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Abstract

We evaluated the possibility of using *Yersinia pseudotuberculosis* as a live vaccine against plague because it shares high genetic identity with *Y. pestis* while being much less virulent, genetically much more stable, and deliverable orally. Forty-one *Y. pseudottuberculosis* strains were screened by PCR for the absence of the High Pathogenicity Island, the superantigens YPM and the type IV pilus and the presence of the pYV virulence plasmid. One strain (IP32680) fulfilled these criteria. This strain was avirulent in mice upon intragastric or subcutaneous inoculation, and persisted for two months in the mouse intestine without clinical signs of disease. IP32680 reached mesenteric lymph nodes, spleen and liver, without causing major histological lesions and was cleared after 13 days. The antibodies produced in vaccinated animals recognized both *Y. pseudotuberculosis* and *Y. pestis* antigens efficiently. After an sc challenge with *Y. pestis* CO92, bacteria were found in low amounts in the organs and unfrequently in the blood of vaccinated animals. One oral IP32680 inoculation protected 75% of mice and two inoculations induced much higher antibody titers and protected 88% of mice. Our results thus validate the concept that an attenuated *Y. pseudotuberculosis* strain can be an efficient, cheap, safe and easy-to-produce live vaccine for oral immunization against bubonic plague.

Introduction

Yersinia pestis, the etiologic agent of bubonic and pneumonic plague, is a Gram-negative bacterium with an extraordinary pathogenicity. Transmitted by infected fleas, the bacteria cause a hyperplasia of the draining lymph node (bubo), followed by septicemia and the patient's death within one week if an effective antibiotherapy is not administered on time. Although the bubonic form of the disease is by far the most frequent in the world (45), a primary bubonic infection sometimes develop into a secondary pneumonic infection highly contagious from man to man via the generation of aerosols. This form of the disease is almost systematically fatal within 3 days and there is a concern that *Y. pestis* could be used this way as a weapon in bioterrorism (20). Additionally and of great public-health concern was the recent identification of a *Y. pestis* strain naturally resistant to eight antibiotics, including those recommended for plague treatment and prophylaxis (16). The widespread diffusion of the transmissible plasmid conferring this resistance suggests that other resistant strains are very likely to appear (43). Against such multidrug resistance in *Y. pestis*, one of the only alternatives would be mass vaccination, but no safe and efficient vaccine against plague is currently available.

The live attenuated *Y. pestis* strain EV76 efficiently protected against bubonic plague and induced high titers of antibodies, but did not confer long-lasting immunity and induced mild to severe side reactions (27). Furthermore, the immunogenicity and virulence of the EV76 vaccine preparations used in different countries were found to be highly variable, most likely because of the genetic drift of the bacteria used (27, 51). Indeed, a high genomic plasticity is observed in *Y. pestis* (5, 30) and results from frequent chromosomal rearrangements between the numerous copies of

insertion sequences (IS) present in its genome. Killed whole cell *Y. pestis* vaccines has been produced previously in the USA (USP) and in Australia (CSL). They are also known to be reactogenic in humans and induce a shorter and less effective protection than EV76 (27). The USP killed vaccine was recently discontinued in the USA.

A recent effort has been made to develop new recombinant subunit vaccines against human plague. Most of these subunit vaccines use *Y. pestis*-specific capsular antigen F1, and/or the low calcium response antigen V (LcrV) that is present in the three pathogenic *Yersinia* species. Such vaccines were found to efficiently protect mice against bubonic and pneumonic plague and are well-tolerated in humans (3, 4, 18, 23, 37, 41, 47, 48). However, vaccines composed of a limited number of antigens may not be able to protect against F1-negative strains (49) or strains harboring LcrV variants (5). The technical expertise required for the production of recombinant vaccines is also an obstacle for developing countries, and injectable vaccines always raise the problem of contamination via used syringes.

Live vaccines offer several advantages over recombinant vaccines. Their high antigenic complexity guarantees a response against a broad range of antigenic targets. In live vaccines, antigens are present in their native forms with normal glycosylations, they are produced *de novo* as long as the bacteria persist, which provides a prolonged stimulation of the immune system, and there is no need for an adjuvant since bacterial antigens (LPS and other pathogen–associated signatures) naturally stimulate the innate immune system. They generally induce both antibody production and a cell-mediated response, and once developed and validated, these vaccines can be produced locally, allowing more economically feasible mass production.

82 *Yersinia pseudotuberculosis* is the recent ancestor of *Y. pestis* (1) but is much less virulent
83 and usually causes enteric diseases that are rarely fatal. The two species are very close with more
84 than 95% genetic identity and virulence plasmids that share a conserved colinear backbone (8).
85 The genome of *Y. pseudotuberculosis* is however much more stable than that of *Y. pestis* because
86 it contains fewer IS copies (5, 8, 51). We report that oral inoculation with a *Y. pseudotuberculosis*
87 strain, selected for its very low virulence, induces an efficient immunity against bubonic plague
88 without causing adverse reactions. This demonstrates that a live attenuated *Y. pseudotuberculosis*
89 can be a promising vaccine against bubonic plague.

Materials and methods

Yersinia strains and culture media.

The *Y. pseudotuberculosis* strains used in this study are listed in Table 1. The fully virulent *Y. pestis* strain CO92 whose genome has been sequenced (30) was used for animal challenge and the attenuated strain EV76 (27) was used as a control vaccine. Bacteria were usually grown at 28°C on Luria-Bertani agar plates supplemented with 0.2% hemin (LBH) for 48h before use. A selective medium containing Irgasan (0.1 g/µl: LBHI) was defined to quantify yersiniae in ex-vivo samples. For inoculation in mice, bacteria grown at 28°C for 48h on LBH plates were collected and resuspended in sterile saline for immediate use. Bacterial concentrations were evaluated by spectrometry at 600nm and confirmed by cfu counts on LBH plates.

PCR.

DNA was obtained by thermolysis of bacteria in a solution containing 50 mM NaOH and 0.25% SDS. The primers (Proligo France) amplified the *int* gene of the High Pathogenicity Island (HPI, (24), the *yopM* (33) and *yopK* (forward 5'-CTGCTCATTTACTGAGAACG-3', reverse: 5'-CTATAAGTAGAGAGTTTTTCGG-3'), genes of the pYV virulence plasmid, the *pilR* and *pilN* genes of the type IV pilus locus (10) and the *ypmA/C* or *ypmB* genes coding for YPM superantigens (15). The house-keeping gene *ureB* encoding an urease subunit was used as an internal positive control for the PCR reactions. PCR amplification reaction mixtures contained 1 unit of AmpliTaq polymerase (Applied Biosystems), 10mM dNTPs, and 10µM of each primer. A PCR program working for all gene fragments was defined, involving one step at 95°C for 5 min, followed by 30 cycles of amplification consisting in 3 steps each: (i) 94°C for 30 s, (ii) 55°C for

30 s, and (iii) 72°C for 90 s. Strains used as positive controls for the various PCR primers were: IP32953 for *int*, *yopM* and *yopK*, IP33053 for *pilN* and *pilR*, IP32887 and IP31411 for *ypmA/C*. These strains were taken from the National Reference Center strain collection except strain 1093 positive for *ypmB*, a kind gift of Prof. M. Simonet, Institut Pasteur of Lille. The amplification reactions were done in a PTC-100 thermocycler (Bio-Rad).

***In vivo* analyses**.

Female OF1 mice, 6 to 8 weeks old, were purchased from Charles River (L'Arbresle, France). All animal care and experimentations were conducted according to the guidelines of the Institut Pasteur. Mouse infections with virulent bacteria were performed in a BSL3 animal facility. Inoculation of bacteria via the intragastric (ig) route (200 µl in saline) was done with a curved feeding needle and subcutaneous (sc) inoculations (100 µl) were performed in the abdominal skin. Mice were not fasted before intragastric inoculation. Mortality, body weight, behavior and fur appearance were recorded daily for 3 weeks.

To follow the kinetics of bacterial dissemination *in vivo*, groups of three mice per time point were humanely euthanized after one or two ig inoculations. Various organs (Peyer's patches, mesenteric lymph nodes, liver, spleen), as well as feces (two fecal pellets taken in the large intestine) were collected aseptically and homogenized in sterile PBS, using 3 mm glass beads and an electric mixer mill (Retsch, Germany). Serial dilutions of the homogenates were plated on LBH plates for counting.

For histological analysis, organs were fixed with buffered formaldehyde for 48h, embedded in paraffin, cut in 5 micrometers sections, and stained with hematoxylin–eosin or Giemsa stain. The severity of lesions caused to tissues by *Y. pseudotuberculosis* strains were observed on blinded

histological sections and quantified using a newly defined lesions score ranging from 0 to 10. 0: no inflammation; 1: minimal inflammation (congestion, microhemorrhages); 2: less than 2 abcesses in a given organ; 3: from 2 to 5 microabcesses; 4: from 5 to 10 microabcesses; 5: from 10 to 20 microabcesses; 6: from 20 to 50 microabcesses; 7: confluent intratissular necrosis zone; 8: organ destruction below 50%; 9: organ destruction from 50 to 80%; 10: total destruction and very abundant polymorphonuclear cells (PMN). At least 10 fields per slide were read on 2 slides per mouse.

Vaccination using the IP32680 strain consisted of 1 intragastric inoculation with $2x10^9$ cfu in saline or 2 inoculations at 30 days interval. Vaccination using the *Y. pestis* EV76 strain consisted of a single subcutaneous (sc) injection of 10^7 cfu as described by Flashner (14).

To obtain immune sera, mice were anesthetized and blood was collected from the retro-orbital vein. To analyze IgA in intestinal lavages, mice were humanely euthanized and the gut section extending from the stomach to the caecum was flushed with 10 ml cold PBS containing protease inhibitors (*Complete®,* Roche). After centrifugation, the supernatant was filtered and frozen until use.

Immuno-assays

To produce the *Y. pseudotuberculosis* or *Y. pestis* antigenic solutions used for coating in the ELISA assays, either strains IP32680 or CO92 was grown at 37°C on LB Agar for 48h, centrifuged, resuspended in cold PBS containing protease inhibitors and sonicated (Branson sonicator). Centrifuged supernatant was sterilized by filtration (0.22 μm), and its protein content was measured using a Bradford assay (Bio-Rad). ELISA plates (NUNC) were coated overnight

157 with the antigenic solution (5 μg/ml) in PBS. The recombinant LcrV fusion protein for LcrV-
158 specific ELISA was produced by fusion of the *Y. pestis* CO92 *lcrV* gene with the *Schistosoma*
159 *japonicum* Glutathione-S-Transferase (GST) gene (39). Briefly, the *lcrV* gene amplified by PCR
160 was cloned in frame with the GST gene into the pGEX B expression vector under control of the
161 lactose promoter. *E. coli* strain BL21 was transformed with the vector and expression of the
162 recombinant protein was induced by addition of IPTG (Sigma). The recombinant protein was
163 purified from the *E. coli* bacterial lysate using Glutathione-agarose beads (Sigma). Serum and
164 intestinal lavage samples were serially diluted in PBS containing 0.1% BSA for determination of
165 Ab titers by ELISA. Secondary rat antibodies directed against mouse IgG, IgG1, or IgG2a were
166 from Becton-Dickinson Pharmingen. Secondary goat antibodies directed against mouse IgG2b,
167 IgG3 or IgA were from Caltag Laboratories. All secondary antibodies were coupled to
168 horseradish peroxydase (HRPO) used with TMB substrate (OptiEIA, BD Pharmingen). Titers
169 were determined by reading the sample dilution giving half of the maximal signal on the titration
170 curve (generally OD_{450} = 1). The risk that the very low IgA titers obtained could result from a
171 defect of the HRPO-coupled anti-IgA 2^{nd} antibody was excluded by demonstrating that this
172 reagent works in an ELISA designed to quantify antigen-specific IgA in bronchoalveolar lavages
173 of *Bacillus anthracis* – vaccinated mice (data not shown; courtesy of Dr P. Goossens, Institut
174 Pasteur).

175

176 **Evaluation of mouse protection against a challenge with *Y. pestis*.**

177 The fully virulent *Y. pestis* strain CO92 was grown at 28°C and a suspension in saline containing
178 1000 cfu (≥100 times the LD_{50}) was injected sc in the *linea alba* of IP32680-vaccinated and non-
179 vaccinated mice 30 days after the last vaccine dose. Animal survival was followed for 21 days. *Y.*

pestis dissemination in the body and septicaemia were assessed two days after the challenge with CO92 by homogenizing various organs and plating serial dilutions of organ samples or blood.

Statistical analysis

Spearman's rank test was used to evaluate the correlation between antibody titers against *Y. pestis* and *Y. pseudotuberculosis*. Fisher's exact test was used to evaluate the protection in mice and Student's t test to compare bacterial loads in challenged mice groups.

Results

Selection of avirulent *Y. pseudotuberculosis* strains.

Our criteria to select a *Y. pseudotuberculosis* strain suitable for use as a vaccine were that this strain should be: (i) avirulent, (ii) able to persist for at least one week in the intestine without causing severe lesions, and (iii) capable of inducing an adaptative immune response. Forty-one *Y. pseudotuberculosis* strains of serotype II (Table 1), known to lack the HPI (12), were tested for the presence of major virulence factors. Absence of the HPI was confirmed by PCR amplification of the HPI-borne gene *int* (24). Absence of other known virulence factors such as the superantigens YPM A/B/C (15), the type IV pilus encoded by the *Yersinia* Adhesion Pathogenicity Island (YAPI, (9)) and presence of the pYV virulence plasmid was checked by PCR. Strains had to possess the pYV plasmid to be selected because this element is essential for the bacterial persistence in the host and encodes antigens of interest for vaccination (41). Among 41 strains tested by PCR (Table 1), seven (listed in Table 2) met our criteria.

To eliminate virulent isolates, high doses of bacteria were inoculated sc (10^9 cfu) or ig ($2x10^9$ cfu) to groups of 5 mice. Strains IP32680 and IP32721 were the only isolates completely avirulent at these doses (Table 2).

Persistence of these two strains in the intestine of infected mice was checked at different time points (days 1, 2, 3, 6, 13) after inoculation. The fully virulent *Y. pseudotuberculosis* strain IP32953 (8) was used as positive control. While IP32721 was rapidly cleared from the intestinal tract (data not shown), IP32680 was able to persist over the 13 day survey period in the feces of infected mice (Fig. 1A) and was detectable for up to 2 months in some mice (data not shown).

Because of its avirulence and prolonged persistence, strain IP32680 was selected for further analyses.

Infectivity of strain IP32680 for the mouse.

In a first set of experiments, the infectivity of strain IP32680 given orally was analyzed by comparison with the highly virulent IP32953 strain that was given at the same dose. Since the lethal dose 50% (LD_{50}) of IP32953 via the ig route is $1.6x10^7$ cfu, a dose of 10^8 cfu was injected to induce high bacterial loads and lesions in the organs without immediately killing all mice. Mice that received IP32680 ig did not develop any clinical signs of infection (normal fur and behavior, no body weight loss) up to day 60, as opposed to those infected with IP32953 (Fig. 2) which were sick and often died.

To quantify tissue colonization, groups of mice infected ig with IP32680 or IP32953 were sacrificed at various time points post-infection and the bacterial content of their Peyer's patches, mesenteric lymph nodes, liver and spleen was measured. IP32680 was present and persisted in the Peyer's patches and mesenteric lymph nodes longer than the two-week survey period (Fig. 1B), showing bacterial colonization of these tissues. In these intestinal lymphoid tissues, the number of IP32680 cfu at the initial phase of infection (first week) was lower than that of IP32953, though the number of cfu became similar at later stages (but only survivors of IP32953 infection could then be examined). In contrast, IP32680 invaded deep organs much less efficiently than IP32953 (Fig. 1C). Bacterial counts of both strains reached a peak between days 3 and 6, yet the number of IP32680 cfu in the spleen and liver of infected animals was 100 to 1000 times lower than that of IP32953. Furthermore, the spleen and liver of the animals infected with IP32680 were cleared of

the infection at day 15, while the spleen of the mice surviving infection with IP32953 still contained live bacteria (Fig. 1C).

Histological analysis showed a massive necrosis at day 6 (peak of infection) in the Peyer's patches of IP32953 infected mice whereas only some marginal polymorphonuclear cells were present in those infected with IP32680 (Fig. 3A). Similarly, IP32953induced numerous abscesses and abundant inflammatory polymorphonuclear infiltration in the spleen and liver whereas IP32680 caused only rare micro-abcesses (Fig. 3A). To quantify the extent of organ lesions, samples from six mice per group were assessed using a numeric scoring system. The mean scores indicated that lesions caused by IP32680 were very mild (Fig. 3B) whereas, as a comparison, mouse infection with 10 times less IP32953 resulted in much higher scores in surviving animals (Fig. 3B).

In a second set of experiments, the infectivity of IP32680 was evaluated using a vaccination protocol (two ig inoculations of $2x10^9$ cfu of IP32680 at a 30 day interval). As previously observed, mice did not show signs of stress and did not loose weight (data not shown). Similarly, upon the first inoculation, IP32680 colonized the gut and reached the deep organs (Fig. 1D). After the second inoculation, colonization of the gut and Peyers' patches was similar to that observed after the first inoculation. Bacteria were however recovered in lower numbers from the mesenteric lymph nodes and the liver, and were absent from the spleen. Lesions generated after a second inoculation of IP32680 (Fig. 3B) were significantly milder in the liver ($p=0.02$) and not significantly different in the other organs tested. At least during the 60 day observation period, the bacteria could be found in feces, showing that mice develop a carrier state.

Antibody response elicited by IP32680 against *Y. pseudotuberculosis* and *Y. pestis*

252 The capacity of strain IP32680 to induce an antibody response after ig administration either once
253 or twice at a 30 day interval (prime/boost strategy; $2x10^9$ cfu per dose) was evaluated. Blood was
254 collected 30 days after the last inoculation and serum antibody titers against *Y.*
255 *pseudotuberculosis* antigens were analyzed by ELISA. After a single inoculation of IP32680 (Fig.
256 4A), moderate titers ($< 10^3$) of IgG were detectable. The second IP32680 inoculation induced a
257 marked increase (10 times) of these antibody titers. The IgG2a and IgG2b isotypes predominated
258 after both the first and the second inoculations, suggestive of a type 1 immune response (13).
259 Immunization via the intestinal route was also expected to induce a mucosal type of immune
260 response, characterized by high amounts of IgA released in mucosal secretions and subsequently
261 by the presence of IgA in blood. Unexpectedly, *Y. pseudotuberculosis*-specific IgA production
262 was low (titers < 50) in both the intestinal secretions and the serum of mice infected once with
263 IP32680. The IgA titers increased moderately (titers $\leq$ 100) in the double-inoculation regimen
264 (data not shown).

265 Since the pYV-encoded LcrV antigen has previously been shown to induce an immune response
266 and a protection against plague (3, 41), we examined whether antibodies were raised against LcrV
267 in sera of mice vaccinated with IP32680 given once. IgG titers varied over a large range from
268 animal to animal, with values from more than 8000 to undetectable (data not shown). Because the
269 number of vaccinated animals dying upon plague challenge was low, it was not possible to
270 analyze statistically the relationship between the anti-LcrV titer and survival. However, the fact
271 that several mice, in which very low anti-LcrV titers were recorded, survived the *Y. pestis*
272 challenge, suggests that anti-LcrV IgG might not be an essential component of the protective
273 immune response raised, in agreement with the high number of possible antigenic targets present
274 on live bacteria.

The antibodies elicited by IP32680 also strongly recognized *Y. pestis* antigens (Figure 4B), and titers directed against the two species were closely correlated (Spearman's Rho = 0.94; p = 0.0002), confirming the extensive antigenic identity between *Y. pestis* and *Y. pseudotuberculosis*.

Protection conferred by IP32680 against bubonic plague

Mice vaccinated orally once or twice at a 30 day interval with IP32680 were exposed to a sc lethal challenge (1,500 cfu = 150 LD_{50}) with the fully virulent *Y. pestis* strain CO92 30 days after the last immunization. Negative controls included groups of unvaccinated mice and positive controls groups of mice vaccinated with the EV76 live vaccine (14, 27). While all unvaccinated mice died, 75% of animals vaccinated with a single inoculation of IP32680 survived ($p = 0.007$). The protection conferred by two inoculations of IP32680 was superior (88% survival, $p \leq 0.001$; Fig. 5A), although not statistically different from the protection conferred by one inoculation. By contrast, the protection conferred by the conventional EV76 vaccine strain was limited, with a mortality of 50% (Fig. 5A).

Y. pestis CO92 bacteria were found in high amounts in the liver, spleen, inguinal lymph nodes and blood of the unvaccinated mice at day 2 post injection (Fig. 5B) and the animals started to die at day 4. In contrast, *Y. pestis* was only rarely found in the organs and blood of IP32680 vaccinated mice, and in most of these animals there was a complete absence of bacteria in the organs (Fig. 5B). The difference in bacterial loads was statistically highly significant ($p \leq 0.00003$) in all organs tested.

Therefore, ig inoculation of IP32680 conferred a protection against plague superior to that obtained with the conventional *Y. pestis* strain EV76, and two inoculations further increased this protection.

297 **Discussion**

298 This study demonstrates that vaccination against bubonic plague can be obtained using an
299 attenuated *Y. pseudotuberculosis* strain given orally. A live *Y. pseudotuberculosis* could thus be
300 an interesting alternative to the live attenuated *Y. pestis* strain EV76, which causes severe side-
301 effects (27) and is genetically unstable. Vaccination with IP32680 given once or twice did not
302 cause any visible symptoms in the vaccinated animals, and protection against bubonic plague
303 induced by one (75%) or two (88%) inoculations of IP32680 (88%) was higher than that induced
304 by EV76 (50%). An avirulent *Y. pseudotuberculosis* is therefore an interesting alternative to the
305 use of a live attenuated *Y. pestis,* such as EV76, against bubonic plague.
306
307 Two ways of producing live vaccines have historically been employed: attenuation of the original
308 pathogen (for example development of the BCG tuberculosis vaccine) or selection of a natural
309 avirulent strain that is closely related to the pathogen (for example the *vaccinia* strain used in
310 Smallpox vaccines). The option of attenuating *Y. pestis* by genetic engineering has been used by
311 several laboratories, for instance by deletion of the *yopH* or *pcm* genes (6, 14), or by addition of
312 an *E. coli lpxL* gene to modify the LPS (28). All of these mutations led to a strong attenuation of
313 virulence and a good protection against bubonic plague. Still, the use of *Y. pestis* presents a
314 number of drawbacks. The major one is its genome instability (5, 30) caused by the high number
315 of IS present in its genome, which are known to favor genetic rearrangements. *Y.*
316 *pseudotuberculosis* has a much more stable genome due to its small number of IS (5, 8, 51).
317 Finally, a *Y. pseudotuberculosis* live attenuated vaccine would be less problematic than a *Y. pestis*
318 if accidentally released in the environment.
319

The main pitfall with a live vaccine is its ability to cause adverse reactions. IP32680 did not cause any visible clinical symptoms in the vaccinated mice. According to the concept of "danger" proposed by Matzinger (26), self or commensal material is tolerated because it does not cause inflammation, and vaccines must be able to trigger a certain level of inflammation in order for an immune response to start. The fact that IP32680 was able to colonize deep organs, to persist in situ for some weeks and to induce mild lesions was most likely a key factor in the development of an effective immune response. Whereas an adjuvant is added to particulate vaccines to induce this inflammation, bacterial signatures like LPS are likely to play this adjuvant role in our live vaccine.

Immunity against bubonic plague has previously been obtained in the mouse by sc inoculation of live *Yersinia enterocolitica* serotype O3 (2), which infects humans but is not very virulent in the mouse. *Y. pseudotuberculosis* is genetically closer to *Y. pestis* than *Y. enterocolitica* (1, 8), therefore, the immune response raised against *Y. pseudotuberculosis* is expected to be more efficient against *Y. pestis* than that induced by *Y. enterocolitica*. Vaccination using live virulent *Y. pseudotuberculosis* has been reported in the '50s by Wake (42) who vaccinated mice with a strain of *Y. pseudotuberculosis* (10^2 cfu) delivered via the sc route. The weaker protection obtained against bubonic plague (50%) was possibly due to the low dose of bacteria given, imposed by the virulence of the strain. Very recently, efficient protection against bubonic plague was also obtained using a *dam* mutant of *Y. pseudotuberculosis* IP32953 (clone C18) delivered orally (40). The isolate used was cured of the virulence plasmid pYV because the *dam* mutation resulted in a high rate of loss of the pYV plasmid, indicating that protective immunity can be raised against chromosome-encoded targets. Their observation that the pYV plasmid is not required is

intriguing because pYV-encoded YopE and YopH toxins are required for survival in the intestinal tissue (25). However it is possible that the HPI present in the *dam*-mutated pYV-cured C18 clone (and not in IP32680) -or other virulence factors to be identified- confers this ability by a different mechanism. Previously, Simonet et al. had reported that intravenous injection of a *Y. pseudotuberculosis* strain, attenuated by loss of pYV, provided a 50% protection against bubonic plague (36).

Attenuated live bacterial vaccines mimic natural infection and are expected to provide sustained exposure to target antigens, resulting in a stronger immune response. The vaccinia vaccine against smallpox and the BCG against tuberculosis are the most famous of attenuated vaccines, and other such vaccines recently evaluated in humans include *Salmonella*, *Shigella, Vibrio cholerae* and *Listeria* (22). As compared to subunit vaccines of limited antigenic complexity like those using the LcrV and F1 antigens, a live *Yersinia* is expected to elicit an immune response against a wide array of antigens, thus conferring protection against various natural or genetically modified variants of *Y. pestis.* For example the LcrV antigen contains a region whose hypervariability may allow it to escape the immune response (35). *Y. pestis* strains lacking the F1 antigen have a similar virulence as F1-positive strains (44) and would escape an anti-F1 immune recognition. The risk that a mutated strain bypasses the immunity stimulated by a live vaccine is, on the contrary, very unlikely due to the high number of alternative targets. Protection induced by IP32680 can be ascribed to the efficient recognition of *Y. pestis* antigens, in agreement with genetic identity between *Y. pestis* and its ancestor.

An attractive rationale for choosing oral inoculation is the avoidance of syringes which are a major source of disease transmission, a problem frequently mentioned by WHO in its vaccination guidelines (46). Oral vaccination with *Y. pseudotuberculosis* takes advantage of its capacity to colonize and persist in the gut for at least a few weeks, exerting a prolonged stimulation of the immune system. This local stimulation of mucosal immunity was expected to result in a protection against infection starting at other mucosal surfaces (19), and thus to potentially protect against pneumonic plague. However, our preliminary experiments of *Y. pestis* infection by aerosols in mice inoculated twice with IP32680 revealed a poor protection against pneumonic plague. Only 30% of the animals survived a nose-only exposure to aerosolized CO92 (8000 cfu found in lungs 1 h after exposure; data not shown). The low IgA production observed in vaccinated mice may contribute to explain the low mucosal protection against pneumonic plague. Little information regarding IgA production in response to *Y. pseudotuberculosis* infection in the mouse is available. Whereas mouse immunization using an oral *Salmonella* –F1/V vaccine (29) as well as several active "probiotic" or commensal bacteria succeeded in inducing IgA production in the gut (11), intestinal infection with virulent *Y. enterocolitica* O:8 failed to induce IgA production in either BALB/C or C57BL/6 mice (7). This low IgA response to live yersiniae could result from the fact that pathogenic yersiniae use their type three secretion system (TTSS) to induce apoptosis of antigen-presenting cells in Peyers' Patches, as reported for macrophages (reviewed by Zhang; (50). Whereas the fast elimination of IP32680 from the spleen after the second inoculation may be ascribed to a successful systemic immune response, the low IgA response in the gut may explain how the bacteria persist for several weeks in the gut as detected in feces, resulting in a "carrier" state. It also suggests that the immune response induced by IP32680 did not occur in the Peyer's Patches, and thus was mainly systemic and not mucosal.

388

389 The serum immunoglobulin isotype profile (dominance of IgG2a & b) found in mice vaccinated
390 with IP32680 suggest that the immune response was oriented toward the Th1 type (13). This type
391 of response is characterized by the production of cytokines such as IFNγ and TNFα which support
392 phagocyte activation, an event favorable to the capture and destruction of bacteria. Peyer's Patches
393 rather favor the development of a Th2 or regulatory T cells of response (21). Lymphocytes and
394 antibodies most likely work together to eliminate bacteria by providing help to phagocytes (38)
395 because transfer of either lymphocytes (31) or antibodies (17) from immunized mice can protect
396 against pneumonic plague. The protection against bubonic but not pneumonic plague reported
397 here may therefore reflect the presence of IgG and the lack of IgA to collaborate with cells (32).

398

399 This work demonstrates the potential of using a live attenuated strain of *Y. pseudotuberculosis* as
400 an oral vaccine against bubonic plague. Bubonic plague is by far the most frequent form of the
401 disease and naturally occuring pneumonic plague usually starts from a primary bubonic plague
402 case (34, 45). Vaccination against bubonic plague should thus reduce the frequency of both forms
403 of the disease in endemic countries. Strain IP32680 cannot be used for human vaccination
404 because the causes of its attenuation are not known and the possibility of its reversion to full
405 pathogenicity cannot be excluded. Therefore, development of live attenuated strains of *Y.*
406 *pseudotuberculosis* harboring defined, irreversible mutations is necessary. Also, addition of *Y.*
407 *pestis*-specific antigens to the *Y. pseudotuberculosis* vaccinal strain could improve its efficiency
408 against plague in both its bubonic and pneumonic forms. The most obvious antigen in that regard
409 is F1, which is the main component of the pseudo-capsule and therefore the most abundant
410 antigen in *Y. pestis* in vivo. Indeed, the *caf-1* operon that encodes for F1 has been previously

411 transferred efficiently in a *Salmonella* strain (29). Such a genetically-engineered oral *Y.*
412 *pseudotuberculosis* plague vaccine would represents a promising approach for mass vaccination
413 in endemic countries.

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417 *pseudotuberculosis* strains.

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573 **Legends to figures**

574

575 Figure 1: *Y. pseudotuberculosis* IP32680 colonizes the digestive tract and transiently penetrates
576 into deep organs.

577 Strains IP32680 or IP32953 (10^8 cfu) were inoculated ig to groups of three mice that were
578 sacrificed at different time points. Bacteria recovered from either feces (A), the intestinal tract (B)
579 or deep organs (C) were counted. Given are the cfu per 2 fecal pellets (feces), per g (liver), per
580 whole organ (spleen, mesenteric lymph nodes) or per 2 Peyers' patches. In a second set of
581 experiments (vaccination protocol), mice received twice $2x10^9$ bacteria ig at a 30 day interval
582 (arrows) and bacterial counts were performed similarly (D). Shown are the means of Log cfu +
583 SEM from three mice per point of one representative experiment out of two. nd: not detected.

584

585 Figure 2: Evolution of mouse weight after ig inoculation of yersiniae.

586 Strains IP32680 or IP32953 (10^8 cfu) or saline (control group) were inoculated ig to groups of 8
587 mice and their individual weight was measured daily. Shown are means ± SD of 8 mice per group
588 for IP32680 and saline. Because IP32953 killed some mice, the mean is not given and only three
589 curves from individual animals are shown as examples, with death indicated by crosses. One
590 representative experiment out of two.

591

592 Figure 3: Intragastric inoculation of IP32680 causes minor lesions in mouse organs.

A. Groups of 6 mice from 2 pooled experiments were treated as described in legend of figure 1 and organs taken at day 6 (maximal bacterial load) were stained with haematoxylin-eosin and May-Grünwald-Giemsa. Shown are representative pictures of Peyers' patches, liver and spleen of animals inoculated with IP32953 or IP32680. Arrowheads show necroses and arrows show abscesses. L: gut lumen, WP: white pulp, RP: red pulp. Scale bar : 100 μm.

B. Tissue lesion scores recorded at day 6 after first or second oral inoculations (vaccination protocol) of IP32680 to 6 mice (mean ± SD). As a comparison, scores observed for mice having survived a single IP32953 inoculation (3 out of 6) are given.

Figure 4: Humoral immune response of IP32680-inoculated mice against *Y. pseudotuberculosis* and *Y. pestis*

A: Serum antibody titers against IP32680 in mice inoculated ig once or twice at a 30 day interval with $2x10^9$ cfu. Blood samples were collected 30 days after each inoculation. A filtered sonicate of the IP32680 strain was used for coating in ELISA plates. Shown are mean Ig titers ± SD from 12 mice per group, pooled from 2 independant experiments.

B : Correlation between the IgG titers against IP32680 and CO92 extracts in the sera of mice inoculated once or twice with IP32680. Shown are individual titers from 8 mice per group. The linear regression analysis line is drawn and the Rho value obtained using Spearman's rank test is given ($p = 0.0002$).

Figure 5: Protection against bubonic plague conferred by oral vaccination with IP32680.

A: Groups of 8 mice received either saline (unvaccinated), 1 sc inoculation of 10^7 cfu of EV76 (positive control group), or 1 or 2 ig inoculations of $2x10^9$ cfu of IP32680 at a 30 day interval. All mice were challenged sc with a lethal dose of 10^3 cfu of *Y. pestis* CO92 at d30 (mice with EV76 or IP32680 given once) or d60 (mice with IP32680 given twice) and mortality was followed thereafter daily for 3 weeks. ***: $p \leq 0.005$, **: $p \leq 0.01$, ns: not significant. One representative experiment out of 2.

B: Enumeration of CO92 bacteria in organs of challenged mice. Organs and blood from unvaccinated mice and from mice having received the IP32680 strain twice were taken to determine the bacterial load two days after a *Y. pestis* challenge performed as in A. Given are the cfu per g (liver), or per whole organ (spleen, 2 inguinal lymph nodes) or per ml of blood. Results from 2 independant experiments were pooled and shown are the means of Log cfu ± SEM of a total of 16 vaccinated mice and 21 unvaccinated mice. ND: not detected. ****: $p \leq 0.00003$.

Table 1

SCREENING BY PCR OF SEROTYPE II *Y. PSEUDOTUBERCULOSIS* STRAINS EVALUATED FOR USE AS VACCINE FOR THE PRESENCE OF VIRULENCE DETERMINANTS.

		pYV		Type IV pilus		HPI	Superantigen	
Strain	Origin	*yopK*	*yopM*	*pilN*	*pilR*	*int*	*ypmA/C*	*ypmB*
IP32554	Human blood	-	-	-	-	-	-	-
IP32555	Human blood	-	-	+	+	-	-	-
IP32576	Human blood	-	-	-	-	-	-	-
IP32584	Pig feces	-	-	-	-	-	-	-
IP32585	Antelope LN	-	-	-	-	-	-	-
IP32589	Human LN	-	-	-	-	-	-	-
IP32596	Rabbit	-	-	+	+	-	-	-
IP32598	Human blood	-	-	-	-	-	-	-
IP32621	ND	+	+	-	-	-	-	-
IP32628	Veterinary	-	-	+	+	-	-	-
IP32642	Bird feces	+	+	+	+	-	-	-
IP32643	Parrot	-	-	-	-	-	-	-
IP32661	Veterinary	-	-	-	-	-	-	-
IP32672	Hare	+	+	+	+	-	-	-
IP32678	Hare	-	-	-	-	-	-	-
IP32679	Hare	-	-	-	-	-	-	-
IP32680	Hare	+	+	-	-	-	-	-
IP32681	Hare	-	-	+	+	-	-	-
IP32692	Hare	+	+	+	+	-	-	-
IP32721	Hare	+	+	-	-	-	-	-
IP32828	Human feces	+	+	+	+	-	-	-
IP32860	Human blood	+	+	-	-	-	-	-
IP32870	Hare liver	+	+	+	+	-	-	-
IP32873	Hare liver	+	+	+	+	-	-	-
IP32874	Hare liver	+	+	+	+	-	-	-
IP32876	Hare blood	+	+	+	+	-	-	-
IP32892	Monkey	-	-	-	-	-	-	-
IP32896	Human	+	+	-	-	-	-	-
IP32908	Bird liver	+	+	-	-	-	-	-
IP32913	Monkey lung	-	-	+	+	-	-	-
IP32926	Hare spleen	+	+	+	+	-	-	-
IP32934	Hare liver	+	+	+	+	-	-	-
IP32969	Monkey liver	-	-	-	-	-	-	-
IP33006	Human blood	-	-	-	-	-	-	-
IP33047	Hare	-	-	-	-	-	-	-
IP33054	Human blood	-	-	-	-	-	-	-
IP33067	Human LN	+	+	+	+	-	-	-
IP33098	Human Feces	+	+	-	-	-	-	-
IP33286	Human blood	+	+	+	+	-	-	-
IP33293	Human Feces	-	-	-	-	-	-	-
IP33306	Hare	-	-	-	-	-	-	-

Table 2

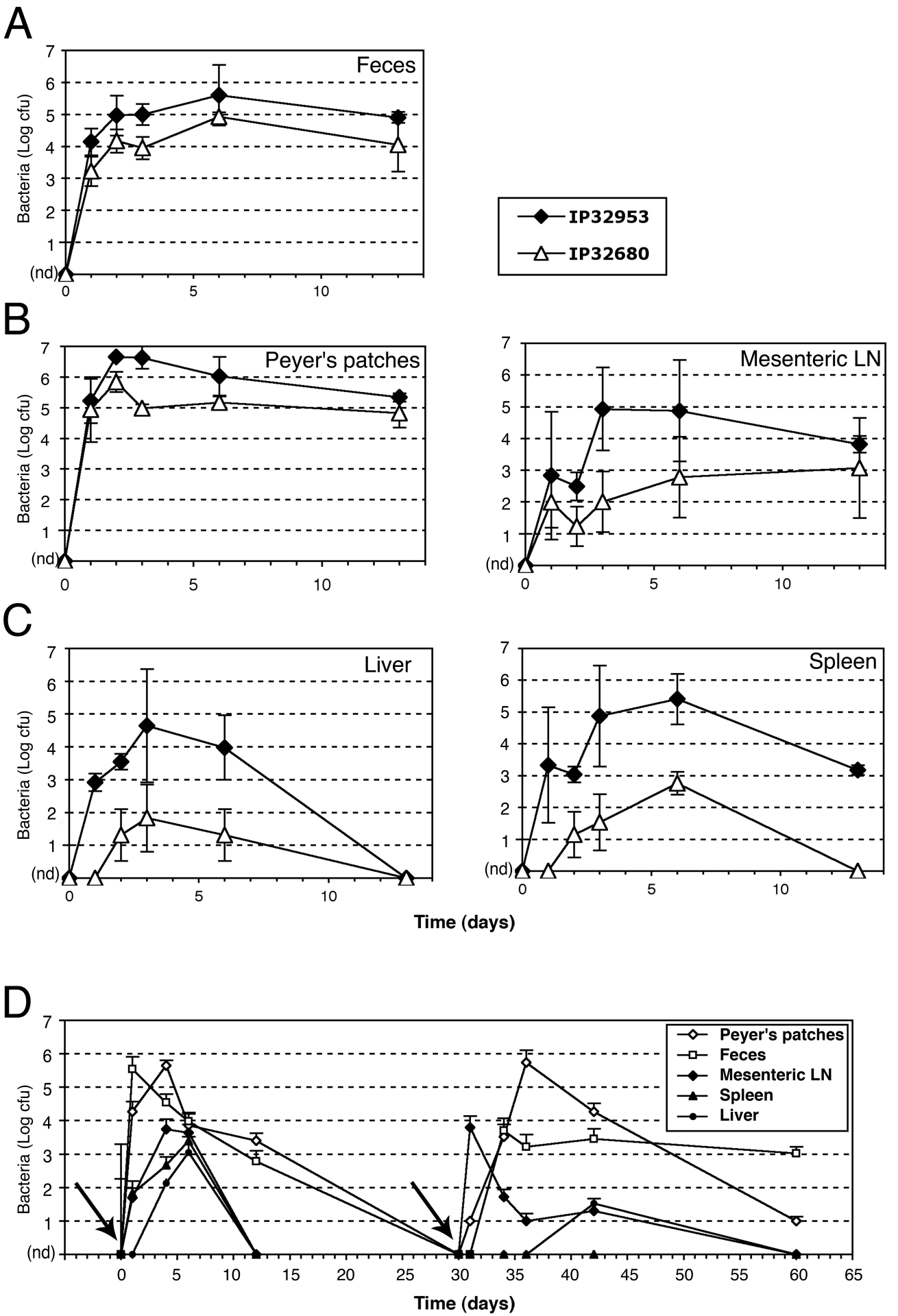
COMPARISON OF VIRULENCE IN THE MOUSE OF THE 7 SELECTED *YERSINIA PSEUDOTUBERCULOSIS* STRAINS AND THE VIRULENT STRAIN IP32953

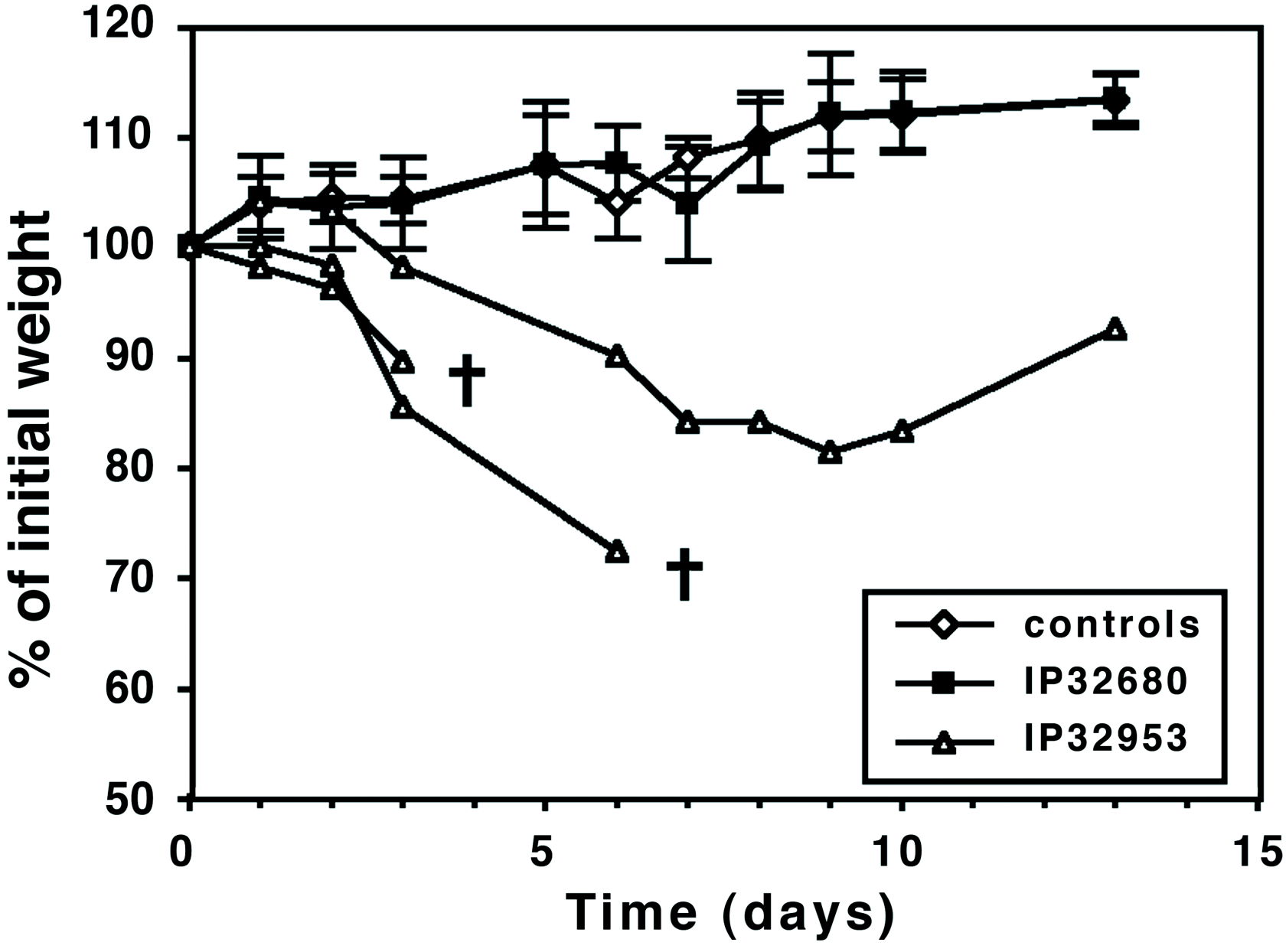
Strains	Serotype	Mortality* after sc inoculation**	Mortality* after ig administration***
IP32953	I	8/10	9/10
IP32896	II	2/5	2/5
IP33098	II	0/5	5/5
IP32908	II	1/5	3/5
IP32621	II	2/5	0/5
IP32860	II	0/5	2/5
IP32721	II	0/10	0/10
IP32680	II	0/10	0/30

* Mortality (dead mice/total) was followed daily for 3 weeks. For strains killing no mice in both conditions, experiments were reproduced to confirm the absence of virulence and results were pooled.

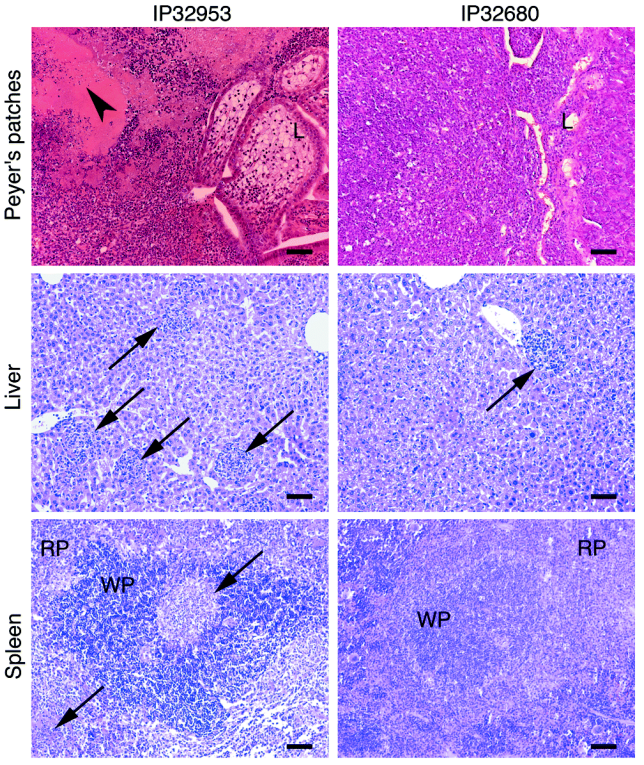
** 10^9 cfu were inoculated subcutaneously in ventral skin

*** $2x10^9$ cfu were given intragastrically using a feeding needle


A
Feces
Bacteria (Log cfu)
(nd)
IP32953
IP32680
B
Peyer's patches
Mesenteric LN
C
Liver
Spleen
Time (days)
D
Peyer's patches
Feces
Mesenteric LN
Spleen
Liver
Time (days)


120
110
100
90
80
70
60
50
% of initial weight
0
5
10
15
Time (days)
†
†
controls
IP32680
IP32953

A



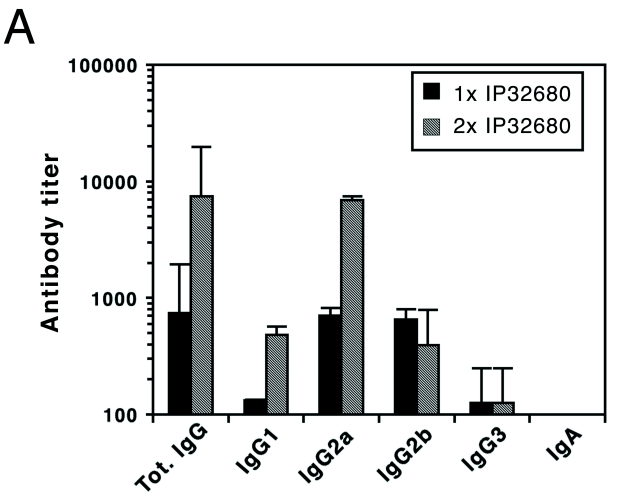
B

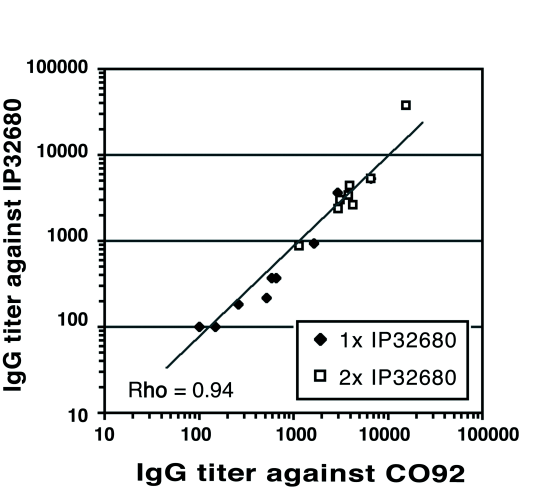
Lesion scores in target organs

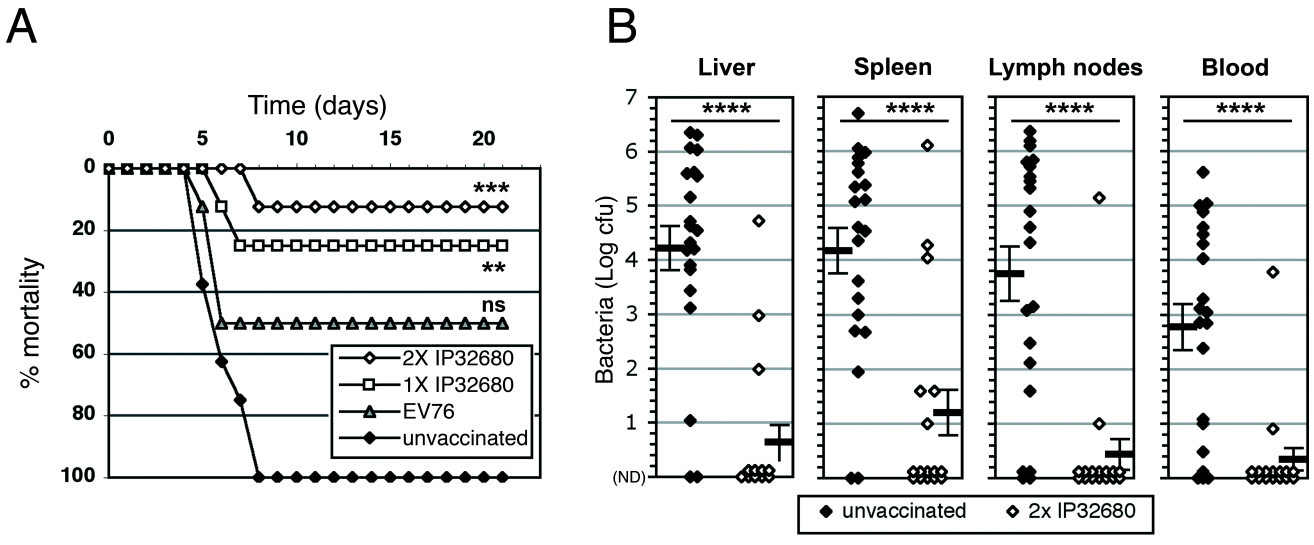
	IP32953 10^8 cfu (a)	IP32680 10^9 cfu (b)	
	-	1st inoculation	2nd inoculation
Liver	≥ 6	2.1 ± 1.3	1.0 ± 1.4
spleen	≥ 4	2.1 ± 1.2	1.6 ± 1.5
Peyer's P.	≥ 4	1.5 ± 1.3	1.0 ± 1.7
MLN	≥ 7	ND	0.7 ± 1

(a): scores from 3 survivors out of 6 mice inoculated once
(b): mean scores ± SEM of 6 mice

Figure 4


A
100000
10000
1000
100
Antibody titer
1x IP32680
2x IP32680
Tot. IgG
IgG1
IgG2a
IgG2b
IgG3
IgA


B
100000
10000
1000
100
10
IgG titer against IP32680
1x IP32680
2x IP32680
Rho = 0.94
10
100
1000
10000
100000
IgG titer against CO92


A
Time (days)
0
5
10
15
20
% mortality
0
20
40
60
80
100
***
**
ns
2X IP32680
1X IP32680
EV76
unvaccinated
B
Liver
Spleen
Lymph nodes
Blood
Bacteria (Log cfu)
7
6
5
4
3
2
1
(ND)
****
****
****
****
unvaccinated
2x IP32680