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A continuous epitope from transmissible gastroenteritis virus S protein fused to *E. coli* heat-labile toxin B subunit expressed by attenuated *Salmonella* induces serum and secretory immunity

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Abstract

Antigenic site D from the spike protein of transmissible gastroenteritis virus (TGEV), which is a continuous epitope critical in neutralization, has been expressed as a fusion protein with *E. coli* heat-labile toxin B subunit (LT-B) in attenuated *S. typhimurium*. Synthetic peptides containing the sequence of site D induced TGEV neutralizing antibodies when inoculated subcutaneously in both rabbits and swine. A synthetic oligonucleotide encoding residues 373–398 of TGEV S protein, including antigenic site D, was cloned in frame with the 3' end of LT-B gene, into a plasmid used to transform *S. typhimurium* Δ asd χ 3730. A collection of 6 recombinant plasmids designated pYALTB-D I–VI encoding LTB-site D fusions with a variable number of site D sequences were selected. Four of the 6 LTB-site D fusion products expressed in *S. typhimurium* χ 3730 formed oligomers (pentamers) that dissociated at $> 70^\circ$. *S. typhimurium* χ 3730 (pYALTB-D) V and VI expressed the oligomer forming products with higher antigenicity. Partially purified LTB-site D fusion product expressed from *S. typhimurium* χ 3730 (pYALTB-D) V induced anti-TGEV neutralizing antibodies in rabbits. Recombinant vaccine strain *S. typhimurium* Δ cya Δ crp Δ asd χ 3987 transformed with plasmid pYALTB-D V expressed constitutively products that formed oligomers presumably containing 20 copies of site D, and showed a high stability *in vitro*. This recombinant strain was orally inoculated in rabbits and induced TGEV specific antibodies in both serum and intestinal secretion.

Keywords: Transmissible gastroenteritis virus; TGEV; Coronavirus; Mucosal immunity; *Salmonella typhimurium*

1. Introduction

Transmissible gastroenteritis virus (TGEV) is a coronavirus that causes enteric disease in swine of all ages. The disease is especially severe in newborn animals less than 2 weeks old, in which

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mortality approaches 100% (Saif and Wesley, 1992). TGEV spike protein (S) is the major inducer of neutralizing antibodies (Garwes et al., 1978; Jiménez et al., 1986; Laude et al., 1986). In this protein four antigenic sites (A, B, C and D) have been defined (Delmas et al., 1986; Jiménez et al., 1986; Correa et al., 1988; Correa et al., 1990; Delmas et al., 1990), site A being antigenically dominant. Sites A and D, and to a minor extent site B, have been involved in the neutralization of TGEV (Delmas et al., 1986; Jiménez et al., 1986). Sites A and B are conformational and glycosylation dependent, while sites C and D are continuous and glycosylation independent, although a small effect of glycosylation has been observed in site D (Correa et al., 1988; Correa et al., 1990; Posthumus et al., 1990a; Posthumus et al., 1990b; Gebauer et al., 1991).

Passive immunity is of primary importance in providing newborn piglets with immediate protection against TGEV infection (Saif and Wesley, 1992). This type of protection can be achieved by the induction of lactogenic immunity (Hooper and Haelterman, 1966; Stone et al., 1977; Wesley et al., 1988; de Diego et al., 1992), which can be stimulated by presentation of selected antigens to the immune system in gut associated lymphoid tissues (GALT) (Montgomery et al., 1974). One possible approach to deliver antigens to the GALT is the use of vectors with tropism for Peyer's patches, such as *Salmonella* (Curtiss, 1990). *S. typhimurium* Δ *cya* Δ *crp* mutants, which are deficient in the synthesis of adenylate cyclase and the cyclic AMP (cAMP) receptor protein (CRP), are both avirulent and immunogenic in orally immunized mice and swine, while retaining their ability to colonize and persist in the GALT (Curtiss and Kelly, 1987; Coe and Wood, 1992). The expression of heterologous antigens as fusion products with *E. coli* heat-labile toxin B subunit (LT-B) in attenuated *Salmonella* has been described as an optimal strategy for the induction of secretory immunity against foreign proteins (Pierce et al., 1980; Spangler, 1992). LT consists of 5 non-covalently bound B subunits and 1 A subunit. The non-toxic B subunits are responsible for binding the protein to GM₁ ganglioside located on the surface of intestinal epithelial cells,

while the A subunit bears enzymatic activity. LT and its B subunits are potent oral immunogens which can function also as oral adjuvants eliciting high-titer serum and secretory antibodies when administered into the gut (Houghten et al., 1985; Lipscombe et al., 1991). Several *asd*⁺ LT-B⁺ plasmids allowing fusions of foreign genes to the C terminus of LT-B have been constructed (Jagusztyn-Krynicka et al., 1993). The *asd*⁺ plasmids ensure stable in vivo maintenance in attenuated Δ *asd* *S. typhimurium* vaccine strains in the absence of external selection. This is because the Δ *asd* mutation confers a requirement for diaminopimelic acid (DAP) (which is not present in animals), such that loss of the vector leads to cell death (Nakayama et al., 1988; Galán et al., 1990).

A short sequence representing site D from TGEV S protein was chosen to be fused to the LT-B gene. Several continuous epitopes have been identified in TGEV S protein, but only antigenic site D has shown to be critical in neutralization (Posthumus et al., 1990a; Gebauer et al., 1991; Lenstra et al., 1992). Site D has been identified between residues 378–395 of the S protein measuring the binding of specific MAbs to short overlapping peptides derived from the S amino acid sequence (the PEPSCAN method) (Posthumus et al., 1990a; Gebauer et al., 1991). Antigenic site D elicits antibodies with four epitope specificities defined by MAbs 1D.G3, 8D.H8, 57.57 and 57.51 (Sánchez et al., 1990; Posthumus et al., 1990a). All TGEV strains that have been tested maintain one or more of these epitopes, which makes unlikely the appearance of escaping mutants for all the epitopes in one virion. In this work, the construction of attenuated *S. typhimurium* Δ *cya* Δ *crp* vectors expressing TGEV site D sequences as fusion products with LT-B and their potential use as bivalent oral vaccines is described.

2. Results

In previous experiments we have shown that synthetic peptide D containing the sequence of site D from TGEV S protein (CYTVSDSSFF-SYGEIPFGVTDGPRYC) induced TGEV specific neutralizing antibodies when subcutaneously

inoculated into rabbits (Posthumus et al., 1991). This peptide includes residues 373–398 of TGEV S protein and contains the core sequence SFF-SYGEI recognized by neutralizing MAbs specific for site D of the S protein (Posthumus et al., 1990a). It was of interest to determine if site D would also elicit TGEV specific antibodies in swine, since this animal is the objective of a potential TGEV vaccine. Peptide D was oxidized with $(\text{NH}_4)_2\text{CO}_3$ to form cyclic monomers via a disulfide bridge between the N- and C-terminal cysteines and used to immunize rabbits and swine without coupling to a carrier protein. Animals were subcutaneously inoculated with 400 μg of peptide in Freund's complete adjuvant (FCA) on day 1 and received subsequent boosters of 200 μg of peptide on days 35 and 57. Samples of sera were taken on day 65 and assayed by RIA against TGEV and peptide D as described (Sanz et al., 1985). Both rabbit and swine sera bound to the synthetic peptide D and to TGEV (Table 1). All the animals seroconverted. Furthermore, the specific TGEV antibodies raised in both animal species had neutralizing activity against the virus. Neutralization index was measured as previously

described (Jiménez et al., 1986). Both for the neutralization and RIA assays the strain PUR46-MAD of TGEV was used (Sánchez et al., 1990).

LT-B was selected to be fused to site D sequence to enhance the immunogenicity of this epitope for oral administration. It was also of interest to express multiple repeats of site D fused to LT-B to increase the immunogenicity of this epitope. Two synthetic complementary oligonucleotides encoding residues 371–400 of TGEV S protein, corresponding to site D sequence, were hybridized, cloned into the *asd*⁺ LT-B expression plasmid pYA3048 (*asd*⁺ LT-B⁺) (Jagusztyn-Krynicka et al., 1993), and electroporated into the restriction negative, modification positive strain *S. typhimurium hsdLT hsdSA hsdSB Δ asd χ 3730* (pYA232) (Curtiss et al., 1988). The vector:insert ratio used in the ligation was 1:10 in order to obtain a collection of recombinant plasmids in which the LT-B genes were fused to a variable number of sequences of site D combined in the two possible orientations. The nucleotide sequence of site D that was cloned (5'-ATCTCCT-GTTACACCGTTTCCGACTCCTCCTTCTTCT-CCTACGGTGAAATCCCGTTCGGTGTTAC-CGACGGTCCGCGTTACTGCTACGTT-3') was not exactly the same as that of the S gene from TGEV genome, since some triplets present in the viral sequence were substituted for other synonymous triplets of high frequency of use in Enterobacteriaceae. The fusion of site D sequence to LT-B was done to the carboxyl end of this molecule through a linker coding for 6 amino acids containing two prolines, in order to preserve the molecular properties of LT-B, such as pentamerization and GM1 binding (Jagusztyn-Krynicka et al., 1993).

Transformants were induced with isopropyl β -D-thiogalactopyranoside (IPTG) 1 mM and screened by colony blot using a mixture of MAbs specific for site D. Recombinant plasmids expressing LTB-site D fusions were designated pYALTB-D and classified into 6 families according to the molecular mass of the expression product (Fig. 1A). The families were designated pYALTB-D I–VI, pYALTB-D I being the plasmid encoding the smallest expression product (15 kDa) and pYALTB-D VI the one encoding the largest re-

Table 1
TGEV and peptide D specific antibody titers and neutralization index in sera from peptide D immunized animals^a

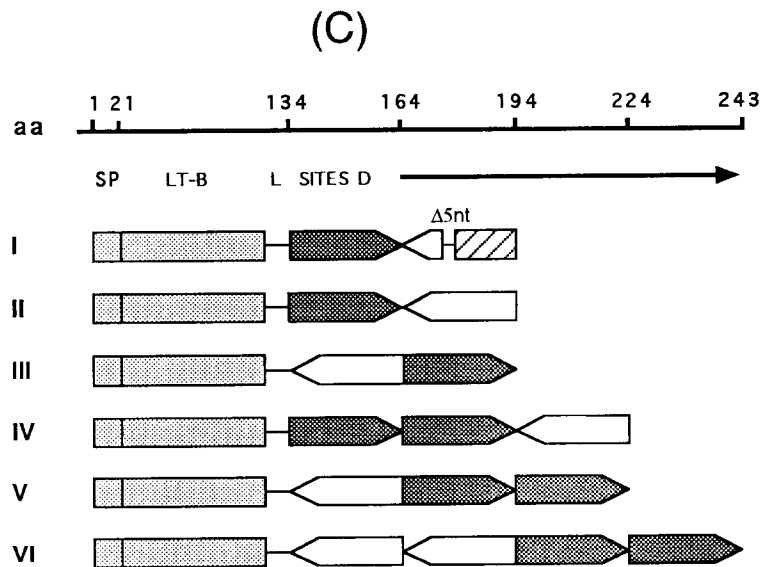
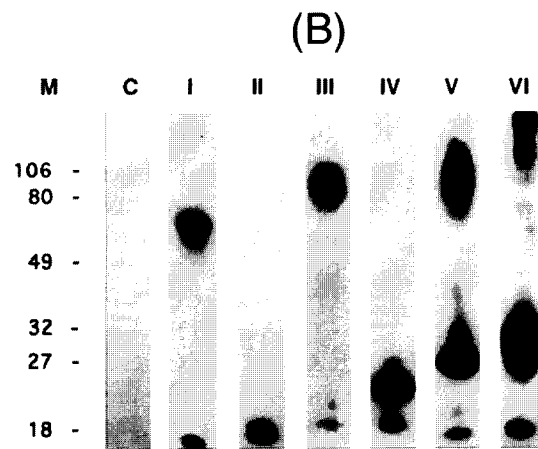
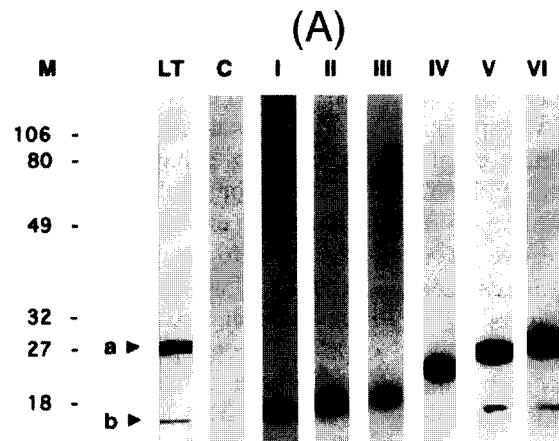
Animal ^b	Titer, RIA ^c		N.I. for TGEV ^d
	TGEV	Peptide D	
Control rabbit	<1.0	<1.0	<0.1
Rabbit	4.1	4.5	1.0
Control swine	<1.0	<1.0	<0.1
Swine	3.3	4.2	1.0

^aTwo-month old rabbits (New Zealand White) and 2-month old haplotype defined miniature swine (Sachs et al., 1976) were used.

^bSamples of sera (day 65) were pooled in each case ($n = 3$).

^cRIA titer is expressed as the $-\log_{10}$ of the last dilution that bound 3-fold the background radioactivity.

^dNeutralization index was determined by dividing the number of PFU of virus per ml mixed with non-immune serum by the number of PFU of virus per ml in the presence of immune serum. N.I. is expressed as the \log_{10} of this ratio.



combinant product (30 kDa). The ability of these recombinant products to form pentamers was analyzed by Western blot using samples that had not been previously heated (pentamers dissociate into constituent monomers only after heating to $> 70^{\circ}\text{C}$). Fusion proteins expressed by pYALTB-D I, III, V and VI kept their ability to form heat-labile oligomers (pentamers) (Fig. 1B). However, products from pYALTB-D II and IV did not form oligomers.

Plasmids pYALTB-D I–VI were sequenced to determine the structure of the 6 recombinant LTB-site D products (Fig. 1C). Plasmids pYALTB-D I–III contained one single site D sequence in the right orientation while pYALTB-D IV, V and VI contained two right oriented site D sequences. Recombinant products pYALTB-D II and IV, which did not form oligomers, had in common the expression of one site D reverse sequence at the carboxyl end of the chimerical protein.

The immunogenicity of LTB-site D expression products was also determined. The fusion product encoded by pYALTB-D V was selected to study its immunogenicity since it has shown to be the most antigenic by Western blot. pYALTB-D V expression product was partially purified from a saturated culture of *S. typhimurium* $\chi 3730$ (pYALTB-D) V induced with IPTG. The purification was carried out by $(\text{NH}_4)_2\text{SO}_4$ precipitation and preparative PAGE-SDS (Fig. 2A). Rabbits were inoculated with 150 μg of partially purified recombinant product that was distributed by subcutaneous, intradermal and intramuscular routes. Animals received a first dose of antigen emulsified in FCA and subsequent boosters on days 17 and 32 with antigen emulsified in Freund's incomplete adjuvant (FIA). Sera from day 39 (from 3 animals) were analyzed by RIA against TGEV, syn-

thetic peptide D and LT. All inoculated animals developed serum antibodies that at day 39 bound to TGEV, peptide D and LT. These sera also showed neutralizing activity against TGEV. RIA titers and neutralization index (N.I.) for pooled sera are shown in Table 2.

Plasmid pYALTB-D V was electroporated into the attenuated *S. typhimurium* $\Delta\text{cya}\Delta\text{crp}\Delta\text{asd}$ $\chi 3987$ (Curtiss and Kelly, 1987) strain in order to obtain recombinant vectors constitutively expressing LTB-site D hybrid proteins. Transformants were selected by plating on LB agar without DAP and constitutive expression of the recombinant product was studied by Western blot analysis using site D-specific MAbs. *S. typhimurium* $\chi 3987$ (pYALTB-D) V selected colonies showed a similar level of LTB-site D expression than that observed in the inducible strain $\chi 3730$. The recombinant product expressed constitutively, also associated to form heat-labile oligomers (Fig. 2B).

The stability of the expression of $\chi 3987$ (pYALTB-D) V was studied in vitro. Recombinant bacteria were grown during 50 generations and a representative number of colonies was analyzed at 25 and 50 generations. In these colonies the expression of the recombinant product was determined by Western blot using site D-specific MAbs. Recombinant *S. typhimurium* $\chi 3987$ (pYALTB-D) V showed a high degree of stability in vitro, since 100% of the bacteria analyzed after 50 generations maintained the expression of the recombinant product. $\chi 3987$ (pYALTB-D) V was selected to be tested as a bivalent vector to induce immunity against *Salmonella* and TGEV.

A group of rabbits were orally inoculated with *S. typhimurium* $\chi 3987$ (pYALTB-D) V. Control animals were orally inoculated with *S. typhimurium* $\chi 3987$ (pYA3048). An RIA assay was

Fig. 1. Recombinant *S. typhimurium* $\chi 3730$ (pYALTB-D) expression products. (A) Families of LTB-D fusion proteins expressed from IPTG induced $\chi 3730$ (pYALTB-D) colonies were analyzed by Western blot with site D-specific MAbs after heating the samples at 70°C in the presence of SDS and 2-mercaptoethanol. (B) Analysis of the formation of LTB-site D pentamers by Western blot of the same samples without heat treatment. (C) Structure of LTB-site D fusion products determined by restriction enzyme analysis and sequencing of pYALTB-D plasmids. LT-B has been represented as rectangles to the left, and site D monomers as repetitive units to the right. Shaded units represent site D monomers in the correct orientation. Units in white represent site D monomers in reverse sense. Striped areas are not expressed. I–VI, recombinant *S. typhimurium* $\chi 3730$ (pYALTB-D) I–VI; C, $\chi 3730$ (pYA3048); LT, AgNO_3 stained A and B subunits of purified *E. coli* heat-labile toxin (ICN, Ohio); M, molecular mass markers; SP, signal peptide; L, linker; D, deletion of a number of nucleotides shown to the right of this symbol.

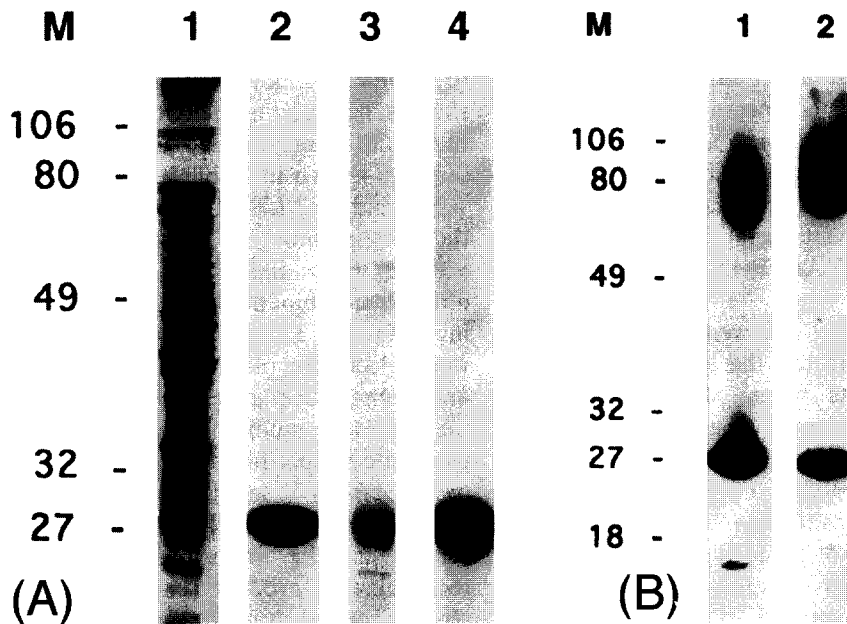


Fig. 2. Characterization of a purified LTB-site D recombinant product and its constitutive expression in *S. typhimurium*. (A) Purification of pYALTB-D V expression product by preparative PAGE-SDS and electroelution from IPTG induced *S. typhimurium* χ 3730. The total amount of protein was determined in every step of the purification procedure by PAGE-SDS and AgNO₃ staining (lanes 1 and 3). In parallel, the presence of pYALTB-D expression product in each sample was determined by Western blot analysis with site D-specific MAbs (lanes 2 and 4). Lanes 1 and 2, total protein after precipitating a lysate of the recombinant bacteria with (NH₄)₂SO₄ (100 mg/ml); lanes 3 and 4, total protein after cutting and electroeluting a polyacrylamide band containing expression product. (B) Constitutive expression of LTB-site D in *S. typhimurium* χ 3987. This strain was electroporated with recombinant plasmid pYALTB-D V and the expression of the LTB-site D fusion product was analyzed by Western blot with site D-specific MAbs (1). *S. typhimurium* χ 3730 (pYALTB-D V) was used as a positive control (2). *S. typhimurium* χ 3987 (pYA3048) was used as a negative control and it was not recognized by site D-specific MAbs (results not shown). Samples were not heated in order to study oligomer formation. M, molecular mass markers.

performed in order to evaluate both the IgG and IgA response. Briefly, 0.25 μ g of partially purified TGEV (PUR 46-MAD strain) was adsorbed to

each well of a polyvinyl disposable flat-bottom plate (Titertek, Flow Laboratories) diluted in 50 μ l of PBS, by overnight incubation at 37°C.

Table 2

Serum antibody response in rabbits immunized with partially purified pYALTB-D V expression product^{a,b}

Antigen	Titer, RIA ^d			
	TGEV	Peptide D	LT	N.I. for TGEV ^e
Control ^c	0.1	0.1	0.1	0.1
pYALTB-D V	1.9	1.8	2.06	1.75

^aTwo-month old rabbits (New Zealand White) were used.

^bSamples of sera (day 39) were pooled in each case ($n = 3$).

^cControl animals were immunized with an equivalent amount of material electroeluted from polyacrylamide containing no protein emulsified in FCA (first dose) or FIA (boosters).

^dRIA titer is expressed as described in Table 1.

^eNeutralization index was determined as described in Table 1.

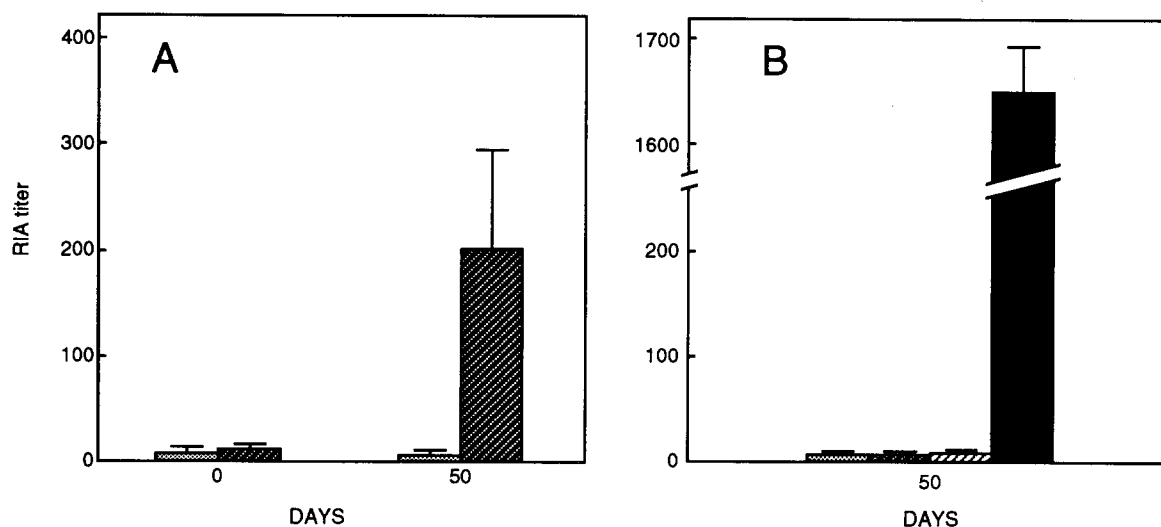


Fig. 3. TGEV specific antibody response in rabbits orally inoculated with *S. typhimurium* χ 3987 (pYALTB-D) V. Six animals were orally immunized with 2×10^9 cfus of *S. typhimurium* χ 3987 (pYALTB-D) V on days 1, 5, 10 and 40. As control, 2 rabbits were inoculated with a similar dose of *S. typhimurium* χ 3987 (pYA3048) on the same days. Sera and intestinal secretion from the immunized animals were collected on day 50 and the antibody response to TGEV was assayed by RIA. (A) TGEV specific IgG response in serum and (B) IgG and IgA response in intestinal secretion. Reported values are the mean \pm standard deviation of RIA titers from the two animals that responded to TGEV (or from animals used as controls). RIA titers were determined as described in Table 1. Symbols: () IgG and () IgA response in rabbits immunized with *S. typhimurium* χ 3987 (pYA3048), respectively; () IgG and () IgA response in rabbits immunized with *S. typhimurium* χ 3987 (pYALTB-D) V.

Plates were blocked by adding 200 μ l of 5% bovine serum albumin (BSA) in PBS to each well and incubating for 2 h at 37°C. Fifty μ l of several dilutions of sera or intestinal secretion from the inoculated rabbits were added to wells and incubated at room temperature for 3 h. Direct incubation with 125 I-labeled protein A was used to determine the IgG response. Duplicates of the intestinal secretion samples were incubated with goat anti-rabbit sIgA (Cappel) as a second antibody prior to incubation with 125 I-labeled protein G in order to determine the IgA response. One third of the rabbits that received the recombinant bacteria showed TGEV specific antibodies in both serum and intestinal secretion (Fig. 3A and Fig. 3B), indicating that both systemic and secretory immunity had been induced in these animals. In serum the response was of the IgG isotype, while in intestinal secretion only IgA was induced. The antibodies induced in intestinal secretion showed a neutralization index of one logarithmic unit above the control, while in serum no neutralizing

activity was detected, probably due to the low antibody titer. Samples of intestinal secretions were diluted 1:10 in PBS supplemented with fetal calf serum 2% and used in the neutralization assay as it has been previously described (Jiménez et al., 1986). Both strains of bacteria used in the experiment also induced *Salmonella* specific antibodies in serum and secretions of all the inoculated animals (results not shown)

In this study, recombinant bacteria expressing a TGEV epitope fused to LT-B has induced antibodies that recognize viral particles in orally inoculated rabbits. A similar epitope derived from site D fused to *E. coli* CS31 capsule-like antigen also induced TGEV specific antibodies when the purified recombinant product was used to immunize mice. Live bacteria expressing this hybrid protein, however, failed to induce antibodies that recognized virus particles when inoculated intraperitoneally in mice (Bousquet et al., 1994). An effective vaccine against TGE requires the induction of secretory immunity in swine. The recombi-

nant vectors tested in this work induced a TGEV neutralizing response in intestinal secretions which makes them suitable candidates to be tested as oral vaccines in swine. The immunogenicity of these recombinant vectors in orally inoculated swine will be investigated using containment facilities for large animals with a P3 safety level.

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