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NOTES

Expression of Thin Aggregative Fimbriae Promotes Interaction of *Salmonella typhimurium* SR-11 with Mouse Small Intestinal Epithelial Cells

SOILA SUKUPOLVI,[1] ROBIN G. LORENZ,[1] JEFFREY I. GORDON,[2] ZHAO BIAN,[3] JOHN D. PFEIFER,[1] STAFFAN J. NORMARK,[3] AND MIKAEL RHEN[3]*

Center for Immunology, Department of Pathology,[1] and Department of Molecular Biology and Pharmacology,[2] Washington University School of Medicine, St. Louis, Missouri 63110, and Microbiology and Tumor Biology Center, Karolinska Institute, S-171 77 Stockholm, Sweden[3]

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The factors that mediate binding of *Salmonella typhimurium* to small intestinal epithelial cells have not been fully characterized. In this paper we demonstrate that elimination of production of thin aggregative fiber by a transposon insertion within the gene encoding the subunit protein of the fiber reduced binding of *S. typhimurium* SR-11 to a conditionally immortalized proximal small intestinal epithelial cell line established from transgenic mice. This binding defect could be overcome by transcomplementation with a wild-type allele. The conditionally immortalized cell line should prove useful in identifying the epithelial cell receptor for bacterial attachment since expression of its bacterial binding activity can be induced by manipulating the line's proliferative status.

Salmonella typhimurium causes a systemic disease in mice that closely resembles human typhoid fever. To disseminate within the host, the bacterium must first cross the intestinal epithelium. During the early phases of infection, *Salmonella* may interact with small intestinal enterocytes (33) or with the M cells of Peyer's patches (8, 22, 25, 33). There is evidence for initial entry of *S. typhimurium* into the intestinal mucosa through M cells (19, 25). However, since only 10 to 20% of the cells in the murine follicle-associated epithelium are specialized M cells (22, 28), bacterial entry is likely to occur also by attachment to and invasion of enterocytes (33).

Surface organelles in a variety of bacteria are thought to act as virulence factors by mediating attachment to eukaryotic cells (2, 20, 24). *Salmonella* produces a variety of such organelles (1), but their precise role(s) in mediating binding to intestinal epithelial cell targets and in initiating salmonellosis is to be determined. Thin aggregative fimbriae, first described for *Salmonella enteritidis* (12), are able to bind soluble fibronectin (11) and mediate plasminogen activation (29). The corresponding curli organelles expressed by *Escherichia coli* are known to induce bradykinin release from H-kininogen present in human plasma (1a). The *agfA* gene is part of an operon containing three contiguous genes, *agfBAC*, all of which are involved in the production of thin aggregative fimbriae (10). These fibers are composed of AgfA subunits and are located on the bacterial cell surface. *S. enteritidis agfA* encodes a 151-residue protein that is 74% identical to the *E. coli* CsgA subunit protein of curli organelles (26, 27).

S. typhimurium SR-11 χ4666 constitutively expresses thin aggregative fimbriae on its surface (30). In the present study, we have investigated the role of thin aggregative fimbriae in cell adhesion by performing transposon Tn*1731*-mediated inactivation of the *S. typhimurium* SR-11 χ4666 *agfA* gene. A comparison of isogenic SR-11 χ4666 strains with wild-type and mutant *agfA* alleles revealed that these fimbriae are required for binding to a conditionally immortalized small intestinal epithelial cell line generated from transgenic mice.

Construction of *S. typhimurium* strains lacking a functional *agfA* gene. The *agfA* gene was cloned from *S. typhimurium* SL2965 (31) by standard methods. The resulting pUC18-based plasmid, pHUB100, was then subjected to Tn*1731* mutagenesis by the method described by Ubben and Schmitt (35). The positions of transposon insertions were defined by digestion with appropriate restriction enzymes and then by nucleotide sequence analysis. A Tn*1731* insertion in codon 17 of *agfA* was used in the experiments.

pHUB100 containing *agfA* with this Tn*1731* insertion was linearized with *Xho*I and transformed into *S. typhimurium* LB5010 (5). Tetracycline-resistant, ampicillin-sensitive transformants were isolated, and their whole-cell DNA was subjected to hybridization analysis with an *agfA*-specific probe. Integration of Tn*1731* into the *agfA* locus was confirmed by the appearance of two reactive *Hin*dIII bands rather than the single *Hin*dIII fragment present in the isogenic *agfA*$^+$ strain (data not shown). No plasmid DNA was recoverable from the *agfA*::Tn*1731* strain, indicating that its antibiotic resistance phenotype was not simply due to an extrachromosomal copy of pHUB100.

A bacteriophage P22 lysate was grown on the *S. typhimurium* LB5010 derivative carrying *agfA*::Tn*1731*. This mutation was then transduced into SR-11 χ4666. The tetracycline-resistant transductants that were obtained had a smooth colony morphology when cultured at 37°C on Luria agar. This phenotype was clearly different from the rugose colony morphology of wild-type SR-11 χ4666 cells. Immunoelectron microscopy with

* Corresponding author. Mailing address: Microbiology and Tumor Biology Center, Karolinska Institute, S-171 77 Stockholm, Sweden. Phone: 46-8-728 7173. Fax: 46-8-342651. E-mail: Mikael.Rhen@mtc.ki.se.

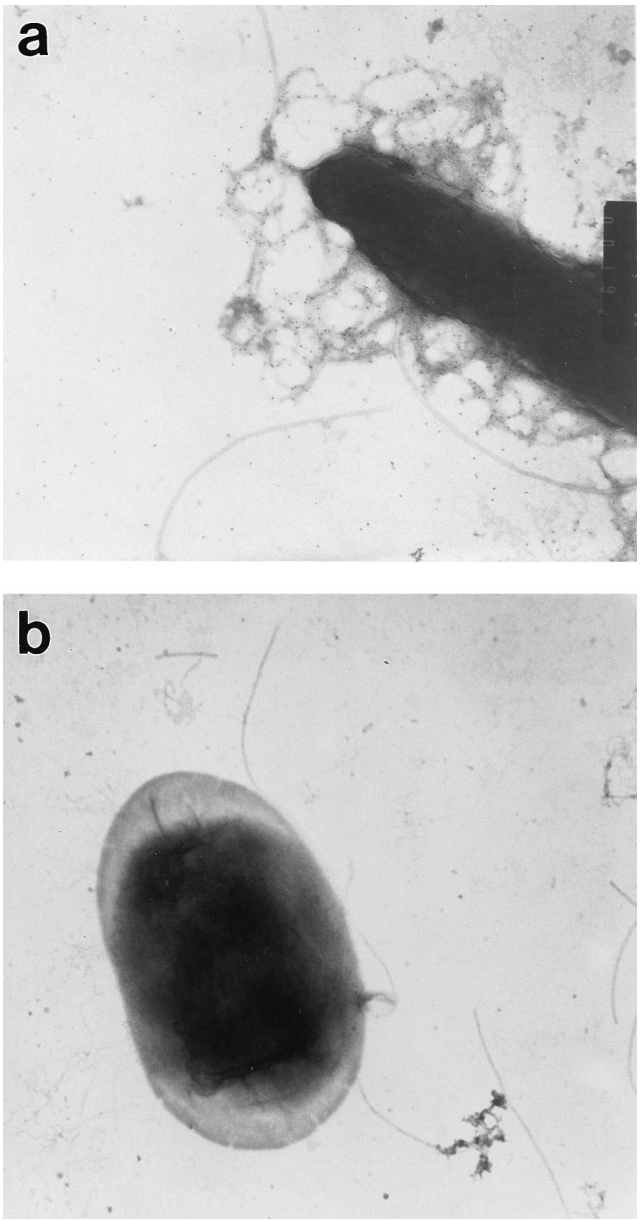

FIG. 1. Immunoelectron microscopy of isogenic SR-11 χ4666 *agfA* (A) and *agfA*::Tn*1731* (B) strains. A polyclonal antibody raised against the CsgA protein of *E. coli* was used to visualize thin aggregative fimbriae. Magnification, ×30,000.

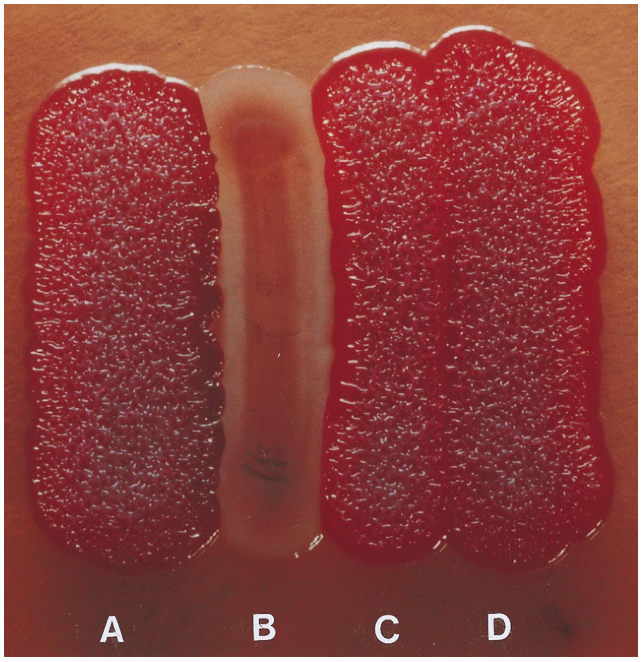

FIG. 2. Extracellular formation of *E. coli* thin aggregative fibers on *S. typhimurium* SR-11. Bacterial strains were seeded as parallel streaks on a CFA agar plate supplemented with Congo red as follows: streak A, *S. typhimurium* AgfA$^-$ AgfB$^+$; streak B, *E. coli* CsgA$^+$ CsgB$^-$; streak C, *S. typhimurium* AgfA$^-$ AgfB$^-$; and streak D, *S. typhimurium* AgfA$^-$ AgfB$^+$. After bacterial growth at 28°C for 2 days, an intensified red zone was observed on streak A on the side facing streak B, indicating CsgA fiber formation on the AgfA$^-$ *S. typhimurium* strain. The intense red zone was not observed between the *E. coli* CsgA donor (streak B) and an AgfA$^-$ AgfB$^-$ strain (streak C).

rabbit antiserum generated against the corresponding subunit protein of *E. coli* curli organelles, the CsgA protein, confirmed the lack of thin aggregative fibers in the SR-11 χ4666 *agfA*::Tn*1731* mutant, in contrast to wild-type SR-11 χ4666.

The isogenic *agfA*$^+$ and *agfA*::Tn*1731* strains were harvested during stationary phase in Luria-Bertani medium, Western blots of total bacterial proteins were prepared, and the blots were probed with a rabbit polyclonal antiserum raised against *E. coli* CsgA. An 18-kDa band was present in *agfA*$^+$ lysates but not in *agfA*::Tn*1731* lysates (data not shown).

To rule out the possibility that the *agfA*::Tn*1731* strain had acquired mutations affecting its auxotrophic properties or lipopolysaccharide structure, we tested the growth of this strain on minimal medium and its sensitivity to lipopolysaccharide-specific bacteriophages (37). The SR-11 χ4666 *agfA*::Tn*1731* mutant was prototrophic (as is *agfA*$^+$ SR-11 χ4666). Bacteriophage testing showed the presence of a wild-type *S. typhimurium* O antigen in both strains. The growth rate of the *agfA*::Tn*1731* mutant was the same as that of the wild-type SR-11 χ4666 strain.

The *S. typhimurium* AgfA protein is functionally similar to the CsgA protein of *E. coli*. Sequence analysis of the cloned *agfBA* operon of *S. typhimurium* SL2965 allowed us to compare the primary structure of *S. typhimurium* Agf proteins to the previously published sequences of *S. enteritidis* AgfA and AgfB (10) and *E. coli* CsgA and CsgB (17). As expected, the *S. typhimurium* proteins showed >70% identity with the *E. coli* proteins and >90% identity with the *S. enteritidis* proteins.

Our nucleotide sequence analysis disclosed that *agfB* is located immediately upstream of *agfA* in the *S. typhimurium* *agfBA* operon. Southern blots indicated that the SR-11 χ4666 *agfA*::Tn*1731* mutant contained an intact *agfB*$^+$ allele. The presence of *agfB* and the absence of detectable AgfA protein allowed us to use this strain to determine whether *E. coli* CsgA is functionally similar to AgfA in an intercellular complementation assay. SR-11 χ4666 *agfA*::Tn*1731* cells were cultured together with the *E. coli* mutant strain MHR261, which produces CsgA but not CsgB (18). In the absence of CsgB this *E. coli* strain secretes CsgA monomers into the medium. The CsgA protein monomers secreted by *E. coli* were polymerized to functional fibers, detectable by Congo red binding in the region of growth of SR-11 χ4666 *agfA* *agfB*$^+$ cells facing the *E. coli* strain (Fig. 2). This phenotype was not detected when a *Salmonella* mutant lacking both AgfA and AgfB was used. Together, these results suggest that *Salmonella* Agf proteins are functionally equivalent to the *E. coli* Csg proteins. The

polymerization of CsgA protein has been shown to occur extracellularly (18) through a self-assembly process that depends on CsgB acting as a nucleator. The AgfB protein of *S. typhimurium* was able to act in an analogous fashion when *S. typhimurium* was grown with a $CsgB^-$ $CsgA^+$ mutant of *E. coli*, thus demonstrating the functional similarity between the corresponding fibers of *S. typhimurium* and *E. coli.*

Establishing small intestinal epithelial cell lines. The normal adult mouse small intestinal epithelium contains four principal cell types: columnar absorptive enterocytes, mucus-producing goblet cells, a complex population of enteroendocrine cells, and Paneth cells, which elaborate antimicrobial peptides. Enterocytes are the predominant cell type, comprising >80% of the epithelial cell population. Terminal differentiation of these lineages occurs as cells migrate along crypt-villus units (for a review, see reference 16). Studies with transgenic mice have shown that nucleotides −596 to +21 of the rat liver fatty acid binding protein gene (*Fabpl*) can be used to direct expression of foreign products to each of the four principal small intestinal epithelial lineages throughout the course of their differentiation (23, 34). These transcriptional regulatory elements are not active in any intestinal mesenchymal cell population. Conditionally immortalized cytokeratin-positive epithelial cell lines were generated from the proximal half of the small intestine of FVB/N mice containing a transgene made up of *Fabpl* and the gene encoding the temperature-sensitive simian virus 40 (SV40) large T antigen (TAg^{tsA58}).

A 2.76-kb DNA fragment encoding SV40 TAg^{tsA58} [3, 6, 7, 21, 36]) was ligated to a *Bam*HI-digested, pGem11Z-based plasmid containing nucleotides −596 to +21 of *Fabpl* (32), yielding pLFTAgtsA58. A 3.3-kb *Fabpl*-SV40 TAg^{tsA58} fragment was released from pLFTAgtsA58, purified by agarose gel electrophoresis followed by QIAEX (Qiagen, Chatsworth, Calif.) extraction, and injected into FVB/N oocytes (Taconic Farms). Oocytes were transferred to pseudopregnant Swiss Webster recipients. Three transgenic founders were identified among the 48 live-born mice screened. A pedigree was established from one of these founders (G_o17) and maintained through matings to normal FVB/N littermates. Northern blots established that adult (6- to 7-week-old) transgenic animals from line 17 expressed SV40 TAg^{tsA58} mRNA along the length of the gastric-colonic axis.

These mice were used, with modifications of a protocol developed by Evans et al. (13), to establish conditionally immortalized small intestinal epithelial cell lines. The proximal half of the small intestine of a 6-week-old animal was removed, opened along its cephalocaudal axis, and minced finely with scissors. Tissue fragments were washed with Hanks' balanced salt solution and then incubated on a rotating shaker for 30 min at room temperature in 20 ml of Hanks' balanced salt solution containing 0.1 mg of collagenase (type XI, from *Clostridium histolyticum*; specific activity = 1.7 U/mg; Sigma) per ml and 0.6 mg of *Bacillus polymyxa* protease (0.5 U/mg; Sigma) per ml. The mixture was passed through a 1-ml pipette to mechanically dissociate epithelial and mesenchymal elements. Mesenchymal elements were allowed to settle for 1 min. The supernatant was removed, adjusted to 2% sorbitol, and centrifuged at 200 × *g* for 4 min at 25°C. The resulting supernatant fraction was discarded and the epithelial pellet was resuspended in D20 medium (Dulbecco's modified Eagle's medium [DMEM] plus 20% fetal calf serum; HyClone) supplemented with 50 U of penicillin per ml and 50 μg of streptomycin (Life Technologies) per ml. Cells were plated in 24-well tissue culture dishes (Costar). Cultures were maintained at 33°C under a humidified atmosphere of 5% CO_2–95% air. The medium was initially changed after 1, 7, and 14 days. Thereafter, the medium was replaced every 3 days (after trypsin digestion of the adherent cell population).

Cell lines were cloned by picking single cells under a dissecting microscope. Cloned cells were allowed to proliferate at 33°C in DMEM–20% fetal calf serum. After reaching confluency, cells were harvested from their wells by adding trypsin (0.05% [wt/vol]) and EDTA (0.5 mM) and then replated at lower density. Differentiation was induced by switching the temperature to 39.5°C without altering any other culture conditions. To assess proliferative status after the temperature shift, 5′-bromo-2′-deoxyuridine (BrdU; final concentration = 10 μM; Sigma) was added to the medium 3 h before the cells were harvested and fixed for immunohistochemical studies. SV40 TAg was detected with rabbit antibodies (kindly supplied by Doug Hanahan, University of California at San Francisco; final dilution = 1:1,000) and Cy-3-conjugated sheep anti-rabbit immunoglobulin (Ig) (final dilution = 1:500; Jackson Immunoresearch). BrdU was detected with goat anti-BrdU (9) (final dilution = 1:2,000) and Cy-3-conjugated donkey anti-goat Ig (final dilution = 1:500; Jackson Immunoresearch).

Single-label and multilabel immunohistochemical analyses. Glycoconjugate production is a sensitive marker of small intestinal epithelial differentiation programs (4, 14). Glycoconjugates can be readily detected by using histochemical methods and a previously characterized panel of lectins (14). For this assay confluent cultures of proliferating cells, or confluent cultures of differentiated cells in 96-well tissue culture plates (Costar), were fixed in Bouin's solution (10 min at room temperature), washed in phosphate-buffered saline (PBS) (three times, 5 min/cycle), and pretreated for 15 min at room temperature with blocking buffer (2% bovine serum albumin, 0.3% Triton X-100) in PBS. Cells were then incubated with lectins that had been used previously to characterize the differentiation programs of the four principal small intestinal epithelial lineages in normal adult FVB/N mice (14). This lectin panel included *Griffonia simplifolica* I (carbohydrate specificity, α-Gal/α-GalNAc epitopes; cellular specificity in FVB/N mouse small intestine, differentiated Paneth cells and enterocytes); Jacalin (Galα6Gal and Galβ3GalNAc; enterocytes), *Maclura pomifera* agglutinin (Galβ3GalNAc and Galα6Glc; goblet cells and enterocytes), *Trichosantes kirilowii* agglutinin (β-Gal; Paneth, goblet, and enteroendocrine cells), *Dolichos biflorus* agglutinin (GalNAcα3GalNAc and GalNAc; reacts with all cells but those of endocrine lineage), *Wisteria floribunda* agglutinin (GalNAcα3GalNAc and GalNAcβ3/4Gal; enterocytes), *Griffonia simplifolica* II [$(GlcNAc\beta4)_{2\text{-}3}$ and GlcNAcα4Galβ4GlcNAc; Paneth and goblet cells], pokeweed agglutinin [$(GlcNAc\beta4)_{2\text{-}5}$ and $(Gal\beta4GlcNAc)_n$; reacts with all cells but those of enteroendocrine lineage], *Ulex europaeus* agglutinin type I (Fucα1,2Galβ; Paneth, goblet, and enteroendocrine cells), and the cholera toxin B subunit [GalNAcβ4(Neu5Acα2,3)Galβ; enterocytes]. The sources of these lectins are described by Falk et al. (14).

For the binding assays the epithelial cells were allowed to proliferate at a permissive temperature (33°C). Proliferation was then suppressed, and differentiation was induced by raising the temperature to 39.5°C, which causes the mutant SV40 TAg to become unstable. The cell lines contained readily detectable amounts of immunoreactive SV40 TAg^{tsA58} at 33°C (Fig. 3A). After a 10-day incubation at 39°C, the mutant SV40 TAg was no longer detectable (Fig. 3C) and proliferation was completely suppressed, as judged by their lack of incorporation of BrdU. Cessation of proliferation was associated with a change in cellular morphology from small and oval to a larger, flatter phenotype. In addition, there was increased accumulation of GalNAcβ4(Neu5Acα2,3)Galβ-containing glycoconjugates rec-

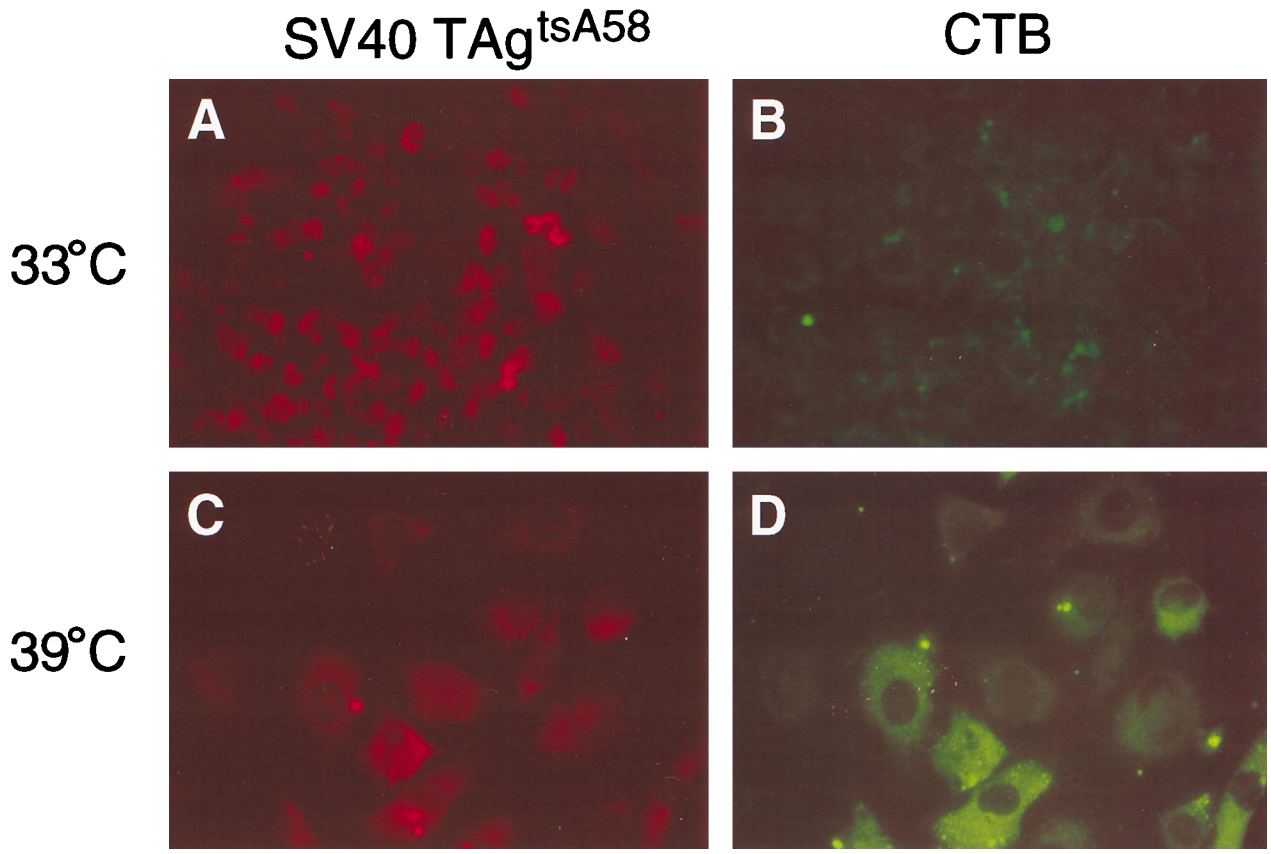

FIG. 3. Temperature-dependent changes in the accumulation of SV40 TAg^{tsA58} and glycoconjugates recognized by the cholera toxin B subunit lectin in a conditionally immortalized mouse proximal small intestinal epithelial cell line. (A and B) H10 cells were cultured at 33°C and then incubated with rabbit anti-SV40 TAg serum, Cy-3-conjugated sheep anti-rabbit Ig, and fluorescein isothiocyanate-labeled cholera toxin B subunit (CTB). At this temperature, the mutant TAg is readily detectable in the nuclei of these proliferating cells (red immunoreactive protein in panel A). Glycoconjugates recognized by CTB are barely detectable (green staining material in panel B). (C and D) Double labeling of cells 10 days after the incubation temperature was raised to 39.5°C. The mutant viral protein was now barely detectable (C), while glycoconjugates recognized by CTB were now abundantly represented in the cytoplasms of the nonproliferating cells (D). The variation in glycoconjugate levels seen between the cells diminishes over time after a shift to the nonpermissive temperature (data not shown). The magnification for all panels was ×336.

ognized by the cholera toxin B subunit (compare Fig. 3B and D). These carbohydrate epitopes are normally produced only by differentiated villus-associated enterocytes in FVB/N mice (14).

Thin aggregative fimbriae mediate binding of *S. typhimurium* SR-11 to cultured mouse small intestinal epithelial cells. The adhesion assays were performed essentially as described previously (15). Monolayers (80 to 90% confluent) were cultured with *S. typhimurium* SR-11 χ4666 strains that had been grown overnight on Luria agar at 37°C (10^7 CFU of either *agfA*$^+$ or *agfA*::Tn*1731* bacteria/well). Before the assay, the bacteria were collected from the plates and suspended in PBS. To minimize the effect of autoaggregation of SR-11 χ4666 cells during the binding assay, the suspension was allowed to stand on the bench for 15 min. The upper part of the suspension was then used for the assay, thus avoiding the aggregated bacteria. Following a 30-min incubation at 37°C in 5% CO_2–air, nonadherent bacteria were removed by washing the cell monolayer five times with DMEM (1ml/well/wash and 2 min/wash at 23°C). Two hundred microliters of DMEM, supplemented with trypsin and EDTA, was applied to each well containing a washed monolayer. After a 5-min incubation at 37°C, 0.8 ml of PBS was added and detached cells were collected with a pipette, serially diluted in PBS, and plated onto Luria agar. The number of CFU was determined after an overnight incubation at 37°C.

Cell line H10 bound *agfA*$^+$ bacteria with highest efficiency among the cloned cell lines tested (mean = 12% of total added bacteria after a 30-min incubation [Table 1]).

H10 was selected for further analysis of the role of thin aggregative fimbriae in mediating bacterium–epithelial-cell interactions. Tn*1731* insertional mutagenesis of *agfA* produced

TABLE 1. Adhesion of isogenic *S. typhimurium* SR-11 χ4666 strains containing *agfA*$^+$ and disrupted *agfA*::Tn*1731* alleles to cloned conditionally immortalized mouse small intestinal epithelial cells

SR-11 χ4666 genotype	% Adhesion to[a]:	
	Differentiated H10 cells	Nondifferentiated H10 cells
agfA$^+$	11.8 (8.1–14.5)	0.5 (0.2–0.7)
agfA::Tn*1731*	0.2 (0.1–0.5)	0.2 (0.2–0.3)
agfA::Tn*1731* (pHUB100)	2.7 (2.2–3.4)	Not done

[a] Means and ranges from six samples are shown. The results are representative of three separate experiments. Binding is expressed as a percentage of the total amount of bacteria added to the cells.

marked, statistically significant reductions in binding to differentiated H10 cells (mean = 0.2% of added bacteria [$P < 0.05$] [Table 1]). Neither the fiber-producing parent strain, SR-11 χ4666, nor the SR-11 χ4666 *agfA*::Tn*1731* mutant showed any significant binding to nondifferentiated proliferating H10 cells grown at 33°C (Table 1).

Plasmid pHUB100 carrying the wild-type *S. typhimurium* *agfA* gene was introduced into SR-11 χ4666 *agfA*::Tn*1731* bacteria by transformation. All of the resulting transformants had the rugose colony morphology characteristic of *agfA*$^+$ SR-11 χ4666 cells. Furthermore, introduction of pHUB100 into the *agfA*::Tn*1731* mutant resulted in the reappearance of the 18-kDa AgfA protein (detected by Western blotting of bacterial protein extracts) (data not shown). The presence of pHUB100 in the *agfA*::Tn*1731* mutant partially restored the ability of this strain to bind to nonproliferating H10 intestinal epithelial cells (Table 1). The incomplete restoration of the adhesive phenotype by pHUB100 might reflect some difference in the expression of thin aggregative fimbriae from the plasmid construct from the genome-based constitutive expression in SR-11 χ4666.

Conclusions. We have developed a genetically well-defined and physiologically relevant model system for examining the effects of *Salmonella* surface fibers on bacterial tropism and attachment to the mouse intestinal epithelium. Insertional mutagenesis of *agfA* in *S. typhimurium* SR-11 χ4666 abolished the assembly of cell surface organelles and attachment to conditionally immortalized proximal small intestinal epithelial cells.

The generation of isogenic strains of *S. typhimurium* SR-11 χ4666 with wild type and disrupted *agfA* alleles and a cloned conditionally immortalized mouse proximal small intestinal epithelial cell line (H10) allowed us to assign a function for thin aggregative fimbriae in epithelial binding. Temperature-induced destabilization of the mutant SV40 TAg TAg*ts*A58 results in cessation of proliferation of H10 cells; a pronounced increase in GalNAcβ4(Neu5Acα2,3)Galβ-containing glycoconjugates, which are normally restricted to differentiated villus enterocytes in the normal mouse small intestine; and in binding of bacteria expressing thin aggregative fimbriae. The surface epithelial structures responsible for thin aggregative fimbria-dependent bacterial binding have yet to be defined. For example, we do not know whether binding involves the induced GalNAcβ4(Neu5Acα2,3)Galβ-containing glycoconjugates produced by H10 cells. However, several features of this genetically engineered in vitro model of host-microbe interaction should facilitate identification and characterization of the epithelial cells' thin aggregative fimbria recognition apparatus: (i) the >50-fold induction of bacterial binding in the H10 cell line when it is allowed to differentiate, (ii) the availability of isogenic bacterial strains with and without thin aggregative fimbriae that do and do not bind the differentiated cell line, and (iii) the ease of purification of the bacterial adhesive organelle in question. Additional molecular insights about the role of thin aggregative fimbriae in mediating *S. typhimurium* pathogenesis may be obtained when these strains are used to infect mice and/or when they are used to study attachment to human enterocytes.

Nucleotide sequence accession number. The nucleotide sequences of the *agfA* and *agfB* genes have been deposited in the EMBL database under accession no. AJ000514.

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