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Rational Live Oral Carrier Vaccine Design by Mutating Virulence-Associated Genes of *Yersinia enterocolitica*

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Three different *Yersinia enterocolitica* serotype O8 strains harboring mutations in virulence-associated genes coding for *Yersinia* adhesin A (YadA), Mn-cofactored superoxide dismutase (SodA), and high-molecular-weight protein 1 were analyzed for their ability to colonize and persist in tissues after orogastric immunization of C57BL/6 mice. We demonstrated that all three *Yersinia* mutant strains were markedly impaired in their ability to disseminate into the spleens and livers of immunized mice but were able to colonize the Peyer's patches for at least 12 days, resulting in the induction of significant antibody titers against *Yersinia* outer proteins (Yops) and in the priming of *Yersinia* antigen-specific CD4$^+$ Th1 cells isolated from spleens. The high level of attenuation did not diminish the immunogenic properties of the mutant strains. In fact, mice immunized with a single oral dose of any of the mutant strains were protected against a lethal oral-challenge infection with wild-type *Y. enterocolitica*. Moreover, adoptive transfer of *Yersinia*-specific antibodies from sera of mice immunized with the mutant WAP-314 *sodA* revealed that this protection could be mediated by *Yersinia*-specific immunoglobulins.

Live replicating bacteria are being considered as attractive antigen delivery vectors. A variety of attenuated *Salmonella typhimurium*, *Yersinia enterocolitica*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes* mutant strains have been evaluated as potential carrier vaccines to present heterologous antigens to the immune systems of vaccinated mice (1, 12, 14, 25, 33).

Despite the progress in the development of new bacterial live carrier vaccines, it has become increasingly clear that new strategies are needed. For example, instead of knocking out genes that result in auxotrophic mutations (e.g., Δ*aroA* or Δ*aroCD*) (9, 23, 48) or interference in global gene expression and regulation (e.g., Δ*phoP* or Δ*phoQ*) (16, 22), an attractive alternative might be to mutate genes that code for virulence-associated factors of bacteria, leading to newly designed vector strains with tissue tropism and restriction.

Y. enterocolitica causes enteritis and lymphadenitis in humans and rodents (17). In mice, yersiniae preferentially bind to M cells, thereby promoting bacterial uptake and transepithelial transport to the Peyer's patches. Both dissemination into the spleen and liver and further proliferation within these organs mark the initiation of a symptomatic infection. The virulence is controlled by chromosomally encoded (Inv, Ail, and the siderophore yersiniabactin) and plasmid-encoded (*Yersinia* outer proteins and *Yersinia* adhesin A) determinants (11). These virulence factors and the pathogenesis of *Y. enterocolitica* have been extensively studied (5, 19, 24, 38–40).

Y. enterocolitica has evolved a strategy to survive and multiply within the lymphoid tissue, predominantly extracellularly (27, 29, 44). This strategy might be an advantageous feature for a carrier vaccine strain. The extracellular location may help the host's immune system to eliminate the recombinant strain after a decent time interval post-oral immunization and thus prevent a chronic colonization.

In our laboratory, we have previously described three *Y. enterocolitica* O8 mutant strains (34, 35, 37): (i) the *yadA-2* mutant, obtained by substituting tyrosine residues for two histidine residues in the YadA protein, which is a plasmid-encoded surface protein that mediates binding to extracellular-matrix proteins, adherence to host cells, and resistance to complement lysis and is essential for virulence of yersiniae; (ii) the Mn-cofactored superoxide dismutase (*sodA*) mutant, which is deficient in resistance to exogenous oxygen radicals produced by phagocytes; and (iii) the *irp-1* mutant, lacking the 384.6-kDa high-molecular-weight protein 1, which is part of the siderophore yersiniabactin biosynthesis apparatus. The aim of this study was to assess the capacity of these three isogenic *Y. enterocolitica* O8 strains carrying mutations in virulence-associated genes to act as potential live oral vaccine candidates in mice.

The *Yersinia* strains used in this study and their construction were described previously (34, 35, 37). Strain WA-314 is a clinical isolate of *Y. enterocolitica* serotype O8 and bears the virulence plasmid pYVO8. This isolate was used as the parental strain for the construction of WA(pYVO8-A-2) and WA-314 *sodA*. Strain WA(pYVO8-A-2) was constructed by site-directed mutagenesis, resulting in the substitution of tyrosine residues for histidine-156 and histidine-159 of the YadA protein. In strain WA-314 *sodA*, the wild-type *sodA* gene has been replaced by *sodA*::Km. To construct WA *irp1*, the previously described cointegrant pRK290B8-5::pO8 was mobilized into the virulence plasmidless mutant WA-CS *irp1*::Kan^r (20).

The significance of the differences among control and experimental groups in all experiments was determined by the Student *t* test. *P* values of <0.05 were considered statistically significant.

Determination of the course of colonization and persistence in mouse tissues. The virulence of the *Y. enterocolitica* mutant

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strains was tested in the orogastric mouse infection model as described previously (37). Prior to infection of 6- to 8-week-old C57BL/6 mice, *Yersinia* stock suspensions were thawed and washed twice in sterile phosphate-buffered saline (PBS; pH 7.4). After appropriate dilution, bacteria were fed to groups of eight C57BL/6 mice by the use of a microliter pipette. The actual number of bacteria administered was determined by plating serial dilutions on Mueller-Hinton agar and counting CFU after incubation for 36 h at 27°C. Control mice were given an equal volume of sterile PBS. At various days postinfection (p.i.), mice were sacrificed. After aseptic removal of the organs, the Peyer's patches, spleen, and liver of each mouse were homogenized in 1, 5, and 5 ml, respectively, of sterile PBS containing 0.1% Tergitol TMN 10 (Fluka, Buchs, Switzerland) and 0.1% bovine serum albumin (E. Merck AG, Darmstadt, Germany) by the use of tissue homogenizers, whereas the small intestine was washed with 10 ml of ice-cold PBS.

The course of immunization was determined by counting the numbers of surviving bacteria, as CFU, in the lumen of the small intestine, the Peyer's patches, the spleen, and the liver on days 2, 5, 7, 12, and 21 postimmunization. The results are summarized in Fig. 1. Two days after orogastric immunization, the mutant strains and the wild-type strain colonized the small intestine and the Peyer's patches (Fig. 1A). The course of infection with WA-314 was progressive, with dissemination of the bacteria into the spleen (mean ± standard deviation, $5.7 \times 10^5 \pm 5.5 \times 10^5$ CFU) and the liver ($5.0 \times 10^5 \pm 5.1 \times 10^5$ CFU) by day 5 (Fig. 1B). At this time point, only the mutant strain WA(pYVO8-A-2) was detected in the spleen ($2.2 \times 10^2 \pm 0.9 \times 10^2$ CFU) and the liver ($2.4 \times 10^2 \pm 2.5 \times 10^2$ CFU), whereas no dissemination of WA-314 *sodA* and WA *irp1* into these organs was observed. In contrast, all three mutant strains profoundly colonized the gut and the Peyer's patches (Fig. 1B). On day 7 p.i., half of the mice infected with the wild-type *Y. enterocolitica* strain died due to the high bacterial load in the spleen and liver leading to a septic course of infection (Fig. 1C). In contrast, bacterial counts of WA(pYVO8-A-2) in the spleen ($3.1 \times 10^3 \pm 3.3 \times 10^3$ CFU) and liver ($1.8 \times 10^3 \pm 2.1 \times 10^3$ CFU) were more than 100 times lower than those of the wild-type strain. The mutant strain WA-314 *sodA* colonized both organs in smaller numbers (10 to 200 CFU per organ), whereas WA *irp1* could not be reisolated from the spleen or liver throughout the investigated period of time, although this strain was able to colonize the Peyer's patches ($1.7 \times 10^4 \pm 0.5 \times 10^4$ CFU on day 7). While all mice infected with wild-type strain WA-314 died between days 6 and 10 p.i., all mice immunized with mutant strains showed markedly reduced signs of illness and survived. By day 12 p.i., WA(pYVO8-A-2) and WA-314 *sodA* were eliminated from the spleens and livers of immunized mice (Fig. 1D). The latter strain was reisolated from Peyer's patches ($3.5 \times 10^3 \pm 3.5 \times 10^3$ CFU) and the small intestine ($3.4 \times 10^3 \pm 3.6 \times 10^3$ CFU) in numbers that were 10 times higher than those for WA(pYVO8-A-2) or WA *irp1*. Twenty-one days after oral inoculation of the mutant strains, only WA-314 *sodA* was still able to colonize the Peyer's patches, although it did so in small numbers (~40 CFU per organ). In addition, this mutant strain was reisolated from the small intestine at a 100-fold-higher concentration ($3.2 \times 10^3 \pm 3.3 \times 10^3$ CFU) than WA(pYVO8-A-2) or WA *irp1*. Thus, all three mutant strains were able to colonize the Peyer's patches of immunized C57BL/6 mice, to different degrees, for at least 2 weeks but were markedly impaired in their ability to disseminate into the spleen and liver compared to the fully virulent parental strain.

Antibody responses against *Yersinia* outer proteins. In the next set of experiments, it was investigated whether the *Yersinia* mutant strains were able to elicit humoral immune responses against *Yersinia* outer proteins, for which the acronym Yop is used. *Yersinia*-specific anti-Yop antibodies in sera of immunized mice were detected by a *Yersinia*-specific enzyme-linked immunosorbent assay (ELISA) as described previously (21, 28, 42). *Yersinia* outer proteins, at a concentration of 10 μg/ml in PBS, were used to coat 96-well microtiter plates (Greiner, Frickenhausen, Germany). Serial dilutions of sera from each of eight mice per group were carried out in PBS containing 0.5% Tween 20 (Merck, Darmstadt, Germany) and 2% bovine serum albumin BSA. Alkaline phosphatase-conjugated anti-mouse immunoglobulin G (IgG), IgA, and IgM (Sigma, Deisenhofen, Germany) were diluted 1:1,000 with PBS containing 0.5% Tween 20 and used as secondary antibodies. Disodium *p*-nitrophenylphosphate (Sigma) was used as the substrate. Optical densities were measured with an ELISA reader (Flow Laboratories, Meckenheim, Germany) at a wavelength of 405 nm. Five duplicates of sera from nonimmunized control mice were tested as negative controls to obtain cutoff values. The cutoff value in this study was defined as the mean absorbance of the negative controls plus 2 standard deviations.

Groups of five mice were immunized with a single oral dose of 10^8 organisms of one of the various strains, and blood samples were collected on days 7, 12, 23, 35, and 90 after immunization. The results are shown in Fig. 2. All mice immunized with the mutant strains showed the highest serum IgA and IgM antibody titers on day 12 p.i. and the highest serum IgG antibody titers on day 23 p.i. Thereafter, a continuous decline of the titers was observed. Over the course of 90 days, the mutant strains differed in the magnitude of *Yersinia*-specific IgG, IgA, and IgM responses elicited in sera of mice. On day 23, WA(pYVO8-A-2) elicited a 23-fold-higher titer (1:14,000) and WA-314 *sodA* elicited a 17-fold-higher titer (1:10,000) of *Yersinia*-specific serum IgG antibody than WA *irp1* (1:600) ($P < 0.05$) (Fig. 2). Mice immunized with WA-314 *sodA* elicited a 10-fold-higher titer (1:600) of serum IgG antibody than those given WA(pYVO8-A-2) (1:60) 90 days after the immunization ($P < 0.05$). At this time point, significant *Yersinia*-specific IgG titers were no longer detectable in sera from mice immunized with WA *irp1*.

Induction of *Yersinia*-specific splenic T cells. To investigate the abilities of the three mutant strains to elicit *Yersinia*-specific T-cell responses, mice were orally immunized with 10^8 yersiniae. Eight days after immunization, spleens were removed and single-cell suspensions were prepared as described previously (4). Purified T cells (26) were stained with a fluorescein isothiocyanate-coupled anti-CD3ε (145 2C11) (Becton Dickinson, Heidelberg, Germany) monoclonal antibody (MAb) and analyzed by flow cytometry (Epics XL-MCL; Coulter Electronics, Krefeld, Germany) as described previously (31). For proliferation assays, 2×10^5 purified T cells, 2×10^5 irradiated (3,000 rads) syngeneic splenic cells, as antigen-presenting cells, and 10 μg of antigen/ml were incubated in 96-well microtiter plates (Nunc, Wiesbaden, Germany) with 200 μl of culture medium per well (Click-RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 2 mM L-glutamine, HEPES, 5×10^{-5} M 2-mercaptoethanol, 10 μg of streptomycin/ml 100 U of penicillin/ml and 10% heat-inactivated fetal calf serum). The following antigens were used at a concentration of 10 μg/ml of culture medium: heat-killed whole bacterial cells of *Y. enterocolitica* O8 (4), recombinant purified *Yersinia* heat shock protein 60 (HSP60) (31), and recombinant purified *Yersinia* outer proteins (YopD, -E, -H, and V antigen) (36). After incubation for 3 days, the cultures were pulsed with [^{3}H]thymidine and the uptake of [^{3}H]thymidine was determined with a liquid scintillation counter (Pharmacia) (31). Proliferative responses were

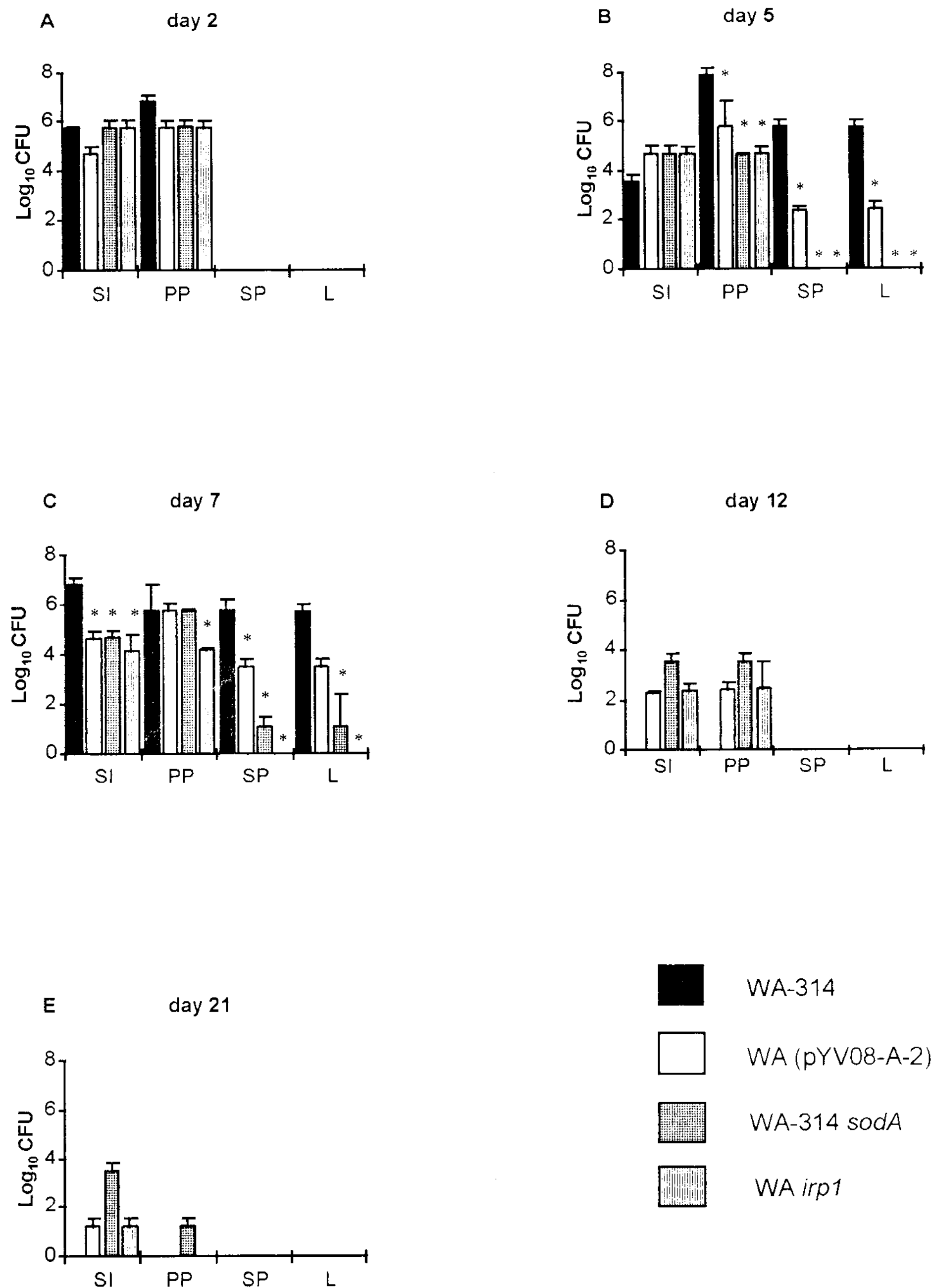

FIG. 1. Time course of colonization and persistence of *Y. enterocolitica* in the liver (L), spleen (SP), Peyer's patches (PP), and small intestine (SI). C57BL/6 mice were orally immunized with 10^8 *Y. enterocolitica* O8 mutant or isogenic wild-type organisms. Two, 5, 7, 12, and 21 days later, the mice were killed and the numbers of bacteria (CFU) present in the different mouse tissues were determined. Four of the eight mice immunized with *Yersinia* wild-type strain WA-314 succumbed on day 7 p.i., whereas the rest of the group died between days 8 and 10. Values are means for eight animals, with standard errors of the means indicated by error bars. *, value differs from that of mice infected with the *Yersinia* wild-type strain ($P < 0.05$).

expressed as stimulation indices (SI), which were calculated as follows: SI = [^{3}H]thymidine uptake (in counts per minute) in the presence of the indicated antigen/[^{3}H]thymidine uptake in the absence of that antigen. All experiments were repeated at least three times for verification.

The proliferation of T cells depended on the presence of antigen-presenting cells (APC) and antigen. Heat-killed *Y. enterocolitica*, *Yersinia* heat shock protein (HSP60), and recombinant *Yersinia* outer proteins (YopD, YopE, YopH, and V antigen) induced significant proliferative responses, as summarized in Fig. 3. Upon antigenic stimulation, T cells isolated from mice immunized with WA(pYVO8-A-2) or WA-314 *sodA* showed proliferative responses almost equivalent to those of T cells from mice infected with *Yersinia* wild-type strain WA-314. In contrast, T cells obtained from mice immunized with WA *irp1* exhibited significantly weaker proliferative

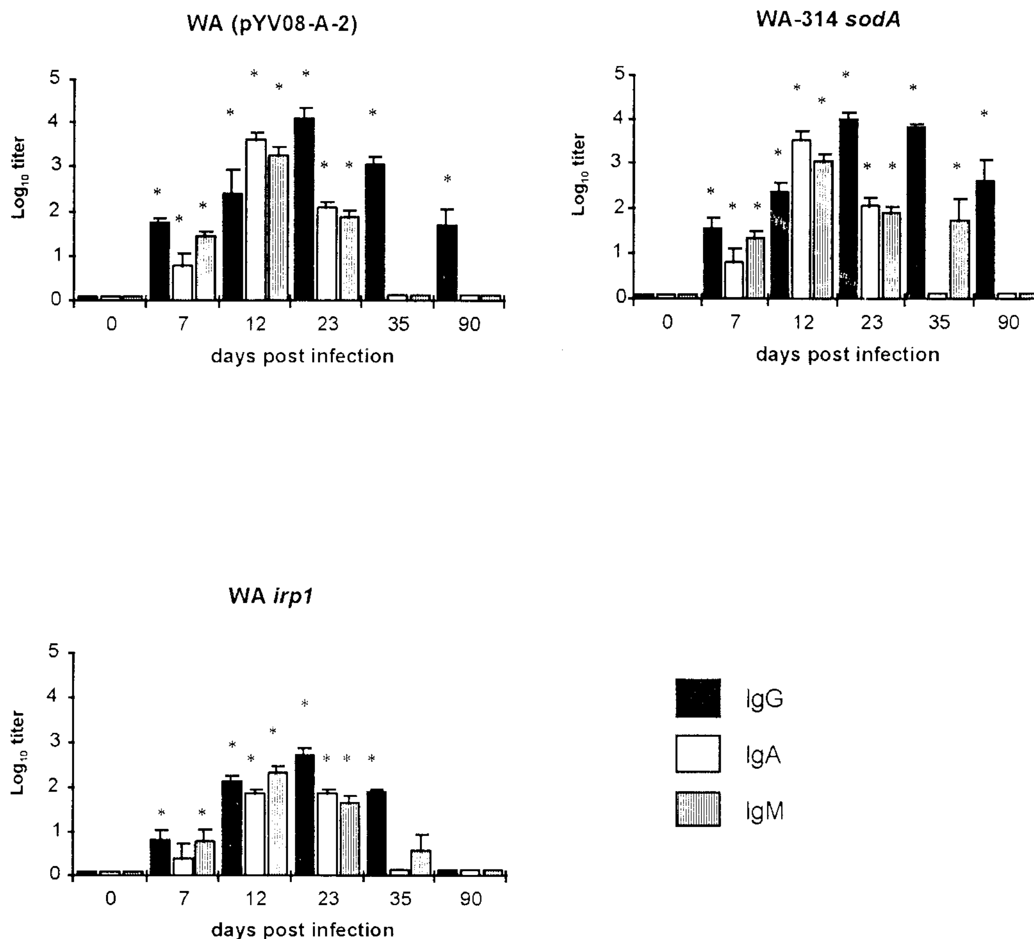

FIG. 2. Serum IgG, IgA, and IgM antibody responses of C57BL/6 mice prior to immunization (day 0) and 7, 12, 23, 35, and 90 days after oral immunization with 10^8 organisms of the indicated *Yersinia* mutant strains, as determined by using a *Yersinia* outer protein-specific ELISA. Columns represent means and standard deviations of results ($\log_{10}$ titer) obtained from eight mice. *, value differs from that of a control serum obtained prior to oral immunization ($P < 0.05$).

responses to the different antigens ($P < 0.05$) than T cells isolated from mice inoculated with either of the two other *Yersinia* mutant strains investigated in this study. Comparisons of proliferative responses to different *Yersinia* antigens revealed distinct patterns. T cells showed only a weak proliferative response to HSP60, YopE, and the V antigen (SI < 10), whereas stimulation with YopH induced a moderate proliferation (SI = 10 to 20). A strong T-cell proliferative response was observed upon stimulation with heat-killed *Y. enterocolitica* or YopD (SI = 25 to 45).

Cytokine production by *Yersinia*-specific T cells. For determination of cytokine production, the supernatants of T cells stimulated with heat-killed *Yersinia* organisms were collected and used in cytokine assays. Gamma interferon (IFN-γ) levels were determined by capture ELISA as described previously (2, 4). Briefly, ELISA microtiter plates were coated with anti-IFN-γ MAb (AN-18.17.24). Biotin-labeled anti-IFN-γ MAb (R4-6A2) and avidin-biotin-alkaline phosphatase complex (Vectastain ABC-AP kit; Camon Wiesbaden, Germany) were used, and the optical densities at wavelengths of 405 and 490 nm were determined with an ELISA reader. In parallel, an interleukin-4 (IL-4)-specific ELISA, including the anti-IL4 MAbs 11B11 (biotin labeled) and BVD6 24G2, was carried out as described for IFN-γ.

Determination of IFN-γ levels revealed that restimulated T cells from mice immunized with the mutant strains produced significant quantities of IFN-γ by day 8 after oral immunization compared to the control group of nonrestimulated T cells from immunized mice ($P < 0.05$) (Fig. 4). However, the amounts of IFN-γ produced by T cells from mice immunized with the mutant strains were lower than those of mice immunized with the wild-type *Y. enterocolitica* strain. In contrast, no significant quantities of IL-4 were detected after immunization with the wild-type or mutant strains (Fig. 4).

Protective immunity. Groups of eight mice were orally immunized with a single dose of 10^8 attenuated *Yersinia* mutant organisms. Ten weeks after this immunization, mice were challenged with 5×10^8 wild-type *Y. enterocolitica* WA-314 organisms (10 times the 50% lethal dose [LD_{50}]) by the oral route. To determine the extent of protection, the bacterial loads in the lumen of the small intestines, the Peyer's patches, the spleens, and the livers of infected animals were determined as described above.

In comparison to the control group of nonimmunized mice, no signs of illness were observed in mice immunized with the attenuated *Yersinia* strains. Five days after the oral challenge, mice were sacrificed and the colonization and persistence of wild-type *Y. enterocolitica* WA-314 in vivo was investigated

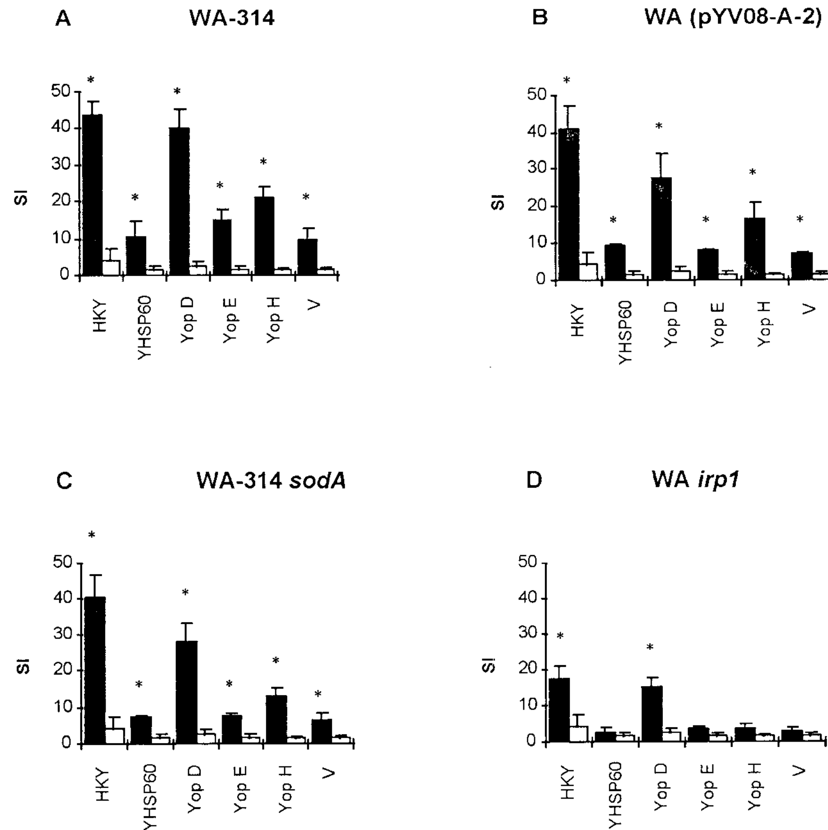

FIG. 3. Proliferative T-cell responses to various *Yersinia*-specific antigens after oral immunization with wild-type or mutant *Yersinia* strains. Splenic T cells were stimulated with 10 μg of heat-killed *Y. enterocolitica* (HKY), *Yersinia* heat shock protein (YHSP60), and recombinant *Yersinia* outer proteins (YopD, YopE, YopH, and the V antigen) or without *Yersinia* antigen. Proliferative responses are expressed as SI. Solid bars represent SI values of T cells stimulated with the indicated antigen, whereas open bars represent SI values of T cells exhibiting non-antigen-stimulated (spontaneous) proliferation. Values are the means of triplicate cultures, with standard deviations indicated by error bars. *, value differs from that of the control ($P < 0.05$).

(Table 1). Three of the eight mice in the nonimmunized group died due to the challenge on day 5. In the remaining five mice, wild-type yersiniae were present in large numbers in the lumen of the small intestine ($3.4 \times 10^5 \pm 1.7 \times 10^5$ CFU), the Peyer's patches ($1.8 \times 10^8 \pm 0.9 \times 10^6$ CFU), the spleen ($4.1 \times 10^5 \pm 0.8 \times 10^5$ CFU), and the liver ($5.9 \times 10^4 \pm 1.2 \times 10^4$ CFU). In contrast, the wild-type *Y. enterocolitica* strain was not isolated from the spleen or the liver of any immunized mouse. In addition, mice immunized with WA(pYVO8-A-2) or WA-314 *sodA* had 1,000- to 10,000-fold-lower bacterial counts in the small intestine and the Peyer's patches than nonimmunized mice ($P < 0.05$). In mice immunized with WA *irp1*, 100- to 1,000-fold-lower numbers of wild-type *Yersinia* were detected in the latter organs ($P < 0.05$).

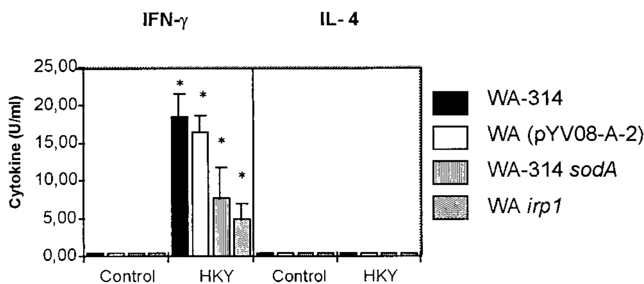

FIG. 4. IFN-γ and IL-4 production by splenic T cells of mice after oral immunization with wild-type or mutant *Y. enterocolitica* strains. T cells were stimulated with 10 μg of heat-killed *Y. enterocolitica* (HKY) per ml. Supernatants were used in an IFN-γ- and IL-4-specific ELISA. The optical density values revealed in the ELISA are expressed as units of IFN-γ and IL-4 per milliliter according to the linear portion of the standard curve. Results are the means ± standard deviations (error bars) of values for five animals. *, value differs from that of the control (nonstimulated T cells) ($P < 0.05$).

Adoptive transfer of *Yersinia*-specific antibodies. To determine whether *Yersinia* antiserum from mice orally immunized with the *Yersinia* WA-314 *sodA* mutant strain can mediate protection against an intravenous challenge with a lethal dose of wild-type *Y. enterocolitica*, adoptive-transfer experiments were carried out. Four weeks after oral inoculation of 10^8 WA-314 *sodA* organisms, hyperimmune sera from mice were collected. After ammonium sulfate precipitation, the immunoglobulins were dialyzed against PBS overnight at 4°C and the protein content was determined as described previously (49). C57BL/6 mice (eight per group) were each treated intravenously with 400 μg of a mouse immunoglobulin-rich fraction 1 day prior to an intravenous (i.v.) challenge with 10 times the LD_{50} of *Y. enterocolitica* O8 wild-type strain WA-314. Five and 11 days after the challenge, bacterial counts in spleens of mice were determined.

As demonstrated in Table 2, the group of mice treated with the WA-314 *sodA* antiserum showed a 100-fold-lower bacterial load in their spleens than the group of mice treated with the purified serum from naive mice ($P < 0.05$) 5 days after the

TABLE 1. Protection against wild-type *Y. enterocolitica* infection

Immunizing strain	No. of bacteria in tissues of mice 5 days after challenge[a]			
	Small intestine	Peyer's patches	Spleen	Liver
WA(pYVO8-A-2)	$(4.4 \pm 0.2) \times 10^2$*	$(3.0 \pm 1.4) \times 10^2$*	0	0
WA-314 *sodA*	$(1.9 \pm 0.6) \times 10^2$*	$(6.3 \pm 1.5) \times 10^2$*	0	0
WA *irp1*	$(8.9 \pm 1.2) \times 10^3$*	$(8.3 \pm 0.8) \times 10^3$*	0	0
Control[b]	$(3.4 \pm 1.7) \times 10^5$	$(1.8 \pm 0.9) \times 10^6$	$(4.1 \pm 0.8) \times 10^5$	$(5.9 \pm 1.2) \times 10^4$

[a] Groups of eight mice were orally immunized with 10^8 organisms of the indicated *Yersinia* mutant strain. Ten weeks after immunization, mice were orally challenged with 5×10^8 (10 times the LD_{50}) *Yersinia* wild-type strain (WA-314) organisms. Five days after the challenge, the bacterial loads in organs of the mice were determined. Values are means ± standard deviations of data for eight animals. Values that differ from the control values at $P < 0.05$ are marked with asterisks.
[b] Nonimmunized mice.

challenge. Moreover, in the former group, all mice survived the *Yersinia* infection and no bacteria were isolated from spleens on day 11 after the challenge, whereas all mice of the latter group died between days 6 and 9 p.i.

Y. enterocolitica has been recognized as a potential bacterial live carrier by several groups. Sory et al. made use of *Y. enterocolitica* strains to induce mucosal and serum antibody responses against the cholera toxin B subunit and the cytoplasmic CRA protein of *Trypanosoma cruzi* (46, 47). However, in those studies, the authors did not carry out the experiments with attenuated *Yersinia* strains but rather used *Y. enterocolitica* serotype O9 strains (biotype II), which are less virulent in mice (low-pathogenicity group) than those of serotype O8 (biotype I B; high-pathogenicity group). In contrast, Bowe et al. (7) and O'Gaora et al. (32, 33) constructed a highly attenuated *Y. enterocolitica* O8 *aroA* mutant strain which was found to persist in the Peyer's patches, mesenteric lymph nodes, spleen, and liver for only 3 days after oral infection. Consequently, mice immunized orally with a single dose of this mutant strain were not protected against a lethal wild-type infection. More recently, Dorrell et al. described a *Y. enterocolitica* O8 *ompR* mutant strain which did not cause a lethal course of infection in orally immunized BALB/c mice (13). Spleens and livers of infected mice were colonized with the mutant strain for 21 days. Mice orally immunized with a single dose of the O8 *ompR* mutant strain were partially protected against an oral challenge with the virulent *Y. enterocolitica* parent strain.

The *Yersinia* mutant strains investigated in this study were capable of translocating from the intestinal lumen to the Peyer's patches, where they persisted for at least 12 days. All three mutant strains were markedly impaired in their ability to disseminate from Peyer's patch tissue into the spleen and liver, resulting in survival of all infected mice. The *yadA-2* mutant strain WA(pYVO8-A-2) was reisolated from these organs on

TABLE 2. Adoptive transfer of *Yersinia*-specific antibodies

Source of serum[a]	No. of bacteria in the spleen[b]	
	Day 5	Day 11
WA-314 *sodA* mice	$(6.4 \pm 0.2) \times 10^5$*	0
Naive mice	$(4.0 \pm 0.2) \times 10^7$	—[c]

[a] C57BL/6 mice were each treated intravenously with 400 μg of purified serum from mice orally immunized with WA-314 *sodA* or from nonimmunized mice 1 day prior to intravenous challenge with 10^5 *Y. enterocolitica* O8 wild-type strain organisms (200 times the LD_{50}).
[b] Five and 11 days after the challenge, bacterial counts per spleen were determined. Values are means ± standard deviations of data for eight mice per group. Values that differ from the control group (naive mice) values at $P < 0.05$ are marked with asterisks.
[c] All C57BL/6 mice which were given serum from naive mice died between days 6 and 9 after the challenge.

days 5 and 7 p.i. 100 times less than the isogenic wild-type strain, whereas the mutant strain WA-314 *sodA* was detected in the spleen and liver only on day 7 p.i. even 1,000 times less than the wild-type *Yersinia* strain. Surprisingly, the mutant strain WA *irp1* did not colonize the latter organs at any time during the course of immunization.

A *yadA* null mutant of *Y. enterocolitica* is characterized by an impaired ability to colonize the intestinal mucosa (27). Previously, we showed that the substitution of tyrosine residues for two histidine residues in YadA resulted in abrogation of binding to various extracellular-matrix proteins (42, 43) and of adherence to HEp-2 cells, whereas autoagglutination (45) and serum complement resistance (10) remained unaffected. However, the collagen-binding function appeared to not be required for translocation of WA(pYVO8-A-2) from the gut lumen to the Peyer's patches.

The attenuation of *Y. enterocolitica* resulting from the mutation of the *sodA* gene is due to the additive effects of the reduced detoxification abilities of metabolically produced bacterial superoxide and of the increased susceptibility to killing by polymorphonuclear leukocytes (35). The *sodA* gene was found to be upregulated under conditions of aerobiosis and iron starvation in *Escherichia coli* (15). Such conditions are encountered by extracellular yersiniae in the spleen and liver. In contrast, *sodA* is known to be downregulated under anaerobic or microaerophilic conditions, such as in the gut or in abscesses of Peyer's patches during *Yersinia* infection (3, 17, 30). In fact, WA-314 *sodA* colonized the small intestine and the Peyer's patches for at least 3 weeks after the orogastric immunization, indicating that the *sodA* gene is not required for intraluminal growth.

Highly pathogenic *Y. enterocolitica* strains possess a chromosomal cluster of iron-regulated genes located in the high-pathogenicity island. This gene cluster carries genes for the biosynthesis and uptake of the *Yersinia* siderophore yersiniabactin (*irp1-9* and *fyuA*), which is a high-affinity ferric iron uptake system that significantly contributes to the virulence of yersiniae (8, 18). WA *irp1* was able to translocate from the intestinal lumen to the Peyer's patches, but its abilities to cause a systemic infection and to colonize the spleen and liver were totally impaired. As mentioned above, the evasion strategy of wild-type *Y. enterocolitica* in the host eventually results in extracellular survival and multiplication in the spleen and liver. Evidently, the siderophore yersiniabactin is essential for the ability of yersiniae to survive and multiply within these organs, whereas presumed accessory iron uptake systems in these organisms are sufficient to mediate survival during the first stage of colonization of the host.

The investigation of humoral and cellular immune responses to *Yersinia*-specific antigens elicited by the *Yersinia* mutant strains revealed that WA(pYVO8-A-2) and WA-314 *sodA*

were able to induce significantly higher IgG, IgA, and IgM antibody titers against *Yersinia* outer proteins than WA *irp1*. It is conceivable that the prolonged colonization of the spleen and liver by the former strains provoked a stronger humoral immune response. On the other hand, it appears that the higher bacterial load of WA-314 *sodA* in the Peyer's patches and the small intestine at 21 days p.i. contributed to the 10-fold-higher IgG antibody titer compared to WA(pYVO8-A-2) at 90 days p.i.

The mutant strains WA(pYVO8-A-2) and WA-314 *sodA* elicited stronger cellular immune responses against a variety of *Yersinia*-specific antigens than WA *irp1*. These data suggest that the transient and weak colonization of the spleens and livers of infected mice by attenuated *Yersinia* strains enhances *Yersinia*-specific antibody and T-cell responses. However, this is not an essential prerequisite of an effective *Yersinia* live oral carrier vaccine, because WA *irp1* was also able to induce significant humoral and cellular immune responses against *Yersinia*-specific antigens. On the other hand, we cannot exclude the possibility that much larger numbers of yersiniae eventually reached the spleen and liver but were rapidly killed and thus did not appear in the CFU counting assay.

It has been previously shown that T cells from C57BL/6 mice immunized with a sublethal dose of wild-type *Yersinia* produced significant levels of IFN-γ upon exposure to antigen (heat-killed *Y. enterocolitica*), while they did not produce IL-4 (2). T cells from mice immunized with the *Yersinia* mutant strains showed the same pattern of cytokine production. Thus, like wild-type *Y. enterocolitica*, the mutant strains induced pronounced Th1 responses. IFN-γ-producing Th1 cells are known to provide help for cell-mediated immune responses which are crucial for the defense from intracellular pathogens (6).

A single oral immunization of a mouse with any of the mutant strains resulted in full protection against a lethal wild-type *Yersinia* infection. Moreover, in experiments involving adoptive transfer of *Yersinia*-specific antibodies from sera of mice immunized with WA-314 *sodA*, we were able to demonstrate that this protection could be mediated by *Yersinia*-specific immunoglobulins. Thus, the high level of attenuation did not diminish the immunogenic properties of the mutant strains.

The mutant *Yersinia* strains investigated in this study elicited pronounced humoral and cellular immune responses against *Yersinia* outer proteins, which are effector proteins of *Yersinia*'s type III secretion system. Recently, Rüssmann et al. showed that the delivery of viral epitopes through the *S. typhimurium* type III secretion system resulted in efficient stimulation of MHC class I-restricted protective antiviral immune responses in vaccinated mice (41). The use of *Yersinia* outer proteins as carriers for heterologous antigens in attenuated *Yersinia* strains may be an attractive strategy to stimulate both humoral and cellular immune responses.

We have shown, based on designed mutations of virulence-associated genes, that rationally attenuated *Y. enterocolitica* O8 strains have a great potential to serve as safe and effective live oral carrier vaccines for the delivery of heterologous antigens in future studies.

E.I.I. and H.R. contributed equally to this work.

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