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An *Escherichia coli* CS31A fibrillum chimera capable of inducing memory antibodies in outbred mice following booster immunization with the entero-pathogenic coronavirus transmissible gastroenteritis virus

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CS31A fibrillae are thin, flexible, heteropolymeric proteinaceous appendages exposed as a capsule-like material around the cell surface of certain Escherichia coli *strains. Two antigenic peptides of the S spike glycoprotein (TGEV-S) amino acids (aa) 363–371 and 521–531 of the transmissible gastroenteritis virus (TGEV) were tandemly introduced in the loop-structured, variable region aa 202–218 of the major ClpG subunit protein composing the bulk of CS31A. The resulting hybrid fibrillae with a 25 aa heterologous peptide were produced at the cell surface. Using a monoclonal antibody (Mab) specific for the TGEV epitopes, purified hybrid fibrillae were analysed in Western blotting under native conditions, which showed that the two viral epitopes were recognized immunologically as an integral part of the hybrid fibrillae, and therefore that they were antigenically active. The immunogenicity of the fusion construct was evaluated with live recombinant bacteria, purified hybrid ClpG monomers, and purified chimeric CS31A polymers. Whatever the form of hybrid used as antigen, intraperitoneally immunized outbred mice elicited serum anti-TGEV peptides antibodies (Abs) with significant titres and capable of recognizing native TGEV particles, indicating that the epitopes are exposed in an immunogenic conformation in all cases. However, virus neutralization titres were only obtained after immunization with either purified polymers or monomers. Furthermore, 4 months after an ultimate immunization with 20 μg of hybrid fibrillae mice developed a strong anamnestic Ab response against the two TGEV peptides following booster inoculation with virions. We conclude that CS31A fibrillae carrying a combination of TGEV epitopes as insert can induce an immunological memory in outbred animals infected with TGEV, and therefore that hybrid CS31A fibrillae may prove efficient as components of a subunit vaccine.*

Keywords: CS31A fibrillae; TGEV coronavirus; recombinant DNA; carrier-delivery system; immune responses

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The fibrillum CS31A is a plasmid-encoded heteropolymeric protein previously identified among animal and human enterotoxigenic or septicemic *Escherichia coli* strains[1–3]. Comparison of the amino acid (aa) sequences of the major subunit proteins from CS31A-related fibrillae, namely K88 and F41, revealed a variable region in ClpG (aa 191–218) forming a flexible loop structure, as assessed by the topological model of ClpG[4]. This region contains a hydrophilic domain and several accessible continuous immunodominant epitopes, one of which (aa 189–194) is exposed on the CS31A polymer

(unpublished data). The aa 191–218 region of ClpG has been demonstrated to accept various small heterologous antigenic peptides without affecting fibrillae formation[5]. All these features make this region interesting to presenting a foreign antigen as repeating units along the CS31A polymer.

Transmissible gastroenteritis virus (TGEV), that belongs to the *Coronaviridae* family of enveloped positive-stranded RNA viruses, causes gastroenteritis resulting in severe diarrhea, dehydration, high mortality and morbidity in piglets under 2 weeks of age[6] by replicating selectively in the differentiated enterocytes covering the villi of the small intestine[7]. Coronavirions contain at least four structural proteins: the S spike glycoprotein (TGEV-S); the N nucleoprotein; the M membrane glycoprotein; and the sM protein. TGEV-S has been shown to mediate attachment of virions to the host cell receptor, to be involved in cell-to-cell fusion, to be the major inducer of TGEV neutralizing antibodies (Abs), and to bear virulence determinants[7–9]. Because transmissible gastroenteritis disease continues to cause major economic losses to the swine industry, and to date effective vaccines or treatments have remained elusive[10], there is a demand for the development of a new type of vaccine against TGEV. Here we describe the exploitation of a carrier-expression system based on the CS31A fibrillae of *E. coli*[5,11] for presenting two antigenic peptides from TGEV to the immune system.

In this paper we use recombinant DNA techniques to introduce in tandem the sites C (TGEV-C) (aa 363–371) and A (TGEV-A) (aa 521–531) of TGEV-S into the aa 202–218 part of ClpG. TGEV-C, recognized by the Mab 3b.5[12], elicits neutralizing Abs[12,13]. TGEV-A, recognized by the Mab 1A.F10[14], is part of an immunogenic conformational antigenic region that, in contrast to TGEV-C, is highly conserved among enteric and respiratory isolates as well as, between porcine, canine and feline coronaviruses[15], and confers lactogenic protection in pregnant sows[16]. In addition, this region consists of the major neutralizing site that also interacts with the TGEV receptor[8]. Therefore, it was hypothesized that adequate presentation to the immune system of pregnant sows of one or several epitopes from this region would provide a protective immune response against TGEV in piglets, and that these epitopes should be used as vaccine candidates[16].

The present study shows that mice immunized with purified hybrid CS31A fibrillae developed a systemic Ab response reactive towards the free synthetic TGEV peptides, native TGEV particles and TGEV infectivity, indicating that C and A epitopes were immunogenic in the CS31A-associated conformation. In addition, a memory Ab response against the CS31A-fused TGEV epitopes was elicited following booster inoculation with TGEV virions, making CS31A fibrillum a good vector candidate for recombinant vaccines design.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The *E. coli* K-12 strain used in this work was DH5α (BRL, Life Technologies, Inc.). Bacteria were grown at 37°C in Luria-Bertani (LB) broth or LB agar supplemented with tetracycline (12 μg ml^{-1}) or chloramphenicol (30 μg ml^{-1}). Plasmid pEH524[17] (*Figure 1*A) carries the CS31A fibrillae-encoding *clp* gene cluster on the 8.5-kb *Eco*RI-*Hin*dIII fragment in the low-copy number vector pHSG575[18] containing pSC101 replicon. The pEH524-determined *clp* gene cluster contains seven structural genes encoding all the secretory proteins required for CS31A biogenesis (*Figure 1*A). They include the major ClpG subunit protein and several accessory proteins involved in the stabilization, transport and assembly of ClpG. The *clpG* gene codes for the ClpG precursor whose processing results in a mature polypeptide of 257 aa[19]. To perform DNA manipulations in *clpG* without affecting the rest of the operon, *clpG* and *clp* helper-genes were cloned into two separate compatible plasmids (*Figure 1*B). The first, pDSPH524[11], contains the *clp* gene cluster with *clpG* deleted, and the second, pPSX83[5], *clpG* only. Plasmid pDSPH524 was constructed by deleting the *Sph*I-*Sph*I fragment from pEH524 and religating. Plasmid pPSX83 was made by cloning *clpG* from pEH524 as a *Pst*I-*Hpa*I fragment dowstream of the *lac* promoter in the *Pst*I-*Sma*I sites of the high-copy number vector pSelect-1 (Promega Corporation) carrying ColE1 replicon. In *trans*-complementation experiments, pDSPH524 and pPSX83 were co-selected on the basis of their chloramphenicol and tetracycline resistance, respectively. The insertion vector pPSX10S (*Figure 2*b) was constructed in two steps from pPSX83 after two consecutive rounds of oligonucleotide-directed site mutagenesis as previously described[5], resulting in the creation of unique *Spe*I and *Bgl*II sites in *clpG* (*Figure 2*b). The engineered *Spe*I and *Bgl*II sites allow subsequent replacement of the aa 202–217 region of ClpG as described in *Figure 2*.

In vitro DNA manipulations and DNA sequencing analysis

Plasmid DNAs were extracted by alkaline lysis and column-purified (Qiagen GmbH, Germany). DNA fragments obtained from digestion with restriction endonucleases were purified from agarose gels with "Prep-A-gene" DNA purification kit (Bio-Rad Laboratories Ltd) before ligation. All other standard techniques were performed essentially as described[20]. Buffers, reaction conditions, restriction enzymes and DNA modifying enzymes were used as recommended by the suppliers. DNA sequencing was performed on denatured double-stranded plasmid DNA with the dideoxynucleotide chain termination method[21] using internal primers, [^{35}S]dATP as the label and the USB Sequenase Version 2.0 DNA sequencing kit.

Oligonucleotides and oligopeptides

The oligonucleotides used in this study were synthesized and, when necessary, PAGE-purified and 5′-phosphoryled (Eurogentec, Belgium). Synthetic peptides were obtained from Neosystem (Strasbourg, France) and their purity was 75% as determined by high-performance liquid chromatography.

Preparation and purification of proteins

For preparation of crude CS31A fibrillae extracts, bacteria grown overnight on LB agar with the appropriate antibiotics were carefully scraped and suspended in PBS (pH 7.2). This suspension was then vigorously

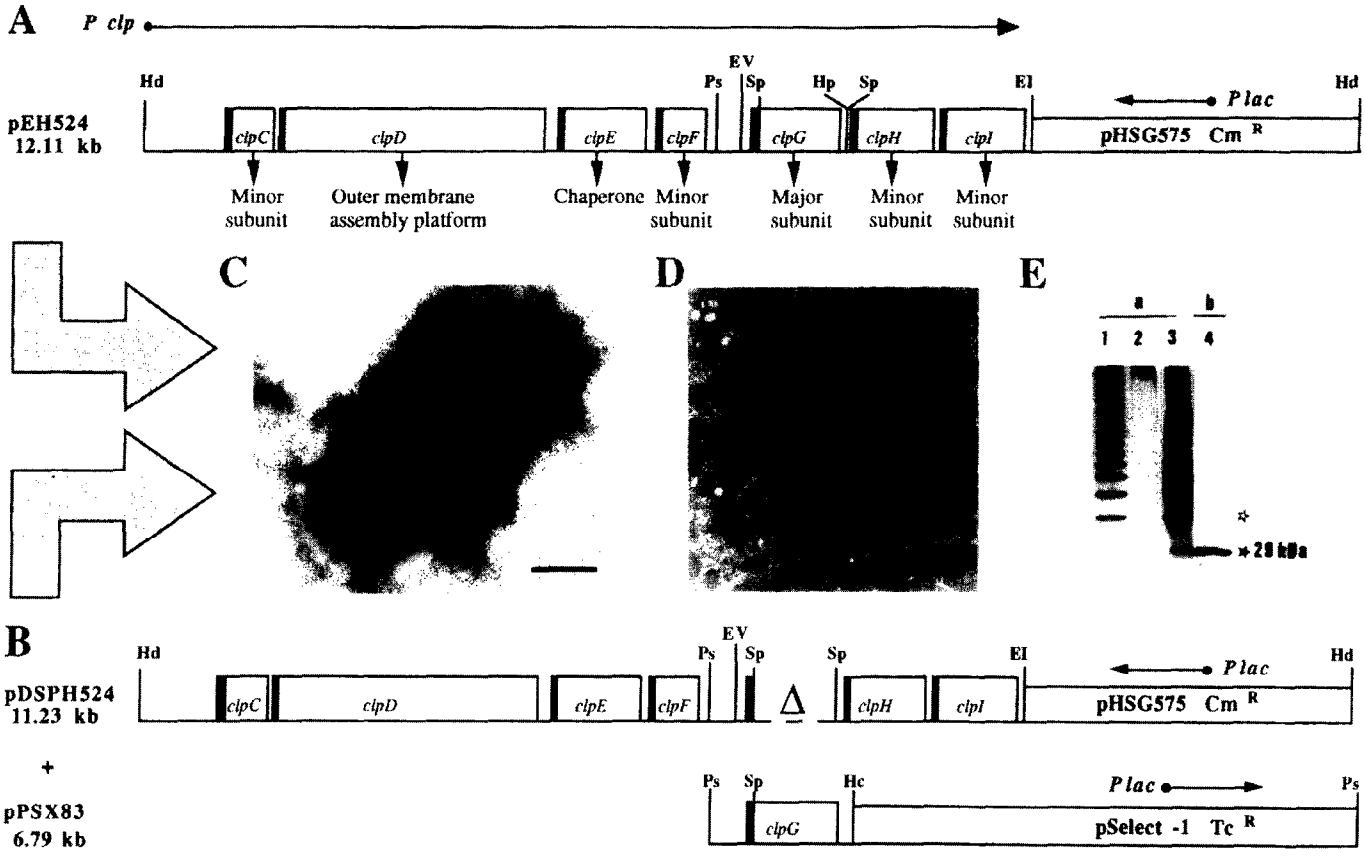

Figure 1 The CS31A system. (A) Genetic organization of the *clp* operon with brief descriptions of the function of the seven structural gene products required for CS31A fibrillum biogenesis. (B) Construction of the CS31A *trans*-complementation system. Black boxes indicate parts of genes coding for signal peptides. Only relevant restriction sites are indicated: EI, *Eco*RI; EV, *Eco*RV; Hc, *Hin*cII; Hd, *Hin*dIII; Hp, *Hpa*I; Ps, *Pst*I; Sp, *Sph*I. Δ, deletion. (C) Display of the major ClpG subunits detected by gold-labelled ClpG-specific polyclonal Abs on CS31A fibrillae expressed by *E. coli* DH5α [pEH524]. The bar represents 0.4 μm. (D) Negatively stained preparations of purified native CS31A fibrillae. The bar represents 0.15 μm. (E) Western blot of CS31A protein extracts separated by using 10% PAGE under (a) native or (b) denaturing conditions, and developed by using anti-CS31A (lanes 1–3) and anti-ClpG (lane 4) antisera. Extracts were either crude thermo-eluted CS31A fibrillae (lane 1), or purified CS31A fibrillae (lane 2) or purified ClpG monomers (lanes 3 and 4). Extracts in sample buffer were either incubated at room temperature (lanes 1–3) or boiled for 5 min (lane 4). Open star, dimer; full star, ClpG monomer

agitated for 1 min with a top mix shaker, and placed at 60°C for 20 min (thermo-elution of CS31A polymer). After centrifugation at 12000*g* for 10 min, the supernatant containing the free fibrillar polymers was recovered for analysis. Purifications of native CS31A polymers and ClpG monomers were performed as previously described[1].

Detection of surface exposed hybrid proteins

Production of hybrid CS31A polymers was detected by *in situ* colony immunoblotting, Western immunoblotting following PAGE under native or denaturing conditions, and electron microscopy.

Colony blots analysis. Single colonies were streaked on a solid agar LB plate containing appropriate antibiotics. After overnight incubation at 37°C, a nitrocellulose filter (pore diameter, 0.45 μm; Schleicher and Schuell) was carefully applied on agar surface. Blots were blocked and washed with 1% BSA–0.1% Tween 20 in PBS until the bulk of bacteria was removed. The filters were incubated with appropriate primary Abs in PBS–1% BSA. Bound primary Abs were detected by incubation of the filters with either horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary Ab, and developed with H_2O_2-α-chloronaphtol.

Western blots analysis. Aliquots of either crude CS31A extracts, or purified CS31A polymers, or purified ClpG subunits mixed with an equal volume of 2× incomplete Laemmli buffer were either boiled for 5 min or incubated at room temperature to obtain the monomeric and multimeric forms of the proteins, respectively. Samples were applied to a 10–15% PAGE and semi-dry electro-transferred onto nitrocellulose[22]. Western blots were then treated as described above.

Electron microscopy analysis. Electron microscopy of purified CS31A fibrillae stained negatively with 1% phosphotungstic acid and of 10-nm colloidal gold immunolabelled intact CS31A on *E. coli* cells were carried out essentially as previously described[1].

Polyclonal and monoclonal antibodies

ClpG subunit-specific rabbit antiserum (anti-ClpG) and native CS31A polymer-specific rabbit antiserum (anti-CS31A) were obtained as previously described[1]. The Mabs 3b.5 and 1A.F10 directed against the TGEV-C and -A epitopes of TGEV-S on native TGEV coronavirus, were described by Delmas *et al.*[12] and Gebauer *et al.*[14], respectively.

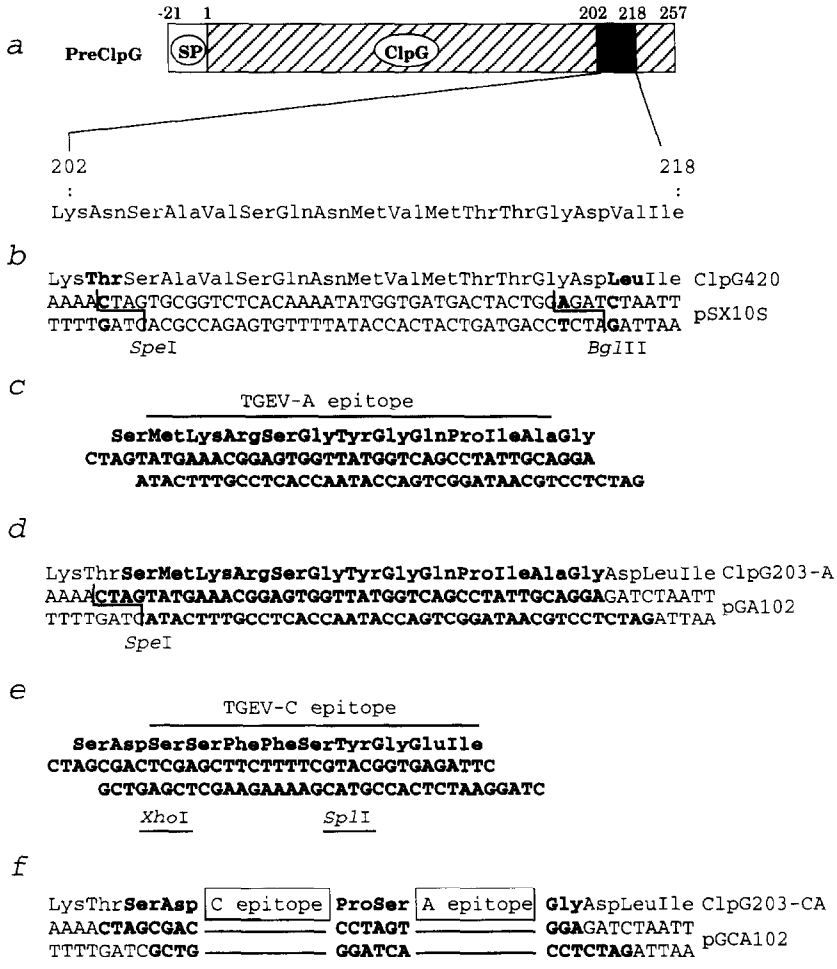

Figure 2 Hybrid protein constructions. (a) Overview of the ClpG precursor with the aa sequence of the target region of interest for viral epitope insertions; SP, signal peptide. (b) The nucleotide and aa sequences of the sites of mutation in pSX10S and ClpG420 protein, respectively. The two highlighted residues represent aa changes (Asn to Thr, and Val to Leu at positions 203 and 217, respectively) generated as a result of the introduction of the *SpeI* and *BglII* sites. (c) The synthetic double-stranded oligonucleotide coding for peptide A of TGEV-S protein (aa 521–531). (d) The nucleotide and aa sequences of the mutated region in pGA102 and ClpG203-A protein, respectively. Residues in bold type correspond to the 13 aa extension including the 11 aa of peptide A. (e) The synthetic double-stranded oligonucleotide specifying the peptide C of TGEV-S protein (aa 363–371). (f) The nucleotide and aa sequences of the modified region in pGCA102 and ClpG203-CA protein, respectively

Mice

The outbred Swiss OF1 and inbred DBA/2 (H-2^d), C57BL/6 (H-2^b), CBA/J (H-2^k), SJL (H-2^s) mice 8–12 weeks of age used in this work for experimental immunization were purchased from IFFA-Credo (Les Oncins, France).

ELISA assays

For the detection of Abs directed against the CS31A and ClpG carriers, 96-well microtitre trays (Falcon) were coated with purified CS31A protein on dissociated form at 5 μg ml^{-1} in 50 mM carbonate buffer (pH 9.6). For the detection of Abs directed against viral epitopes, highly activated 96-well microtitre plates Immulon II (Dynatech) were coated with synthetic peptides containing TGEV-C sequence (TVSDSSFFSYGEIPF) or TGEV-A sequence (SMKRSGYGQPIAG) at 20 μg ml^{-1} in 50 mM carbonate buffer (pH 9.6) containing 10 mM dithiothreitol and incubated for 16 h at 4°C. Detection was achieved by incubation with 2-2′-azinobis (3-ethylbenzothiasoline-6-sulphonate) (ABTS) and 2 nM H_2O_2 in phosphate-citrate buffer for 20 min in the dark at room temperature. ELISA assays were performed in duplicate and the reactive titre of each antiserum was expressed as the reciprocal of the highest dilution which showed a twofold increase in optical density (O.D.) at 405 nm over that obtained with the negative control. Irrelevant peptides derived from capside protein VP1 of foot-and-mouth disease virus (FMDV) (51-RYKQKIIAPAQKGG-65) and from capside protein VP1 of poliovirus (91-YDNPASTTN KDKLFA-105) were used as negative controls in the peptide-specific ELISA.

For anti-TGEV particles titre determination, individual mouse sera were tested as follows: 96-well microtitre plates were coated overnight at 37°C with purified TGEV Purdue-115 virus[23] at 5–10 μg ml^{-1} in PBS, washed with TBS buffer (pH 8.1) and incubated with gelatin at 15 mg ml^{-1} in TBS for 1 h at 37°C. After washing in TBS–0.1% Tween 20, antiserum diluted in TBS–0.1% Tween 20 was added and incubated for 1 h at 37°C. After washing in TBS–0.1% Tween 20, bound Abs were detected by incubation with alkaline phosphatase-labelled anti-mouse IgG conjugate in TBS–0.1% Tween 20 for 1 h at 37°C, then after washing, with *p*-nitrophenyl phosphate (PNPP) at 1 mg ml^{-1} in diethanolamine buffer for 30 min at 37°C. The coloured reaction was stoped by 2 N NaOH. Anti-virus titres were expressed as the reciprocal of the highest dilution giving an O.D. $\geq$0.2 at 405 nm. A reference serum was included in each experiment. Negative controls were sera

of mice hyperimmunized with hybrid CS31A fibrillae carrying a rotavirus VP6 epitope[5]. Mean background of non-specific Ab titres in the sera of hyperimmunized mice was 80 ($\log_2$=6.3).

Virus seroneutralization

TGEV neutralization was determined using a limiting dilution microassay[23]. Briefly, serial twofold or threefold dilutions of antisera were mixed with a virus suspension containing 500 p.f.u. of TGEV Purdue-115 strain. After incubation for 1 h at 37°C, 4×10^4 trypsinized swine testis (ST) cells in 50 μl Eagle's medium supplemented with 15% newborn calf serum were added. Neutralization titres were determined 40 h later, and calculated as the mean of the highest dilution that neutralized 100% of the cytopathic effect in duplicate experiments. A reference serum was included in each experiment.

RESULTS

Structural features of CS31A fibrillae

Electron microscopy of gold-immunolabelled CS31A-producing *E. coli* cells suggests a capsular organization of CS31A[1] (*Figure 1*C), while that of the purified form of CS31A reveals an abundance of very fine fibrillar organelles of 2 nm in diameter[1] (*Figure 1*D). In Western immunoblotting experiments, using a CS31A-specific antiserum, native CS31A appears as a ladder of bands of regulary increasing molecular mass corresponding to the multimeric form of CS31A (*Figure 1*E), and denatured CS31A migrates as a single band consisting essentially of the major 29Kd-ClpG subunit monomers, which can be autonomously reassociated *in vitro* into oligomeric reaggregated complexes[1] (*Figure 1*E).

Construction and expression of hybrid fibrillae

TGEV-C and TGEV-A peptides were tandemly inserted into the aa 202–218 region of the major ClpG subunit of CS31A (*Figure 2*a) in two steps from pPSX10S expressing the mutant ClpG420 protein (*Figure 2*b). In the first step, synthetic double-stranded oligonucleotide specifying the TGEV-A epitope (*Figure 2*c) was ligated with *SpeI*/*Bgl*II-digested pPSX10S, leading to the plasmid pGA102 expressing the ClpG203-A hybrid protein (*Figure 2*d) as previously reported[5]. In the second step, synthetic double-stranded oligonucleotide encoding the TGEV-C epitope (*Figure 2*e) was ligated with *Spe*I-digested pGA102, resulting in the plasmid pGCA102 expressing the ClpG203-CA hybrid protein with an insert of 25 extra aa (*Figure 2*f).

To allow expression of CS31A hybrid fibrillae, pGCA102 was transferred into *E. coli* DH5α [pDSPH524]. One of the transformants screened by *in situ* colony-immunoblotting and reactive towards the TGEV-C site-specific Mab 3b.5, the TGEV-A site-specific Mab 1A.F10, and the ClpG-specific antiserum, was selected. Electron microscopy analysis of the immunogold-labelled *E. coli* cells bearing pGCA102 confirmed the production of the CS31A hybrid fibrillae at the cell surface (*Figure 3*A). Hybrid fibrillae were extracted by the thermo-elution procedure as described in , and separated by 10–15% PAGE under denaturing or native conditions for Western blot analysis (*Figure 3*). The denatured form of hybrid CS31A, consisting of the

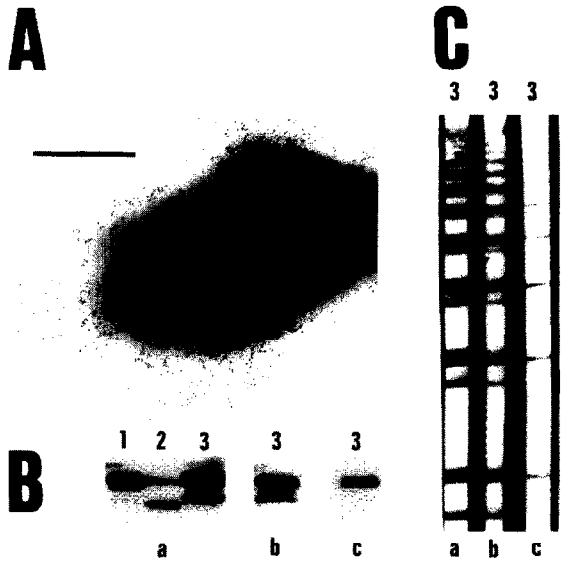

Figure 3 Expression of fibrillae and their isolation from *E. coli*. (A) Immuno-electron micrograph of *E. coli* DH5α [pDSPH524, pGCA102]. Cells were labelled with anti-ClpG antiserum and 10-nm colloidal gold-conjugated goat anti-rabbit IgG. The bar represents 0.5 μm. (B) and (C) Western blot analysis. Crude thermo-eluted fibrillae chimeras were separated by (B) 15% or (C) 10% PAGE under (B) denaturing or (C) native conditions. Blots were revealed by immunoperoxydase staining using as primary Abs either (B, a) anti-ClpG, or (C, a) anti-CS31A, or (b) 3b.5 or (c) 1A.F10. Lanes 1–3 show fibrillae encoded by plasmids; lane 1, pPSX83; lane 2, pGA102; lane 3, pGCA102

ClpG203-CA monomers, exhibited two protein bands reacting with anti-ClpG antiserum (*Figure 3*B). The upper band represents the expected full-length hybrid protein since immunologically revealed additionally by 3b.5 and 1A.F10 and since migrating slightly slower than the wild-type ClpG protein. By contrast, the lower band migrating faster than ClpG was recognized by anti-ClpG and 3b.5, but not by 1A.F10. The ability of hybrid ClpG203-CA subunit to polymerize into a chimeric CS31A fibrillum structure was evidenced from Western blots using PAGE under non-denaturing conditions (*Figure 3*C). Hybrid CS31A fibrillum appeared as a ladder of oligomeric bands of regulary increasing molecular mass constituting the polymeric form of the CS31A chimera. Each oligomer migrated as a double protein band, the major upper band reacting with anti-CS31A, 3b.5 and 1A.F10, and the minor lower band only with anti-CS31A and 3b.5. Altogether, these findings demonstrated the normal antigenic properties of the two viral epitopes exposed on the CS31A hybrid fibrillae at the *E. coli* cell-surface. However, these results also showed that the full fusion construct was partially cleaved, likely by an undefined bacterial protease, and that, unlike TGEV-C, TGEV-A in the truncated form of hybrid was antigenically inactive.

Mouse haplotype effect on antibody responses to the hybrid protein

To test the possibilities that immune responsiveness to TGEV-A and TGEV-C inserted into ClpG is modulated by the mouse haplotype, we compared the immunogenicity of ClpG203-CA protein in five strains of inbred mice and, also, in outbred Swiss mice (*Figure 4*). Groups of five animals belonging to each of the six different strains of mice, were intraperitoneally (i.p.)

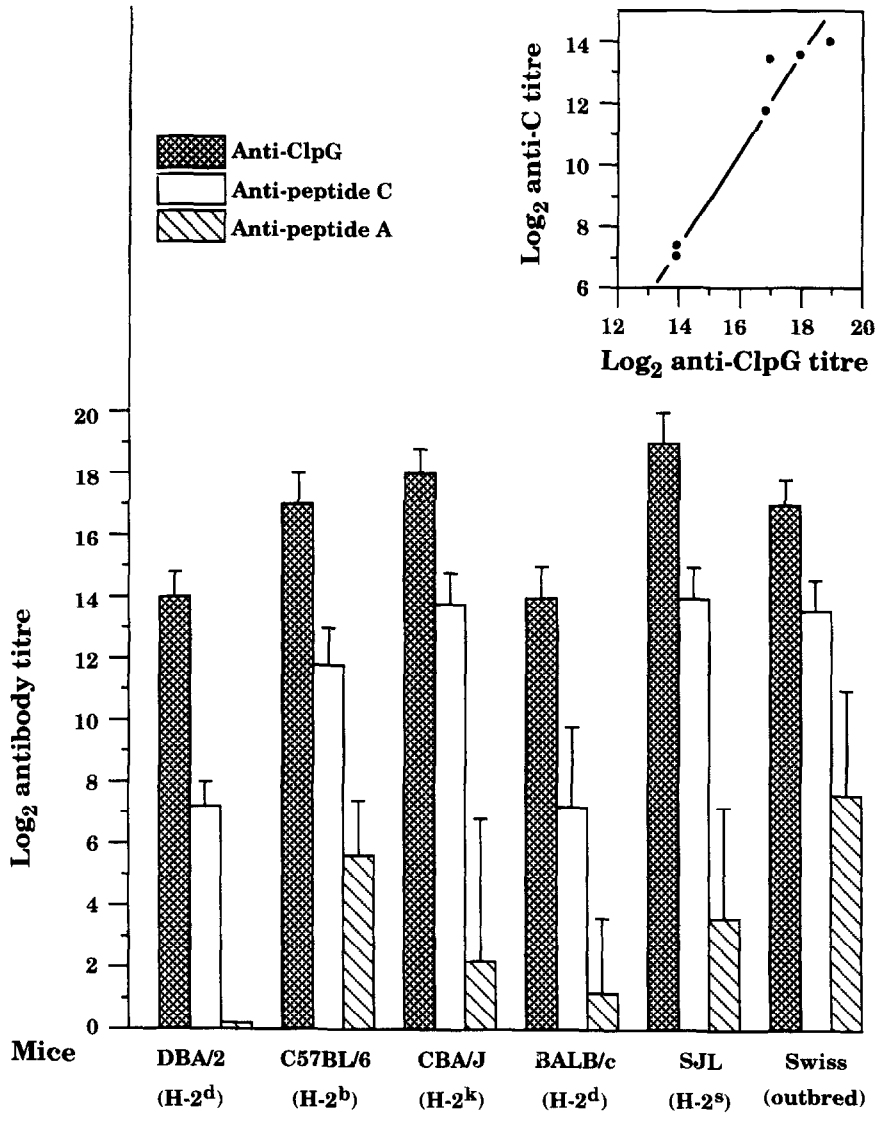

Figure 4 Production of anti-ClpG carrier and anti-TGEV peptides Abs in five strains of inbred mice and in outbred Swiss mice. The inset shows the mean anti-ClpG titres plotted against the mean anti-peptide TGEV-C titres. Mice (five per group) were i.p. immunized on days 0 and 22 with 20 μg of purified GlpG203-CA monomer proteins in incomplete Freund's adjuvant, and bled at day 35. Titres ± standard deviation are calculated as the arithmetic mean of $\log_2$ titres

immunized with purified ClpG203-CA monomers. In all cases, immunization resulted in the elicitation of Ab responses against ClpG and TGEV-C, but not against TGEV-A which was restricted essentially to H-2^b haplotype. Moreover, only the anti-ClpG and anti-peptide C titres were positively correlated (*Figure 4*). Mice with H-2^d haplotype clearly appeared to be the lowest Ab producers whatever the antigen tested. While the production of anti-ClpG and anti-peptide C was better in C57BL/6 (H-2^b), CBA/J (H-2^k), SJL (H-2^s) and Swiss mice, only the former and the latter appeared as the best responders to the ClpG-fused A peptide. Because outbred mice give high immune responses, we chose them to carry on immunological studies. In addition, their outbred status seemed more satisfying than that of inbred mice which artificially select immunological responses restricted to a laboratory animal haplotype.

Immunogenic potential of the fusion construct

To evaluate the immunogenic potential of the chimeric construction we compared the anti-ClpG, anti-peptide A, anti-peptide C, anti-TGEV particles and virus-neutralizing Ab responses following i.p. immunization of outbred Swiss mice with either CS31A hybrid-expressing bacteria, or purified CS31A hybrid polymers, or purified ClpG203-CA monomers (*Figure 5*). It was found that mice developed serum Abs with significant titres and capable of recognizing the ClpG carrier protein, the free synthetic TGEV peptides, and the virus particles, with anti-virus titres that are equivalent whatever the type of hybrid-containing preparation used for immunization. Expression of TGEV-S sites A and C, as a fusion protein on the surface of *E. coli* led to induction of TGEV-neutralizing Abs when purified recombinant antigen was used as immunogen, but not when live vector was administered. Ab responses in mice immunized with bacteria are lower than in mice immunized with purified proteins probably because lower yields of hybrid proteins associated to the bacteria compared to the 20 μg inoculated with purified proteins as previously discussed[5]. Nevertheless, these results indicate that the two TGEV peptides are immunogenic in the native ClpG-, and CS31A fibrillae-associated conformations.

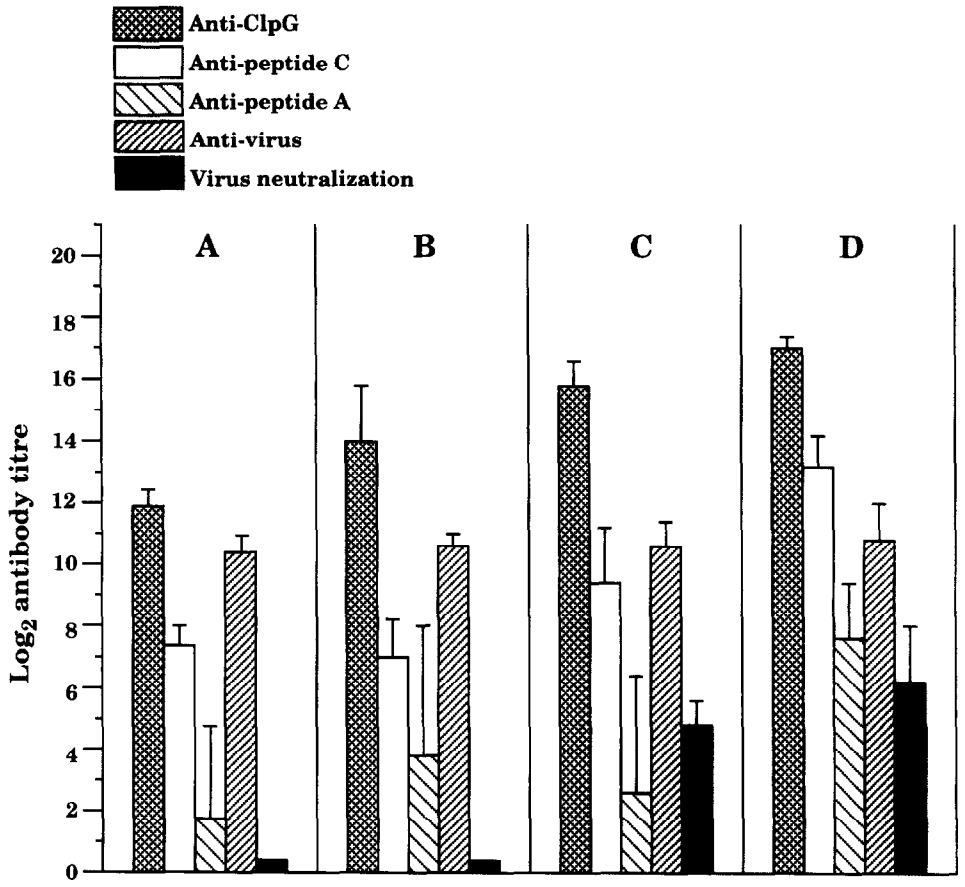

Figure 5 Anti-ClpG, anti-TGEV peptides, anti-TGEV and TGEV-neutralizing Ab responses in Swiss mice. (A and B) Immune responses induced by live *E. coli* DH5α [pDSPH524, pGCA102]; on day 0, five mice were injected i.p. with 6×10^8 bacteria in (A) saline or (B) incomplete Freund's adjuvant (IFA), then with 2×10^8 bacteria in (A) saline or (B) IFA at days 22 and 35, and bled at day 45. Preparation of bacteria as immunogens was carried out as previously described[5]. (C) Immune responses elicited by purified hybrid fibrillae; on days 0 and 22, five mice were immunized i.p. with 20 μg of fibrillae in 30% Al(OH)3, and bled at day 35. (D) Immune responses induced by purified ClpG203-CA monomer proteins; on days 0 and 22, five mice were inoculated with 20 μg of hybrid subunit proteins in IFA, and bled at day 35. Preparation of purified proteins as immunogens was performed as previously described[5]. Titres ± standard deviation are calculated as the arithmetic mean of $\log_2$ titres from sera collected at day 45 (A and B) or 35 (C and D)

Induction of immunological memory by purified hybrid fibrillae

To investigate whether fibrillae-associated TGEV epitopes were capable of generating memory B-cells, Swiss mice were immunized with purified hybrid fibrillae and then maintained at least for 100 days before booster either with homologous hybrid fibrillae (*Figure 6*A) or with TGEV virions (*Figure 6*B). Six days later, an expected strong memory Ab response was induced against the two TGEV peptides and the carrier protein following booster inoculation with the homologous hybrid fibrillae (*Figure 6*A). More interesting, a memory Ab response against these viral peptides was also induced following booster inoculation with virus 6 days after the last immunization, with anti-peptide A and anti-peptide C titres that increased 35- and 15-fold, respectively (*Figure 6*B). Anamnestic effects were specific to sites A and C since, in the same conditions of immunization and booster, control mice inoculated with a different CS31A hybrid, which carried the rotavirus VP6 epitope instead of TGEV epitopes, produced no anti-peptide A and only a weak response against C peptide following inoculation with TGEV (*Figure 6*C). The anti-peptide C titre elicited by the control mice at day 150 was 120-fold lower than that elicited by mice immunized with CS31A fibrillae carrying TGEV epitopes. Therefore, TGEV-A and TGEV-C under their CS31A fibrillae-associated form of fusion to the major ClpG subunit induced memory Abs, likely through B-cells activation by TGEV epitopes under their natural viral conformation.

DISCUSSION

Compared to other bacterial proteins used as exposure vectors for heterologous antigenic determinants (see Ref. 24 for a review), fibrillae (or fimbriae) have several advantages that favour their use as vaccines: being extracellular appendages their production and purification are a quick, easy and economical means to obtain a vaccine component of reproductible high quality[25]; being polymeric they allow to a foreign antigen previously inserted into the major fibrillar subunit to be exposed repeatedly along the fibrillum length and presented in great quantities on the entire bacterial cell surface, which may enhance immunogenicity[26]; they are good immunogens, exhibiting only low-level toxicity and since they are capable of binding to specific receptors on mucosal surface, they may be of great value in targeting the immunogen to those locations[27]. Many fimbriae[24], and CS31A fibrillae[5,11] have been used as a carrier system before. The obtained recombinant polymer proteins described in these studies were antigenic, and some

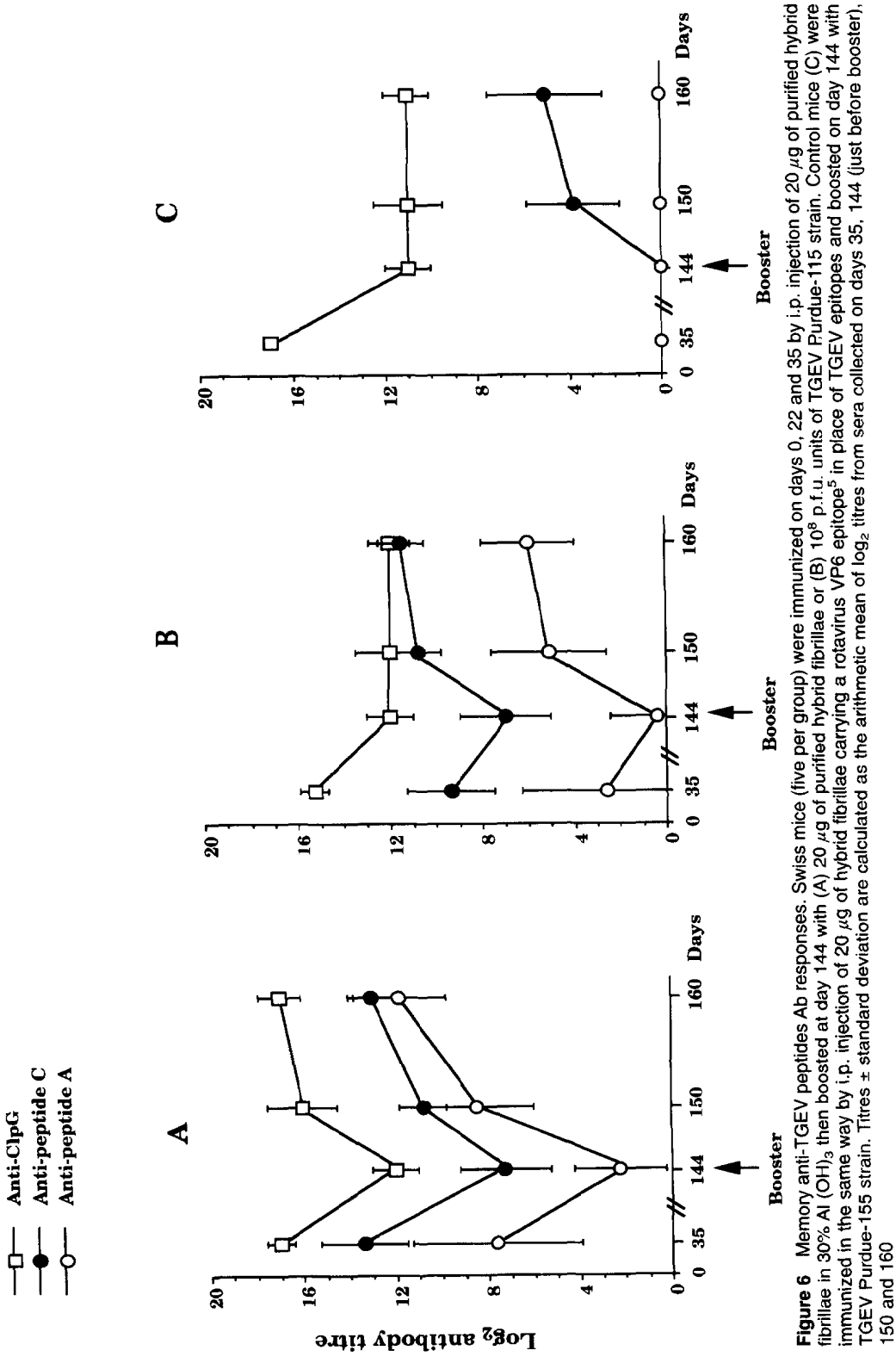

Figure 6 Memory anti-TGEV peptides Ab responses. Swiss mice (five per group) were immunized on days 0, 22 and 35 by i.p. injection of 20 μg of purified hybrid fibrillae in 30% Al $(OH)_3$ then boosted at day 144 with (A) 20 μg of purified hybrid fibrillae or (B) 10^8 p.f.u. units of TGEV Purdue-115 strain. Control mice (C) were immunized in the same way by i.p. injection of 20 μg of hybrid fibrillae carrying a rotavirus VP6 epitope[5] in place of TGEV epitopes and boosted on day 144 with TGEV Purdue-155 strain. Titres ± standard deviation are calculated as the arithmetic mean of $\log_2$ titres from sera collected on days 35, 144 (just before booster), 150 and 160

induced a serological response, but up till now no immunological memory effect of immunization with purified hybrid fimbriae has been described. The present work was undertaken to test whether an effective vaccine against TGEV could be envisaged based on the exploitation of the *E. coli* CS31A fibrillae as a delivery system. For this purpose, two antigenic peptides of the protein S of TGEV, namely TGEV-C (aa 363–371) and TGEV-A (aa 521–531), were tandemly inserted into the aa 202–218 part of the major fibrillar ClpG subunit. We have shown previously that introduction of different foreign epitopes in this ClpG region resulted in an effective assembly of the hybrid subunits into CS31A fibrillae[5], suggesting that aa 202–218 region would be the most favourable region for insertion. From results reported here, we provide evidence that the CS31A carrier-delivery system offers a realistic epitope-based strategy for recombinant TGEV vaccine development since:

(1) insertion of TGEV-A plus TGEV-C, consisting in 25 extra aa, does not interfere with fibrillae formation;
(2) the two viral epitopes are exposed in an immunogenic conformation in the ClpG subunit, CS31A fibrillae, and *E. coli* contexts;
(3) ClpG carrier is highly immunogenic;
(4) hybrid CS31A fibrillae elicit systemic Ab responses reactive against TGEV peptides, native virus particles and virus infectivity; and
(5) TGEV peptides fused to CS31A fibrillae induced Abs from memory B-cells which were activated by TGEV epitopes under their natural viral conformation in an outbred mouse population; it is unclear whether this memory response results from a cooperative effect of TGEV-A and TGEV-C peptides, the one acting as a T helper epitope and the other as a B determinant or from a B memory only[28].

Here, the immunogenicity of the recombinant bacteria, purified hybrid ClpG monomers or chimeric CS31A polymers was studied in i.p. immunized mice. Since, mucosal immunity is essential to provide protection against TGEV infections, we are evaluating also the secretory immunoresponses to various live *E. coli* producing different CS31A fibrillae chimeras with TGEV-A or/and TGEV-C epitopes inserted into different permissive sites of ClpG. Currently, we have some data supporting IgA responses. Thus, although results from bacteria expressing the TGEV-C::TGEV-A fusion peptide at the 202–218 region of ClpG, to which this work was focusing, are not available yet, data from bacteria presenting only TGEV-C epitope at this same region indicate that such recombinant bacteria are capable of eliciting IgA responses in mice. In this case, when five outbred Swiss mice were inoculated orally with 2×10^9 live bacteria on days 0, 14 and 35, specific IgA Abs against the CS31A fibrillum carrier and TGEV-C peptide were detected 10 days later in intestinal fluids with a mean titre of 1/180 (1/128–1/256) and 1/16 (<1/16–1/32), respectively. Therefore, we think that CS31A fibrillae chimeras may prove efficient as components in combination with a mucosal adjuvant or as live oral vaccines, especially since chimeric CS31A fibrillae can induce immunological memory and mucosal immunity in outbred animals, and since CS31A can also be expressed in the attenuated *Salmonella typhimurium* SL3261 strain[5].

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