Mucosal and Systemic Immune Responses to Chimeric Fimbriae Expressed by *Salmonella enterica* Serovar Typhimurium Vaccine Strains

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Recombinant live oral vaccines expressing pathogen-derived antigens offer a unique set of attractive properties. Among these are the simplicity of administration, the capacity to induce mucosal and systemic immunity, and the advantage of permitting genetic manipulation for optimal antigen presentation. In this study, the benefit of having a heterologous antigen expressed on the surface of a live vector rather than intracellularly was evaluated. Accordingly, the immune response of mice immunized with a *Salmonella enterica* serovar Typhimurium vaccine strain expressing the *Escherichia coli* 987P fimbrial antigen on its surface (Fas⁺) was compared with the expression in the periplasmic compartment (Fas⁻). Orally immunized BALB/c mice showed that 987P fimbriated *Salmonella* serovar Typhimurium CS3263 (*aroA asd*) with pCS151 (*fas*⁺ *asd*⁺) elicited a significantly higher level of 987P-specific systemic immunoglobulin G (IgG) and mucosal IgA than serovar Typhimurium CS3263 with pCS152 (*fasD* mutant, *asd*⁺) expressing 987P periplasmic antigen. Further studies were aimed at determining whether the 987P fimbriae expressed by serovar Typhimurium χ 4550 (*cya crp asd*) could be used as carriers of foreign epitopes. For this, the vaccine strain was genetically engineered to express chimeric fimbriae carrying the transmissible gastroenteritis virus (TGEV) C (379-388) and A (521-531) epitopes of the spike protein inserted into the 987P major fimbrial subunit FasA. BALB/c mice administered orally serovar Typhimurium χ 4550 expressing the chimeric fimbriae from the *tet* promoter in pCS154 (*fas*⁺ *asd*⁺) produced systemic antibodies against both fimbria and the TGEV C epitope but not against the TGEV A epitope. To improve the immunogenicity of the chimeric fimbriae, the *in vivo* inducible *nirB* promoter was inserted into pCS154, upstream of the *fas* genes, to create pCS155. In comparison with the previously used vaccine, BALB/c mice immunized orally with serovar Typhimurium χ 4550/pCS155 demonstrated significantly higher levels of serum IgG and mucosal IgA against 987P fimbria. Moreover, mucosal IgA against the TGEV C epitope was only detected with serovar Typhimurium χ 4550/pCS155. The induced antibodies also recognized the epitopes in the context of the full-length TGEV spike protein. Hence, immune responses to heterologous chimeric fimbriae on *Salmonella* vaccine vectors can be optimized by using promoters known to be activated *in vivo*.

Infectious diarrhea remains a major cause of mortality and morbidity in neonatal and recently weaned piglets (U.S. Department of Agriculture National Swine Survey: Morbidity/Mortality and Health Management of Swine in the United States [1992] and Swine '95 Study, Part III: 1990–1995 Changes in the U.S. Pork Industry [1997]; NAHMS_INFO@aphis.usda.gov). Transmissible gastroenteritis virus (TGEV) and enterotoxigenic *Escherichia coli* (ETEC) are among the leading causative agents of diarrhea in piglets (48, 58). TGEV is a coronavirus and has three major structural proteins (S, N, and M) (40). The spike (S or E2) protein, located on the surface of the virus, elicits antibodies that can neutralize virus and protect animals against infection (37, 80, 83). Four sites (A, B, C, and D) have been defined by analysis with monoclonal antibodies and sites C (positions 379 to 388) and A (positions 521 to 531) have been identified as targets for neutralization of TGEV (18, 19, 28). Both C and A are continuous epitopes and are glycosylation independent (28, 68). This feature makes them suitable for being displayed by carrier proteins as antigenic epitopes to induce anti-TGEV immunity.

Enteroadhesive fimbriae play a critical role in the pathogen-

esis of ETEC. The binding of fimbriae to intestinal receptors ensures optimal mucosal colonization by the bacteria and efficient enterotoxin delivery to the enterocytes. Fimbriae can serve as an effective vaccine to induce an immune response against ETEC infections. For example, piglets of dams injected with purified 987P fimbriae were protected against experimental infection with 987P-fimbriated ETEC, and this protection was correlated with the presence of specific anti-987P antibodies in the colostrum (34, 49, 51). Veterinary vaccines based on fimbrial proteins have been used successfully for many years (48), and fimbriae are considered major antigens of currently tested vaccines to protect humans from ETEC (2, 41, 62). Passive immunization of animals with anti-fimbria antibodies protects animals by blocking fimbria-mediated enteroadhesion of ETEC (33, 43). Passive immunity is also of primary importance in providing newborn piglets with immediate protection against TGEV (59, 60).

In order to take advantage of the excellent immunogenicity of fimbriae, several investigators have modified fimbriae genetically to create chimeric organelles displaying foreign epitopes (54). Recently, the CS31 and the 987P fimbriae of *E. coli* were engineered to present TGEV epitopes (20, 46, 56). Both purified chimeric fimbriae were shown to induce anti-TGEV and anti-fimbria specific antibodies in mice and rabbits. Protection against neonatal infectious agents such as TGEV or 987P-ETEC is currently best obtained by passive immunization

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i>		
987	O9:K103:987P (prototype F6)	35, 50
SE5000	MC4100 <i>recA56</i> (Fim ⁻)	67
χ6212	<i>ΔasdA1</i> derivative of DH5α	52
BL21	<i>lon ompT</i>	Novagen, Madison, Wis.
SG396	<i>htpR lonR9</i>	29
Serovar Typhimurium		
χ4487	<i>Δ(crp-cysG)-10 Δ(zhc-1431::Tn10) Δasd1 Δ(zhf-4::Tn10) Δcya-12 Δ(zid-62::Tn10)</i>	R. Curtiss
χ4550	<i>gyrA1816 ΔasdA1 Δ(zhf-4::Tn10) Δcrp-1 Δcya-1</i>	66
SL3261	<i>ΔaroA</i>	B. Stocker
CS3263	<i>ΔaroA ΔasdA1</i>	This study
Plasmids		
pYA3332	<i>asd</i> -containing vector	R. Curtiss
pLG339	pSC101 derivative, Km ^r	70
pDMS167	pBR322 <i>fas</i> ⁺	64
pDMS203	pDS167 <i>fasD</i> (Fas ⁻)	24, 64
pRS234	pDMS167 with TGEV C epitope in <i>fasA</i> (Fas ⁺)	56
pCS101	pRS234 with the TGEV A epitope in <i>fasA</i> (Fas ⁺)	This study
pCS110	pCS101 with <i>PacI</i> site changed to <i>Bam</i> HI site (Fas ⁺)	This study
pCS112	pCS110 with <i>Afl</i> III site changed to <i>Bam</i> HI site (Fas ⁺)	This study
pCS114	pCS101 with <i>asd</i> gene in <i>Bam</i> HI site (Fas ⁺)	This study
pCS150	pLG339 with the TGEV C and A epitopes inserted into <i>fasA</i> of pCS112 (Fas ⁺)	This study
pCS151	pDMS167 with <i>asd</i> in its <i>Bam</i> HI site (Fas ⁺)	This study
pCS152	pDMS203 with <i>asd</i> in its <i>Bam</i> HI site (Fas ⁻)	This study
pCS154	pCS150 <i>asd</i> (Fas ⁺)	This study
pCS155	pCS154 with the <i>nirB</i> promoter upstream <i>fasA</i> (Fas ⁺)	This study

of piglets after induction of colostral antibodies in the sow (48, 59, 60). Colostral antibodies can be induced by the oral delivery of protective antigens activating the gut-associated lymphoid tissues (GALT) of sows (9, 60). One method of delivering antigens to the GALT is by the use of vectors possessing tropism for Peyer's patches, such as *Salmonella enterica*. In the last two decades, various *Salmonella* vectors have been tested for their capacity to deliver antigens and induce the GALT to mount protective immune responses (14, 17). More-recent studies have been aimed at optimizing antigen expression by using promoters like the *nirB* or *htrA* promoters of *S. enterica* which are activated by specific environmental conditions found in the host (11, 12, 27, 57). Typically, the delivered antigens were expressed intracellularly by attenuated *S. enterica* serovar Typhimurium mutants (15, 32). Recently, oral administration of attenuated serovar Typhimurium expressing either human or farm animal ETEC fimbriae was shown to elicit specific immunoglobulin A (IgA) and IgG responses and even to induce protective immunity in a model using mice to study diarrhea (3, 85). Although *Salmonella* fimbrial proteins have also been engineered to present foreign epitopes (81, 84), the possibility that this alteration attenuated the vaccine strains to the point where they were no longer able to elicit a protective mucosal immune response was not investigated.

In this study, we found that various live attenuated strains of serovar Typhimurium can be made to express chimeric 987P fimbriae. Moreover, these fimbriated strains were shown to elicit both systemic and mucosal immune responses against both the 987P fimbriae and the foreign epitopes, namely, TGEV epitopes. Most interestingly, the best immune responses against the TGEV epitopes were obtained with a construct utilizing the *nirB* promoter for fimbrial expression, suggesting that inducible promoters can be used in vivo to optimize expression of chimeric fimbriae.

MATERIALS AND METHODS

Mice. Five-week-old female BALB/cByJ mice were obtained from Jackson Laboratory and housed in filter-top cages in an air-conditioned animal facility. Water and food was provided ad libitum. Mice were adapted for one week after arrival before being used for immunization.

Bacterial strains, media, and reagents. The *E. coli* and serovar Typhimurium strains used in this study are listed in Table 1. Serovar Typhimurium CS3263 strain is a *ΔasdA1* derivative of SL3261 (serovar Typhimurium WRAY *hisG aroA*) and was constructed by generalized transduction, using phage P22Htint and χ4487 as the donor strain. *E. coli* 987 was grown in minimal medium E supplemented with pantothenic acid and glycerol, as described previously (26). Strains SE5000 and SL3261 were grown in L medium, whereas strains χ6212, χ4487, χ4550, and CS3263 were grown in L medium with 50 μg of diaminopimelic acid (Sigma, St. Louis, Mo.) per ml. When necessary, media were supplemented with the following antibiotics: ampicillin (200 μg/ml), tetracycline (10 μg/ml), or kanamycin (45 μg/ml). Medium components were purchased from Difco (Detroit, Mich.). Restriction and modification enzymes were from New England Biolabs, Inc. (Beverly, Mass.). Unless specified, reagents were purchased from Sigma.

Plasmid constructs. Standard procedures were used to construct the following plasmids (Fig. 1). In order to add a second epitope of TGEV to the major subunit FasA of 987P fimbriae carrying already epitope TGEV C, plasmid pRS234 was linearized with *Xma*I and ligated with a previously annealed 39-bp pair of oligonucleotides (5'-CCGGGTATGAAACGTTCCGGTTACGGTACGCCGATCG CT-3' and 5'-CCGGAGCGATCGGCTGACCGTAACCGGAACGTTTCATA C-3') which encodes the TGEV A epitope, resulting in plasmid pCS101. Correct orientation and in-frame insertion of the TGEV A segment in pCS101 was confirmed by DNA sequencing. Plasmids pCS151, pCS152, and pCS114 were constructed by inserting a gel-purified 1.8-kb *Bgl*II fragment of pYA3332 containing the *asd* gene into *Bam*HI-linearized pDMS167, pDMS203, or pCS101, respectively. A gene cluster of pCS101 encoding all the structural genes for fimbriation (*fasA* to *fasG*) but missing the 5' end of *fasH*, was flanked in two steps by *Bam*HI restriction sites. For this, a 12-mer *Bam*HI linker (d(CGCGGATCCGCG) was inserted into Klenow enzyme-treated *Pac*I-linearized pCS101 to obtain pCS110; second, a similar linker was inserted into Klenow enzyme-treated *Afl*III-linearized pCS110 to obtain pCS112. The 7.76-kb *Bam*HI fragment of pCS112 encoding the *fasA* to *fasG* genes with the two TGEV epitopes in *fasA* was inserted into the low-copy-number plasmid pLG339 to obtain pCS150. The *Bgl*II-fragment of pYA3332 containing the *asd* gene was inserted into the *Bam*HI site at the 3' end of *fasG* in pCS150 to obtain pCS154. pCS155 was constructed by inserting an annealed 70-bp pair oligonucleotides (5'-GATCAGGTAAATTTGATGTAC ATCAAATGGTACCCCTTGCTGAATCGTTAAGGTAGCGGTAAGTAC

FIG. 1. Physical maps of the expression plasmids used in the immunization experiments. Plasmids pCS151 and pCS114 are derivatives of pBR322 carrying all the 987P genes (*fasABCDEFG* and *fasH*). Plasmid pCS152 is a derivative of pDMS151 which expresses intracellular 987P fimbrial components because a portion of the gene for the usher protein FasD was deleted. In these plasmids, expression of *fasA* is under the control of the transcriptional activator *fasH*. Plasmids pCS154 and pCS155 are derivatives of pLG339 expressing the genes for the 987P export and assembly (*fasABCDEFG*) under the control of the tetracycline promoter (pCS154) and the *nirB* promoter (pCS155). Plasmid pCS151 expresses wild-type 987P fimbriae, while plasmids pCS114, pCS154, and pCS155 express chimeric 987P fimbriae with the TGEV C and A epitopes in the major subunit FasA.

TG-3' and 5'-GATCCAGATCTTACCGCTACCTTAACGATTACGAAGGGGTACCATTTGATGTACATCAAAATTACCTG-3'), encoding the *nirB* promoter with its FNR binding site, into the *Bam*HI site of pCS154, upstream *fasA*.

Seroagglutination and antibodies. Slide seroagglutination tests were performed with rabbit anti-987P antiserum (63) previously preadsorbed with non-fimbriated phase variants of strain 987, with 987P quaternary structure-specific monoclonal antibodies (63), with an anti-TGEV C epitope antiserum (56), and/or with an anti-TGEV A epitope antiserum. Anti-TGEV A epitope antibodies were induced in rabbits by subcutaneous injections of 200 μ g of TGEV A peptide cross-linked to keyhole limpet hemocyanin (56) in complete Freund's adjuvant, followed by three booster injections of 200 μ g of the same antigen in incomplete Freund's adjuvant at 2-week intervals. Seroagglutination was evaluated semi-

quantitatively (+++, immediate very strong agglutination; ++, strong agglutination after 10 s; +, weak agglutination after 10 s; -, no agglutination for 1 min), as described previously (65).

Peptides and fimbriae. The TGEV C and A peptides of the spike protein, corresponding to amino acid residues 379 to 388 and 521 to 531, respectively, were both synthesized with a cysteine added to their carboxy termini (SSFFSYGEIPC and MKRSGYGQPIAC) at the Protein Chemistry Laboratory of the University of Pennsylvania School of Medicine. Fimbriae expressed on the bacterial surface were prepared by heat extraction, as described previously (39).

SDS-PAGE and Western blotting. Bacterial pellets, isolated fimbriae, or the baculovirus TGEV S protein lysate R2-2 and baculovirus Sf9 mock protein (kind gift from Dr. Linda Saif) were resuspended in sample buffer, boiled for 5 min, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE). Western blots were probed with sera of immunized mice or with rabbit anti-987P fimbriae antibodies as controls using horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence (ECL) for detection (64). The relative steady-state amount of FasA protein in different constructs was evaluated by comparing proteins densitometry with NIH Image Software (Division of Computer Research and Technology, National Institutes of Health, Bethesda, Md.). Amounts of extracts were qualitated by using the same number of bacteria (CFU).

Immunization and sampling. For each immunization, a single colony of *Salmonella* serovar Typhimurium was grown in L broth without any antibiotics at 37°C on a rotary shaker at 150 rpm overnight. The bacterial cells were gently washed once and resuspended in sterile phosphate-buffered saline (PBS; 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl; pH 7.2) at a concentration of 1×10^{11} to 5×10^{11} CFU/ml. Viable counts were performed on all inocula. Before immunization, the mice were deprived of food and water for 4 h. The mice were intubated with feeding needles for intragastric delivery of 200 µl of bacterial suspensions and fasted for an additional 30 min. The mice were immunized at days 0 and 26. For each immunized group of mice, pooled fecal pellets were collected biweekly. Approximately 500 mg of feces was added to tubes containing 2 ml of a protease inhibitor solution (PBS with 0.5% bovine serum albumin (BSA) and a cocktail of protease inhibitors [Complete; Boehringer Mannheim] using the manufacturer's recommended concentration). The fecal pellets were soaked in ice for 15 min, and the tubes were agitated vigorously for 5 min twice on a vortex mixer at maximum speed. The suspensions were centrifuged at $13,000 \times g$ for 15 min, and the supernatants were stored at -20°C. To collect serum samples, intestinal secretions, and bile, mice were anesthetized with Metofane (methoxyflurane; Mallinckrodt Veterinary, Inc., Mundelein, Ill.) at between 6 and 8 weeks postimmunization. Blood and bile were collected by heart and gallbladder punctures, respectively. Whole small intestines, from the duodenum to the ileocecal junction, were excised, and luminal contents were carefully collected with the help of 3 ml of protease inhibitor solution introduced into intestinal lumens. Recovered intestinal contents were vortexed vigorously for 5 min. After centrifugation at $13,000 \times g$ for 15 min at 4°C, supernatants were collected and stored at -20°C.

ELISA. Individual mouse serum, intestinal secretion, bile, and group-pooled fecal pellet extract were tested for IgA, IgG, IgG1, and/or IgG2a antibodies against 987P fimbriae by enzyme-linked immunosorbent assay (ELISA) essentially as described earlier (63). Briefly, 96-well ELISA plates (Immulon-4; Dynatech Laboratories, Inc., Chantilly, Va.) were coated with isolated wild-type 987P fimbriae (0.2 µg in 100 µl of 0.1 M carbonate buffer, pH 9.6, per well) overnight at 4°C or with TGEV C peptide (1 µg in 100 µl of 0.1 M carbonate buffer, pH 9.6, per well) by using a household microwave oven at 145 W, twice for 10 s, followed by overnight incubation at 4°C (86). 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 body fluid samples 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 or IgA antibodies at 37°C for 1 h. After the last washing step, bound antibodies were detected by using *o*-phenylenediamine as the chromogenic reagent and then reading the absorbance at 450 nm.

Dot blot assay. Nitrocellulose strips were spotted with 3 µl of dilutions of TGEV C peptide (2, 0.4, and 0.1 µg) or TGEV A peptide (2 and 0.4 µg) or with isolated 987P fimbriae (0.1 µg). All dilutions were made in TNS buffer (0.01 M Tris, pH 7.3; 0.15 M NaCl; 0.1% Nonidet P-40). The strips were air dried and blocked with 3% BSA in TNT buffer (0.01 M Tris, pH 7.3; 0.9% NaCl; 0.05% Tween 20) at room temperature for 1 h. The strips were incubated with the diluted sera or pooled fecal extracts for 2 h at room temperature with shaking. The blots were developed with HRP-conjugated with goat anti-mouse IgG or IgA antibodies and then visualized by ECL.

Statistical analysis. Antibody titers were compared by using the unpaired Student's *t* test, as described elsewhere (47). Statistical significance was assessed at *P* values of <0.05, <0.01, or <0.001.

RESULTS

Construction of *Salmonella*-based vaccines with stable expression of *E. coli* 987P fimbriae. To stabilize antigen expression and to optimize the immunogenicity of 987P encoded by multicopy-number plasmids, we used the *asd*⁺ balanced lethal system originally developed with *Salmonella* *crp cya* strains. Accordingly, the *asd* gene from pYA3332 was inserted into the tetracycline-resistance gene of pDS167, which carries all the genes for 987P fimbriation (*fas* genes), creating pCS151. *E. coli asd* strain χ 6212 transformed with pCS151 stably expressed 987P fimbriae on its surface in vitro in the absence of ampicillin. All 20 tested colonies of transformed bacteria grown overnight on L agar plates, and all subcultures in L broth were fimbriated in the absence of antibiotics, as shown by seroagglutination assays (+++/+++), with antibodies recognizing

FIG. 2. SDS-PAGE of isolated 987P fimbriae (A) and Western blot analysis of whole bacterial cells with anti-987P antibody (B) of *E. coli* SE5000 or serovar Typhimurium CS3263 with different plasmids. Lane 1, SE5000/pDMS167 (*Fas*⁺); lane 2, CS3263/pCS152 (*fasD*); lane 3, CS3263/pCS151 (*Fas*⁺); lane 4, CS3263/pCS114 (*Fas*⁺); lane 5, protein molecular mass standards in kilodaltons. Densitometric analysis suggests that in comparison to strain CS3263/pCS151 (*Fas*⁺), strain CS3263/pCS152 (*fasD*) expresses 22% of the amounts of FasA. Molecular masses (in kilodaltons) of standard proteins are shown on the right.

only fully assembled fimbriae. In contrast, none of 20 tested colonies of *Salmonella* *crp cya asd* vaccine strain χ 4550 transformed with pCS151 expressed these fimbriae, suggesting that catabolite repression was regulating 987P expression not only in the wild-type strain 987, as described previously (26), but also when the appropriate genes were cloned in multicopy-number plasmids. This was confirmed by constructing an *asd* derivative of the *Salmonella aroA* vaccine strain SL3261, designated CS3263, and showing that *crp*⁺ and *cya*⁺ strain CS3263/pCS151 stably expressed the 987P fimbriae in vitro.

Improved mucosal and systemic immune responses with a surface-exposed antigen. In order to determine whether an antigen is a better immunogen when expressed intracellularly or exported to the bacterial surface, the immunogenicity of the major 987P antigen FasA was compared when FasA was expressed by *Salmonella* vaccine strain CS3263 carrying either pCS151 (*fas*⁺ *asd*⁺) or pCS152 (*fasD* mutant, *asd*⁺). Use of pCS151 results in FasA export and bacterial fimbriation, as shown by seroagglutination and SDS-PAGE (Fig. 2A) of heat-extracted preparations of fimbriae. In contrast, salmonellae do not export FasA or express 987P fimbriae when the usher protein FasD is missing, as observed with strain CS3263/pCS152. Nevertheless, the latter strain still synthesized the major 987P antigen FasA, albeit at a lower level (approximately 22%) than strain CS3263/pCS151, as determined by Western blotting with rabbit anti-987P fimbriae serum (Fig. 2B). BALB/c mice (eight per group) were immunized with 3×10^{10} *Salmonella* sp. strains CS3263/pCS151 or CS3263/pCS152, respectively. Both strains could be isolated from the feces for at least 4 days. All tested fecal isolates (20 colonies) of strain CS3263/pCS151 (*fas*⁺) were 987P fimbriated, as determined by seroagglutination, suggesting the absence of in vivo selection of nonfimbriated plasmid-carrying salmonellae. All mice from each group were euthanized at between 6 and 8 weeks postimmunization, and samples were used for evaluating the systemic and mucosal humoral immune responses against 987P antigen. Although mice immunized with CS3263/pCS152 showed serum antibodies to 987P, the mice immunized with

FIG. 3. 987P fimbria-specific total serum IgG, serum IgG1, and IgG2a (A) and serum IgA (B) responses. BALB/c mice were immunized orally with two doses (26-day interval) of serovar Typhimurium CS3263/pCS152(*fasD*) expressing fimbrial antigen in the periplasm (hatched bars) or serovar Typhimurium CS3263/pCS151 expressing 987P fimbriae on the surface (crossed bars). The sera collected before immunization (solid bars) were used as controls. Means and standard deviations of serum IgG and IgA titers of mice tested 6 to 8 weeks postimmunization are shown. Serum antibody titers elicited by serovar Typhimurium CS3263/pCS151 were significantly higher than the titers induced by serovar Typhimurium CS3263/pCS152 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

CS3263/pCS151 developed significantly higher titers of 987P-specific serum IgG, including both IgG1 and IgG2a (Fig. 3A) and IgA (Fig. 3B). Similarly, mucosal secretory IgA titers determined in stool (Fig. 4A), gut wash (Fig. 4B) and bile (Fig. 4C) were also significantly higher with CS3263/pCS151. Interestingly, in Fig. 3 the IgG2a titers were significantly higher than the IgG1 titers for the mice immunized with CS3263/pCS152 ($P < 0.05$), suggesting that this construct induced a predominant Th1 response, whereas there was a mixed response in the mice immunized with CS3263/pCS151. How much these results relate to the total amount of steady-state subunit antigen or to the export and assembly status of the subunit expressed by the respective constructs remains undetermined. However, a system capable of exporting an overexpressed antigen to the cell surface may diminish the intracellular degradation of this antigen or may prevent negative feedbacks that decrease either antigen expression or bacterial growth.

Construction of chimeric 987P fimbriae carrying two epitopes of the TGEV S protein. The 987P fimbrial subunit gene *fasA* was previously genetically engineered to use as a polymeric surface display system for immunogenic foreign epitopes (56). Having shown above that 987P can be expressed on salmonellae, we first showed that pRS234 (56), a plasmid that harbors a modified 987P gene cluster containing the TGEV C epitope between residues 2 and 3 of *FasA*, also assembles fimbriae on salmonellae, as shown by the seroagglutination (+ + + + +). We next showed that plasmid pCS101 containing a second epitope, TGEV A, added at the carboxy terminus of

the C epitope (Fig. 5A) directed the expression of chimeric fimbriae on the surface of *E. coli* SE5000/pCS101, as demonstrated by seroagglutination with anti-987P, anti-TGEV C, and anti-TGEV A epitope antibodies. The TGEV C and A peptide segments were chosen for their known immunogenic properties, with both continuous epitopes being recognized by neutralizing monoclonal antibodies (19). No more than two epitopes consisting of a total of 27 residues could be added to the permissive site of *FasA* without interfering with fimbriation (data not shown). We also found that, consistent with a previous study (46), insertion of the TGEV A epitope into fimbriae mediated partial proteolytic cleavage of the chimeric fimbrial subunit when using K-12 *E. coli* SE5000 with a wild-type phenotype for protease production, as shown by SDS-PAGE (Fig. 5B). By using other *E. coli* host strains known to lack certain proteases, such as BL21 (71) or SG396 (29), subunit cleavage was not significant, although the total amounts of detectable subunits were also lower (Fig. 5B). Most importantly, significant amounts of only full-length subunits were detectable when pCS101 was in the *Salmonella* sp. strain SL3261 (Fig. 5B), indicating that most subunits in the chimeric fimbriae carried both TGEV epitopes and that this construct was suitable for immunization studies.

FIG. 4. 987P fimbria-specific mucosal IgA responses in pooled stools (A), gut washes (B), and bile (C). BALB/c mice were immunized orally with two doses (26-day interval) of serovar Typhimurium CS3263/pCS152 (*fasD*) expressing fimbrial antigen in the periplasm (hatched bars) or serovar Typhimurium CS3263/pCS151 expressing 987P fimbriae on the surface (crossed bars). The fecal pellets were collected biweekly. The gut washes and bile were collected before immunization and at 6 to 8 weeks after immunization. The samples collected before immunization (solid bars) were used as controls. The IgA in the stools were measured from pooled fecal pellets. IgA levels in gut washes and bile were measured individually, and error bars represent standard deviations of the values for eight mice. No antibodies were detected in fecal pellets from preimmunized mice. IgA titers elicited by serovar Typhimurium CS3263/pCS151 in the gut washes and bile were significantly higher than the titers induced by serovar Typhimurium CS3263/pCS152 (*, $P < 0.05$; **, $P < 0.01$).

FIG. 5. Expression product of the allelic FasA proteins by different plasmids. (A) Amino acid sequences of the amino terminus of the allelic FasA proteins. pDS167, pCS151, and pCS152 encode the genes for the wild-type 987P fimbriae; pRS234 encodes the genes for expressing chimeric fimbriae with the FasA-TGEV C protein (epitopes in boldface); and pCS101, pCS114, pCS154, and pCS155 encode the genes for expressing chimeric fimbriae with the FasA-TGEV C-TGEV A proteins (epitopes in boldface and italic, respectively). (B) SDS-PAGE of isolated wild-type or chimeric 987P fimbriae from different *E. coli* or serovar Typhimurium strains. Lane 1, protein molecular mass standards in kilodaltons; lane 2, *E. coli* SE5000/pDMS167; lane 3, *E. coli* SE5000/pRS234; lane 4, *E. coli* BL21/pCS101; lane 5, serovar Typhimurium SL3261/pCS101; lane 6, *E. coli* SE5000/pCS101; lane 7, *E. coli* SG396/pCS101. SE5000 (wild type for protease production) cleaved the chimeric FasA protein; BL21 (*lon ompT*), SG396 (*htrp lonR9*), and SL3261 did not cleave the chimeric FasA protein.

Immunogenicity of chimeric 987P with a *Salmonella aroA asd* mutant. The *asd* balanced-lethal system was used to stabilize plasmid maintenance for in vivo experiments. Accordingly, an *asd* gene was introduced into the tetracycline-resistance gene of pCS101, generating pCS114, and *Salmonella* sp. strain CS3263, an *asd* mutant of strain SL3261, was prepared by generalized transduction. *Salmonella* sp. strain CS3263/pCS114 was found to stably express chimeric fimbriae without antibiotics. Expression of the chimeric fimbriae was shown to be under the control of the 987P transcriptional regulator *fasH* and to require CRP-Cya (data not shown), as in the original ETEC strain 987 (25, 26). The immunogenicity of the chimeric fimbriae expressed by CS3263/pCS114 was tested 6 to 8 weeks after oral administration of the salmonellae to eight BALB/c mice. Although the fimbriae elicited serum IgG and IgA (Fig. 6A) and mucosal IgA (Fig. 6B) specific for 987P epitopes, the antibody titers were generally low. Only a low titer (1:100) of anti-TGEV C epitope IgG was detected by dot blot assay in the sera of two of seven mice, and no anti-TGEV epitope IgA was detectable (data not shown).

Induction of mucosal response with a *Salmonella cya crp asd* mutant expressing chimeric fimbriae. 987P fimbrial expression is regulated by catabolite repression, and it has been proposed that these fimbriae are only expressed in the distal portion of

the small intestine (25, 26). Thus, it is possible that the poor immune response to the TGEV epitopes may be related to suboptimal expression of the fimbrial antigen in the intestines. To circumvent this potential problem, plasmid pCS154 was constructed. This plasmid expresses the chimeric FasA protein carrying both TGEV epitopes, as well as the FasB to FasG proteins, under the control of the tetracycline promoter from the low-copy-number pLG339 vector. In vitro-grown χ 4550/pCS154 produced significant amounts of chimeric 987P, as shown by seroagglutination with anti-TGEV epitope and anti-987P antibodies. The antigen specific humoral response of eight BALB/c mice was investigated 6 to 8 weeks after oral administration of χ 4550/pCS154 (Fig. 7). Compared to the CS3263/pCS114-immunized animals, the χ 4550/pCS154-immunized mice developed higher titers of systemic and mucosal antibodies against 987P fimbriae, but the differences were not statistically significant. Moreover, anti-TGEV antibodies were again only detected against the TGEV C epitope as serum IgG (Fig. 7E and Fig. 8A).

Enhanced immunogenicity with the *nirB* promoter directing the expression of chimeric fimbriae. In an attempt to improve the production of chimeric fimbriae in the intestines of mice, the *nirB* promoter was introduced into pCS154, just upstream of the putative ribosomal binding site for the chimeric *fasA* gene. The resulting plasmid, pCS155, thus contains a promoter

FIG. 6. Responses of 987P fimbria-specific serum IgG and IgA (A) and mucosal IgA in stools (B). BALB/c mice were immunized orally with two doses of serovar Typhimurium CS3263/pCS114 in a 26-day interval. Sera were collected before and at 6 to 8 weeks after the first immunization. Serum IgG (solid bars) and IgA (hatched bars) were measured individually. Error bars represent the standard deviations of the values for seven mice. The IgA levels in stools were measured from the pooled fecal pellets. No antibodies were detected in fecal pellets from preimmunized mice.

FIG. 7. Responses of 987P fimbria-specific serum IgG (A), serum IgA (B), mucosal IgA in gut washes (C) and stools (D), and TGEV C peptide-specific serum IgG (E). BALB/c mice were immunized orally with two doses (26-day interval) of 987P-fimbriated serovar Typhimurium χ 4550/pCS155 (crossed bars) or serovar Typhimurium χ 4550/pCS154 (hatched bars). Samples collected before immunization (solid bars) were used as controls. Serum IgG, serum IgA, and IgA in gut washes were measured 6 to 8 weeks postimmunization. Error bars represent the standard deviations of the values for eight mice. Stool IgA levels were measured from pooled fecal pellets. No antibodies were detected in fecal pellets from preimmunized mice. Serum IgG and mucosal IgA titers elicited by serovar Typhimurium χ 4550/pCS155 were significantly higher than the titers induced by serovar Typhimurium χ 4550/pCS154 (*, $P < 0.05$; **, $P < 0.01$).

typically activated in the anaerobic intestinal or intracellular environment. Comparison of the immune response with χ 4550/pCS155 versus χ 4550/pCS154 yielded results that differed significantly in only some of the parameters measured (Fig. 7). χ 4550/pCS155 elicited significantly higher levels of anti-987P IgG (Fig. 7A) in the serum and of IgA in gut washes (Fig. 7C) of orally immunized BALB/c mice. Addition of the *nirB* promoter enhanced the immune response to the TGEV epitopes, as best visualized in dot blot assays (Fig. 8A), with serum anti-TGEV A epitope IgG being induced in the mice immunized with χ 4550/pCS155 (Fig. 8A). Moreover, mucosal IgA against the TGEV C epitope was only developed in the mice immunized with χ 4550/pCS155 (Fig. 8B). These results indicated that use of the *nirB* promoter improved the immunogenicity of chimeric 987P fimbriae delivered by salmonellae. Having shown that the specific antibodies obtained reacted with the short TGEV C and TGEV A peptides, we further determined whether they were also able to recognize the full-length protein, namely, the recombinant TGEV Spike protein. Western blotting showed that the antibodies reacted with this protein (Fig. 8C), indicating that the TGEV epitopes remained accessible to the antibodies in the context of the TGEV S protein.

DISCUSSION

In addition to the panoply of better-studied attenuated *S. enterica* serovar Typhimurium vaccine strains, new mutants continue to be evaluated for their use and advantages as

antigen delivery vectors. Construction of chimeric proteins containing heterologous sequences, the expression of these proteins by *Salmonella* vectors, and the evaluation of the recombinant strains as potential vaccines have been reported by many groups (11, 42, 45, 53). In most studies, the foreign antigens were expressed and maintained intracellularly (15, 32). In some cases, to facilitate direct interaction of the expressed antigen with the host's immune system, the antigens were exported to the bacterial surface by constructing fusions with genes for outer membrane proteins, flagellin, or fimbria (1, 30, 78, 79, 81). The goal of the present study was the development of a multivalent vaccine for swine diarrhea. Thus, an attenuated *Salmonella* strain has been made to express antigens of two different porcine enteropathogens: the 987P fimbriae of a porcine ETEC carrying antigenic epitopes of the porcine TGEV. To our knowledge, this is the first report describing a prototype *Salmonella* vaccine delivering antigens as foreign chimeric fimbriae.

Several permissive sites in the major subunit of 987P were recently identified by random linker insertion mutagenesis of the subunit gene *fasA* (56). A site near the amino terminus of the processed *FasA* was characterized as the most tolerant for additional foreign epitope insertion. In the current study, two epitopes of the TGEV S protein, designated epitope C and A, were found to be tolerated by the 987P biogenesis machinery since, as shown with epitope-specific antibodies, chimeric fimbriae displayed both epitopes on the bacterial surface. This construction resulted in the insertion of 27 additional amino

FIG. 8. Dot blots and Western blot demonstrating systemic and mucosal antibody response to the TGEV C or A epitope. (A) TGEV C epitope-specific serum IgG were detected by dot blot assay after oral immunization of four different BALB/c mice with serovar Typhimurium χ 4550/pCS154 (lanes a to d). TGEV C and A epitope-specific serum IgG were detected after oral immunization of four different BALB/c mice with serovar Typhimurium χ 4550/pCS155 (lanes e to h). Rabbit anti-wild-type 987P serum was used as negative control (lane n), and rabbit anti-chimeric 987P-TGEV C serum was used as positive control (lane p). The sera from the immunized mice were diluted 1:100. The antigen in rows 1, 2, and 3 was TGEV C peptide at 2, 0.4, and 0.1 μ g per spot, respectively. The antigen in rows 4 and 5 was TGEV A peptide at 2 and 0.4 μ g per spot, respectively. The antigen in row 6 was purified wild-type 987P fimbriae at 0.1 μ g per spot. (B) TGEV C epitope-specific IgA was detected in pooled fecal pellets (diluted 1:10) by a dot blot after oral immunization of BALB/c mice with serovar Typhimurium χ 4550/pCS155 (lane b) but not after oral immunization with serovar Typhimurium χ 4550/pCS154 (lane a). The antigen in rows 1 and 2 was TGEV C peptide at 2 and 0.4 μ g per spot, respectively. The antigen in rows 3 and 4 was TGEV A peptide at 2 and 0.4 μ g per spot, respectively. The antigen in row 5 was purified wild-type 987P fimbriae at 0.1 μ g per spot. (C) Western blot with serum from one representative mouse immunized with serovar Typhimurium χ 4550/pCS155. The anti-TGEV epitope antibody can recognize the epitope in the context of the TGEV S protein R2-2 expressed by recombinant baculovirus (lane a). Sf9 is the baculovirus mock protein used as negative control (lane b). Molecular masses (in kilodaltons) of standard proteins are shown on the right.

acid residues into FasA. A third TGEV epitope could not be added to this site (data not shown), suggesting that successful fimbria export and assembly constrain the length of an inserted segment, as proposed previously (4, 7, 73, 76). Moreover, as observed with the CS31A fimbriae (7, 46), the addition of the TGEV A epitope resulted both in decreased fimbria production and in significant cleavage of chimeric subunits. However, by using certain *E. coli* hosts like BL21, which does not express the OmpT protease (71), subunit cleavage was significantly decreased. Most importantly and fortuitously, this cleavage was also minimal in the *Salmonella* strains used in this study.

Various constructions were prepared for 987P expression by

an *aroA* or a *cya crp* serovar Typhimurium mutant. 987P expression in the *aroA* mutant was regulated by *fasH*, the 987P transcriptional regulator and, therefore, by catabolite repression. In the *cya crp* mutant, 987P fimbriae were directly expressed from the plasmid-encoded tetracycline promoter. Although oral immunization of mice with these two strains resulted in significant systemic and mucosal humoral responses against 987P, the responses against the TGEV epitopes were weak or not detectable. Regulation by catabolite repression was previously proposed to be involved in the expression of 987P only in distal segments of the small intestine (25, 26). It was previously shown that the level of the immune response in animals was proportionally related to the amount of antigens expressed in the *Salmonella* vectors used (16, 82). Thus, we reasoned that it may be possible to improve the immune response by increasing the level of fimbriation expressed in vivo. For this, we used the *nirB* promoter, known to be activated by Fnr under anaerobic conditions as found in the intestinal environment or intracellularly (12, 27, 57). This approach led to better systemic and mucosal immune responses to the chimeric fimbriae. Moreover, antibodies were elicited against both TGEV epitopes, although mucosal antibodies were only detected for the TGEV C epitope. That this epitope is more immunogenic than the TGEV A epitope is consistent with findings by others (20, 46).

Fimbria proteins typically are highly immunogenic, and this property has been attributed mainly to their polymeric structure (54). They share their immunogenic advantage with aggregated proteins which make better antigens than soluble ones. That the particulate structure and highly repetitive nature of some antigens enhances their immunogenicity was recently demonstrated with cross-linked protein crystals which induced a higher level of antibodies than the soluble form of the same protein (69). Since each fimbria thread consists of several hundred subunits and each bacterium expresses hundreds of fimbria filaments, the abundance of these proteins makes fimbriae major cell-associated antigens of fimbriated bacterial vaccines (54). Our results with *Salmonella* vaccine strains CS3263/pCS151 and CS3263/pCS152 clearly show that fimbria subunits are better immunogens when exported and assembled than when retained intracellularly by salmonellae. The in vitro data may suggest that this observation results mainly from the increased amounts of subunit proteins detectable under in vitro conditions. However, it remains likely that other variables, such as the location and multimericity of the displayed subunits contributed in vivo to the results obtained. For example, a malaria antigen elicited comparable immune responses when expressed in the periplasm or on the surface of salmonellae despite a 10- to 100-times-higher expression of periplasmic antigen (30). Similarly, better protection was achieved when the p60 protein of *Listeria monocytogenes* was secreted in the phagocytic vacuole containing the *Salmonella* host vector than when the protein was kept intracellularly (31).

Some researchers claim that the key point to an active immune response is the initial amount of antigens that prime the GALT (10, 16, 55). Consistent with this, our data suggest that increased amounts of antigen delivered by salmonellae in vivo, with the use of the *nirB* promoter enhanced the humoral immune response. However, because the genetic background and the nature of the attenuation of different *Salmonella* vaccine strains has been shown to have a profound influence on immune responses (6, 23, 74, 77, 87), other researchers propose that a longer persistence of salmonellae in the mucosal immune system, especially in the Peyer's patches, is the critical issue for the induction of mucosal immune responses (23, 72).

Thus, vaccine strain viability may also have influenced some of our data.

A major attribute of the 987P fimbria as a foreign epitope carrier is its enteroadhesive property. It was recently demonstrated with the F4 fimbriae of porcine ETEC strains that the fimbria-induced immunity is receptor dependent (75). This suggests that, like the mucosal adjuvant effect of the B subunit from cholera toxin, a fimbria-receptor interaction can amplify a mucosal immune response. Since BALB/c mice lack 987P receptors (22), it will be most relevant to study the immune responses of piglets immunized with attenuated bacteria expressing chimeric 987P fimbriae. Moreover, unlike many other ETEC fimbriae whose enteroadhesion is mediated by their major subunits (5, 8, 21, 36, 44, 61), enteroadhesion of the 987P fimbriae is essentially mediated by a minor subunit, the FasG protein (13, 38, 39). Therefore, an advantage of the 987P carrier over other fimbrial systems is the ability to genetically engineer the major subunit FasA as a carrier molecule without affecting the enteroadhesive property of the fimbriae.

In conclusion, we have demonstrated that the 987P antigen exposed on the surface of attenuated *Salmonella* strains, delivered orally to animals, elicits both systemic and mucosal immune responses. The fimbriae are capable of presenting foreign epitopes to the immune system and can induce a specific anti-foreign epitope immune response after oral administration of the 987P-expressing *Salmonella* strains. Furthermore, the fimbria-based polymeric display system expressed by salmonellae can be optimized by using a promoter which is known to be induced in vivo. The enhanced immunogenicity of chimeric fimbriae expressed by such strains may present significant opportunities for vaccine development.

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