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Short communication

A live attenuated strain of *Yersinia pestis* Δ*yscB* provides protection against bubonic and pneumonic plagues in mouse model

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ABSTRACT

To develop a safe and effective live plague vaccine, the Δ*yscB* mutant was constructed based on *Yersinia pestis* biovar *Microtus* strain 201 that is avirulent to humans, but virulent to mice. The virulence, immunogenicity and protective efficacy of the Δ*yscB* mutant were evaluated in this study. The results showed that the Δ*yscB* mutant was severely attenuated, elicited a higher F1-specific antibody titer and provided protective efficacy against bubonic and pneumonic plague in mouse model. The Δ*yscB* mutant could induce the secretion of both Th1-associated cytokines (IFN-γ, IL-2 and TNF-α) and Th2-associated cytokines (IL-4 and IL-10). Taken together, the Δ*yscB* mutant represented a potential vaccine candidate based on its ability to generate strong humoral and cell-mediated immune responses and to provide good protection against both subcutaneous and intranasal *Y. pestis* challenge.

1. Introduction

Plague is a zoonotic disease caused by Gram-negative bacterium *Yersinia pestis*, which is usually transmitted to humans from infected rodents via the bite of an infected flea [1]. Historically, plague was a fatal infectious disease afflicting human populations, leading to millions of deaths. Recently, plague has been classified as a re-emerging infectious disease by the World Health Organization [2] and has attracted a considerable attention because of its potential misuse as an agent of biological warfare or bioterrorism [3]. Thus, it is imperative to develop an ideal plague vaccine for human use.

Construction of live attenuated vaccines is considered as one of the most effective strategies to develop plague vaccines. Effector proteins (Yops) of the type III secretion system (T3SS) play an essential role in the pathogenesis of *Y. pestis* [4]. Regulation of Yop secretion is dependent on the expression of at least five proteins YopN and its chaperones SycN and YscB, TyeA and LcrG [5]. Mutational inactivation of *yscB* induces secretion of Yops in the presence or absence of calcium and before host cell contact, and secretes reduced level of YopN in comparison to the parent strain [6]. These results signify that the *yscB* mutant might result in an avirulent or highly attenuated phenotype. In this study, we constructed the Δ*yscB* mutant based on a strain of *Y. pestis* biovar *Microtus* strain 201 which is avirulent in larger mammals and humans, and investigated the possibility of using the mutant as a live attenuated plague vaccine.

2. Methods

2.1. Construction of the Δ*yscB* mutant

The *Y. pestis yscB* mutant (Δ*yscB*) was constructed by using one-step inactivation method based on the λ-Red-mediated recombinant system [7]. Briefly, the *yscB*::*kana* mutagenic cassette was amplified from pRS551 using the primer pairs *yscB-kana*-F (5′-CTAAAAAACTTGGCAGCCAGTTTAGGAAGAAAACCGTTT*GAGATTGC-AGCATTACACG*-3′) and *yscB-kana*-R (5′-TTAATTCCACCCCACGCGAG-ACGCTACAGAAAATGGTGTTT*GTAACGCACTGAGAAGC*-3′). The underlined sequences are homologous to the 13–52 and 375–414 nucleotides of the *yscB* sequence and the italic sequence is used as primers to amplify Km-resistance cassette.

2.2. Determination of virulence for the Δ*yscB* mutant

Groups of 6-week-old female BALB/c mice (6 mice per group) were challenged with the Δ*yscB* mutant by subcutaneous or intranasal route. The actual challenge doses were calculated by counting CFUs on agar plates. The challenged animals were observed for 14 days after injections, and the dose that killed 50%

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of the mice (LD_{50}) was calculated using the Reed–Muench method [8].

2.3. Animal immunizations

The Δ*yscB* mutant was cultivated at 26 °C to mid-log phase, and then the bacterial cultures were shifted to 37 °C for another 3 h. Groups of ten female BALB/c mice were injected s.c. in the groin with one dose of the Δ*yscB* mutant (1.63×10^4 CFU), the vaccine EV76 (1.6×10^4 CFU) or the same volume of PBS (0.1 ml).

2.4. F1-specific antibody assays

Sera collected from the immunized and control mice were assayed for the presence of F1-specific IgG by a modified ELISA. The titer of specific antibody was estimated as the maximum dilution of the serum with an OD value of 0.2 units over background. Background values were obtained from serum samples collected from the animals only receiving PBS. Antibody endpoint titer per immunization group is presented as the GMT to F1 antigen.

2.5. Elispot assay of cytokines

Enzyme-linked immunospot (ELISPOT) assays were performed for INF-γ, IL-2, TNF-α, IL-4 and IL-10 cytokines using commercially available murine Elispot kits (R&D Systems) according to the manufacturer's instructions. The stimulants include F1 peptide pool (10 μg/ml), LcrV peptide pool (10 μg/ml), Concanavalin A (5 μg/ml, positive control), the Δ*YscB* mutant of *Y. pestis* strain 201 (10^5 CFU) or medium RPMI 1640 (negative control). Results are expressed as number of spot-forming cells (SFC)/10^6 cells.

2.6. Challenge with Y. pestis

Challenge experiments were carried out with the *Y. pestis* 141 strain (Antigua biovar) that was isolated from *Marmota himalayana* in Qinghai-Tibet plateau and has a median lethal dose (MLD) of 5.6 or 4375 colony-forming unit (CFU) for BALB/c mice by the subcutaneous or intranasal route. The immunized mice were challenged on week 6 after the primary immunization with 1.24×10^6 CFU by the subcutaneous or intranasal route, and then closely observed for 14 days.

2.7. Statistical analysis

Differences in immune responses between the treatment group and control group were analyzed with the Student's *t*-test. A probability value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. The ΔyscB mutant highly attenuated and effective against plagues

Y. pestis biovar *Microtus* strains are virulent to *Microtus* and mice, but they seem to be avirulent to larger mammals, such as guinea pigs, rabbits and humans. The *Microtus* strains might have a good potential to develop an attenuated vaccine for humans, because these strains have all known protective antigens and are avirulent for humans [9,10]. To avoid eliciting ecological disaster, an ideal vaccine must be avirulent in both animals and humans. Therefore, we constructed the Δ*yscB* mutant from a *Microtus* strain 201 and investigated the possibility of using the mutant as a live attenuated plague vaccine candidate. Our results show that the LD50 of the Δ*yscB* mutant in BALB/c mice is estimated to be more than 10^6 CFU,

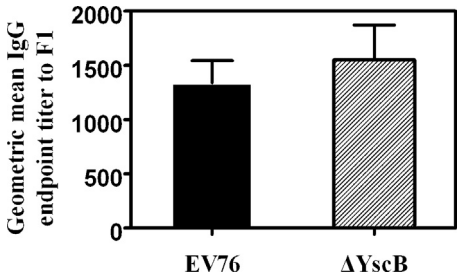

Fig. 1. Development of IgG titers to F1 in BALB/c mice immunized with the Δ*yscB* mutant and wild-type *Y. pestis* strain 201 six weeks post immunization.

which is more than 400,000-fold higher than 3 CFU of the wild-type strain. It is clearly demonstrated that the *Microtus* strain of *Y. pestis* 201 can be highly attenuated by deletion of *yscB* gene in mouse infection model.

Although antibody level does not correlate with protective efficacy, humoral immunity plays an important role in protection against plague [11]. The antibody response to F1 antigen from each group of 10 animals on week 6 after primary immunization was determined and shown in Fig. 1. There was no significant anti-F1 IgG titer difference between the immunized animals with the Δ*yscB* mutant and those with EV76 ($p > 0.05$), whereas no anti-F1 IgG was detected in the control animals that only received PBS buffer. When groups of 10 immunized mice were challenged with 1.24×10^6 CFU of virulent *Y. pestis* strain 141, the Δ*yscB* mutant provided 87.5% or complete protection by the subcutaneous or intranasal route,

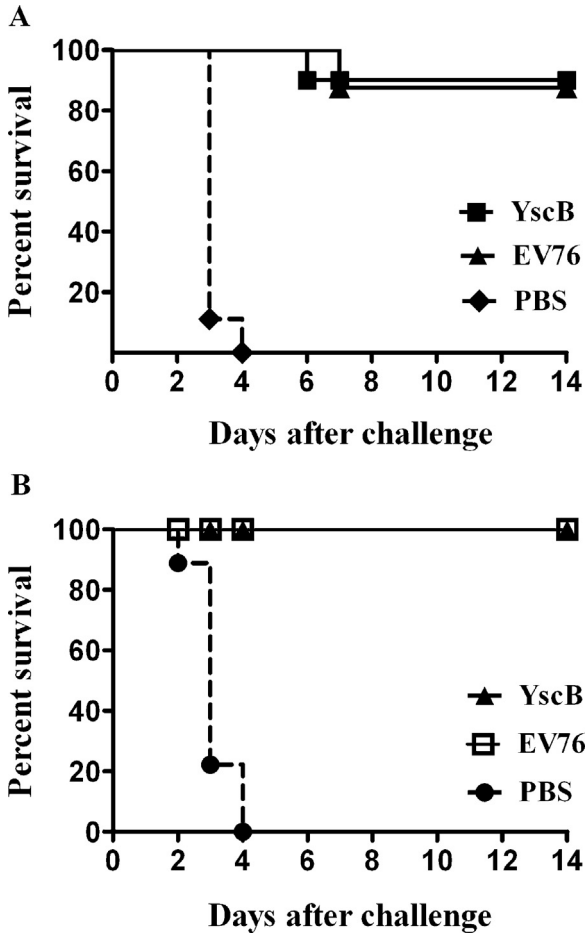

Fig. 2. Survival of the mice immunized with the Δ*yscB* mutant after infection subcutaneously (A) or intranasally (B) with virulent *Y. pestis* strain 141.