and alveolar spaces of the lungs. In contrast, leptospire were not detected in tissues of guinea pigs infected with the loa22- strain at postchallenge day 6 (Figure 4B, C and E, middle panel) and
20 21. In sectioned kidneys and livers from guinea pigs infected with the complemented TK2 strain (Figure 5), immunohistochemical analyses identified leptospire in numbers observed for wild-type infections. Antiserum to Loa22 (Figure 6) stained all wild-type and TK2 leptospire found in kidney and liver sections, showing that this protein is expressed during acute leptospirosis.

DISCUSSION

The recent completion of the genome sequences of pathogenic *Leptospira* strains has led to the identification of putative determinants that may play a role in virulence
30 [9,10,11]. One such determinant, loa22, was found to be a lipoprotein with an OmpA domain [14] which is up-regulated during host infection [17] and strongly recognized by sera from leptospirosis patients [15]. Furthermore Loa22 is conserved among

pathogenic *Leptospira* [14,15,16], suggesting that it may play a specific role in disease pathogenesis. However, its role has not been elucidated until this moment since targeted mutagenesis was not feasible in pathogenic *Leptospira*. Recently, we showed that the Himar1 mariner transposon permits random mutagenesis in the pathogen *L. interrogans* [13]. In search of mutants that might be affected in virulence, we identified a *L. interrogans* mutant exhibiting Himar1 insertion into *loa22*. By analysis of the *loa22*- strain, we now show that *Loa22* is required for virulence of the pathogen within animal models and fulfill the molecular Koch's postulates [12] as a virulence factor.

Complementation of the virulence phenotype of the *loa22*- strain by chromosome insertion of *loa22* demonstrated that the virulence defect was due to the inactivation of *loa22* and not to a second-site mutation. Transcriptional data and sequence analysis of the transposon insertion sites in the mutant and complemented strains further confirm that Himar1 insertion did not affect another gene that could be involved in virulence (data not shown). The parental and mutant strains of *L. interrogans* showed similar cell morphology and growth characteristics in vitro, therefore demonstrating that *loa22* is not essential for in vitro growth. Although there was no statistical difference in death rates among animals challenged with wild-type and complemented strains, infections with the complemented strain did not cause death in all animals. The inability to restore complete virulence (100% lethality) in the complemented strain did not appear to be due to instability of the construct, as the complemented strain resulted from chromosome insertion. The complemented strain reisolated from animals expressed *Loa22*. In addition, infection with another strain, TK1, for which the *loa22* gene was complemented at another chromosomal site, caused death rates in guinea pigs (6 of 8 guinea pigs; challenge dose, 4×10^8 bacteria /ml), which was not significantly different to results obtained for infections with the TK2 strain (Table 1). The complemented strains were subjected to more in vitro passages than the wild-type strain due to electroporation-mediated transformation followed by plating onto solid medium.

Leptospire are known to lose their virulence phenotype with prolonged in vitro culture passages [20], although the mechanism for this loss is not well understood. It is also possible that the complemented loa22 gene did not attain the optimal level of expression required for the virulence phenotype.

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Infection with *L. interrogans* produces a lethal infection in the standard hamster and guinea pig model and mimics the clinical presentation of severe leptospirosis in patients (i.e., jaundice, pulmonary hemorrhage) [1,2,3]. In guinea pigs, infection with the loa22- strain did not induce death but was recovered from blood and tissues.

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This suggests that the loss of Loa22 did not affect the ability of the pathogen to establish a bacteraemia or induce a persistent colonization, but attenuated the ability of leptospire to cause disease and fatal outcomes in the animal models. Consistent with the lack of clinical manifestations, mildly abnormal or no histological changes were observed in tissue organs of guinea pigs infected with loa22- strain. The

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absence of leptospire in the tissues of guinea pigs infected with the mutant loa22-strain six days post-inoculation by histopathologic analysis indicates that loss of the loa22 genotype significantly reduced the pathogen burden during infection and the inflammatory responses elicited by this infecting load. The standard inoculation method used in animal models of leptospirosis, intraperitoneal injection, may not reflect conditions encountered during natural infection since leptospire enter the

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host by penetrating breaks in the skin or traversing the mucosal membranes. Further studies will need to be performed that use subconjunctival or subcutaneous challenge routes to determine whether Loa22 plays a role in the initial steps of infection.

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The process of host infection by pathogens is usually complex and multifactorial. We observed that loss of loa22 genotype was associated with complete loss of virulence in the guinea pig model as indicated by the inability to induce death in these animals. In hamsters, infection with the mutant loa22- strain was associated with significantly

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reduced death rates (10 vs 100%, mutant vs wild-type strain, respectively) but did not lead to complete loss in the ability to produce a lethal infection. The observed

differences may reflect intrinsic differences in susceptibility of the two animal models to infection with the strain, *L. interrogans* serovar Lai, which was used in this study.

Our results demonstrate that Loa22 is exposed on the surface of *L. interrogans*,
5 confirming the localization of the protein to the outer membrane [14]. The structure of Loa22 is composed of a C-terminal OmpA domain of approximately 110 amino acids. This OmpA domain refers to the C-terminus of *E. coli* OmpA, a major outer membrane protein of *E. coli*.

10 Orthologs of the OmpA-domain are found in proteins from a wide range of bacterial species. Predictions for the structure of this C-terminal OmpA domain have ranged from that of a globular domain containing a predicted peptidoglycan-associating motif which is located in the periplasm [21,22], to a domain containing a significant proportion of anti-parallel β sheets that are associated with the outer membrane
15 [23,24]. The N-terminal domain of *E. coli* OmpA was crystallized as a β -barrel-structured porin, which is believed to be inserted into the outer membrane [25]. However, this N-terminal region has no significant sequence similarity to Loa22. Since there is no sequence similarity between Loa22 and other OmpA-like proteins in this N-terminal region, these proteins may be structurally distinct.

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The role of Loa22 during pathogenesis remains to be determined. The protein OmpA of *E. coli* and other gram-negative bacteria are believed to play a multifunctional role in bacterial physiology and pathogenesis. In gram-negative bacteria, OmpA has been shown to be an adhesin [26,27,28] and induce cytokine production by dendritic cells
25 [29,30]. In a recent study, recombinant Loa22 was shown to bind in vitro with components of the extracellular matrix such as plasma fibronectin and collagen type I and IV [5], suggesting that the surface-exposed domain of Loa22 may, in fact, act as an adhesin. Furthermore, the lipopeptide moiety of spirochetes are potent mediators of the inflammatory response [8]. Loa22, which has a lipobox sequence, may
30 therefore induce severe disease manifestations by eliciting the host immunopathogenic responses.

Proteins of the OmpA family have been proposed to play a role in the stabilization of the envelope structure [22]. Although the loa22- strain was recovered from the animal, we cannot rule out that the absence of this protein in the membrane may impact on several steps in host infection such as the stability of the outer membrane and survival of the leptospiral pathogen in vivo and the ability to penetrate tissues during dissemination or adhere during colonization.

In conclusion, this study identified the first virulence factor in pathogenic *Leptospira* and will form the basis for further investigation of the role that Loa22 plays in leptospiral pathogenesis. Furthermore Loa22 is expressed on the leptospiral surface, suggesting that immunization with this protein may elicit bacteriocidal or pathogenesis-blocking immune responses. Bacterin-based vaccines have been used in some countries but they present a number of disadvantages, including adverse reactions, short duration of efficacy and lack of protection against serovars not included in the vaccine preparations [1,2,3]. A better understanding of the role of Loa22 may facilitate identification of defined and more effective sub-unit vaccine candidates for leptospirosis.

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AB096686. Reports Leptospira interr...[gi:30984126]• Features

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 ORGANISM *Leptospira interrogans* serovar Manilae
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 REFERENCE 1
 AUTHORS Koizumi, N. and Watanabe, H.
 TITLE Molecular cloning and characterization of an OmpA family outer membrane lipoprotein of *Leptospira interrogans*
 JOURNAL Unpublished
 REFERENCE 2 (bases 1 to 858)
 AUTHORS Koizumi, N.
 TITLE Direct Submission
 JOURNAL Submitted (21-NOV-2002) Nobuo Koizumi, National Institute of Infectious Diseases, Department of Bacteriology; Toyama-1-23-1, Shinjuku, Tokyo 162-8640, Japan (E-mail:nkoizumi@nih.go.jp, Tel:81-3-5285-1111(ex.2224), Fax:81-3-5285-1163)
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Table 1: Virulence of *L. interrogans* serovar Lai strain Lai and its derivatives in the guinea pig and hamster infection models.

Bacterial strain ^a	No. of animals infected	No. of animals that died	Time to death (days)	<i>P</i> value ^b
Experiment 1 : Guinea pigs; challenge dose, 2x10 ⁸				
<i>wt</i>	14	10	5,5,5,5,5,5,5,5,5,6	-
<i>loa22⁻</i>	14	0	-	0.00015
TK2	14	8	5,5,5,6,6,7,8,9	NS
Experiment 2: Guinea pigs; challenge dose, 4x10 ⁸				
<i>wt</i>	8	8	4,4,4,4,4,4,4,4	-
<i>loa22⁻</i>	8	0	-	0.00015
TK2	8	6	6,6,6,7,8,8	NS
Experiment 3: Hamsters; challenge dose, 10 ⁸				
<i>wt</i>	10	10	7,7,7,7,7,7,7,7,7,8	-
<i>loa22⁻</i>	10	1	8	0.00011
TK2	10	6	9,10,12,17,20,21	NS

^a *wt*: wild-type strain, *loa22⁻*: mutant *loa22⁻*, TK2: complemented strain

^b Fisher test was performed to determine if there was a significant difference in mortality between the *wt* and other strains. NS, non-significant.

WHAT IS CLAIMED IS:

1. An attenuated strain of *Leptospira*, said strain not expressing the Loa22 protein.
2. A composition comprising the strain as defined in claim 1.
3. Use of the strain of claim 1 or the composition of claim 2 in the prevention and/or treatment of a leptospiral disease.
4. A method for the treatment and/or prevention of a subject against a leptospiral disease, the method comprising the step of administering to said subject a strain as defined in claim 1 or a composition as defined in claim 2.
5. Use of a strain as defined in claim 1 in the diagnostic of a leptospiral disease.

Application number / numéro de demande: 2589155

Figures: 1a, b, 2, 3, 4a, b, c, d, e, 5a, b, c,
~~Figure~~ 6a, b

Unscannable item(s)

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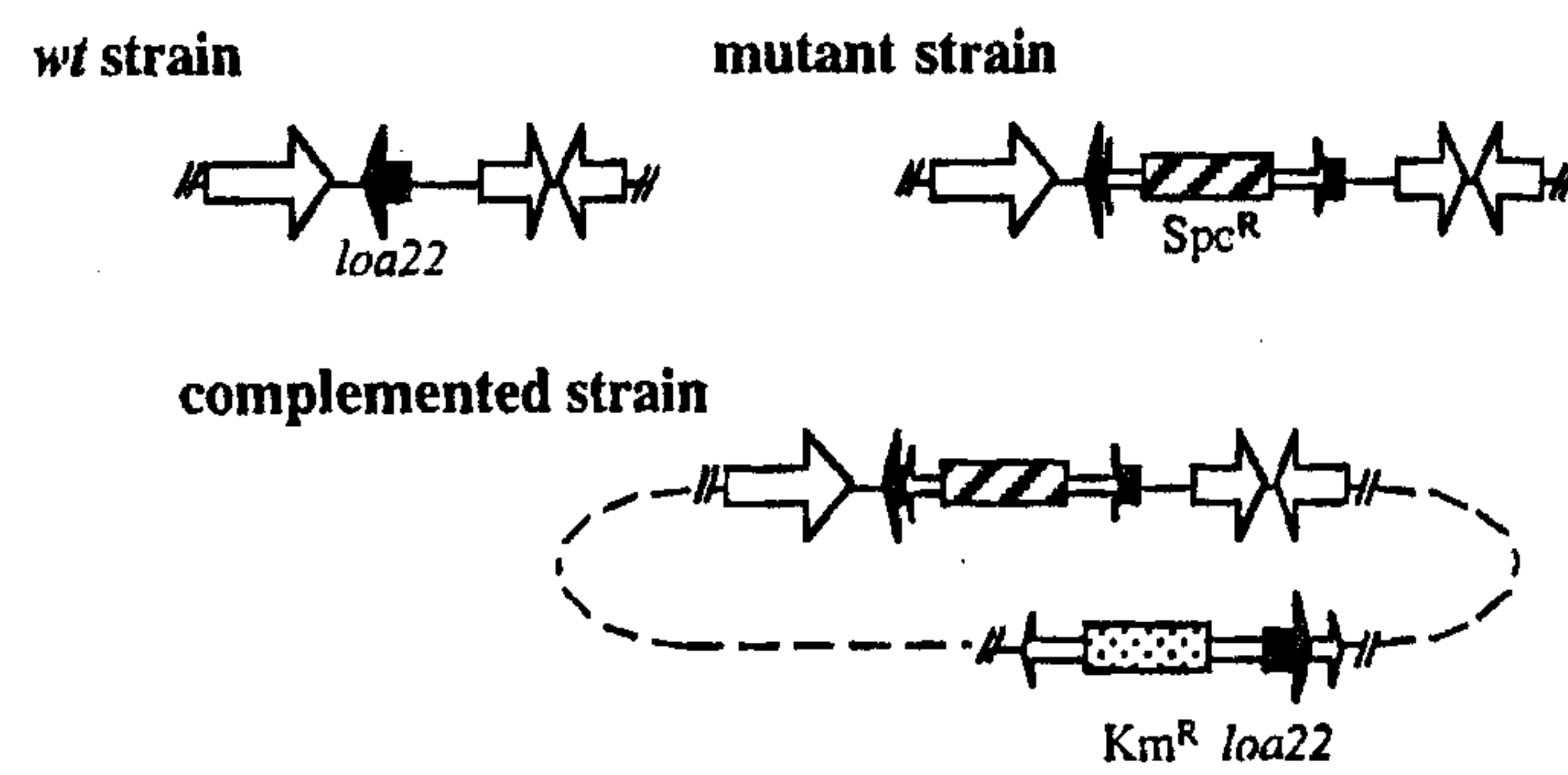
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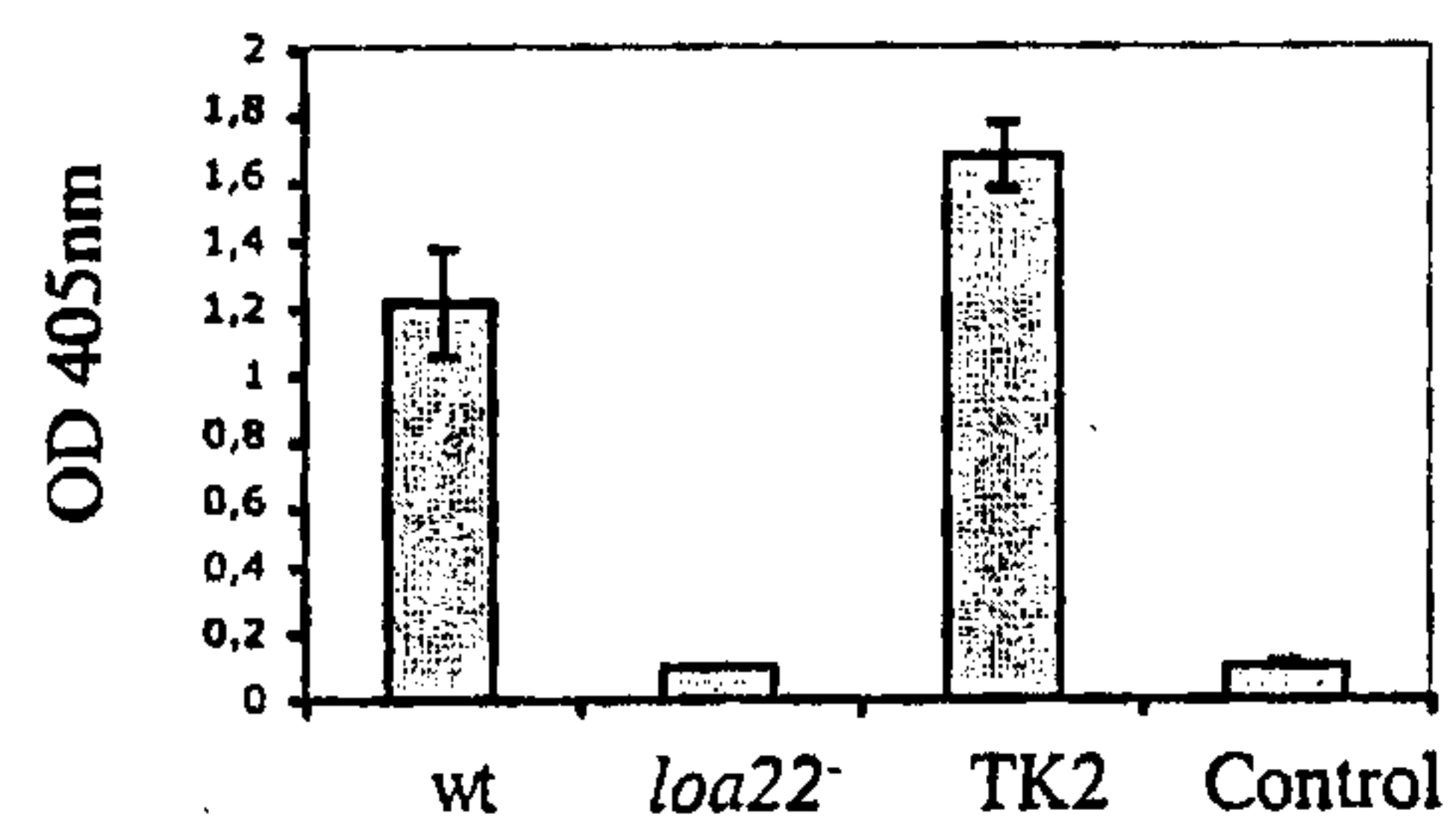


Figure 1