

Levels of Expression and Immunogenicity of Attenuated *Salmonella enterica* Serovar Typhimurium Strains Expressing *Escherichia coli* Mutant Heat-Labile Enterotoxin

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The effects of heterologous gene dosage as well as *Salmonella typhimurium* strain variability on immune response toward both the heterologous antigen, the nontoxic mutant of the *Escherichia coli* heat-labile enterotoxin LTK63, and the carrier *Salmonella* strain have been analyzed. Effects of a single integration into the host DNA and different-copy-number episomal vectors were compared in *S. typhimurium* $\Delta cya \Delta crp \Delta asd$ strains of two different serotypes, UK-1 and SR-11. Expression of the enterotoxin in the different *Salmonella* isolates in vitro was found to vary considerably and, for the episomal vectors, to correlate with the plasmid copy number. LTK63-specific serum immunoglobulin G (IgG) and mucosal immunoglobulin A (IgA) antibodies were highest in mice immunized with the high-level-expression strain. High anti-LTK63 IgG and IgA titers were found to correspond to higher anti-*Salmonella* immunity, suggesting that LTK63 exerts an adjuvant effect on response to the carrier. Statistically significant differences in anti-LTK63 immune response were observed between groups of mice immunized with the attenuated $\Delta cya \Delta crp$ UK-1 and SR-11 derivatives producing the antigen at the same rate. These data indicate that the same attenuation in *S. typhimurium* strains of different genetic backgrounds can influence significantly the immune response toward the heterologous antigen. Moreover, delivery of the LTK63 enterotoxin to the immune system by attenuated *S. typhimurium* strains is effective only when synthesis of the antigen is very high during the initial phase of invasion, while persistence of the *S. typhimurium* strain in deep tissues has only marginal influence.

Enterotoxigenic *Escherichia coli* strains produce a plasmid-encoded heat-labile enterotoxin (LT) (15, 34) related to cholera toxin (CT) (9, 35). LT is composed of two subunits, A and B, which are exported to the periplasmic space, where they assemble into an AB₅ multimeric complex (16). Several mutants of LT-A have been constructed, and in particular, a nontoxic mutant which contains a substitution of serine 63 with lysine (LTK63) has been shown to maintain the structural and immunogenic properties of wild-type LT (21, 27, 28). LTK63 has also been found to display the strong mucosal adjuvant activity pertaining to wild-type LT. Efficient induction of mucosal immune response, specifically in the mouse vagina, has been achieved via the intranasal route of immunization (10). For the development of oral vaccines, however, it would be desirable to exploit the properties of LTK63 for enhancing antigen-specific immune response in the intestinal mucosa by means of oral delivery of the potent mucosal adjuvant.

Oral delivery of antigens by live vaccines is known to lead to a more effective production of antigen-specific antibodies in mucosal secretions than oral administration of the soluble antigen (36, 39). Several antigen delivery systems which use as carriers mutant intracellular pathogens that have lost the ability to persist and produce the disease while retaining limited growth in vivo have been developed. In particular, attenuated

Salmonella mutants are suitable immunological carriers for virulence determinants from other enteric bacteria in that they can induce humoral immune response selectively at the site of colonization, the gut mucosa. Vaccine strains of *Salmonella* have been successfully attenuated by introducing different types of mutations (5, 8, 23, 26). Notably, *Salmonella* strains with a galactose epimerase (*galE*) mutation (18) or deletions in genes for the biosynthesis of aromatic compounds (*aro* mutants) (11, 12, 17, 19) or in the adenylate cyclase (*cya*) and cyclic AMP receptor protein (*crp*) genes (6) are the most extensively characterized.

Delivery of the B subunit of the *E. coli* enterotoxin (LT-B) by a *galE* mutant of *Salmonella typhimurium* has been shown to elicit low levels of anti-LT-B serum and mucosal antibodies. Since the vector used for expression of LT-B was rapidly lost in vivo, i.e., in the absence of the antibiotic required for selection of the plasmid, the level of immune response could be correlated only with the amount of antigen expressed during the initial phase of invasion (3).

Recently, direct comparison between the *aroA aroD/pnrB* and the $\Delta cya \Delta crp \Delta asd/asd^+$ delivery systems for the ability to induce humoral and cellular immunity after a single immunization showed that the former vaccine strain had greater potential as a carrier for antigen delivery (20). However, the balanced lethal *asd* system for in vivo selection of plasmids expressing heterologous antigens in the attenuated $\Delta cya \Delta crp \Delta asd$ strains is still very attractive in that *asd^+* plasmids do not require antibiotic resistance markers for selection while stably maintained in vivo (24). In addition, the $\Delta cya \Delta crp \Delta asd/asd^+$ delivery system has been reported to induce protective immu-

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype or phenotype	Reference or source
Strains		
<i>E. coli</i> χ 6212	Φ 80d <i>lacZ</i> Δ M15 <i>deoR</i> Δ (<i>lacZYA-argF</i>)U169 <i>supE44</i> λ^- <i>gyrA</i> <i>recA1</i> <i>relA1</i> <i>endA1</i> <i>AsdA4</i> Δ [<i>zhf-2::Tn10</i>] <i>hsdR17</i> ($r^- m^+$)	R. Curtiss III
<i>S. typhimurium</i>		
χ 4072 SR-11	pStSR100 ⁻ <i>gyrA1816</i> Δ <i>cya-1</i> Δ <i>crp-1</i> Δ <i>asdA1</i> Δ (<i>zhf-4::Tn10</i>)	24
χ 4217 SR-11	pStSR101 ⁺ <i>gyrA1816</i> Δ <i>cya-1</i> Δ <i>crp-1</i> Δ <i>asdA1</i> Δ (<i>zhf-4::Tn10</i>)	33
χ 3985 UK-1	pStV1 ⁺ Δ <i>cya-12</i> Δ (<i>zid-62::Tn10</i>) Δ <i>crp-11</i> Δ (<i>zhhb::Tn10</i>)	7
χ 3987 UK-1	pStV1 ⁺ Δ <i>cya-12</i> Δ (<i>zid-62::Tn10</i>) Δ <i>crp-11</i> Δ (<i>zhc-1431::Tn10</i>) Δ <i>asdA1</i> Δ (<i>zhf-4::Tn10</i>)	14
χ 4217-10E	χ 4217 with insertion of mini-Tn5 <i>asd elt-K63</i>	2
χ 3987-18A	χ 3987 with insertion of mini-Tn5 <i>asd elt-K63</i>	2
Plasmids		
pBluescript KS-LT	<i>E. coli</i> vector with LT gene inserted into the <i>SmaI-HindIII</i> sites	28
pBluescript KS-LTK63	<i>E. coli</i> vector with mutant LTK63 gene inserted into the <i>SmaI-HindIII</i> sites	28
pYA3074	Low-copy-number <i>Asd</i> ⁺ vector with pSC101 replicon	R. Curtiss III
pYA3149	Medium-high-copy-number <i>Asd</i> ⁺ vector with pBR322 replicon	R. Curtiss III
pYA3137	High-copy-number <i>Asd</i> ⁺ vector with pUC replicon	R. Curtiss III

nity against several pathogens (25, 29, 40). Most of these studies have restricted analysis of the immune response to antigens expressed from the same *asd*⁺ plasmid carried by Δ *cya* Δ *crp* Δ *asd* mutants usually of the same *S. typhimurium* serotype. In this work, we have analyzed the influence of heterologous gene dosage, and thus level of expression, as well as *S. typhimurium* strain variability on immune response toward both the heterologous antigen, a nontoxic mutant of *E. coli* LT, and the carrier *Salmonella* strain. Effects of a single integration into the host DNA and episomal vectors at different copy numbers were compared in *S. typhimurium* strains of two different Δ *cya* Δ *crp* Δ *asd* serotypes, UK-1 and SR-11.

MATERIALS AND METHODS

Strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. The Δ *cya* Δ *crp* Δ *asd* *S. typhimurium* strains were kindly provided by Roy Curtiss III, Washington University, St. Louis, Mo.

The complex medium used for routine bacterial growth was Luria broth (LB) medium. The minimal medium used was that described by Curtiss and Kelly (6). When required, the antibiotics ampicillin, chloramphenicol, tetracycline, and nalidixic acid were added to culture media at concentrations of 100, 50, 30, 12.5, and 40 μ g per ml, respectively. For growth of the Δ *asd* *Salmonella* strains, the amino acids methionine (20 μ g/ml), threonine (8 μ g/ml), isoleucine (20 μ g/ml), and/or diaminopimelic acid (50 μ g/ml) were added to the culture media.

For immunization experiments, bacteria from static overnight cultures were diluted 1:20 in prewarmed LB and grown at 37°C under aeration to give 10⁹ CFU/ml. CFU were determined by plating serial dilutions of cultures of LB agar plates or Difco MacConkey base supplemented with 1% maltose and nalidixic acid for strains χ 4072 and χ 4217.

DNA manipulations. Extraction and purification of plasmid DNA were carried out as described by Sambrook et al. (31). Genomic DNA extraction was performed as described by Rossolini et al. (30). Southern blot experiments were performed by bidirectional transfer of DNA from agarose gels to nylon membranes. Specific DNA probes were obtained amplifying DNA sequences by PCR and labeled with [α -³²P]dATP, using the random-primers method.

The pBluescript KS plasmids containing the wild-type *elt* or the *eltK63* mutated gene (provided by M. Pizza) were described previously (28). The 2-kb *BamHI-SalI* fragment carrying *elt* or *eltK63* was subcloned into the pYA3074, pYA3149, and pYA3137 *asd* vectors (provided by R. Curtiss III). The resulting plasmids were transformed and amplified in *E. coli* χ 6212 and then purified and transformed into the attenuated *S. typhimurium* strains. Transformants were selected in LB medium. The fermentation pattern, auxotrophic requirements, and lipopolysaccharide content were routinely analyzed in all strains. The completely smooth lipopolysaccharide was examined by polyacrylamide gel electrophoresis (PAGE) and silver staining after periodic acid treatment (38).

The number of copies per cell of each construct was determined by Southern blot analysis of total DNA extracted from the different strains, digested with *BamHI* and *SalI*, and hybridized to an *elt*-specific probe and to a probe specific

for the *spvC* gene of *S. typhimurium*. The relative intensity of the *elt*-specific band was calculated by using a densitometer (Ultrascan; LKB).

The nucleotide sequence of mini-Tn5 *asd eltK63* insertions was determined from DNA fragments obtained by inverse PCR. Briefly, *SalI-XhoI*-digested genomic DNA fragments were incubated with T4 DNA ligase overnight at 15°C. Inverse PCR was performed by using an Expand 20 kb^{plus} PCR kit (Boehringer) in a total volume of 50 μ l with 6 ng of DNA (2 μ l of the ligation mixture) as a template and 300 nM each of the following primers: 5'-ACAGACGTGAGCC TGAAAGGTTGG-3' and 5'-CTCCACTACAGGGAGCTGTTATAGC-3' (complementary to the promoter and to the 3' end of the coding region of *eltK63*, respectively).

SDS-PAGE and immunoblot analysis. Bacterial cultures were grown to the logarithmic or stationary phase in LB broth. Cells were collected and lysed by boiling (5 to 10 min) in LSB buffer (2% sodium dodecyl sulfate [SDS], 10% glycerol, 100 mM dithiothreitol, 62.5 mM Tris-HCl [pH 6.8]). Total proteins in cell extracts were quantified with a micro-bicinchoninic acid protein assay reagent kit (Pierce, Rockford, Ill.). Proteins were separated by SDS-PAGE (15% polyacrylamide gel) and transferred to nitrocellulose membranes. Mouse polyclonal anti-LT antiserum was used as the primary antibody. Bands were visualized by enhanced chemiluminescence (Amersham Life Science) or with 4-chloro-1-naphthol (Sigma) as the substrate. Evaluation of the amount of LT produced by the different strains was obtained by densitometric analysis of bands, using purified enterotoxin or CT as a reference.

Immunization and sampling. Groups of five or seven 6- to 8-week-old female BALB/c mice (Charles River, Calco, Como, Italy) were immunized intragastrically with two or four doses containing ca. 8 \times 10⁸ CFU of each *Salmonella* strain. Mice were deprived of water and food for 12 h and fed with 30 μ l of 0.2 M sodium bicarbonate to neutralize stomach acidity 30 min prior to immunization. Groups of mice were immunized on days 0 and 10 to 14 or on days 0, 10, 17, and 24.

Serum samples and intestinal mucus were collected on days 14, 17, 24, 36, 50, and 66. Blood samples were collected from the retro-orbital sinus of each anesthetized mouse. To collect gut secretions, mice were sacrificed, the small intestine was excised, and the mucus was scraped from the luminal surface. Mucus samples were collected in centrifuge tubes containing 5 μ l each of 0.1 M phenylmethylsulfonyl fluoride in ethanol, 1% bovine serum albumin, and 1% sodium azide. After centrifugation at 12,000 \times g for 10 min at 4°C, supernatant fluids were collected, 5 μ l of a solution containing 0.1 M phenylmethylsulfonyl fluoride and 1% sodium azide was added, and samples were stored at -20°C.

Spleens and Peyer's patches were removed from sacrificed animals and processed as described by Curtiss and Kelly (6). After disruption in a Dounce tissue homogenizer, suspensions were diluted in phosphate-buffered saline (PBS) and plated on MacConkey agar containing 1% lactose. The expected phenotype of *S. typhimurium* strains recovered from immunized animals was verified on MacConkey agar supplemented with 1% maltose.

ELISA for LT and *Salmonella*. Individual mouse serum and mucus samples were tested for immunoglobulin A (IgA) and IgG antibodies against LT or whole *Salmonella* cells by enzyme-linked immunosorbent assay (ELISA). A PBS solution of purified LT (a gift from M. Pizza) was absorbed to 96-well ELISA plates (Greiner-GmbH, Kremsmunster, Austria) at 0.2 μ g per well overnight at 4°C. For anti-*Salmonella* antibody titration, bacteria were grown overnight, harvested by centrifugation, and resuspended in PBS at 3 \times 10¹¹ CFU/ml. Bacteria were

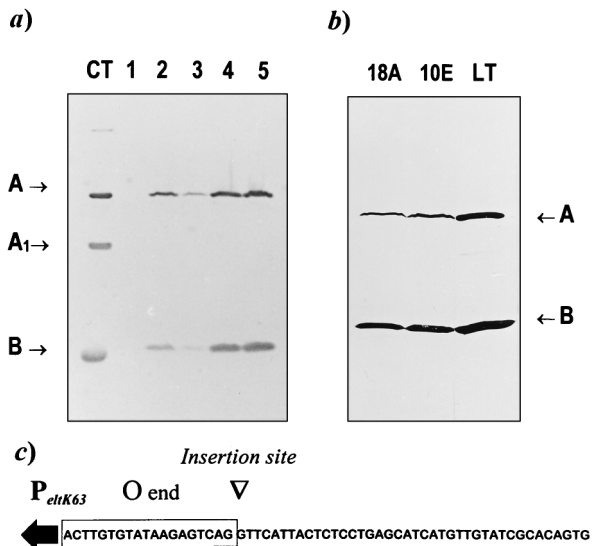


FIG. 1. Western blot analysis of expression of LTK63 in different *S. typhimurium* strains, using an anti-LT antibody. (a) Total-cell extracts of stationary-growth-phase cultures of strains χ 3987 (lane 1), χ 3987-18A (lane 2), χ 3987:pYA3074-LTK63 (lane 3), χ 3987:pYA3149-LTK63 (lane 4), and χ 3987:pYA3137-LTK63 (lane 5); (b) total-cell extracts of late-logarithmic-growth-phase cultures of *eltK63* single-copy integrants. Total proteins in cell extracts were quantified, and 30 μ g of each sample was used for the immunoblot. Purified CT (3 μ g) and LT (3 μ g) were used as a control and as a molecular mass marker for the A, A1, and B subunits of the toxins. (c) Nucleotide sequence of the integration site of the mini-Tn5 *asd eltK63* into the virulence plasmid of strains χ 3987-18A and χ 4217-10E. The sequence of the mini-Tn5 19-bp O end flanking the *eltK63* promoter (P_{eltK63} ; arrow) is boxed.

heat killed for 10 min at 80°C and stored at -20°C. Each sample well was coated with 0.1 ml of this suspension diluted 100-fold in 0.1 M carbonate buffer (pH 9.6). Plates were blocked with PBS containing 0.05% Tween 20 and 1% bovine serum albumin for 2 h at 37°C. After washing with PBS, an anti-isotype secondary antibody (Sigma) was added and plates were incubated for 2 h at 37°C. Serum samples were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (γ -chain specific) antibodies, mucus samples were incubated with biotin-conjugated goat anti-mouse IgA (α -chain specific) antibodies. Plates were washed with PBS, developed with a solution of *p*-nitrophenyl phosphate (1 mg/ml) in 1 M diethanolamine buffer (pH 9.8), and read in an ELISA reader (Titertek Multiscan) at 405 nm. Titers were expressed as the last dilution that gave an optical density at 405 nm of 0.1. Under these experimental conditions, preimmune samples always gave an optical density at 405 nm of <0.1 from the first dilution.

Nucleotide sequence accession number. The nucleotide sequence of the mini-Tn5 insertion in the virulence plasmid described in this work has been assigned GenBank accession no. AF025956.

RESULTS

Influence of copy number on expression level of LT-K63 in vitro. Expression of LTK63 by *S. typhimurium* attenuated strains carrying the *eltK63* gene cloned into the pYA plasmids listed in Table 1 was examined. Western blot analysis of total-cell extracts and periplasmic fractions from logarithmic- and stationary-growth-phase cultures of the different *S. typhimurium* strains showed, as expected, a good correlation between the amount of LTK63 produced by each strain and the copy number of the recombinant plasmid carried by the strain. Expression of the enterotoxin gene, which is under the control of its own promoter in these constructs, was constitutive in all strains analyzed and in all conditions tested.

Representative results are shown in Fig. 1a. A fivefold difference was observed between the amount of toxin synthesized by strain χ 3987:pYA3074-LTK63 (Fig. 1a, lane 3) and that

produced by χ 3987:pYA3137-LTK63 (Fig. 1a, lane 5). The medium-high-copy-number plasmid pYA3149, in turn, could direct synthesis of LTK63 at a level slightly lower than that found for the high-copy-number plasmid pYA3137 in the same strain (Fig. 1a; compare lanes 4 and 5). However, some differences were found between the strains in that χ 4072:pYA3137-LTK63 produced the same amount of enterotoxin as χ 4072:pYA3149-LTK63 but showed an unusual growth curve with a 3-h lag phase followed by an exponential growth phase with the same doubling time as the other strains. Furthermore, it is noteworthy that strain χ 4072:pYA3137-LTK63 lysed during static growth.

To assess if the phenotype conferred to strain χ 4072 by plasmid pYA3137-LTK63 was due to the presence of the high-copy-number plasmid or to toxicity of LTK63, a preliminary analysis of the attenuated strains carrying plasmid pYA3137 with and without the *eltK63* insert was carried out by plating cells from static growth onto selective medium. Lysis was observed only for strain χ 4072 containing the *eltK63* insert in plasmid pYA3137. The plasmid without the insert did not cause lysis in any of the strains analyzed (data not shown). Likely, expression of *eltK63* from the high-copy-number plasmid is toxic for strain χ 4072 during static growth.

Two *S. typhimurium* strains, χ 3987-18A and χ 4217-10E, carrying a single-copy integration of the *eltK63* gene into the genome and producing equally high amounts of LTK63, were also analyzed for expression of the enterotoxin. The two strains were obtained by inserting a single copy of a mini-Tn5 *asd eltK63* minitransposon into the genomes of *S. typhimurium* χ 3987 and χ 4217. It should be noted that strain χ 4217 is an SR-11 derivative (33), while strain χ 3987 is a derivative of the *S. typhimurium* UK-1 strain χ 3985 (14). Insertion of the mini-Tn5 *asd eltK63* was found to have occurred in the same locus of the virulence plasmid in both strains χ 3987-18A and χ 4217-10E. The nucleotide sequence of this locus at the junction of the O end of the minitransposon was determined from DNA fragments obtained by inverse PCR. The locus does not show any homology to known *S. typhimurium* sequences (Fig. 1c).

In Fig. 1a, lane 2, the level of LTK63 production of strain χ 3987-18A is compared with those of the episomal systems used for expression in the same strain. The amount of LTK63 found in the single-copy integrant was threefold greater than that observed for the low-copy-number plasmid pYA3074. Figure 1b shows a direct comparison of the level of LTK63 expression in late-logarithmic-growth-phase cultures of the *eltK63* single-copy integrants.

It was also found that in all strains analyzed, the LT was secreted to the periplasmic space (data not shown), where presumably it is assembled into its heterohexameric form, as it has been shown that assembly of LT takes place in the periplasm in *E. coli* (16).

Serum antibody response induced by *S. typhimurium* χ 3987 expressing different levels of LTK63. Groups of five BALB/c mice were immunized orally with strain χ 3987 containing the low-copy-number plasmids pYA3074-LT and pYA3074-LTK63 or the high-copy-number plasmid pYA3137-LTK63. The same strain carrying the pYA3074 vector without the LTK63 insert and the parental attenuated *asd*⁺ strain χ 3985 were used as control strains. A single oral booster dose of 8×10^8 *S. typhimurium* cells was administered 10 days after the first inoculation. The *Salmonella*-specific IgG titers in serum samples from immunized animals 24, 36, and 66 days after the first dosing were found to differ significantly particularly at day 24, in that strain χ 3987:pYA3137-LTK63 induced an average 10-fold-higher response. A diminished, but still significant, difference was observed at day 66 (Fig. 2). A possible explanation for the

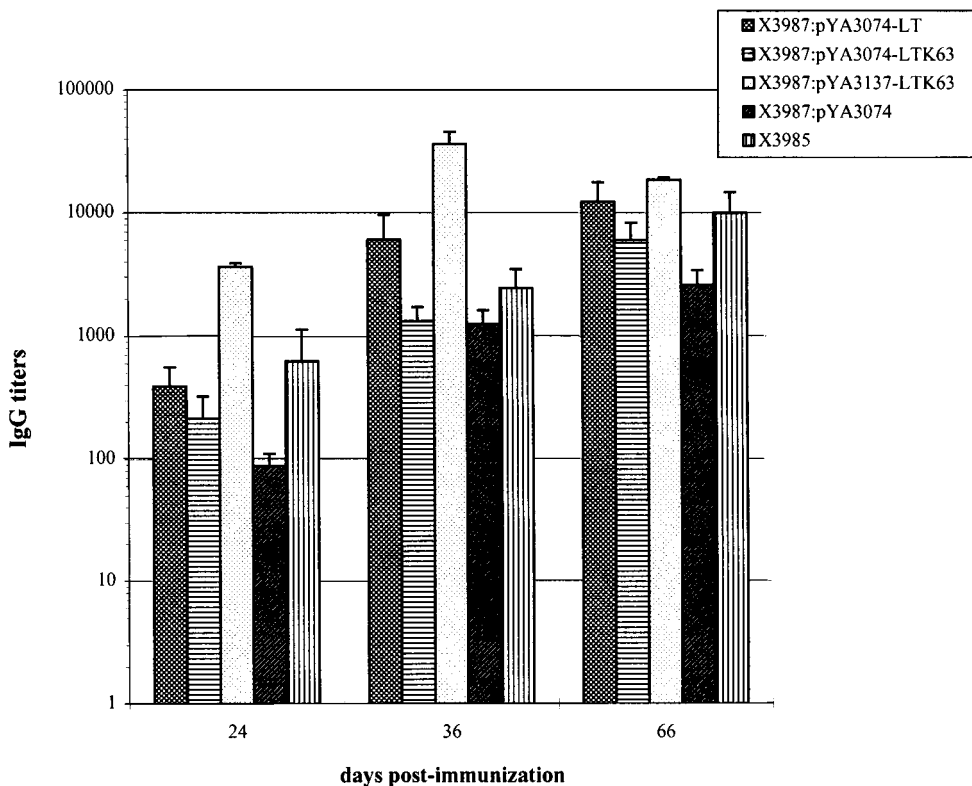


FIG. 2. Influence of amounts of LT or LTK63 synthesized by *S. typhimurium* on anti-*Salmonella* immune response. Serum IgG activity to *S. typhimurium* in mice immunized with two doses of strain χ 3987 containing the low-copy-number plasmids pYA3074-LT and pYA3074-LTK63, the high-copy-number plasmid pYA3137-LTK63, or the control plasmid pYA3074 without the enterotoxin gene insert was determined. The two doses were given to groups of five mice on days 0 and 10. IgG responses were detected by ELISA 24, 36, and 66 days after primary immunization. The standard error of the mean was calculated for all data, and mean values were compared by Student's *t* test. *P* values of ≤ 0.0001 were considered statistically significant.

less pronounced difference at day 66 in mice receiving the high-copy-number plasmid strain could be that the higher anti-*Salmonella* titers elicited by this strain after the first immunization result in a reduced number of organisms able to colonize after the second inoculum.

No significant difference was observed between the antibody responses induced by strains χ 3987:pYA3074-LT and χ 3987:pYA3074-LTK63. When the anti-*Salmonella* IgG titers induced by the two control strains are compared, it should be noted that the *asd* mutation present in strain χ 3987 further attenuates *S. typhimurium*. Thus, the higher anti-*Salmonella* IgG titers elicited by the *asd*⁺ strain χ 3985 are presumably due to a higher persistence of this strain in deep organs (Table 2).

Analysis of LT-specific IgG titers in the same serum samples was carried out only for the three χ 3987 strains containing the LT or LTK63 construct. IgG titers were found to be negligible with both low-copy-number constructs, while samples from mice immunized with the high-copy-number plasmid-bearing strain χ 3987:pYA3137-LTK63 showed high anti-LT IgG titers at days 24 (1:10,310), 36 (1:31,310), and 66 (1:20,970).

Influence of *Salmonella* genetic background on the induction of serum antibody response. A similar immunization scheme was extended to include oral inoculation of groups of seven BALB/c mice with 8×10^8 cells of the three *S. typhimurium* strains carrying a single-copy insertion of the mini-Tn5 *asd eltK63* minitransposon into the genomes of *S. typhimurium* χ 3987 and χ 4217 (Table 1).

The LT-specific and *Salmonella*-specific IgG titers determined in serum samples from mice immunized with these

strains are shown in Fig. 3A. While the anti-*Salmonella* serum responses were similar for the two strains, a significant difference was observed in the anti-LT titers, particularly at day 66 from immunization. Sera from mice immunized with strain χ 3987-18A showed LT-specific IgG titers 10- to 100-fold higher than those in samples from the other group of mice. It is interesting that this occurs in spite of the fact that persistence of this *S. typhimurium* strain in deep organs 50 days after immunization was found to be over 10-fold lower than that of strain χ 4217-10E (Table 2) and that the levels of LTK63 pro-

TABLE 2. Persistence of *S. typhimurium* strains in deep organs^a

Strain	No. of bacteria			
	Peyer's patches		Spleen	
	24 dpi	50 dpi	24 dpi	50 dpi
χ 3985	1.1×10^4	0.8×10^1	1.7×10^4	2.4×10^3
χ 3987-18A	5.2×10^3	0	6.3×10^3	7.5×10^1
χ 4217-10E	4.6×10^3	1.2×10^2	9.9×10^3	9.5×10^2
χ 3987:pYA3137/LT-K63	1.1×10^3	1.0×10^{1b}	2.7×10^3	1.3×10^{2b}
χ 3987:pYA3149/LT-K63	1.8×10^3	0 ^b	1.4×10^3	0 ^b
χ 4072:pYA3149/LT-K63	2.9×10^3	0.3×10^{1b}	1.7×10^2	0.2×10^{1b}

^a Groups of seven BALB/c mice were inoculated intragastrically with two doses of 8×10^8 *S. typhimurium* organisms. Peyer's patches and spleens were removed at specified days postimmunization (dpi), homogenized in PBS, and plated on MacConkey agar containing 1% lactose.

^b Recovery of *S. typhimurium* from Peyer's patches and spleens of mice immunized with four doses of 8×10^8 *S. typhimurium* organisms.

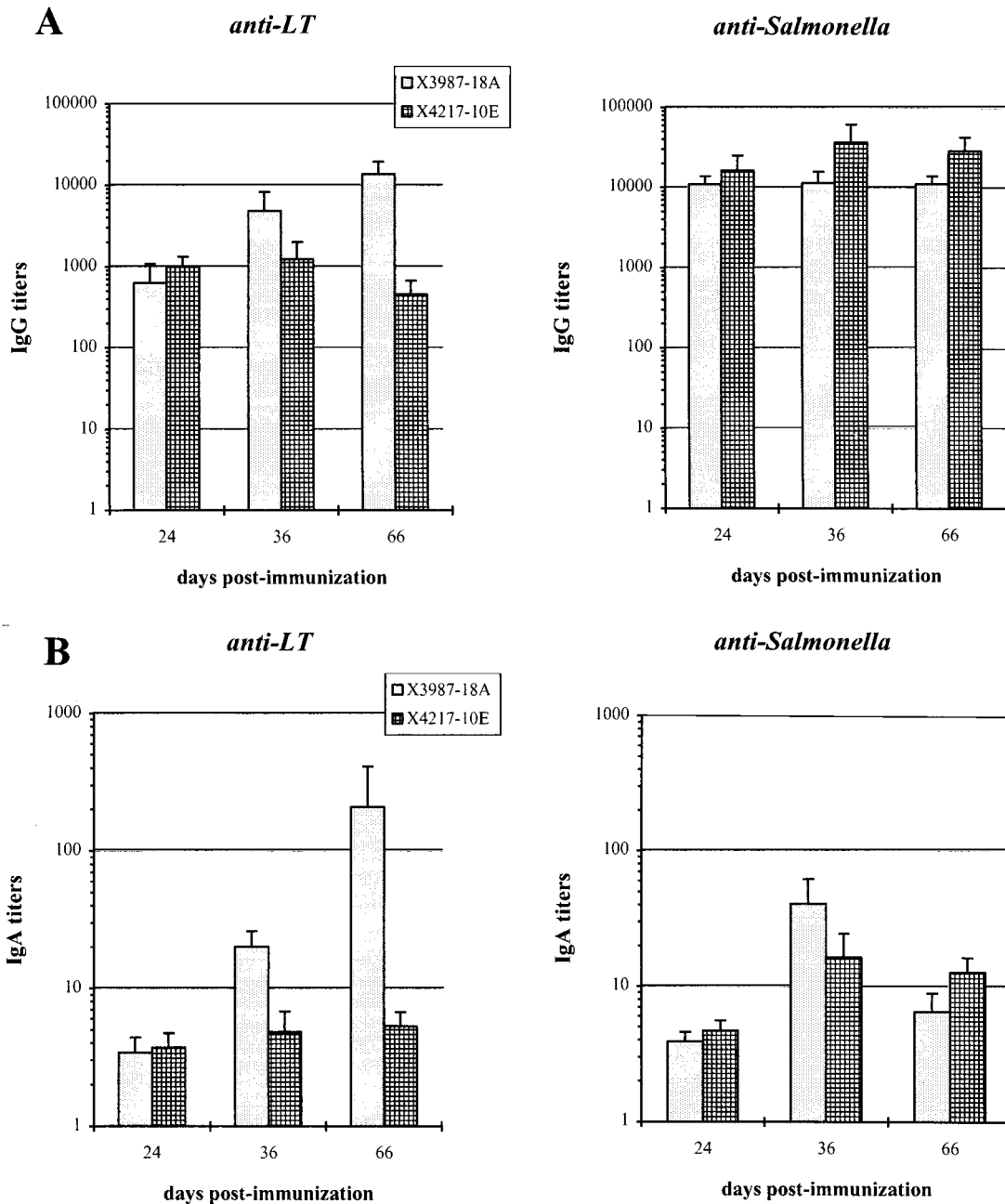


FIG. 3. ELISA analysis of immune response induced by two different genetic backgrounds carrying LTK63 single-copy integrations. Groups of seven mice were inoculated with the two *S. typhimurium* strains on days 0 and 14. Samples were collected at 24, 36, and 66 days after primary immunization. (A) Serum anti-LTK63 and anti-*Salmonella* IgG antibody response; (B) mucosal anti-LTK63 and anti-*Salmonella* IgA titers. Mean values of each group were compared by Student's *t* test. *P* values of ≤ 0.05 were considered statistically significant.

duced in vitro were equally high for the two strains (Fig. 1b). Possibly, this may be attributed to a difference in the level of expression of LTK63 in vivo during the initial steps of invasion.

Differential induction of serum antibody response by high LTK63 expression. Since the amount of enterotoxin produced by *S. typhimurium* seemed the most crucial parameter in determining a good immune response, an immunization experiment was carried out with four doses of the *S. typhimurium* strains containing the pYA3149-LTK63 and pYA3137-LTK63 constructs. The strains carrying the medium-high-copy-number

plasmid were included in this experiment since strain $\chi 4072$: pYA3137-LTK63 lysed after growth in static culture. In addition, in order to compare this immunization scheme with the previous ones, groups of seven mice were inoculated with only two doses of the same plasmid-bearing *S. typhimurium* strains used for immunizing mice with four doses. The immune response in these animals was evaluated only at day 66 from the first inoculum. The results presented in Fig. 4A show that both the anti-LTK63 and the anti-*Salmonella* IgG titers were highest for the group of mice immunized with strain $\chi 3987$: pYA3137-LTK63.

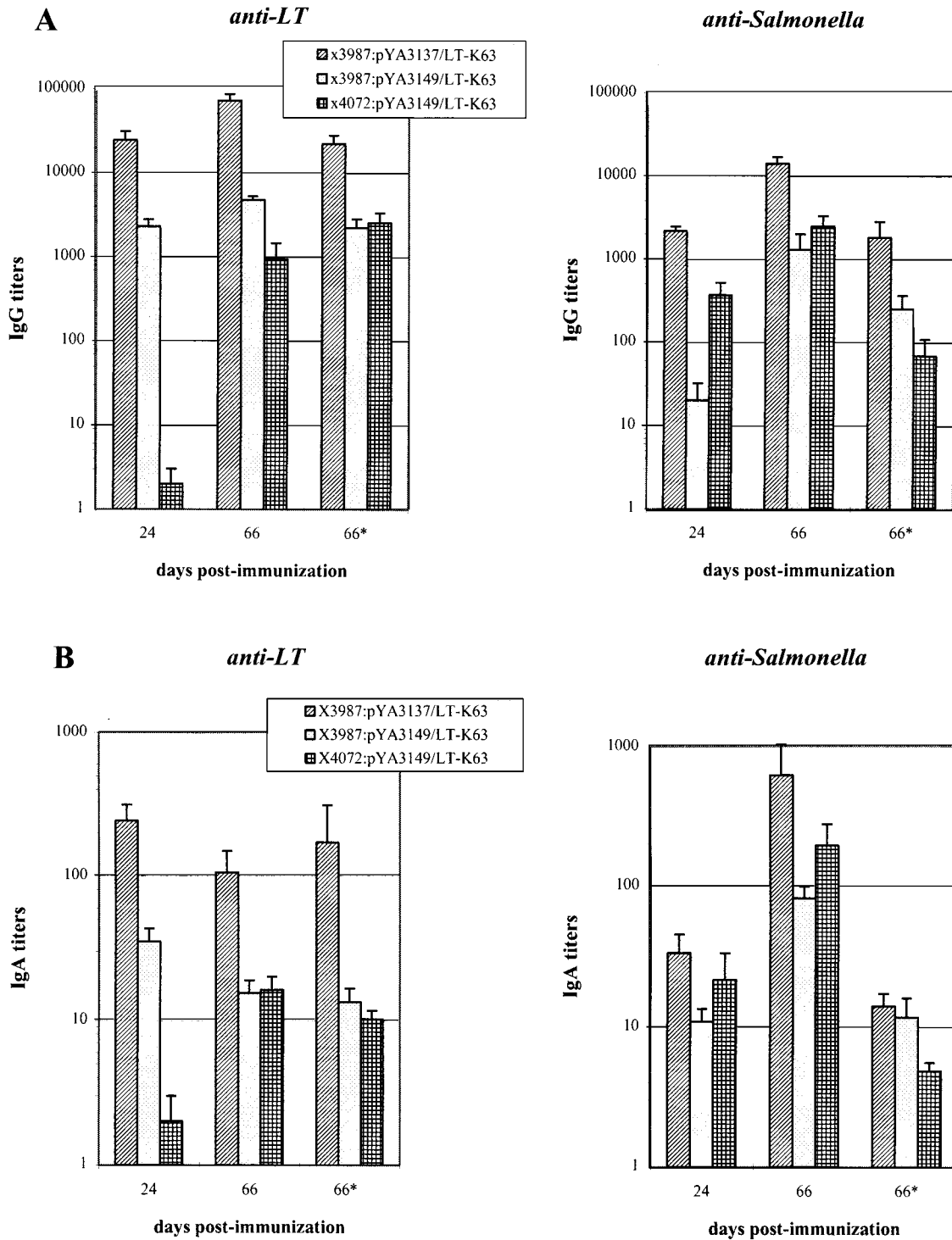


FIG. 4. ELISA analysis of immune response induced by UK-1 (χ 3987) and SR-11 (χ 4072) strains expressing high levels of LTK63. Groups of seven mice were immunized with two (on days 0 and 10) or four doses (on days 0, 10, 17, and 24) of strains χ 3987:pYA3137-LTK63, χ 3987:pYA3149-LTK63, and χ 4072:pYA3149-LTK63. Samples were collected at day 66 after primary immunization from mice immunized with two doses (66*) and at 24 and 66 days from mice immunized with four doses. (A) Serum anti-LTK63 or anti-*Salmonella* total IgG titers; (B) mucosal anti-LTK63 or anti-*Salmonella* total IgA titers. Mean values of each group were compared by Student's *t* test. *P* values of ≤ 0.001 (A) and ≤ 0.01 (B) were considered statistically significant.

Analysis of mucosal antibody response. Intestinal mucus samples from mice immunized with the *S. typhimurium* strains reported in Table 2 were analyzed for LTK63-specific and *Salmonella*-specific IgA responses. The mean IgA titers of each group of seven mice are shown in Fig. 3B and 4B. The anti-LTK63 secretory response was clearly higher in mice inoculated with the medium-high- and high-episomal-expression constructs (Fig. 4B) than in mice immunized with the *eltK63* integrants (Fig. 3B), with the exception of the group immunized with strain χ 3987-18A. The high anti-LTK63 IgA titers in the latter group of mice correlate well with the anti-LTK63 serum IgG titers found in the same group (Fig. 3A), although the variation of titers in individual mice was greater for the IgA samples than for the IgG samples.

A correlation was observed also between the anti-*Salmonella* IgG (Fig. 4A) and IgA titers (Fig. 4B) of mice immunized with four doses of the strains carrying plasmid pYA3149-LTK63 or pYA3137-LTK63. Animals inoculated with strain χ 3987:pYA3137-LTK63 always showed higher systemic and local immune responses than the other two groups of mice. Moreover, after the fourth immunization, higher IgG titers were paralleled by higher IgA titers in all groups (Fig. 4, day 66).

The stability in vivo of the *eltK63* constructs was assessed by Southern blot analysis of genomic or plasmid DNA extracted from bacteria isolated from Peyer's patches and spleens of immunized animals. All strains were found to have maintained the *eltK63* construct. Western blot analysis of cell extracts from the same *S. typhimurium* isolates also confirmed that the level of LTK63 synthesis was unchanged after the passage in vivo (data not shown).

DISCUSSION

As most studies on attenuated *Salmonella* carriers have analyzed differences in the induction of immunity between levels of antigen expression (3, 4), selection for vector maintenance in vivo, or attenuation of the delivery system (20, 37), we have compared the immune responses induced by the same antigen when delivered by different strain-plasmid combinations. In particular, we have analyzed a single integration into the host DNA and multicopy episomal vectors in three Δ *cya* Δ *crp* Δ *asd* strains of different genetic backgrounds expressing a nontoxic mutant of the *E. coli* LT.

A first comparison of variation in antigen delivery by the same *S. typhimurium* strain in relation to different expression levels of the antigen was made by analyzing the systemic response in mice immunized with strain χ 3987 containing the low- and high-expression constructs. The lack of an LTK63-specific immune response in mice immunized with the low-expression construct, in contrast to high levels of IgG antibodies elicited by the high-expression vector, correlates with a fivefold difference in amount of LTK63 synthesized in vitro by the two constructs. This finding suggests that for this *S. typhimurium* strain, the threshold for induction of anti-LTK63 systemic response is high (see below). Alternatively, expression in vivo of LTK63 from the two plasmids may not correlate with what is seen in vitro, although expression in both constructs is driven by the same promoter.

An interesting finding of this experiment is that strain χ 3987:pYA3137-LTK63 induced anti-*Salmonella* IgG titers significantly higher, during the first 3 to 5 weeks postimmunization, than those induced by control strains or by low LTK63 producers. LTK63 has been previously shown to exert an adjuvant effect on immune response to different antigens administered to mice by different routes (10).

By comparing *S. typhimurium* strains with different genetic

backgrounds and expressing the same amount of LTK63 from a single integration site, the influence of strain variability could be inferred from the anti-LTK63 antibody response, while both systemic and local immune responses to *Salmonella* were not significantly different for the different strains. Only strain χ 3987-18A could elicit high LTK63-specific IgG and IgA titers. Considering that strain χ 4217-10E contains the *eltK63* gene integrated in the same locus of the virulence plasmid as strain χ 3987-18A and can persist longer in deep tissues, this result implies that very early events at the priming of the immune response are particularly critical in the induction of an anti-LT immune response (4). Furthermore, the χ 3987-18A genetic background may be intrinsically more effective in priming anti-LTK63 immune responses. The observation that high expression of the antigen is crucial during the early phase of the induction of the immune response is consistent with the finding by others that oral administration of *Salmonella* strains expressing LT-B induces a dramatic increase in the level of mRNA of some cytokines (e.g., interleukin-12) at mucosal sites within hours following immunization (1).

One of the advantages of the Δ *cya* Δ *crp*/*asd* system is the effectiveness of the *asd* selection for plasmid maintenance in vivo (7, 13, 24). As previously shown by other reports, the stability in vivo of the *asd* vectors used in this study for expression of LTK63 was found to be very high even for the high-copy-number plasmid pYA3137. It should be noted, however, that expression of LTK63 from the latter plasmid conferred an unusual phenotype to strain χ 4072 in vitro: lysis after static growth. For this reason, the analysis of immune response had to be extended to strains containing the medium-high-copy-number plasmid pYA3149. Comparison between both systemic and local antibody responses induced by UK-1 and SR-11 strains expressing LTK63 from the high- or the medium-high-copy-number plasmids clearly showed that, in addition to any influence of the *S. typhimurium* strain delivering the antigen, the level of expression of the heterologous antigen is critical for a high immune response. A 2-fold difference in amount of LTK63 produced by strain χ 3987:pYA3137-LTK63 was sufficient to enhance both IgG and IgA titers 10-fold. An adjuvant effect of LTK63 could be inferred also from these data, since higher *Salmonella*-specific titers correlated with higher expression of LTK63.

In conclusion, our data indicate that the same attenuation in different genetic background *S. typhimurium* strains, expressing the recombinant antigen at the same level, can produce significantly different immune responses. Moreover, delivery of the LTK63 antigen to the immune system by attenuated *S. typhimurium* strains is effective only when synthesis of the antigen is very high during the initial phase of invasion, while persistence of the *S. typhimurium* strain in deep tissues has only marginal influence.

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