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Comparison of a fimbrial versus an autotransporter display system for viral epitopes on an attenuated *Salmonella* vaccine vector

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Abstract

Attenuated *Salmonella* have been used as vectors to deliver foreign antigens as live vaccines. We have previously developed an efficient surface-display system by genetically engineering 987P fimbriae to present transmissible gastroenteritis virus (TGEV) C and A epitopes for the induction of anti-TGEV antibodies with a *Salmonella* vaccine vector. Here, this system was compared with an autotransporter protein surface display system. The TGEV C and A epitopes were fused to the passenger domain of the MisL autotransporter of *Salmonella*. Expression of both the MisL- and 987P subunit FasA-fusions to the TGEV epitopes were under the control of in vivo-induced promoters. Expression of the TGEV epitopes from the *Salmonella typhimurium* CS4552 (*crp cya asd pgtE*) vaccine strain was greater when the epitopes were fused to MisL than when they were fused to the 987P FasA subunit. However, when BALB/c mice were orally immunized with the *Salmonella* vector expressing the TGEV epitopes from either one of the fusion constructs or both together, the highest level of anti-TGEV antibody was obtained with the 987P-TGEV immunogen-displaying vector. This result suggested that better immune responses towards specific epitopes could be obtained by using a polymeric display system such as fimbriae.

Keywords: *Salmonella*; Vaccine; Coronavirus; TGEV; Fimbriae; Autotransporter

1. Introduction

The transmissible gastroenteritis virus (TGEV) is an extremely contagious agent of pigs with high rates of fatal outcomes for piglets [2]. Maternal antibodies, passed to piglets in colostrum and milk, provide protection against TGEV infection. The gut-mammary link of lymphocyte trafficking results in local antibody production in the mammary gland after oral immunization [3]. Consistent with other coronaviruses, the spike (S) protein of TGEV is the major inducer of neutralizing antibodies, and four antigenic sites were identified [4,5]. Among them, epitope C and A [4] are especially attractive since, in addition to being major inducers of neutralizing antibodies, they are also linear epitopes that can be incorporated into carrier molecules that improve their immunogenicity. For example, both purified chimeric CS31 and 987P fimbriae carrying TGEV C and A epitopes have been developed and shown to be immunogenic [6,7]. We have previously reported that oral immunization with a *Salmonella* vaccine vector expressing the chimeric 987P fimbriae, elicits specific serum IgG and mucosal IgA antibody responses to TGEV [8]. We have also observed that the use of in vivo inducible promoters and the addition of a *pgtE* mutation to the *Salmonella* vector significantly enhanced the immune response toward the heterologous antigens [1]. However, because the level of neutralizing antibody elicited against TGEV was viewed as insufficient, the vaccine remained to be improved.

More recently, autotransporter proteins have been found to serve as attractive targets for the insertion and display of heterologous antigens on live bacterial vaccines [9–11]. In *Salmonella*, the MisL autotransporter protein was used for displaying the immunodominant epitope of *Plasmodium falciparum* [10]. Here, we studied the use of this display system for the TGEV C and A epitopes and compared it with the 987P

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fimbrial display system using the *Salmonella pgtE* vaccine vector for oral immunization.

2. Materials and methods

2.1. Mice

Six week-old female BALB/cByJ mice were obtained from the Jackson Laboratory and housed in filter-top cages in an air-conditioned animal facility. Water and food were provided ad libitum. Mice were adapted for a minimum of 1 week after arrival before being immunized.

2.2. Bacterial strains, media and reagents

The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains SE5000 and XL1-Blue were grown in L medium, whereas *Salmonella enterica* serotype Typhimurium, strain CS4552 (*asd cya crp pgtE*), was grown in L medium with 50 μg/ml DL-α,ε-diaminopimelic acid (DAP, Sigma, St. Louis, MO, USA). When necessary, media were supplemented with the following antibiotics: ampicillin (200 μg/ml), chloramphenicol (30 μg/ml) or kanamycin (45 μg/ml). Culture media were purchased from Difco (Detroit, MI, USA). Restriction and modification enzymes were from New England Biolabs Inc. (Beverly, MA, USA). Unless specified, reagents were purchased from Sigma.

2.3. Plasmid constructs

Standard procedures were used to construct the following plasmids. Expression of the MisL-TGEV fusion protein was engineered to be under the control of the in vivo-inducible *spiC* promoter [1]. For this, a DNA fragment containing the *spiC* promoter with the TGEV C and A epitopes at 5′-end of the *fasA* ORF was amplified by PCR using plasmid pCS192 as template and upper primer 5′-GC**TCTAGA**GGATCCA-ATGCTTCCCTCCAGTTG with lower primer 5′-GC**TC-TAGA**ATTTGCCTGGCTGGTGTTGTTTTC. The PCR fragment was cut by *Xba*I and then inserted into the *Nhe*I site of plasmid pnirBLT-MisL [10], resulting in plasmid pCS261. This plasmid was further modified by the addition of genes that stabilize plasmid maintenance in the absence of antibiotics. For this, two different toxin–antitoxin postsegregation systems, the *flmA-flmB* locus [12] and the *ccdA-ccdB* (*letA-letD*) operon [13–15] from the F plasmid of *E. coli* XL1-Blue, were amplified by PCR with the upper primer 5′-CG**GGATCC**GTGGATGGTGCCGAACAAACT, lower primer 5′-GA**AGATCT**CCTGGCAGTCTGGTTGTTCAT and upper primer 5′-CG**GGATCC**GTTAACATAACGAAA-GGTAAAA, lower primer 5′-GA**AGATCT**CTGCAGACT-GGCTGTGTATAAC, respectively. The amplified *flm* locus was cut with *Bam*HI and *Bgl*II and inserted into the *Bam*HI site of pACYC184, resulting in plasmid pCS236. The amplified *ccd* operon was cut with *Bam*HI and *Bgl*II and inserted into the *Bam*HI site of pCS236, creating plasmid pCS238 with the *flm* and *ccd* loci in opposite orientations. Finally, an amplicon containing both loci was prepared with the two flanking primers (lower primers described above) and inserted into the blunted *Eco*RI site of pCS261, creating plasmid pCS263. Similarly, the *asd* gene [16] from plasmid pYA3332 was recovered as a *Bgl*II fragment that was blunted with the T4 polymerase and inserted into the blunted *Eco*RI site of pCS261, creating plasmid pCS265.

2.4. Plasmid stability

To determine the persistence of a plasmid in dividing cells, bacterial cultures were passaged for 20 days, for approx-

Table 1
Virus, bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or Source
Virus		
TGEV	Attenuated Purdue-115 strain	Gift from Linda J. Saif
E. coli		
SE5000	MC4100 *recA*56 (Fim$^-$)	[35]
XL1-Blue	*lac* [F′ *proAB lacI^{q}ZΔM15* Tn*10*(Tc^r)]	Stratagene, La Jolla, CA, USA
S. enterica Serovar Typhimurium		
CS4552	χ4550 *pgtE*::*aphA*	[1,36]
Plasmids		
pYA3332	*asd*-containing vector, p15A *ori*	Gift from Roy Curtiss III
pACYC184	p15A *ori*	[37]
pnirBLTBsp-MisL	p_{nirB} for LTsp-MisL, colE1-like *ori*	[10], gift from Cesar Gonzalez-Bonilla
pCS173	p_{pagC} for TGEVCA-FasA-G, pSC101 *ori*	[19]
pCS192	p_{spiC} for TGEVCA-FasA-G, pSC101 *ori*	[1]
pCS236	pACYC184 with *flmAB*	This study
pCS238	pCS236 with *ccdAB*	This study
pCS261	pnirBLTsp-MisL with p_{spiC} and TGEVCA	This study
pCS263	pCS261 with *flmAB* and *ccdAB*	This study
pCS265	pCS261 with *asd*	This study

imately 200 generations, each passage corresponding to a 10^{-3} dilution of an overnight culture into fresh medium without antibiotics. The stability of the plasmid was estimated by determining the numbers of antibiotic-resistant live bacteria containing the plasmid at each passage (colony-forming units or CFUs on antibiotic containing L agar plates) divided by the total number of live bacteria at each passage (CFUs on L agar plates). This number was multiplied by 100 to obtain a percentage of bacteria containing the plasmid.

2.5. Seroagglutination (SA), indirect fluorescence assays (IFA), SDS-PAGE and Western blotting

Slide seroagglutination tests were performed with rabbit anti-TGEV C and anti-TGEV A epitope antisera [1,6]. For IFA, the bacterial strains were spread on glass slides, fixed with cold acetone, incubated with the rabbit anti-TGEV C antibody, washed three times with PBS, incubated with FITC conjugated goat anti-rabbit-IgG and washed with PBS. Dried slides were examined by fluorescence microscopy. For SDS-PAGE and Western blotting, bacterial pellets were solubilized in SDS sample buffer, boiled for 5 min, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were stained with Coomassie Blue or analyzed by Western blotting. The blotted proteins were probed with rabbit antibodies specific for the TGEV C or TGEV A peptide, using horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (ECL) for detection, as described previously [8]. The relative amounts of expressed fusion proteins were evaluated by densitometry, using the NIH ImageJ software, version 1.37m (http://rsb.info.nih.gov/ij/).

2.6. Immunization

For each immunization, *Salmonella* with the different plasmid constructs were grown overnight in L broth without antibiotics at 37 °C on a rotary shaker at 150 rpm [1]. The bacterial cells were gently washed once and resuspended in sterile phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.2) at the concentration of 1–5 × 10^{11} CFU/ml. Viable bacteria were counted on all the inocula. Before immunization, the mice were deprived of food and water for 4 h. The mice were administered orally 200 μl of bacterial suspensions with feeding needles and fasted for an additional 30 min. Mice were immunized at day 0 and at day 28. At week 8, mice were anesthetized with Metofane (methoxyflurane; Mallinckrodt Veterinary Inc., Mundelein, IL, USA) and exsanguinated by heart punctures.

2.7. ELISA and virus neutralization assay

Individual mouse sera were tested for IgG against the TGEV C or A peptides using enzyme-linked immunosorbent assays (ELISA) [1]. Briefly, TGEV C or A peptide (1.0 μg in 100 μl 0.1 M carbonate buffer, pH 9.6, per well) were coated to 96-well ELISA plates (Immulon 4; Dynatech Laboratories Inc., Chantilly, VA, USA) using a microwave [17] and further overnight coating at 4 °C. The plates were blocked with 0.5% BSA in PBS at 37 °C for 2 h, washed four times with PBS and incubated with serial dilutions of sera in PBS–0.1% BSA–0.05% Tween-20 for 2 h at 37 °C. After the second washing step, plates were incubated with HRP-conjugated anti-mouse IgG antibodies at 37 °C for 1 h. After the last washing step, bound antibodies were detected by using *o*-phenylenediamine and H_2O_2 as substrate and reading the absorbance at 450 nm.

2.8. Statistical analysis

Groups of log-transformed data were compared by using the unpaired Student's *t*-test [18]. Probability (*P*) values of less than 0.05 indicated that the groups were significantly different.

3. Results

3.1. Construction and expression of the MisL-TGEV fusion protein

The *Salmonella* autotransporter protein MisL was employed to direct TGEV antigenic determinants to the surface of a *Salmonella* vaccine vector. As described in Section 2, the TGEV C and A epitopes were fused to the N-terminus of the translocator domain of MisL (Fig. 1A). The fusion protein included the signal sequence of the 987P fimbrial subunit FasA for translocation to the periplasmic space. The TGEV C and A epitopes (TGEVCA) were inserted between the second and third amino acid residue of the mature FasA protein and followed by nine FasA residues before the fusion site with the MisL β domain described previously [10]. The putative MisL β domain included an additional 163 amino acids of the α domain to avoid potential protein misfolding in the periplasm and ensure correct conformation in the outer membrane for transit of the passenger domain through the pore and surface display of its N-terminus [10]. Expression of the TGEVCA-MisL fusion protein was placed under the control of the *nirB* and *spiC* promoters. These in vivo inducible promoters and the use of tandem promoters were previously shown to improve humoral immune responses against heterologous antigens [1,8]. *Salmonella typhimurium* with the plasmid construct pCS261 was found to express a TGEVCA-MisL fusion protein determined by SDS-PAGE and Western blot analysis with anti-TGEV A and C antibodies (Fig. 1B). The protein band suggested a molecular weight of approximately 60 kDa, corresponding to the calculated molecular weight of the construct (59.4 kDa). The TGEVCA-FasA fusion protein migrated and reacted with the TGEV antibodies as described previously [1].

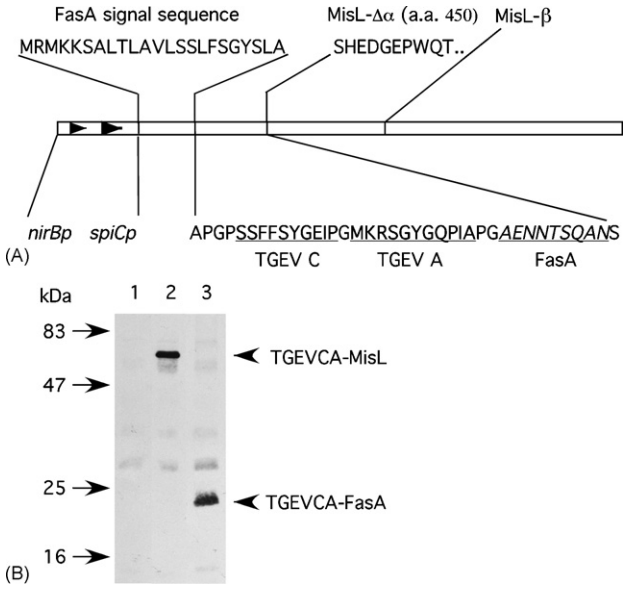

Fig. 1. Characterization of the TGEVCA-MisL fusion protein. (A) Schematic representation of the constructed TGEVCA-MisL expression cassette. The fusion protein is preceded by the *nirB* and *spiC* promoters (*nirBp* and *spiCp*), includes the FasA signal sequence for secretion into the periplasmic space, the TGEV C and A epitopes in tandem (bold and underlined) with a few amino acids of the amino terminus of FasA (italic and underlined) fused to amino acid (aa) 450 of MisL (α domain), and the MisL β domain for transport to the bacterial cell surface. (B) Western blot of *Salmonella* strain CS4552 with (1) pYA3332, (2) pCS261 and pYA3332 or (3) pCS173, using whole bacterial cell lysates probed with a mixture of anti-TGEV C and A antibodies. The TGEVCA-MisL and TGEVCA-FasA fusion products are labeled by arrows on the right.

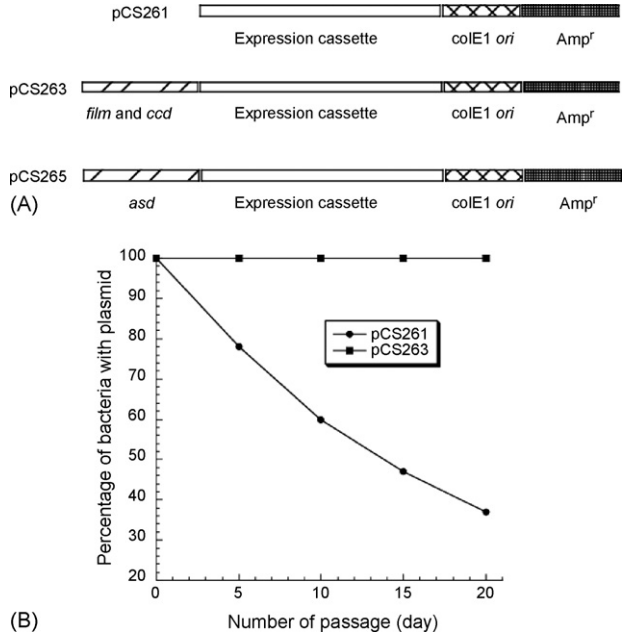

Fig. 2. Plasmid stabilization by postsegregational killing. (A) Schematic representation of the constructed *flm*- and *ccd*- or *asd*-containing expression plasmids. All the plasmids contained the expression cassette, as shown in Fig. 1A, a colE1-like origin of replication (colE1 *ori*) and an ampicillin resistance gene (Amp^r). (B) Plasmid segregational stability curve. The bacteria were grown in LB at 37 °C without any antibiotics, serial passages were conducted daily by diluting the overnight culture 1:1000 into fresh media. The percentage of bacteria with plasmid at each time point was calculated by dividing CFU-recovered from ampicillin plates by CFU-recovered from antibiotic-free plates, and multiplying this number by 100. The bacteria contained either plasmid pCS261 (filled circles) or plasmid pCS263 (filled squares).

3.2. Stabilized plasmids and fusion protein expression

To ensure that the *Salmonella* vector would keep its plasmid in the absence of antibiotic selection after vaccine administration to mammalian hosts, two postsegregational killing systems from the F plasmid of *E. coli* (*flm* and *ccd* genes) were engineered into the TGEV-MisL plasmid construct (Fig. 2A). The efficiency of plasmid maintenance in vitro was investigated by growing *Salmonella* with plasmid in antibiotic-free media over 200 generations. The plasmid-bearing rate was calculated by dividing the number of bacteria recovered from antibiotic plates by the number of bacteria recovered from antibiotic-free plates. As shown in Fig. 2B, plasmid pCS261 which has no stabilization system was gradually lost during the 20 sequential passages, while plasmid pCS263 was kept in all the bacteria. This result demonstrated that the *flm* and *ccd* genes efficiently stabilized plasmid pCS263 in the *Salmonella* vaccine strain. Moreover, this strain was shown to express the TGEVCA-MisL fusion protein from plasmid pCS263, as detected by SDS-PAGE (Fig. 3A) and Western blot analysis with anti-TGEV epitope C (Fig. 3B) and A antibodies (Fig. 3C). Epitope expression was approximately 2.5 times as efficient as that from pCS173, which expresses the TGEVCA-FasA fusion protein, as determined by Western blot densitometry with the TGEV C and A antibodies. Expression from plasmid pCS263 was as efficient as that from plasmid pCS265, which carried the *asd* gene for plasmid stabilization by the balanced-lethal system (Fig. 2A), an alternative plasmid maintenance system that we had used previously [8,16,19]. However, TGEVCA-MisL expression from pCS263 was reduced by approximately one-half when the *Salmonella* vector contained also pCS173 for simultaneous expression of the TGEVAC from surface exposed fimbriae [1]. Expression of the heterologous fimbrial subunit with the TGEV epitopes was also reduced by approximately one-half, indicating that each surface display system inhibited the other display system. These inhibitions suggested regulatory effects such as promoter competition or interference between protein export mechanisms.

3.3. Surface display of the TGEV epitopes on Salmonella

The ability of the recombinant MisL β domain to translocate the TGEV C and A epitopes to the bacterial surface of a *Salmonella* vaccine vector was determined by seroagglutination with specific anti-TGEV C and anti-TGEV A antibodies. The *Salmonella* vector CS4552 containing either plasmid pCS261, pCS263, pCS265 or pCS173 agglutinated well with the anti-TGVE C or the anti-TGEV A epitope antibodies,

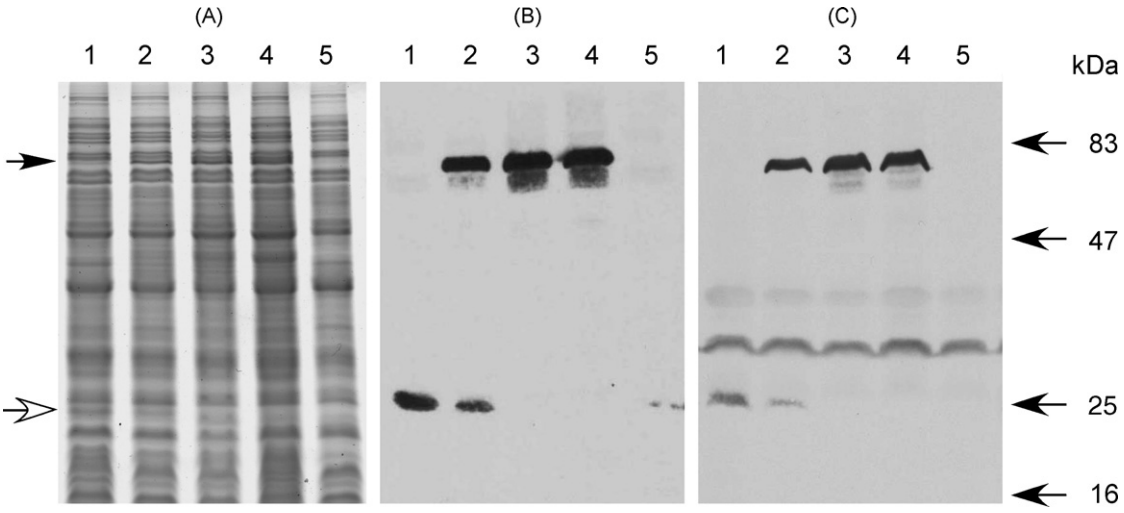

Fig. 3. Expression of the TGEVCA-MisL fusion protein in a *Salmonella* vaccine strain. (A) SDS-PAGE of the whole bacterial cell lysates. Western blotting with (B) anti-TGEV C and (C) anti-TGEV A antibody of the whole bacterial lysates. Lane (1) *Salmonella* CS4552 pCS173; lane (2) CS4552 pCS173 pCS263; lane (3) CS4552 pCS263; lane (4) CS4552 pCS265; lane (5) CS4552. The black arrow on the left indicates the TGEVCA-MisL fusion protein, whereas the white arrow indicates the TGEVCA-FasA chimeric subunit that forms the 987P fimbriae.

whereas CS4552 alone did not (data not shown). Moreover, an indirect fluorescence assay with the rabbit anti-TGEV C antibody and an anti-rabbit IgG-FITC conjugate was used to further confirm the presence of the TGEV epitopes on the bacterial surface. As shown in Fig. 4, the *Salmonella* vector CS4552 fluoresced only when it carried plasmid pCS265 or pCS173. Interestingly, the agglutination of CS4552 pCS173 was much stronger, suggesting better accessibility of the epitopes on 987P than on MisL. These results indicated that the TGEV C and A epitopes translocated efficiently to the bacterial surface whether expressed from within the MisL autotransporter protein or from within the 987P fimbrial subunit FasA.

3.4. Comparison of immune responses to the TGEVCA fusion proteins

The humoral immune responses to four different vaccine constructs were compared after oral administration to groups of 9–10 mice. The *Salmonella* vaccine vector CS4552

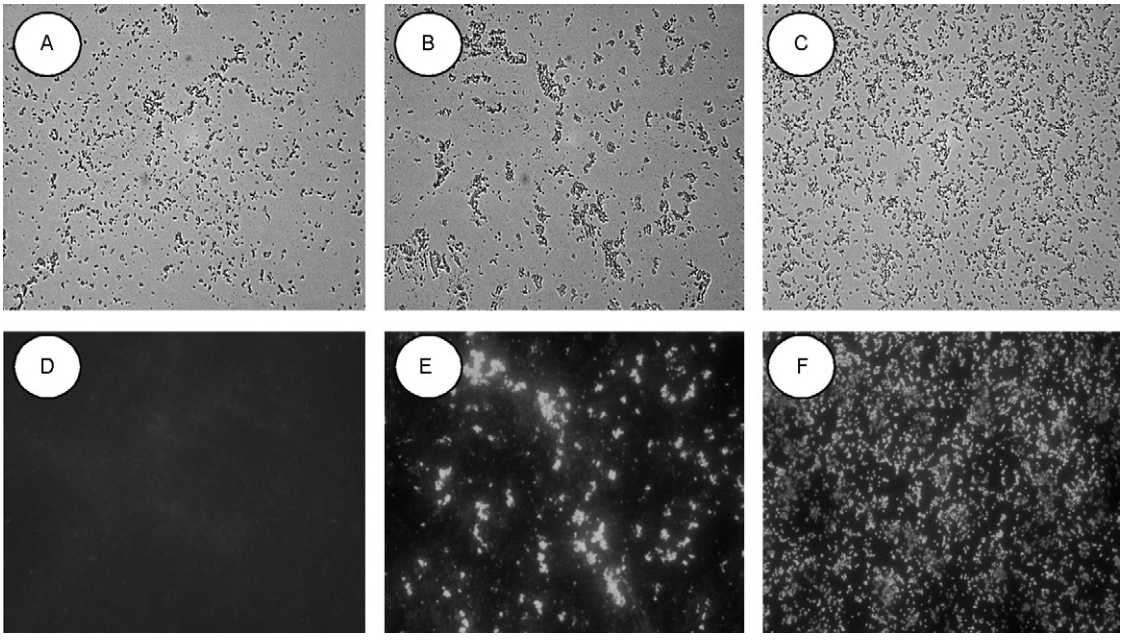

Fig. 4. Surface exposure of the TGEV epitopes on the *Salmonella* vaccine strain. The bacterial cells were labeled with rabbit anti-TGEV C peptide antibody and FITC-conjugated goat anti-rabbit IgG antibody. The labeled bacteria cells were visualized by bright-field light microscopy (BF: panels A, B and C) or by indirect fluorescent microscopy (IFA: panels D, E and F). (A and D) CS4552; (B and E) CS4552 pCS173; (C and F) CS4552 pCS265.

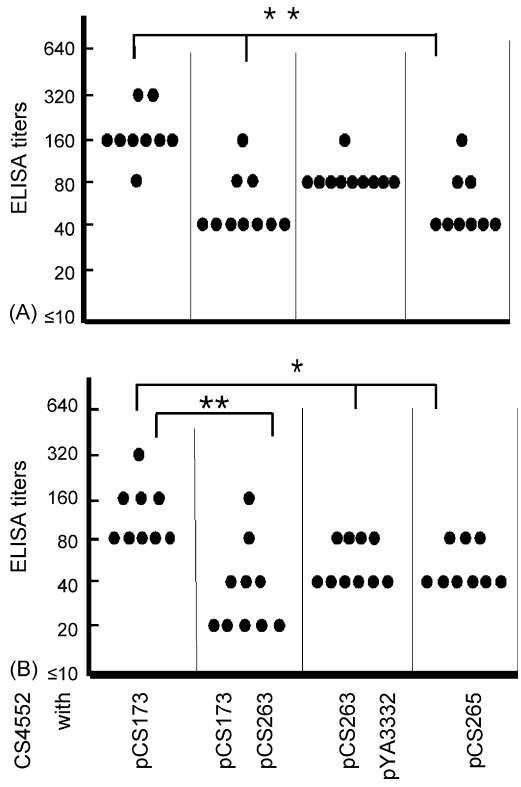

Fig. 5. Systemic anti-TGEV IgG titers after immunization with *Salmonella* vaccines. Groups of 9–10 BALB/c mice were immunized orally with two doses (28-day interval) of the *Salmonella* CS4552 vaccine strain with different plasmids for each group, as shown at the bottom of the figure. IgG titers were determined at 8 weeks postimmunization. Each filled circle represents the serum IgG titer of one mouse. (A) Anti-TGEV C epitope antibody titers elicited by CS4552 pCS173 were significantly higher than the titers induced by CS4552 pCS173 pCS263 ($^{**}p < 0.01$) and CS4552 pCS265 ($^{**}p < 0.01$). (B) Anti-TGEV A epitope antibody titers elicited by CS4552 pCS173 were significantly higher than the titers induced by CS4552 pCS173 pCS263 ($^{**}p < 0.01$), pCS263 pYA3332 ($^{*}p < 0.05$) and pCS265 ($^{*}p < 0.05$).

[1] was transformed with pCS173, pCS263 and pYA3332 together, pCS173 and pCS263 together, or pCS265. All the mice received an oral boost at day 28 and specific serum IgG titers were determined at week 8. As shown in Fig. 5 and comparing the vaccines with the *asd*-stabilized plasmids, *Salmonella* with the TGEV epitopes presented by the 987P fimbriae (CS4552 pCS173) was eliciting significantly higher antibody titers than the *Salmonella* with the TGEV epitopes presented by the MisL protein (CS4552 pCS265). Interestingly, *Salmonella* expressing the TGEVCA-MisL fusion protein from an *asd*-stabilized plasmid (pCS265) or from the *flp-ccd*-stabilized plasmid (pCS263 with pYA3332) induced comparable serum IgG titers, indicating that the stabilization systems did not influence differentially immune responses. The previously observed inhibitory effect between the MisL and 987P display systems for TGEVCA expression was consistent with the immunization results, which showed that CS4552 carrying both pCS173 and pCS263 induced the lowest level of antibody responses (Fig. 5). Neutralizing antibodies were detected in individual mice of each group. However, there were no significant different titers for the vaccines expressing the TGEVCA in fimbriae or in MisL, with 3–4 mice out of 9–10 expressing neutralizing antibody titers of 4–8. Only 1 out of 10 mice immunized with the vaccine expressing both the TGEVCA in fimbriae and in MisL had detectable neutralizing antibodies (titer of 8), consistent with the lower ELISA titers found in this group.

4. Discussion

Immune responses induced by attenuated live vaccines are profoundly influenced by the delivery efficiency of protective epitopes. Epitope density and stability are major influencing factors. Attenuated *Salmonella* have been used for delivery of a variety of foreign viral or bacterial antigens [20,21]. It has been clearly established that immunogens delivered by attenuated *Salmonella* strains induce better systemic and mucosal immune responses when the immunogens are displayed on the bacterial surface [8,22]. A variety of surface display systems have been studied [23]. Most widely used are fimbria and outer membrane proteins, including autotransporter systems [9,11,24,25]. In this study, we compared the efficiency of a fimbrial and an autotransporter display for presenting the same two viral epitopes by an attenuated *Salmonella* vector to the murine immune system. Although immune responses were induced by both display systems, IgG titers were higher with the 987P fimbrial system than with the MisL display. This finding cannot be explained by differences in fusion protein expression or the copy number of the plasmids used, since both would have favored the MisL display system. The *Salmonella* expressed 2.5 times more TGEVCA antigen with the MisL construct, as expected, since the MisL fusion protein was expressed from a higher copy number plasmid (ColE1-like *ori*, 30–50 copies/cell) than the FasA fusion protein (pSC101 *ori*, 6–8 copies/cell). Plasmid maintenance could also not have been an issue, since the observed differences also occurred with the pCS173 and pCS265 plasmids, both being stabilized by the asd-balanced lethal system. In contrast, fluorescence microscopy clearly showed that *Salmonella* expressing the TGEVCA-FasA fusion protein agglutinated significantly better than the ones expressing the TGEVCA-MisL fusion protein. This result suggested the former bacteria presented either a higher density of TGEV epitopes on the bacterial surface or better accessibility to the epitopes. In comparison to the outer-membrane-located autotransporters such as MisL, fimbriae place heterologous epitopes further away from the bacterial surface, promoting easier access and less interference by other bacterial outer-membrane components. Together with a more efficient surface exposure, the multimeric nature of epitope presentation by fimbriae can explain the induction of a better immune response, since surface display of heterologous epitopes on *Salmonella* and of polymerized proteins improve such responses [8,22]. An alternative explanation is

that the 987P fimbriae act as better carrier molecules than MisL for the adaptive immune system of the mice used in this study.

Although the TGEV epitopes induced specific antibodies whether they were presented in the context of 987P or MisL, the antibody titers were relatively low and neutralizing antibodies were only detected in 30–40% of the mice. In an attempt to improve the efficacy of epitope expression by the *Salmonella* vector, constructs expressing the TGEV C and A epitopes simultaneously from the 987P fimbriae and the MisL protein were studied. Since one aim in the development of live vaccines is to exclude antibiotic-resistance genes from the constructs and to avoid the reliance on antibiotic use for plasmid maintenance, alternative approaches were applied. We relied on two compatible plasmids, the fimbrial expression plasmid containing the *asd*-balanced lethal system [8,16,26,27], and the MisL-containing plasmid carrying a postsegregational killing system [28] as a second, *asd*-independent plasmid maintenance system. Since it has been demonstrated that combination of different postsegregational killing loci can enhance plasmid stability [29], two different ones, namely the *flmAB* and the *ccdAB* genes, originating from the F plasmid were integrated into the plasmid. The former genes correspond to the *hok-sok* toxin–antitoxin genes of plasmid R1 in which *hok* (*flmA*) encodes a lethal pore-forming protein and *sok* (*flmB*) is an antisense mRNA that prevents Hok's synthesis [30]. The *sok* mRNA being highly susceptible to degradation by nucleases, *sok* transcription, and thus *hok-sok* carrying plasmids, must be maintained in replicating bacteria to avoid bacterial death by Hok, which is translated from a stable transcript. Hok acts by depolarizing membranes, similar to the action of the holin proteins of bacteriophages before cell lysis. A *hok-sok* system has been used successfully to maintain plasmids in *Salmonella* [31]. Similarly, *ccdB* encodes a stable toxin, while *ccdA* encodes a less stable protease-susceptible antidote [15]. In the presence of the plasmid, both proteins are expressed and the toxic activity of CcdB is reversibly inactivated by CcdA. Upon plasmid loss, CcdB outlives CcdA and kills the bacterial cell by poisoning DNA gyrase in a manner that bears resemblance to the activity of the quinolone antibiotics. By introducing both the *flm* and *ccd* postsegregational killing genes into a TGEVCA-MisL expressing plasmid, we observed essentially no plasmid loss in vitro. In addition, maintenance of this plasmid being independent of the *asd*-balanced system of the TGEVCA-FasA plasmid, both plasmids could be studied together in the same *Salmonella* vector. Surprisingly, steady-state amounts of TGVECA were not increased by the simultaneous use of both plasmids, since the amounts of the individual fusion proteins were each reduced by approximately one-half. Moreover, *Salmonella* displaying TGEVCA from both MisL and from the 987P fimbriae induced lower levels of antibody responses to the TGEV A and C epitopes. Since both systems utilized the nirB promoter in tandem with the pagC or the SpiC promoters, two SPI-2 promoters that are regulated directly or indirectly at the transcriptional level by SlyA and PhoP/PhoQ [32,33], competition for DNA-binding sites was likely. However, other explanations remain plausible, including effects on plasmid copy numbers, regulatory negative feedback mechanisms or competition for the export of the two systems.

In summary, by comparing two bacterial surface display systems for viral epitopes on an attenuated *S. typhimurium* vaccine, we found that a fimbrial system induced higher systemic IgG titers than an autotransporter display system. The two systems were comparable concerning the induction of virus neutralizing titers. The *Salmonella* vector was able to stably express the TGEV epitopes simultaneously in MisL and 987P, but the construct did not improve specific immune responses. Nevertheless, the approach has strong potentials. Autotransporters have the advantage over fimbriae for the export and display of larger heterologous immunogens [9–11,34]. Thus, future prototype vaccines based on the simultaneous use of the MisL and 987P display systems will focus on inserting into MisL larger fragments of the TGEV S protein that include other epitopes known to induce neutralizing antibodies [4,5], as well as the use of different promoters for the two display systems.

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