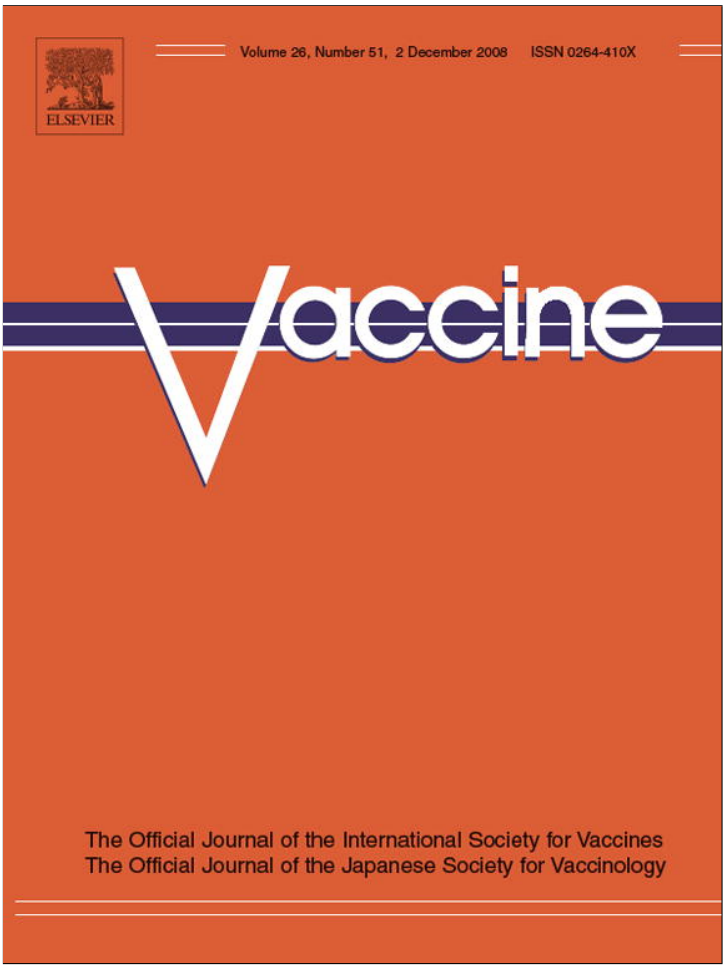

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Short communication

IgA response by oral infection with an attenuated *Yersinia enterocolitica* mutant: Implications for its use as oral carrier vaccine

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ABSTRACT

Yersinia enterocolitica (*Ye*) mutant strain (*sycH-*) is unable to secrete the virulence protein YopH. Mucosal vaccination is often required to induce protection, but stimulating strong IgA response is frequently difficult. Here, we addressed whether *Ye sycH-* might induce IgA response, and investigated its attenuation in TNFRp55−/−, IL-12p40−/− and IL-4−/− mice. We found that *Ye sycH-* colonizes Peyer's patches, and induces higher *Yersinia*-specific IgA levels in feces and in serum compared with *Ye* wild type. The *Ye sycH-* mutant proved to be attenuated and induced IgA in both wild-type and immunodeficient mice. These lines of evidence show the attenuation of *Ye sycH-* and its ability to stimulate an IgA response. This mutant might be useful as an oral vaccine carrier.

1. Introduction

Yersinia enterocolitica (*Ye*) is a Gram-negative pathogen that causes food-borne gastroenteritis. The pathogenicity of *Ye* depends on a virulence plasmid (pYV) which encodes the Yop (*Yersinia* outer protein) virulon, a set of pathogenicity factors that enable extracellular bacteria to disarm cells of the immune system by direct injection of effector proteins [1]. A key virulon protein is YopH, a 468 amino acid protein-tyrosine phosphatase with a C-terminal catalytic domain and a multifunctional N-terminal domain, which binds tyrosine-phosphorylated target proteins [2]. Thus, Yop H impairs the entry of the bacteria into HeLa cells or their phagocytosis by macrophages [3], and blocks T and B lymphocyte activation [2,4]. The Yops require specific Yop chaperone (Syc) for their secretion; thus, Yop H requires the chaperone SycH [1].

During the course of an infection with *Yersinia*, the bacteria reach the intestinal tract, enter through the M cells of the Peyer's patches (PP) [5], then disseminate to the mesenteric lymph nodes (MLN), and subsequently, to the spleen, the liver, and the lung. Innate host defense mechanisms including those mediated by polymorphonuclear leukocytes and macrophages are involved in *Yersinia* control in PP [6]. In addition, clearance of infection involves NK cells, activation of $CD8^+$ T and $CD4^+$ Th1 cells, and cytokines such as tumor necrosis factor (TNF)-α, interleukin-12 (IL-12) and gamma interferon (IFN-γ) [7,8].

In previous studies, we demonstrated that a *Ye* mutant strain which is deficient in the chaperone SycH (*Ye sycH-*) resulting in a functional deficiency in YopH, colonizes intestinal tissues of wild-type and immunodeficient mice, but is attenuated for systemic infection in these mice [9]. Thus, even in immunocompromised hosts, *Ye sycH-* may be suitable for using as live carrier vaccines. This issue is of considerable interest because recent studies have demonstrated that vaccination with attenuated live vaccine may give rise to severe and fatal infection courses in patients with previously unknown immunodeficiency [10]. In addition, immunization experiments with attenuated *Ye* or *Y. pseudotuberculosis* strains as carriers for heterologous antigens (e.g. cholera toxin or antigens derived from *Listeria monocytogenes*) induced a protective immune response against challenge in mice [11,12]. Likewise, attenuated *Ye* live carrier strains were reported to induce both CD8 and some CD4 T cell responses [13].

There is currently great interest in developing mucosal vaccines against a variety of microbial pathogens. Thus, a mucosal route of vaccination seems to be crucial for protection against infections at mucosal surfaces such as *Vibrio cholerae* or enterotoxigenic *Escherichia coli*, in which vaccine-induced protection is mediated mainly, if not exclusively, by locally produced secretory IgA antibodies [14]. But despite the many attractive features of mucosal vaccination, it has often proven difficult in practice to stimulate a strong IgA immune response and protection by mucosal

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administration of antigens [14]. In addition, the mucosal immune response induced by *Ye* mutant strains has not been previously explored. Our previous studies demonstrated that *Ye sycH-* may be the most appropriate mutant in terms of safety. Moreover, this mutant induced a cytokine response in spleen cell culture [9]. However, the immunogenicity of this mutant needs to be investigated because an optimal attenuated vaccine strain should be both safe and immunogenic [9].

In the present work, we extended these studies by investigating whether *Ye sycH-* may induce a mucosal IgA response after oral administration in normal and immunocompromised hosts. We tested IgA response and *Ye sycH-* attenuation in TNFRp55−/− and IL-12p40−/− to evaluate this mutant in conditions of deficiency of a cytokine receptor or a cytokine that play a crucial role in *Yersinia* protection [7–9]. In addition, we tested the infection in IL-4−/− mice since IL-4 is responsible for the generation of Th2 functions in $CD4^+$ T cells that are selectively induced by oral immunization and they might control IgA responses [15]. Our data could contribute to the development of novel oral live carrier vaccines.

2. Materials and methods

2.1. Bacteria

The strains WA-314 wild-type *Ye* serotype O:8; pYV^+ (WAP) [16], and a *Ye* WA-314 mutant *sycH-*; pYV^+, $Spect^R$ (*Ye sycH-*) [17], were used for infection. Bacteria were cultured overnight in Luria broth (LB) at 27 °C, and prepared for the experiments as described previously [9]. Yop profile was analysed [18] in both strains, and it corroborated that *Ye sycH-* is unable to secrete Yop H.

2.2. Mice and infection

TNFRp55−/− [19], IL-12p40−/− [20] and IL-4−/− [21] mice on a C57BL/6 background and C57BL/6 wild-type mice were used in all experiments. Mice were kept under specific-pathogen-free conditions in a positively pressurized cabinet (EHRET, Emmendingen, Germany) and provided with sterile food and water *ad libitum*. Mice 6–8 weeks old were used for the experiments. At least two independent experiments were carried out with five mice per group. Mice

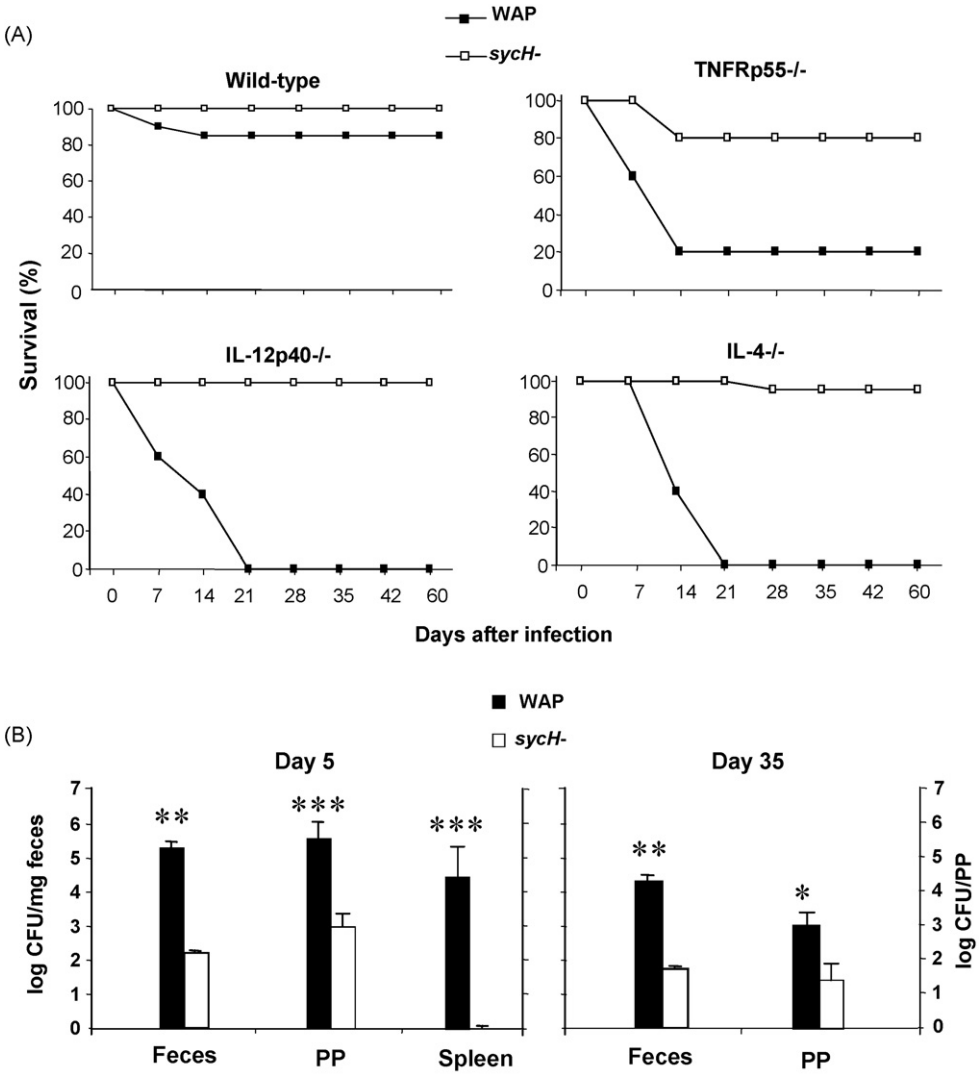

Fig. 1. Susceptibility of mice to *Ye* WAP or *sycH-* infection. (A) Survival curve of wild-type C57 BL/6, TNFRp55−/−, IL-12p40−/−, and IL-4−/− mice after $1–5 \times 10^8$ *Ye* WAP or *sycH-* oral infection. Representative results from two independent experiments with five mice per group are shown. (B) Bacterial load in feces, Peyer's patches (PP) and spleen after *Ye* WAP or *sycH-* infection. The data are representative from three independent experiments and they are the results of five mice per group. $^*P < 0.05$, $^{**}P < 0.02$, and $^{***}P < 0.001$ compared with *Ye sycH* infection. CFU: colony-forming units.

were starved for 16 h and orogastrically infected with 1×10^8 to 5×10^8 yersiniae in 200 μl. The actual number of bacteria administered was controlled for each experiment by plating serial dilutions of the inoculated suspension on Mueller-Hinton agar and counting the colony-forming units (CFU) after incubation at 27 °C for 48 h. The mice were kept on lattices. Each mouse was bled before and weekly after infection by retro-orbital plexus and individually collected sera were frozen at −20 °C until use.

2.3. Survival curve and bacteriologic examination

The survival of the mice was monitored for 2 months. The number of bacteria in feces, PP and the spleen was determined by homogenization of the feces or the organs in PBS, and plating of serial dilutions on Mueller-Hinton agar for spleen, or on cefsulodine-irgasan-novobiocine-agar (CIN; OXOID, Wesel, Germany) for feces or PP homogenates [9].

2.4. IgA extraction from fecal pellets

Before and each 7 days after infection (days 0, 7, 14, 21, 28, 35 and 42), fresh fecal pellets from normal and knockout mice were collected in microtubes and weighted. Cold extraction buffer (30 mM EDTA, 0.1 mg/ml Soybean trypsin inhibitor, 1% bovine serum albumin in PBS) was added at a ratio of 1 ml/mg fecal pellets. The material was vortexed for 2 min at room temperature, centrifuged at 13,000 × g for 15 min at 4 °C. The supernatants were stored at −20 °C until the assays were performed.

2.5. Enzyme-linked immunosorbent assay (ELISA) for specific IgA

A specific IgA response was analysed by enzyme-linked immunosorbent assay (ELISA). Multiwell plates were coated with 100 μl per well of 10 μg/ml of the whole bacteria disrupted by sonication (SON) in PBS, and incubated at 4 °C overnight. After incubation with 1:50 diluted sera or undiluted fecal extracts, bound antibodies were demonstrated by reaction with horseradish peroxidase-conjugated goat anti-mouse IgA (Sigma, St. Louis, USA) followed by the addition of the enzyme substrate (H_2O_2) and the chromogen o-phenylenediamine (Sigma). Optical density (OD) was measured at 492 nm in an ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA). Sera and feces from uninfected mice were used as negative control.

2.6. Comparison of specific IgA response induced by Ye sycH with those induced by cholera toxin (CT) or bovine serum albumin (BSA) after oral immunization

To compare mucosal and systemic specific IgA responses induced by *Ye sycH*- with those induced by other mucosal immunizations, wild-type mice were inoculated with a single oral dose of 5×10^8 *Ye sycH*- or *Ye* WAP, 10 μg cholera toxin (CT) (Sigma), or 200 μg bovine serum albumin (BSA) (Gibco, LifeTechnology, NY, USA). Seven days after inoculation, a specific IgA response was measured in sera and feces by ELISA. For CT-specific IgA response evaluation, ELISA was performed as described previously [22]. Briefly, the plates were coated with GM1 ganglioside (0.5 nmol/ml) followed by CT (0.5 μg/ml). GM1 ganglioside and CT were kindly provided by Dr. José Luis Daniotti, National University of Cordoba, Argentina. For BSA ELISA, the plates were coated with BSA (200 μg/ml). Fecal extracts or sera at 1:2 or 1:50 dilutions, respectively, were added and serial twofold dilutions were performed. The endpoints titers were expressed as the reciprocal $\log_2$ or $\log_{10}$ for fecal extract or serum, respectively, of the last dilution that gave an OD at 490 nm exceeding the mean + 2 SD of the samples from uninoculated mice.

2.7. Statistical analysis

Differences between *Ye* WAP and *sycH*- infections were tested for significance by Mann–Whitney test. A *P* value less than 0.05 was considered statistically significant. All experiments were repeated at least twice and revealed comparable results.

3. Results

3.1. Effect of oral infection with Ye WAP and sycH- on wild-type and immunodeficient mice

The survival of *Ye* WAP or *sycH*- infected mice was monitored for 60 days in wild-type, TNFRp55−/−, IL-12p40−/− and IL-4−/− mice. We confirmed, as previously demonstrated [9] that, using the same inoculum ($1–5 \times 10^8$ yersiniae which is 10 LD50 of *Ye* WAP), *Ye* WAP was pathogenic in wild-type mice (survival 85%), whereas *Ye sycH*- resulted in avirulence (survival 100%) (Fig. 1A). Moreover, WAP was highly virulent for TNFRp55−/−, IL-12p40−/− and IL-4−/− mice (survival 0–20%) (Fig. 1A). In contrast, *Yersinia sycH*- was also attenuated in these immunocompromised mice (survival 80–100%) (Fig. 1A). The mucosal colonization and persistence of *Ye* WAP and *sycH*- strains were determined by counting the number of bacteria in feces and PP (Fig. 1B), on days 5 and 35 after oral infection. *Ye sycH*- efficiently colonized the intestine of wild-type mice since bacteria were detected in feces and PP on day 5 after infection. However, the *Ye* colony counts was significantly higher in feces ($P < 0.02$) and PP ($P < 0.001$) of mice infected with *Ye* WAP compared with the *Ye sycH*- infected mice (Fig. 1B). In contrast, *Ye sycH*- did not colonize the spleen (Fig. 1B). On day 35, lower bacterial number was detected in feces and PP, and again the bacterial counting was higher in feces ($P < 0.02$) and PP ($P < 0.05$) of mice infected

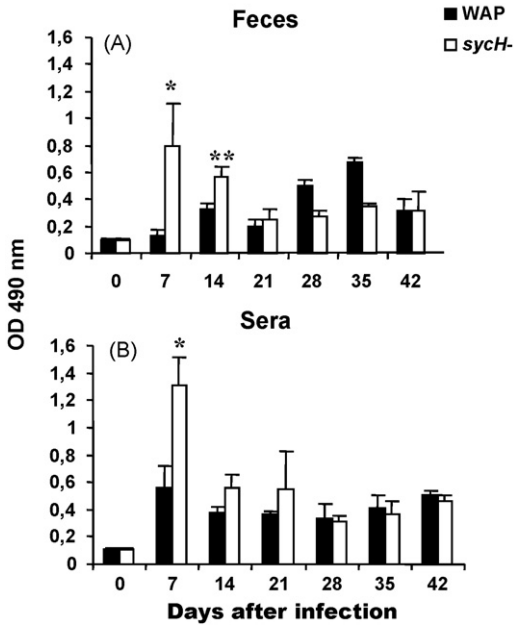

Fig. 2. Systemic and mucosal *Yersinia*-specific IgA responses in wild-type mice after *Ye* WAP or *sycH*- infection. (A) Kinetic of the IgA response in feces obtained weekly after the oral infection and assessed by ELISA. $^{*}P < 0.01$ and $^{**}P < 0.002$ compared with *Ye* WAP infection. (B) Kinetic of the IgA response in sera obtained weekly after the oral infection and assessed by ELISA. $^{*}P < 0.01$ compared with *Ye* WAP infection. Results are expressed as the mean and SD of five mice per group and day.

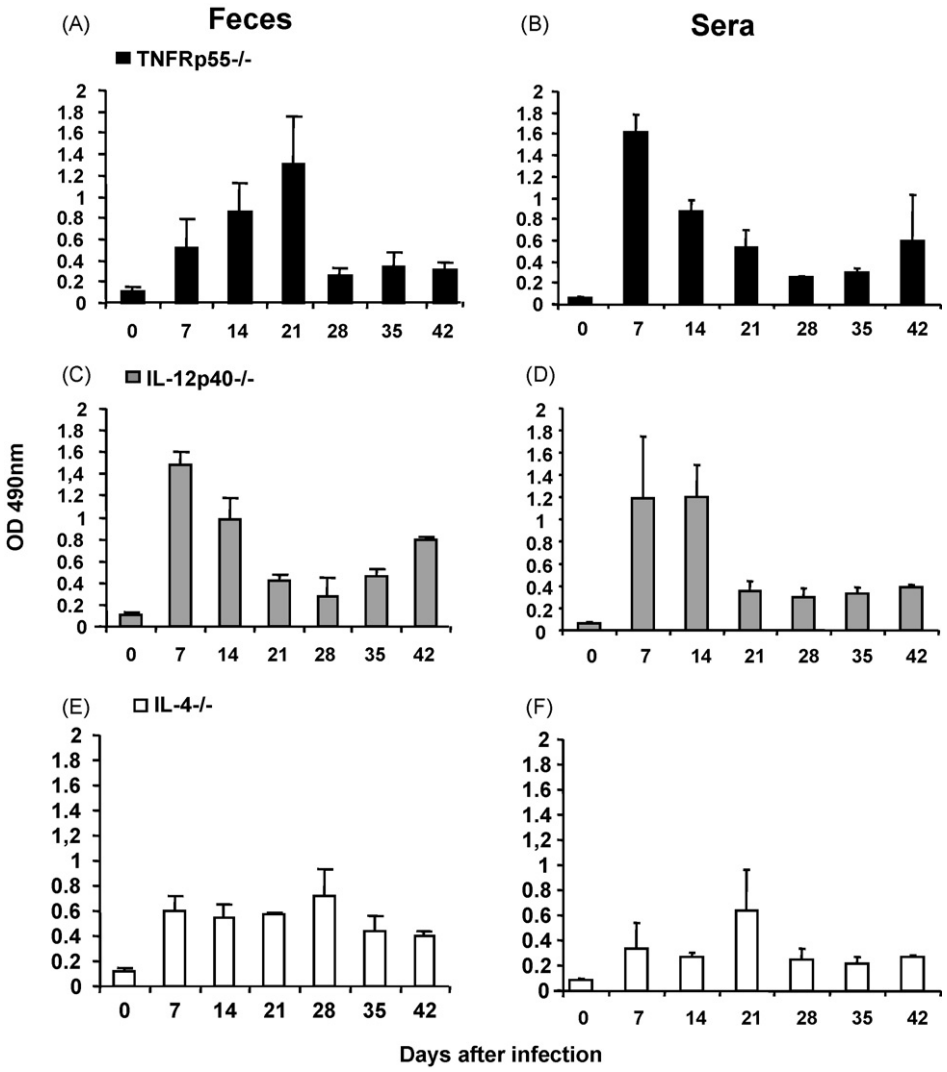

Fig. 3. *Yersinia*-specific IgA responses in feces and sera of immunodeficient mice after *Ye sycH*- infection. Kinetic of the IgA response in feces (A, C and E) and sera (B, D and F) obtained weekly after oral infection of TNFRp55−/− (A and B), IL-12p40−/− (C and D) or IL-4−/− (E and F) mice with *Ye sycH*-. The results are expressed as the mean and SD of five mice per group and day.

with *Ye* WAP compared with counterparts infected with *Ye sycH* (Fig. 1B).

3.2. Systemic and mucosal IgA response in wild-type mice after infection with Ye sycH-

To study the ability of *Ye* WAP and *sycH*- strains to induce mucosal and systemic immune response, we determined the course of the IgA response in serum and feces after the oral infection. We found that *Ye sycH*- at early time after infection (days 7 and 14) induced significantly higher fecal IgA response compared with *Ye* WAP ($P < 0.01$ and $P < 0.002$, respectively) (Fig. 2A). In addition, by day 7, the *Yersinia*-specific IgA response in serum was significantly higher in the wild-type mice infected with *Ye sycH*- compared with *Ye* WAP infected mice ($P < 0.01$) (Fig. 2B).

3.3. IgA response in TNFRp55−/−, IL-12p40−/− and IL-4−/− mice after infection with Ye sycH-

Since TNFRp55−/−, IL-12p40−/− and IL-4−/− mice turned out to be resistant to oral *Ye sycH*- infection, we evaluated the kinetics of intestinal and serum IgA responses. We detected that, although with different kinetics *Ye sycH*- induced *Yersina*-specific IgA responses in sera and feces of the three immunodeficient mice (Fig. 3A–F).

3.4. Specific IgA response induced by Ye sycH- compared with those induced by CT or BSA

In order to determine the potential of *Ye sycH*- to induce mucosal and systemic specific IgA responses, we compared these responses with those induced no only by *Ye* WAP but also by known high and poor mucosal immunogens such as CT and BSA, respectively [23,24]. As shown in Fig. 4, at day 7 after a single oral inoculation, *Ye sycH*- induced significantly higher mucosal and systemic specific IgA responses compared with *Ye* WAP ($P < 0.001$ and $P < 0.02$, respectively) or BSA ($P < 0.001$ and $P < 0.0001$, respectively), and at comparable level to CT.

4. Discussion

Live vaccines induce cell-mediated and antibody mediated immunity and eventually mucosal immune responses. This latter depends on the ability of the vaccine strain to colonize and to multi-

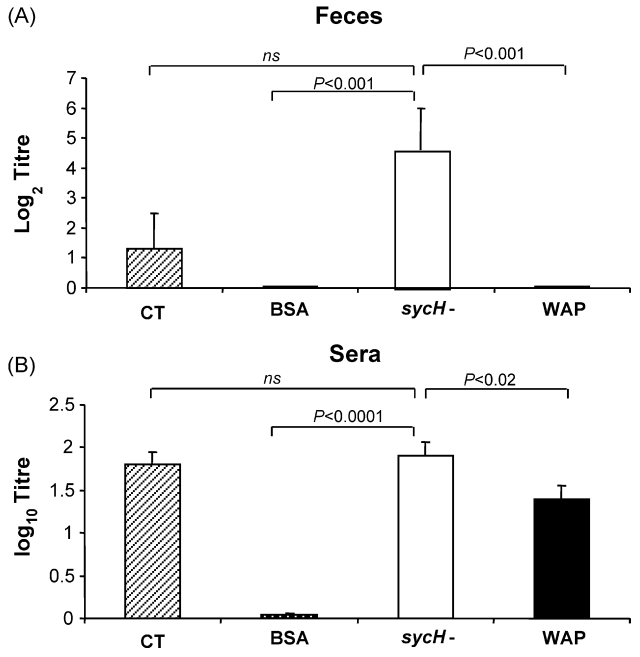

Fig. 4. Comparative study of specific IgA responses induced by *Ye sycH*- with those induced by *Ye* WAP, cholera toxin (CT) and bovine serum albumin (BSA). Specific IgA response measured in feces (A) and sera (B) by ELISA at day 7 after oral inoculation of wild-type mice with a single oral dose of 5×10^8 *Ye sycH*- or *Ye* WAP, 10 μg CT, or 200 μg BSA. Specific IgA responses are showed as the reciprocal $\log_2$ (A) or $\log_{10}$ (B) titers expressed as the last dilution that gave an OD at 490 nm exceeding the mean + 2 SD of the samples from uninoculated mice. The results are expressed as the mean and SD of four mice per group.

ply in the host [25]. In the present work, we evaluated the mucosal immune response of *Ye sycH*- and confirmed that this mutant strain is highly attenuated in normal and immunodeficient mice. On the other hand, a vaccine strain must be sufficiently invasive to induce immune response but not persistent as carrier [26]. In our study, the mutant strain *Ye sycH*- was able to colonize PP early during the infection (day 5) but it was clarified from PP since lower bacterial number was detected on day 35. These results are consistent with studies by Trülzsch et al. [27]. In addition, *Ye sycH*- did not colonize the spleen indicating that this mutant did not induce systemic infection in the mice.

Despite the many attractive features of mucosal vaccination, it has often proven difficult to stimulate strong IgA immune response and protection by mucosal administration of an antigen [28]. In the present study, we found that *Ye sycH*- induced early higher IgA levels in serum and feces compared with *Ye* WAP suggesting that YopH could display mucosal immunomodulator effects during intestinal *Yersinia* infection. In addition, we found that *Ye sycH*- induced also higher specific IgA responses than those induced by a poor mucosal immunogen such as BSA, but these responses were comparable to those induced by a single oral immunization with CT. Since for CT, one of the most potent mucosal adjuvants, immunogenicity and adjuvanticity are closely related phenomena [24,29], the *Yersinia*-specific IgA response induced by *Ye sycH*- could indicate oral adjuvant properties of this mutant to induce IgA secretion. Thus, both M-cell targeting and adjuvant activity could be achieved by *Ye sycH*- mutant suggesting that further studies should be carried out to demonstrate whether this mutant might be a candidate as oral carrier vaccine.

Y. enterocolitica sycH- proved to be attenuated in TNFRp55−/− mice since when compared with *Ye* WAP strain the survival rate of these mice increased from 20 to 80%. Thus, the survival rate was similar to that obtained in immunocompetent wild-type mice infected with the virulent strain *Ye* WAP. Moreover, *Ye sycH*- mutant strain was also attenuated in IL-12p40−/− and IL-4−/− mice since the survival rate in these mice increased from 0 to almost 100%. These results stress the attenuation of this mutant even in immunodeficient mice, and are interesting since several novel human immunodeficiencies have been identified that might introduce limitations to vaccination strategies with live carrier vaccines [10]. In addition, no arthritic symptoms were observed in normal and immunodeficient mice during the 60 days of observation after *Ye sycH*- infection (data not shown). This is also a valuable property in attenuated strains since their use could be limited in certain populations owing the risk of induction of reactive arthritis [30].

This study is the first that provides evidence of the capacity of *Ye sycH*- to induce mucosal IgA response even in TNFRp55−/−, IL-12p40−/− and IL-4−/− mice. Although PP are present in TNFRp55−/− mice, these are reduced in number and size. This PP number reduction may lead to different kinetics for mucosal IgA response observed in our work. This finding agrees with the results obtained in PP null mice [31]. On the other hand, the importance of IL-12 in *Yersinia* infection was confirmed by the marked increased susceptibility detected of IL-12p40−/− mice to *Ye* WAP infection. However, *Ye sycH* was attenuated in these mice, and induced a *Yersinia*-specific IgA response suggesting that IL-12p40 deficiency did not influence this response. Similarly, IL-12p40−/− mice mount anti-*Citrobacter* serum and gut-associated IgA responses [32]. In addition, we evaluated also the *Ye sycH*- induced IgA responses in IL-4−/− mice since IL-4 is responsible for the induction of gut mucosal immune responses [15,22]. However, we found that *Ye sycH*- induced also IgA response in IL-4−/− mice. Probably, other cytokines such as IL-6 and IL-10 support the IgA response [33].

Live attenuated bacterial vaccines are promising candidates for the induction of a broad-based immune response directed at recombinant heterologous antigens and the corresponding pathogen [34]. In summary, our study is the first to show that orally delivered *Ye sycH*- induces systemic and mucosal specific IgA responses. The data from immunodeficient mice confirm that this mutant is attenuated and that the IgA response stimulated by *Ye sycH*- is largely independent of TNFRp55, IL-12p40, and IL-4, and it could be induced in immunocompromised hosts. Future studies should include heterologous antigens in order to further explore the capability of *Ye scyH*- to induce IgA response to these antigens and consequently demonstrate their properties as oral carrier vaccines or adjuvant.

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