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Characterization of transmissible gastroenteritis coronavirus S protein expression products in avirulent *S. typhimurium* Δcya Δcrp: persistence, stability and immune response in swine

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Abstract

The spike protein from transmissible gastroenteritis virus (TGEV) was expressed in attenuated *S. typhimurium* Δcya Δcrp Δasd χ3987. Three partially overlapping fragments of TGEV S gene, encoding the amino-terminal, intermediate, and carboxy-terminal end of the protein, as well as the full length gene were inserted into the asd^+ plasmid pYA292 to generate recombinant plasmids pYATS-1, pYATS-2, pYATS-3, and pYATS-4, respectively, which were transformed into *S. typhimurium* χ3987. Recombinant *S. typhimurium* χ3987 (pYATS-1) and χ3987 (pYATS-4) expressing constitutively a 53 kDa amino-terminal fragment of the S protein and the full length protein (144 kDa), respectively, showed high stability. After 50 generations in vitro 60% and 20% of the bacteria transformed with pYATS-1 and pYATS-4, respectively, expressed the S-protein antigen. Since *S. typhimurium* χ3987 (pYATS-1) showed a better level of expression and stability in vitro, this recombinant strain was selected as a potential bivalent vector to induce both immunity to *Salmonella* and TGEV in swine. In order to study colonization of swine tissues by *S. typhimurium* Δcya Δcrp, a gene conferring resistance to rifampicin was cloned into the chromosome of *S. typhimurium* χ3987, generating χ4509 strain. Both *S. typhimurium* χ4509 (pYA292) and χ4509 (pYATS-1) colonized the ileum of orally inoculated swine with clearance of bacteria between days 10–20 post-infection. The expression of the amino-terminal fragment of the S protein diminished the ability of *S. typhimurium* χ4509 (pYATS-1) to colonize deep tissues. The recombinant strain *S. typhimurium* χ3987 (pYATS-1) induced TGEV specific antibodies in both serum and saliva of orally inoculated swine.

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This strain, as well as *S. typhimurium* χ3987 (pYA292), also elicited both systemic and mucosal immunity to *Salmonella* antigens.

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

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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 two weeks old, in which mortality approaches 100% (Siddell et al., 1983; Saif and Wesley, 1992). TGEV has four structural proteins: the spike (S), the nucleoprotein (N), the membrane protein (M) and the small membrane (sM) protein (Spaan et al., 1988; Spaan et al., 1990; Godet et al., 1992; Enjuanes and Van der Zeijst, 1995). Protein S is the major inducer of TGEV neutralizing antibodies (Garwes et al., 1978; Sturman and Holmes, 1983; Jiménez et al., 1986). In this protein, four antigenic sites (A, B, C and D) have been defined by mutual competition of monoclonal antibodies (mAbs) (Delmas et al., 1986; Jiménez et al., 1986; Correa et al., 1988; Correa et al., 1990; Delmas et al., 1990). Site A is antigenically dominant and has been subdivided into three antigenic subsites: Aa, Ab and Ac (Correa et al., 1988). 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 have been shown to be 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 (Stone et al., 1977; Wesley et al., 1988), 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 III, 1990). A variety of attenuated *Salmonella* strains have been generated in laboratories worldwide (Bacon et al., 1950; Bacon et al., 1951; Germanier and Furer, 1971; Hoiseth and Stocker, 1981). *S. typhimurium Δcya Δcrp* 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, while retaining their ability to colonize and persist in the GALT (Curtiss III and Kelly, 1987). Several heterologous genes from different pathogens have been expressed in *S. typhimurium Δcya Δcrp*. The recombinant bacteria thus generated have been successfully used to deliver the foreign antigens to the GALT and induce secretory and systemic immune responses against both *Salmonella* and the recombinant antigen in different animal species, including swine (Stabel et al., 1990; Stabel et al., 1991; Doggett et al., 1993; Schödel et al., 1993). One main problem with live vector vaccines is the stability and maintenance of recombinant plasmids in vivo, since the use of drug-resistance genes as selection determi-

nants is impractical in this situation. Nakayama et al. (1988) designed a balanced-lethal system in which a gene for β-aspartate semialdehyde dehydrogenase (asd^+) from *Streptococcus mutants* was present in plasmid vectors to complement a lethal Δasd mutation in the chromosome of the *Salmonella* vaccine strain. The *asd*-encoded enzyme is required for the biosynthesis of diaminopimelic acid (DAP), an essential component of the peptidoglycan in the cell wall of Gram-negative bacteria. Growth of *asd* mutants in the absence of DAP leads to cell lysis. The asd^+ plasmids ensure stable in vivo maintenance in attenuated Δasd *S. typhimurium* in the absence of external selection (Nakayama et al., 1988; Galan et al., 1990). In the present work asd^+ plasmid pYA292 (Nakayama et al., 1988; Curtiss III et al., 1990) has been used to express several fragments of TGEV S protein in attenuated *S. typhimurium* Δcya Δcrp Δasd. The recombinant vectors obtained in this way were characterized and their potential use as bivalent oral live vaccines in swine has been evaluated.

2. Materials and methods

Bacterial strains, plasmids and growth conditions. *Escherichia coli* χ6097 (Nakayama et al., 1988) and *S. typhimurium* strains χ3730 and χ3987 (Curtiss III et al., 1990) were used. Plasmid pYA292 (asd^+ $lacZ\alpha$) (Galan et al., 1990) of 3.5 kb was used to express TGEV antigens as fusion proteins with the amino-terminus of β-galactosidase. Both *E. coli* χ6097 and *S. typhimurium* χ3730, used as intermediate strains, were previously transformed with $lacI^{q+}$ plasmids kan^{R+} F' (Yanisch-Perron et al., 1985) or tet^{R+} pYA232 (Nakayama et al., 1988), respectively, in order to regulate expression of recombinant products from pYA292-derived plasmids. In this strain expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma) 1mM. *S. typhimurium* χ4509 was generated from χ3987 by introducing into the chromosome of this strain a gene conferring resistance to rifampicin (Curtiss, unpublished data). All bacterial strains were grown aerobically at 37° on Luria-Bertani (LB) agar or broth and when appropriate, kanamycin (50 μg/ml), tetracycline (15 μ/ml), rifampicin (50 μg/ml) or DAP (50 μg/ml) (Sigma) were added.

Molecular cloning and DNA manipulation

The S gene from the strain PUR46-CC120-MAD of TGEV (Sánchez et al., 1990; Sánchez et al., 1992) had previously been cloned into Bluescript by synthesizing a cDNA from the viral genomic RNA. Three partially overlapping fragments of 1595, 2194 and 1298 bp coding for an amino-terminal, an intermediate and a carboxy-terminal fragment of the S protein, respectively, were obtained. The amino-terminal and carboxy-terminal fragments were subcloned into the expression plasmid pYA292 in frame with the 5′ end of lacZα gene. The intermediate fragment was also cloned into pYA292 by removing a fragment of 0.16 kb containing lacZα gene. Both S gene fragments and the plasmid pYA292 were digested with the appropriate restriction enzymes and purified from agarose gels prior to being ligated and electroporated into *E. coli* Δasd χ6097F'. Recombinant plasmids pYATS-1, pYATS-2 and pYATS-3 containing the 5′ end, intermediate and 3′ end of the S gene, respectively, were obtained (Fig. 1). The full length S gene was also cloned into pYA292 according to the following procedure: The 3′ fragment 3330–4628 was cut with *HindIII*

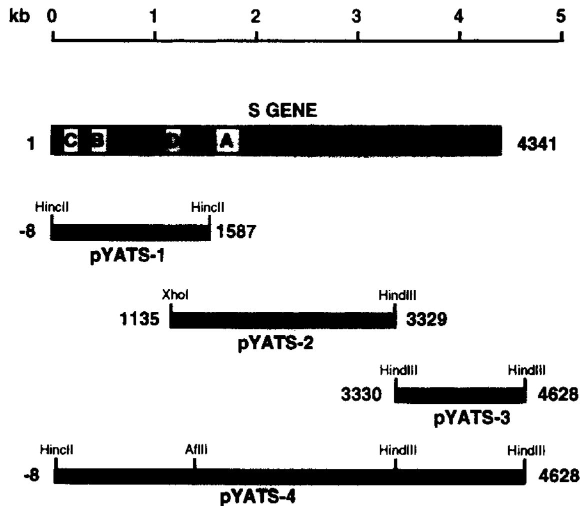

Fig. 1. TGEV S gene fragments cloned in expression plasmid pYA292. Recombinant plasmids pYATS-1, pYATS-2, pYATS-3 and pYATS-4 were obtained by the cloning of three partially overlapping fragments of TGEV S gene (-8-1587, 1135-3329 and 3330-4628) or the full length S gene (-8-4628), respectively. Restriction enzymes used in the cloning of the different fragments are indicated. The relative positions of the 4 dominant antigenic sites on the S gene are also shown (upper part).

from the Bluescript vector containing this insert (Correa et al., 1990). This fragment was cloned into the *HindIII* site of the Bluescript vector that contained the intermediate fragment of the gene (1135–3329), obtaining a recombinant plasmid which included an insert spanning the area 1135–4628 of the S gene. The fragment 1320–4628 was cut from this plasmid using the restriction sites AflII and PstI (from Bluescript polylinker) and ligated to pYATS-1, previously digested with AflII and PstI. The recombinant plasmid generated in this way (pYATS-4) was electroporated into *E. coli* χ6097F′.

Recombinant clones were screened for plasmid DNA by a modification of the Birnboim alkaline minilysate technique (Birnboim and Doly, 1979). Restriction enzyme digestions and ligation reactions were carried out as described by the manufacturers (Boehringer Mannheim, New England Biolabs).

Transformation of *S. typhimurium Δcya Δcrp Δasd.* Recombinant plasmids were purified from *E coli* χ6097F′ transformants and electroporated into the restriction-negative, modification-positive intermediate host *S. typhimurium* χ3730 (pYA232), which also carries the Δ*asd* mutation. Transformants of this strain were selected by plating on LB agar without DAP. The plasmids were then purified from *S. typhimurium* χ3730 transformants and electroporated into *S. typhimurium Δcya Δcrp Δasd* χ3987 or χ4509. Since rough *Salmonella* strains have low immunogenicity, all *S. typhimurium* χ3987 and χ4509 transformants were verified for smooth phenotype by checking bacteriophage P22 sensitivity (Zinder, 1958).

Electrophoretic techniques. DNA was electrophoresed through 0.7% agarose gels (Sigma) by using Tris-acetate-EDTA buffer (Sambrook et al., 1989). Bacteriophage λ DNA fragments generated by digestion with EcoRI/HindIII (B.M.) were used as molecular size standards. Electrophoresis of proteins was performed in SDS- 10% polyacrylamide

Table 1
Reactivity of S protein-specific MAbs with pYATS expression products[a]

Plasmid	TGEV polyclonal antiserum	MAb specificity				
		S protein				N protein
		Site C	Site D		Site A	
		5B.H1	1D.G3	8D.H8	MAb cocktail[b]	3B.H3[c]
pYA292	−	−	−	−	−	−
pYATS-1	+	+	+	+	−	−
pYATS-2	+	−	−	+	±	−
pYATS-3	−	−	−	−	−	−
pYATS-4	+	+	+	+	±	−

[a] +, reactive; ± weakly reactive; − non-reactive.
[b] Mixture of MAbs 6A.C3, 1A.F10, 1G.A7 and 1D.E7.
[c] MAb 3B.H3 was used as a negative control.

gels by the method of Laemmli (1970). Prestained SDS-PAGE standards (Bio-Rad, Ca) were used as molecular mass standards.

Colony immunoblot. Colonies were grown until they reached a diameter of approximately 2 mm and then were adsorbed to a nitrocellulose membrane. Bacteria were lysed by exposing the membrane to chloroform vapors for 20 min at 37°. Filters were washed for 2 h with washing buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Triton X-100) and incubated overnight at 4° with a TGEV-specific rabbit polyclonal antiserum, previously adsorbed with an *E. coli* or *S. typhimurium* lysate. Membranes were washed with washing buffer and incubated with secondary antibodies (goat anti-rabbit immunoglobulin coupled with alkaline phosphatase) diluted as recommended by the manufacturer (Sigma). Membranes were washed and incubated with AP buffer (100 mM TrisHCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for 10 min. Color was developed by exposure of the nitrocellulose to 5-bromo-4-chloroindoxyl phosphate and nitroblue tetrazolium as described (Sambrook et al., 1989).

Western blot analysis. Protein samples were separated by SDS-PAGE and then electrophoretically transferred to nitrocellulose sheets. The membrane was blocked for 2 h in blocking buffer (500 mM NaCl, 20 mM Tris-HCl pH 7.5, 5% bovine serum albumin) followed by overnight incubation at 4° with hybridoma supernatants specific for TGEV (diluted 1:10). The murine monoclonal antibodies (mAbs) used in this work and their specificities are listed in Table 1 and have been previously described (Jiménez et al., 1986; Correa et al., 1988). The nitrocellulose paper was washed, incubated at room temperature for 1 h with a 10^3 dilution of rabbit anti-mouse immunoglobulin (Cappel) preadsorbed with an *E. coli* or *S. typhimurium* lysate, washed, and incubated at room temperature for 2 h with ^{125}I-labeled Protein A (1×10^6 c.p.m./ml; 3×10^7 c.p.m./μg protein). The nitrocellulose paper was washed and subjected to autoradiography.

Measurement of plasmid stability in vitro. Fresh single colonies from LB plates of *S. typhimurium* χ3987 or χ4509 strains carrying *asd*$^+$ recombinant plasmids were inoculated into LB broth without DAP and incubated overnight at 37°. After a 10^{-3} dilution of overnight

culture in LB broth, 10 ml of the dilutent was incubated at 37°. After 10 generations the cultures were diluted and the procedure continued for 50 generations in the same type of medium. At appropriate times, samples of the cultures were diluted and spread on LB agar plates. A representative number of colonies (at least 20) were screened by both western blot and restriction analysis of plasmid DNA in order to determine the fraction of cells that maintained the expression of the recombinant product and a correct recombinant plasmid, respectively.

Determination of optimal conditions to inhibit growth of aerobic flora from swine intestine samples in vitro. Previous experiments in which *S. typhimurium* χ3987 was inoculated orally in swine showed that it was not possible to study colonization of these bacteria in the gut without using a selective method to eliminate most of the intestinal flora from tissue samples. It was of interest to introduce into *S. typhimurium* χ3987 a marker gene conferring resistance to an antibiotic that could inhibit the growth of some of the aerobic intestinal flora of swine in culture. All animals used in these experiments were two-month-old Large White x Belgian Landrace swine kindly provided by Laboratorios Sobrino (Spain). Portions of small intestine from three pigs were placed in cold minimal liquid medium (Curtiss III et al., 1968) with 15% sucrose (MLS) and homogenized using a Potter-Elvehjem tissue grinder. Samples were plated on brilliant green agar, MacConkey 1% maltose agar (ADSA Microbiología, Spain) or LB agar without antibiotic or in the presence of nalidixic acid, streptomycin, tetracycline or rifampicin (Sigma) (50μ/ml in all cases) and incubated overnight at 37°.

Oral immunization of swine. Swine were deprived of food and water 4 h before inoculation. Each pig was inoculated with of 10^{11} CFU of the appropriate strain of *S. typhimurium* suspended in 4 ml of buffered saline with gelatin (BSG) (Curtiss III, 1965). The inoculum was mixed with 4 ml of 10% (wt/vol) sodium bicarbonate prior to inoculation and administered by gastric intubation. Food and water were returned 30 min after inoculation.

Bacteria for inoculation were grown overnight at 37° in LB broth (adding rifampicin when growing *S. typhimurium* χ4509 strains). These cultures were diluted 1:100 into prewarmed LB broth and grown at 37° for approximately 5 h to an optical density at 600 nm of about 0.8 to 1.0. The cells were concentrated 25-fold by centrifugation at 8000 x *g* for 10 min at 4°, followed by suspension in BSG. Dilutions were plated on LB agar for titer determination.

Invasion, colonization, persistence, and immunity studies. Groups of two Large White x Belgian Landrace pigs inoculated with one single dose of *S. typhimurium* χ4509 (pYA292) or χ4509 (pYATS-1), as described earlier, were sacrificed on days 1, 5, 10, and 20 post-oral inoculation. Portions of different parts of the small intestine (duodenum, jejunum, and ileum), liver and spleen were removed from each animal, immediately placed in MLS and kept on ice. Samples were homogenized by using a Potter-Elvehjem tissue grinder, plated on brilliant-green agar containing 50 μg of rifampicin per ml and incubated overnight at 37°. The expression of the amino-terminal fragment of the S protein in *S. typhimurium* χ4509 (pYATS-1) colonies recovered from the inoculated animals was analyzed by western blot as it has been described previously.

For the evaluation of the immunity a group of 9 pigs were inoculated orally with *S. typhimurium* χ3987 (pYATS-1). As a negative control 3 animals were inoculated orally with *S. typhimurium* χ3987 (pYA292). All animals received 4 doses of 10^{11} CFU on days

1, 3, 5, and 31, respectively, as it has been described earlier. Samples of serum and saliva were taken on days 0, 31, and 38 and the anti-*Salmonella* and anti-TGEV specific immune responses were analyzed by radioimmunoassay (RIA).

RIA

Binding of sera from immunized animals to a protein extract of *S. typhimurium* χ3987 or to TGEV was determined by RIA. Briefly, 0.5 μg of *S. typhimurium* χ3987 protein extract or 0.25 μg of partially purified TGEV (PUR 46-MAD strain), respectively, were adsorbed to each well of a polyvinyl disposable flat-bottom plate (Titertek, Flow Laboratories) diluted in 50 μl of PBS, by overnight incubation at 37°. Plates were blocked by adding 200 μl of 5% bovine serum albumin (BSA) in PBS to each well and incubating for 2 h at 37°. Fifty μl of several dilutions of sera or saliva from the inoculated swine 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 (Sanz et al., 1985). Duplicates of the saliva samples were incubated with rabbit anti-swine sIgA (Bethyl Laboratories, Texas) as a second antibody prior to incubation with ^{125}I-labeled protein A in order to determine the IgA response. The titer in RIA was defined as the maximum antibody dilution that bound threefold the background radioactivity.

3. Results

3.1. Cloning of TGEV S gene in the expression plasmid pYA292

Positive colonies of *E. coli* χ6097F′ expressing the amino-terminal, the intermediate fragment, and the whole S protein from recombinant pYA292 plasmids were obtained. Colonies containing the recombinant plasmid coding for the carboxy-terminal fragment did not grow in the presence of IPTG, suggesting that the expression of this product is toxic in *E. coli*. Plasmid DNA was purified from positive clones and analyzed by digestion with restriction enzymes. All the colonies selected by expression carried plasmids which yielded the expected restriction analysis band pattern (results not shown). The recombinant plasmids obtained were designated pYATS-1, pYATS-2, pYATS-3 and pYATS-4, had a size of 5.09, 5.53, 4.79 and 8.13 kb, and contained the 5′ end, intermediate area, 3′ end, and the full length S gene, respectively (Fig. 1).

3.2. Expression of S protein recombinant products in S. typhimurium

The amino-terminal fragment of TGEV S protein, of approximately 53 kDa, was expressed from plasmid pYATS-1 in both *S. typhimurium* χ3730 (pYA232) and χ3987, being expressed to slightly higher levels in the IPTG inducible system (Fig. 2). A slight aggregation of this product was observed in these bacteria. The product expressed from pYATS-2, corresponding to the intermediate fragment of the S protein, had a molecular mass of 70 kDa and was expressed at high level in *S. typhimurium* χ3730 (pYA232). However, the recombinant *S. typhimurium* χ3987 (pYATS-2) had a low level of expression and was unstable. The band of lower molecular mass that appeared in both *S. typhimurium*

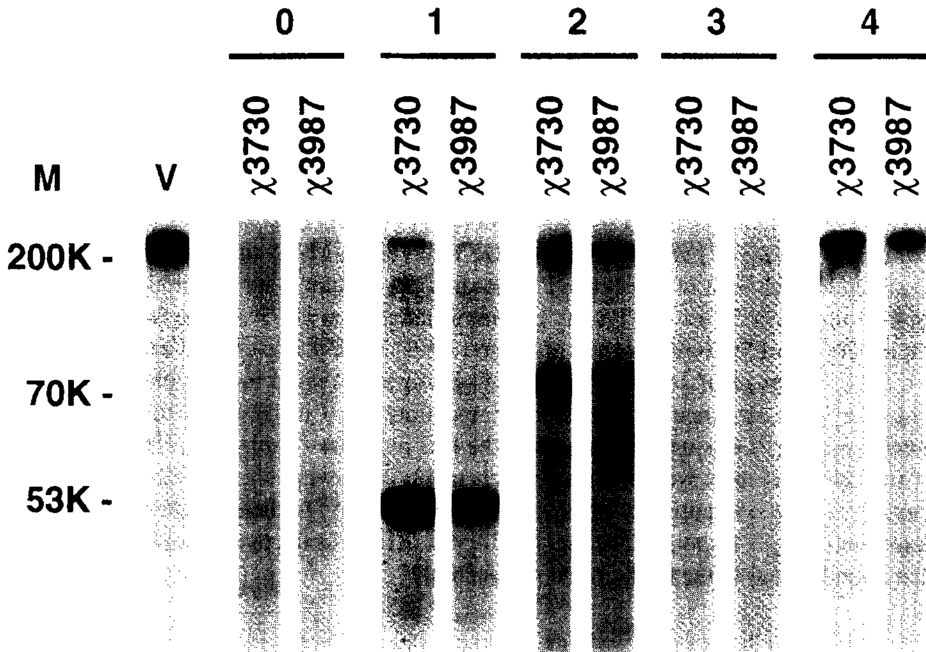

Fig. 2. Western blot analysis of TGEV S protein fragments expressed in *S. typhimurium*. *S. typhimurium* strains χ3730 and χ3987 were transformed with plasmid pYA292, containing no insert (0) or with the recombinant plasmids pYATS-1, pYATS-2, pYATS-3 and pYATS-4 containing the amino-terminal (1), intermediate (2), carboxy-terminal (3) fragments of the S gene, or the full length S gene (4), respectively. Expression of these recombinants was analyzed by western blot using TGEV S protein-specific mAbs. V, TGEV S protein; M, Molecular mass markers.

χ3730 and χ3987 (pYATS-2) (Fig. 2) corresponded to a degradation product of the intermediate fragment, since it was not present in the negative control. pYATS-2 expression product also aggregated in both bacterial systems. The expression of the carboxy-terminal fragment of the S protein was highly toxic in the two bacterial systems, as shown by the lack of growth of *S. typhimurium* χ3730 (pYA232, pYATS-3) in the presence of IPTG or the absence of colonies obtained when electroporating pYATS-3 into *S. typhimurium* χ3987. The whole S protein, with an expected size of 144 kDa, was expressed from pYATS-4 transformed bacteria as an aggregate of high molecular mass. The level of expression of this recombinant protein was also higher in the IPTG inducible strain than in *S. typhimurium* χ3987. Expression of S protein antigenic sites in each of the recombinant products was determined by western blot with specific mAbs and is summarized in Table 1.

3.3. Stability of recombinant S. typhimurium expressing TGEV antigens in vitro

The stability of *S. typhimurium* χ3987 (pYATS-1) and χ3987 (pYATS-4) recombinants showing higher levels of constitutive expression of TGEV antigens was studied in vitro at the level of antigen expression and plasmid DNA. *S. typhimurium* χ3987 (pYATS-1) showed a good level of stability in the expression of the amino-terminal fragment of the S protein, since after 25 and 50 generations 100% and 60% of the colonies, respectively,

Table 2
Sensitivity of swine aerobic intestinal flora to different antibiotics in vitro

	CFU/g of tissue in presence of antibiotic[c]				
Agar	No antibiotic	Nalidixic acid	Rifampicin	Streptomycin	Tetracycline
LB	3.3×10^5	4.2×10^4	2×10^2	2.7×10^5	3×10^5
BG[a]	6.6×10^4	9×10^2	0	2.5×10^3	1×10^5
MacConkey/mal.[b]	3×10^5	5×10^1	0	3×10^5	3×10^5

[a]Brilliant-green agar.
[b]MacConkey agar 1% maltose.
[c]The antibiotic concentration was 50 μg/ml in all cases.

expressed a product of the correct size as determined by western blot. *S. typhimurium* χ3987 (pYATS-4) showed a good but lower stability in the expression of the whole S protein, reflected by the fact that 50% and 20% of the colonies after 25 and 50 generations, respectively, were positive by western blot. The stability at the level of plasmid DNA was apparently much higher: in both cases 90% of the colonies carried plasmids that yielded a correct band pattern by restriction enzyme analysis. Recombinant *S. typhimurium* χ3987 (pYATS-1) was selected to be tested as a possible bivalent vector to induce immunity against *Salmonella* and TGEV in swine.

3.4. Colonization and stability of recombinant S. typhimurium expressing TGEV antigens in swine

Rifampicin was the most effective antibiotic in inhibiting the growth of aerobic flora from swine intestine samples in vitro (Table 2). Practically 100% of the flora that grow in the conditions of the assay were completely inhibited by this antibiotic in the different media that were used. The gene conferring resistance to rifampicin was cloned into the chromosome of *S. typhimurium* χ3987 obtaining strain χ4509 (Curtiss, unpublished data). Recombinant *S. typhimurium* χ4509 (pYATS-1) expressing the amino-terminal fragment of the S protein showed levels of expression and stability similar to those of *S. typhimurium* χ3987 (pYATS-1).

When orally inoculated in swine, both *S. typhimurium* χ4509 (pYA292) and χ4509 (pYATS-1) colonized the ileum to similar levels, which ranged between 10^3–10^4 and 10^2–10^3 CFU/g of tissue or intestinal content at days 1 and 10 after inoculation, respectively. At 20 days post-inoculation, no bacteria could be recovered from the gut of the infected animals. *S. typhimurium* χ4509 (pYA292) also colonized the liver, since 10^2 CFU/g could be isolated from this organ at day 10, but not from the spleen at any time post-infection. The recombinant *S. typhimurium* χ4509 (pYATS-1) did not colonize internal tissues, such as liver or spleen. At 24 h postinoculation 100% of *S. typhimurium* χ4509 (pYATS-1) colonies expressed the recombinant product. This percentage was reduced to 70% at day 5 and 50% at day 10.

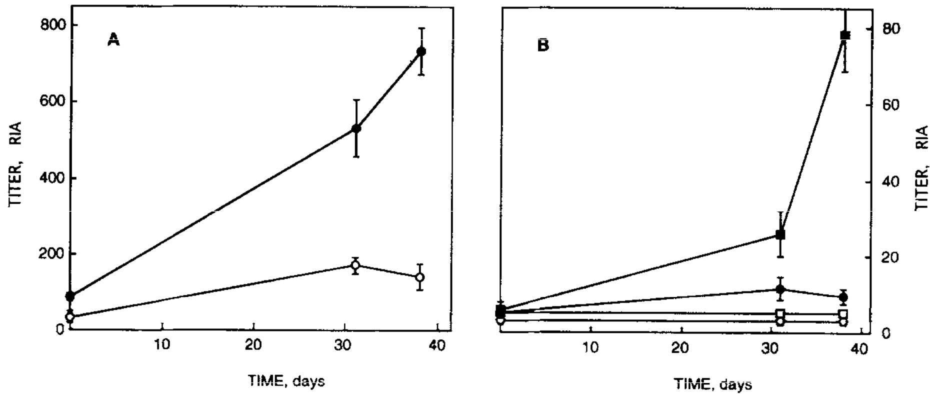

Fig. 3. TGEV specific immune response in sera and saliva of swine orally inoculated with *S. typhimurium* χ3987 (pYA292) and χ3987 (pYATS-1). Sera and saliva from the immunized animals were collected on days 0, 31, and 38 and the presence of TGEV specific antibodies was determined by RIA. A, immune response in serum; B, immune response in saliva. Symbols: (●) IgG and (■) IgA titer in swine immunized with χ3987 (pYATS-1), respectively; (○) IgG and (□) IgA titer in swine immunized with χ3987 (pYA292), respectively. Reported values are the mean of three animals ± standard deviation (only the three animals that responded to TGEV are represented).

3.5. Immune response induced by recombinant S. typhimurium expressing TGEV antigens in swine

One third of the pigs that were inoculated with *S. typhimurium* χ3987 (pYATS-1) showed TGEV specific antibodies in both serum and saliva (Fig. 3), indicating that both systemic and secretory immunity had been induced in these animals. In serum the response was of the IgG isotype, while in saliva only IgA was induced. Both *S. typhimurium* χ3987 (pYA292) and χ3987 (pYATS-1) induced *Salmonella* specific antibodies in serum and secretions of all the inoculated animals with titers in RIA of 10^3 for serum IgG and 10^2 for secretory IgA.

4. Discussion

An attenuated strain of *Salmonella typhimurium* carrying mutations *Δcya* and *Δcrp* has been used to express different constructs based on TGEV S protein, which is the dominant antigen recognized by the porcine immune response against this virus.

Comparison of expression of the four recombinant products (pYATS-1-4) in three strains of bacteria (*E. coli* χ6097, *S. typhimurium* χ3730 and χ3987) showed that the amino-terminal and intermediate fragments encoded by pYATS-1 and pYATS-2, respectively, and the full length S protein encoded by pYATS-4 had similar expression levels in IPTG inducible systems. However, when these constructs were expressed constitutively in *S. typhimurium* χ3987 the expression levels were much lower. This probably indicates that the recombinant products have some toxicity for *S. typhimurium* χ3987, which led to the selection of recombinants with lower levels of expression. The carboxy-terminal fragment of the S protein, encoded by pYATS-3, showed a high degree of toxicity in the three

bacterial systems. The high hydrophobicity of this region could be responsible of its toxicity in bacterial systems (de Groot et al., 1987; Rasschaert and Laude, 1987).

The full length S protein encoded by plasmid pYATS-4 was expressed as a high molecular mass aggregate in both IPTG inducible and constitutive expression systems. It has been reported that glycoprotein S aggregates when expressed in TGEV infected swine testicle (ST) cells in the presence of glycosylation inhibitors, such as tunicamycin (Gebauer et al., 1991). The fact that prokaryotic organisms lack glycosylation systems could explain the observed aggregation of the antigen.

The four recombinant products expressed in *S. typhimurium* χ3987 included sequences from S protein involved in the formation of the different antigenic sites that have been described in this protein. It was of interest to determine the expression of these antigenic sites in each of the recombinant products. According to the sequence of amino acids encoded by each of the recombinant plasmids only pYATS-4 could express the four antigenic sites. Recombinant product pYATS-1 included sequences from sites C, B, and D, while pYATS-2 included sites D and A. Product pYATS-3 containing the carboxy-terminal end of the protein was the only recombinant that did not include any of S antigenic sites. Antigenic sites C and D, which are nonconformational and glycosylation independent (although a small effect of glycosylation has been observed on site D) (Posthumus et al., 1990a; Gebauer et al., 1991), were correctly expressed as determined by western blot. Both site C, located near the amino-terminal end, and site D, located between nucleotides 1134–1185, were recognized in the products encoded by pYATS-1 and pYATS-4. The fact that only site D-specific mAb 8D.H8 reacted with pYATS-2 product (1135–3229) could be due to a partial representation of this site in the intermediate fragment of the protein. This confirmed that mAbs 8D.H8 and 1D.G3 recognized different site D epitopes (Gebauer et al., 1991). Site A, which is conformational and glycosylation dependent, (Correa et al., 1988; Delmas et al., 1990; Gebauer et al., 1991) was weakly detected in recombinant products expressed by pYATS-2 and pYATS-4.

S. typhimurium χ3987 (pYATS-1) was the recombinant which showed higher levels of constitutive expression and stability in vitro. This bacterium was selected to study its potential use as a bivalent vector to induce an immune response in swine against both *Salmonella* and TGEV. The ability of *S. typhimurium* Δcya Δcrp Δasd strains carrying plasmids pYA292 or pYATS-1 to colonize swine tissues, as well as the stability of these bacteria in vivo were also objectives of this work. Since intestinal flora of swine did not seem to contain aerobic bacteria that grew in the presence of rifampicin in vitro, the gene conferring resistance to this antibiotic was cloned into *S. typhimurium* χ3987 chromosome generating strain χ4509. Both *S. typhimurium* χ4509 transformed with pYA292 or pYATS-1 colonized the small intestine of swine when orally inoculated. *S. typhimurium* χ4509 (pYA292) also colonized the liver which could be indicating that the expression of the amino-terminal fragment of the S protein diminishes the invasiveness of the recombinant bacteria. This is in accordance with reported observations in which recombinant *S. typhimurium* Δcya Δcrp χ4064 expressing a 31-kDa protein of *Brucella abortus* was able to colonize only the small intestine and mesenteric lymph nodes of swine, but not the liver or the spleen (Stabel et al., 1991). The inoculated bacteria were cleared from the infected tissues by day 10 to 20, indicating that *S. typhimurium* Δcya Δcrp mutants are less persistent in swine tissues than in mice, in which orally inoculated bacteria can be recovered from

GALT and spleen even at 4 weeks post-inoculation (Curtiss III et al., 1990; Doggett et al., 1993).

All *S. typhimurium* χ4509 (pYA292) colonies recovered from tissues of infected swine maintained a correct pYA292 plasmid as determined by restriction enzyme analysis (data not shown), confirming the stability of the balanced lethal Δ*asd* host-*asd*$^+$ plasmid systems in swine. Recombinant χ4509 (pYATS-1) also showed a high degree of stability in swine, although the fact that 50% of the bacteria recovered from the small intestine at day 10 had lost expression of the S protein amino-terminal fragment suggests that the stability of this recombinant is lower in vivo than in vitro.

One third of the animals inoculated with *S. typhimurium* χ3987 (pYATS-1) developed TGEV specific antibodies. These animals showed both systemic and mucosal immunity. The antibody response in serum was of the IgG isotype as determined by incubating directly with protein A in the RIA assay (protein A binds weakly to swine IgA) (Bennell and Watson, 1980). In saliva, however, only IgA isotype antibodies were detected. The lack of detection of antibodies in saliva when incubating directly with protein A confirms the weak reactivity of this protein with swine IgA. All swine orally inoculated with *S. typhimurium* χ3987 (pYA292) or χ3987 (pYATS-1) developed specific antibodies to *Salmonella* antigens in serum and secretions, which probably indicates that the bacteria have been able to colonize the GALT of the infected animals.

TGEV causes an enteric disease that is especially severe in pigs under ten days of age. The protection against TGEV requires the induction of lactogenic immunity, since piglets are too young to develop an immune response in a short time and must receive passive immunity through the milk. The recombinant *S. typhimurium* χ3987 (pYATS-1) induced a secretory immune response against TGEV in saliva, which makes it a potential vector for the induction of lactogenic immunity. The induction of lactogenic immunity to both *Salmonella* and TGEV by oral inoculation of pregnant sows, on which *Salmonella* could be more invasive, and consequently more immunogenic due to partial immune suppression during pregnancy, with *S. typhimurium* χ3987 (pYATS-1) is currently under study.

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