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RESEARCH LETTER

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Abstract

Escherichia coli is a versatile organism capable of causing a variety of intestinal and extraintestinal diseases, as well as existing as part of the commensal flora. A variety of factors permit specific attachment to host receptors including fimbrial adhesins and outer membrane proteins such as autotransporters. One of the better characterized autotransporters is Antigen 43 (Ag43), the major phase-variable surface protein of *E. coli*. Ag43 is associated with bacterial cell–cell aggregation and biofilm formation. Nevertheless, the precise biological significance and contribution to intestinal colonization remain to be elucidated. Here we investigated the contribution of Ag43 to *E. coli* adherence to intestinal epithelial cells and colonization of the mouse intestine. These investigations revealed that Ag43 increased *in vitro* adherence of *E. coli* to epithelial cells by promoting bacterial cell–cell aggregation but that Ag43 did not promote specific interactions with the mammalian cells. Furthermore, Ag43 did not contribute significantly to colonization of the mouse intestine and expression of Ag43 was lost a few days after colonization of the mouse was established. Unexpectedly, considering its similarity to other adhesins, our findings suggest that Ag43 does not act as a direct colonization factor by binding to mammalian cells.

Introduction

Outer membrane proteins serve a variety of functions essential to survival of Gram-negative bacteria. Many of these proteins have structural roles or are involved in transport, while others are important in pathogenesis and have roles in adhesion to host tissue or evasion of the host immune system (Nikaido, 2003). Some years ago a major outer membrane protein termed Antigen 43 (Ag43) was discovered in *Escherichia coli* (Owen & Kaback, 1978). In the intervening years, several groups have shed light upon the biochemistry and genetic regulation of this protein. Thus, Ag43 was found to exist as a bipartitie protein consisting of two subunits (α^{43} and β^{43}) which are present in *c.* 50 000 copies per cell and in equal stoichiometry (Owen *et al.*, 1987; Caffrey & Owen, 1989). Subsequent investigations revealed that α^{43} was present on the cell surface and was anchored to β^{43}, which is an integral outer membrane protein, through a noncovalent interaction (Caffrey & Owen, 1989). While apparently stable at normal growth temperatures this interaction could be interrupted by brief heating to 60 °C, thus releasing α^{43} from the cell surface (Caffrey & Owen, 1989). Later, Henderson & Owen (1999) revealed that Ag43 was a member of the autotransporter family of secreted proteins. Like all autotransporters, Ag43 possesses a multidomain architecture consisting of a signal sequence that directs translocation across the inner membrane and a β-domain (β^{43}) that forms a β-barrel pore in the outer membrane through which the passenger domain (α^{43}) is translocated to the cell surface (Henderson *et al.*, 1998, 2004). Furthermore, recent *in silico* investigations demonstrated that, like the majority of autotransporter passenger domains, α^{43} adopted a β-helix configuration on the cell surface and belonged to the AIDA-I adhesin subfamily of autotransporters (Henderson *et al.*, 2004; Klemm *et al.*, 2004).

Expression of α^{43} on the cell surface was shown to undergo phase variation with a switching frequency of *c.* 10^{-3} per cell per generation (Owen *et al.*, 1996; Henderson

et al., 1997a). Initial investigations demonstrated that the phase variable switch was due to the competition between deoxyadenosine methylase (Dam) and the cellular redox sensor OxyR for sites within the promoter region of the gene encoding Ag43 (Henderson *et al.*, 1997a; Henderson & Owen, 1999). Subsequent investigations revealed that binding of OxyR to the promoter acted as a transcriptional 'road block' preventing RNA polymerase from transcribing the gene and that this repression could only be relieved by DNA replication and subsequent Dam-mediated methylation of GATC nucleotide motifs within the OxyR-binding site (Haagmans & van der Woude, 2000; Waldron *et al.*, 2002; Wallecha *et al.*, 2002, 2003). Recent investigations have indicated that other factors play a role in the switching events which lead to Ag43 expression (Phase ON) or Ag43 repression (Phase OFF) (Correnti *et al.*, 2002; Lim & van Oudenaarden, 2007).

Many years ago Diderichsen (1980) described a phase variable phenomenon characterized by the ability of one population of *E. coli* to settle in static liquid culture and an isogenic population which would remain in suspension. Henderson *et al.* (1997b) eventually demonstrated that variable expression of Ag43 was responsible for these phenotypes. Thus, when Ag43 is expressed it promotes cell–cell aggregation and the aggregated cells settle out of suspension, and when Ag43 is in the Phase OFF state cells do not aggregate and remain in suspension. Subsequent investigations indicated that Ag43 played an important role in biofilm formation and that the region of the molecule promoting aggregation and biofilm formation could be localized to the N-proximal domain of α^{43} (Danese *et al.*, 2000; Hasman *et al.*, 2000; Kjaergaard *et al.*, 2000a, b; Klemm *et al.*, 2004).

Despite demonstrating a role for Ag43 in cell–cell aggregation and biofilm formation, the precise role of Ag43 in interaction of *E. coli* with mammalian hosts remains uncertain. However, a number of data from previous studies suggest that it may have an important role *in vivo*. These include the facts that (1) α^{43} is surface expressed; (2) α^{43} has homology with certain fimbrial subunits and the autotransporters AIDA-I and ShdA which are known to be involved in adhesion and prolonged faecal shedding (Benz & Schmidt, 1992; Kingsley *et al.*, 2000, 2002, 2003); (3) α^{43}, like many fimbrial subunits and outer membrane adhesins e.g. AIDA-I and pertactin, may be heat released from the cell surface (Leininger *et al.*, 1991; Benz & Schmidt, 1992); (4) Ag43 undergoes reversible phase variation, a property common to other adhesins (Henderson *et al.*, 1999); (5) Ag43 and/or an immunologically related protein is observed in intestinal pathogenic isolates of *E. coli*; and (6) is expressed *in vivo* as determined by analysis of several uropathogenic isolates harvested directly from infected urine (Owen *et al.*, 1996). Thus, in this study we have attempted to determine whether Ag43 contributes to colonization using a variety of *in vitro* and *in vivo* approaches. We have also attempted to determine whether the *in vivo* environment switches Ag43 expression to Phase ON or whether it selects for organisms, which are already expressing Ag43, as one might expect with a protein mediating adhesion to host cells.

Materials and methods

Bacteria, plasmids, cell lines and culture conditions

Escherichia coli ML308-225 was routinely cultured aerobically in 1% (w/v) succinate minimal medium A at 37 °C, and on L-agar. All other strains were cultured on L-broth and L-agar unless otherwise stated. Where appropriate the following supplements were used at the indicated final concentrations: ampicillin (100 μg mL^{-1}), streptomycin (100 μg mL^{-1}), kanamycin (50 μg mL^{-1}) and gentamicin (10 μg mL^{-1}). A streptomycin resistant version of *E. coli* ML308-225, designated *E. coli* ML308-225S, was created by passaging the wild-type organism sequentially in 10, 20, 50 and 100 μg mL^{-1} streptomycin. A suicide plasmid pCVD442 was used for creating isogenic mutants (Mobley *et al.*, 1993). The plasmid pCRII TOPO (Invitrogen) was used for general cloning experiments. Mammalian cells were maintained on either Eagle's Minimal essential medium, RPMI or basal medium essential (Sigma) containing 10% (v/v) foetal calf serum, as appropriate. Cells were routinely grown at 37 °C in a 5% CO_2 atmosphere.

Adherence and invasion assays

The adherence of *E. coli* to cell lines was examined by a modification of the HEp-2 cell assay of Nataro *et al.* (1985). Monolayers were prepared in six-well tissue culture dishes. For light microscopy the monolayers were grown on glass coverslips in the wells of the dishes. Monolayers were washed in phosphate buffered saline (pH 7.2) and incubated in 3 mL of the appropriate medium containing 1% (w/v) D-mannose. After 3 h cells were (1) fixed in 100% acetone, stained with May Grunwald/Giemsa stain and visualized by light microscopy; (2) examined by immunofluoresence; or (3) removed from wells following incubation with 1% (w/v) sodium dodecyl sulphate (SDS) and vigorous shaking and then serially diluted. The total number of adherent bacteria was expressed as a percentage of the total number of bacteria present at the end of incubation. Bacterial numbers were estimated by serial dilution and plating on L-agar. All assays were performed in triplicate with three biological replicates. Inhibition assays were performed using a modification of the adherence assay. Purified α^{43} was added to the monolayers to give a final concentration of α^{43} between 0 and 100 μg mL^{-1}. These were incubated at 37 °C for 3 h.

Subsequently, either (1) bacteria were added and incubation continued as above or (2) the media was replaced with fresh media without purified α^{43} and bacteria were added immediately after and incubation continued as above. The numbers of adherent bacteria were calculated as above.

The invasion assay was carried out as described by Elsinghorst & Kopecko (1992). Monolayers were prepared using 24-well tissue culture dishes. The wells were inoculated with the required number of bacteria and the dishes incubated for 2 h at 37 °C. The monolayers were washed and gentamicin added to kill adherent but not invasive bacteria. Plates were incubated for a further 3 h at 37 °C before bacteria were collected by incubation with 0.1% (v/v) Triton X-100. Lysates were serially diluted and bacterial numbers estimated as above.

Mouse colonization studies

The streptomycin-treated mouse model was performed essentially as described previously (Sweeney *et al.*, 1996; Moller *et al.*, 2003; Miranda *et al.*, 2004). Eleven- to twelve-week-old female BALB/c mice (Charles River Labs, Wilmington, MA) were provided *ad lib* drinking water exclusively containing 5 g L^{-1} streptomycin for 48 h before inoculation and for the duration of the experiment. For inoculation, strains were grown overnight in L-broth, diluted 1 : 500 and incubated to late exponential phase (*c.* 6 h). Bacteria were pelleted by centrifugation, washed once with sterile phosphate-buffered saline (PBS), pH 7.4 and resuspended in 1/10th volume of sterile PBS. Before infection with bacterial strains, mice were given 0.2 mL half-saturated sodium bicarbonate solution orogastrically to neutralize stomach acid. Approximately 15 min later, 0.2 mL of the inoculum was administered orogastrically. Bacterial inocula were quantified by dilution plate counts. Fresh faecal pellets were collected at the indicated time points for up to 8 days postinfection. Faeces were weighed, diluted and homogenized in sterile PBS. Serial dilutions were plated on MacConkey agar (Sigma) with antibiotics. Plates were incubated at 37 °C for 18–24 h before enumeration of bacteria.

DNA manipulation techniques

Standard DNA manipulation techniques were performed as described by Sambrook & Russell (2001). To create ASTAG1 the *agn43* genes were insertionally inactivated in *E. coli* ML308-225S essentially as described previously (Mobley *et al.*, 1993). Regions of *c.* 1 kb upstream and downstream of the *agn43* sequence were amplified using the primers agn43upKO-F, agn43upKO-R, agn43downKO-F and agn43-downKO-R (Table 1) as appropriate. The PCR products were ligated together and cloned into the XbaI and SacI sites of the suicide vector pCVD442 to generate pASTAG. The pASTAG construct was transformed into DH5α*λpir*.

Table 1. Primers used in this study

Primer	Sequence
Knockout primers	
agn43upKO-F	CTGAGCTCCGTGAACAGTTTACCGGTGC
agn43upKO-R	CAGAAGGTCCCGGCCACACCCCCGTTTTTGACA
agn43downKO-F	GGCCGGGACCTTCTGACAGAACCATCGCCTCTC
agn43downKO-R	TTTCTAGATCATCAGGTGTGAATGACAGG
Check-F	GGCAGGTCGTCAAGCCCGGTACAG
Check-R	GTCAGCCCGCAGGAGGCACAGATACG

Subsequently, pASTAG was isolated and transformed into chemically competent SM10λ*pir* for mating with *E. coli* ML308-225S. Mating was performed as described previously and a double crossover event was selected subsequently by plating isolates on LB-streptomycin plates, lacking NaCl, and 5% sucrose (Mobley *et al.*, 1993). Streptomycin-resistant and ampicillin-sensitive mutants were confirmed as lactose-fermenters. Deletion of the *agn43* alleles was verified by PCR using Check-F and Check-R primers (Table 1), sequencing, colony blotting and immunofluorescence. A construct (pAG43) overexpressing Ag43a from *E. coli* ML308-225 was constructed by amplifying the gene using primers Check-F and Check-R and cloning into pCRII TOPO according to the manufacturer's instructions.

Protein preparation and analysis techniques

Quantitation of protein in solution was determined using a modification (Dulley & Grieve, 1975) of the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. SDS-poly acrylamide gel electrophoresis (PAGE) was performed using 12.5% (w/v) acrylamide separating gels and 4.5% (w/v) acrylamide stacking gels as described previously (Laemmli, 1970). Proteins were stained with Coomassie brilliant blue R250. Western blotting and colony blotting were performed as described by Caffrey *et al.* (Caffrey & Owen, 1989). Affinity blotting was performed essentially using the same method described for Western blotting except purified protein [either α^{43} (this study), fibronectin, fibrinogen, collagen, vitronectin, laminin (Sigma)] was used as the primary ligand. Immunoglobulins to the purified proteins, peroxidase-conjugated goat anti-rabbit immunoglobulins and 4-chloro-1-napthol were used as the localizing reagents. Immunofluorescence microscopy was performed essentially as described by Henderson & Owen (1999) except cells were fixed with 100% acetone for 5 min before incubation with anti-α^{43} immunoglobulins. Preparations of purified α^{43} were prepared as described previously (Caffrey & Owen, 1989).

Statistical analysis

Adherence and invasion data were analysed for significance using the paired Student's *t*-test using the online resource

http://www.physics.csbsju.edu/stats/Paired_t-test_NROW_form.html. Mouse colonization data were analysed for significance using the nonparametric Mann–Whitney–Wilcoxon test using the online resource http://elegans.swmed.edu/~leon/stats/utest.cgi.

Results

Ag43 promotes microcolony formation on epithelial cells

Escherichia coli ML308-225 is a derivative of *E. coli* ML originally isolated by Monod's laboratory and was the workhorse for those studying cellular architecture and biochemical processes in *E. coli* during the 1960s and 1970s (Winkler & Wilson, 1966). It was from this strain that Ag43 was first isolated (Owen & Kaback, 1978, 1979) and thus our functional investigations have utilized this strain. To investigate whether *E. coli* ML308-225 possessed the ability to adhere to mammalian cells *in vitro* we performed the standard HEp-2 cell adherence assay. The laboratory strain *E. coli* HB101 was used as a negative control and as anticipated showed relatively little adhesion (data not shown). Similar experiments conducted with Ag43 Phase-ON and Ag43 Phase-OFF cultures of *E. coli* ML308-225 revealed that both displayed adherence to HEp-2 cells in a manner reminiscent of localized adherence (Fig. 1) indicating factors other than Ag43 play a role in adherence. Immunofluorescence micrographs revealed that microcolonies formed on cell lines by Ag43 Phase-OFF cultures contained, as expected, a low percentage of cells expressing Ag43. However, in many instances these did not appear to be in direct contact with epithelial cells (Fig. 1c and e). Furthermore, a consistent observation was that Ag43 Phase-ON populations formed tight microcolonies (Fig. 1b, d and f) whereas Ag43 Phase-OFF cells, while possessing the ability to adhere to cells, formed more diffuse microcolonies (Fig. 1a, c and e).

From the microscopy studies, it was clear that both Phase-ON and Phase-OFF cells possess the ability to adhere to HEp-2 cells. To estimate the relative contribution of Ag43 to the process of adherence we determined the level of adherence of Ag43 Phase-ON and Phase-OFF variants to HEp-2 cells using viable counts; in this case the total number of bacteria binding directly to the cell and to each other is estimated. In addition to populations of the natural variants, we investigated the extent to which an Ag43 null mutant (ASTAG1) and a strain constitutively expressing

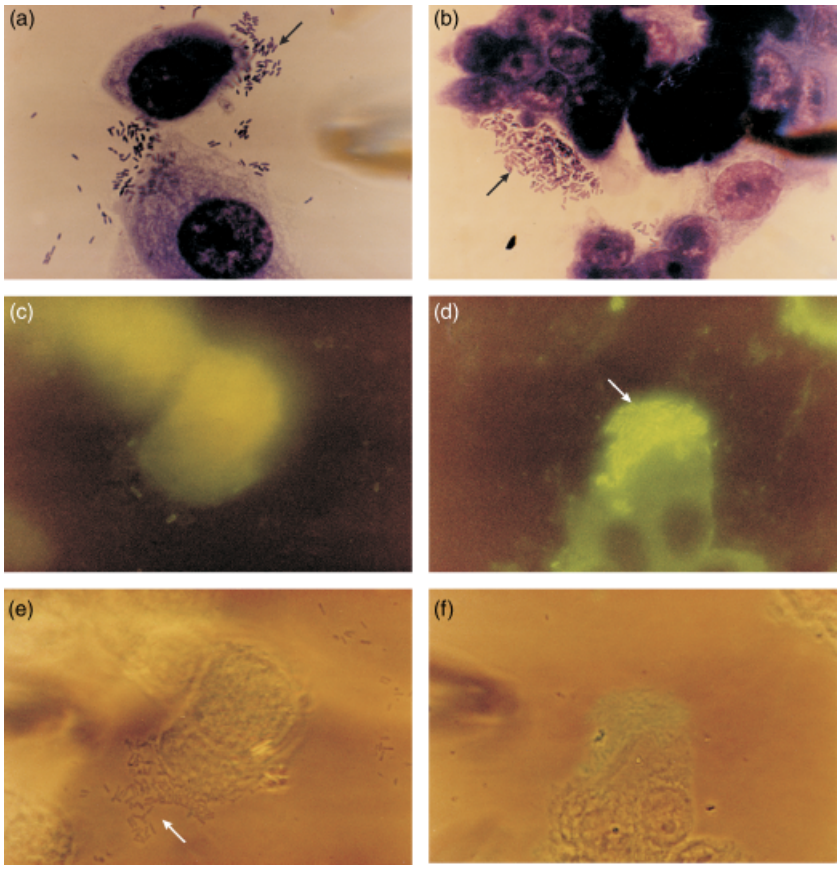

Fig. 1. Adherence of *Escherichia coli* ML308-225 to HEp-2 cells. Ag43 expressing Phase-ON populations are depicted in (b), (d) and (f) whereas Phase-OFF populations are shown in (a), (c) and (e). The formation of microcolonies characteristic of localized adherence is evident in preparations stained with Giemsa (a and b), viewed by immunoflourescence (c and d) or with a combination of phase contrast and immunofluorescence (e and f). Arrows indicate the position of microcolonies. As expected, few Phase-ON cells can be observed in microcolonies formed by Phase-OFF populations (c and e). Phase-ON populations form tighter microcolonies than Phase-OFF populations (d and f).

Ag43 (*E. coli* ML308-225 pAG43) adhered to HEp-2 cells. *Escherichia coli* ML308-225 possesses two copies of *agn43*, which demonstrate > 99% identity at the nucleotide level, to create ASTAG1 both copies were inactivated. The laboratory strain *E. coli* HB101, a negative control, gave the anticipated low levels of adherence (< 0.01%). Equivalent experiments performed with Ag43 Phase-ON and Ag43 Phase-OFF populations of *E. coli* ML308-225 revealed that both adhered to HEp-2 cells at levels considerably higher than *E. coli* HB101 ($P < 0.0001$). ASTAG1 and Phase-OFF populations demonstrated a significantly lower level of adherence (approximately threefold less) than *E. coli* ML308-225 pAG43 and the Phase-ON populations (Fig. 2a).

It is well known that certain mammalian cells express different receptors and varying amounts of the same receptor on their surfaces. With a view to investigating whether Ag43 expressing cells had a greater affinity for certain mammalian cells, adherence assays were performed using Int407, HCT 8, HCT 116 and BHK cell lines. In all cases, Ag43 Phase-ON populations were found to be more adherent than Ag43 Phase-OFF populations; the number of adherent bacteria recovered from the Phase-ON populations was 2.5–6-fold higher than for Phase-OFF populations (data not shown).

To examine whether Ag43 promotes invasion of epithelial cells by *E. coli* ML308-225 we used the standard gentamicin kill assay. Experiments showed *E. coli* ML308-225 were capable of invading HEp-2, HCT 8 and HCT 116 cells at low levels similar to that reported for other noninvasive pathogenic *E. coli* but at a level some three orders of magnitude lower than the positive control *S. enterica* Typhimurium (*c.* 10^7 invading CFU mL^{-1} vs. *c.* 10^4 CFU mL^{-1}). There was no appreciable difference in the levels of invasion between cells expressing Ag43 and those not expressing the protein. Taken together these results argue that Ag43 does not act as a specific invasin.

Ag43 does not promote direct interaction with mammalian cells

As the adherence experiments indicated that Ag43 contributed to the adhesive processes of *E. coli* ML308-225 we investigated whether the level of adherence could be attenuated by preincubation of epithelial cells with purified α^{43}. If Ag43 is a *bonafide* adhesion promoting direct interaction with a mammalian cell receptor then Ag43-mediated interaction of bacteria with mammalian cells should be abrogated by preincubation of mammalian cells with purified α^{43} due to binding of the purified protein to host-cell receptors. Experiments indicated that the adherence of *E. coli* ML308-225 Ag43 Phase-ON cells were significantly reduced when epithelial cells were incubated with purified α^{43} for 3 h before the addition of bacteria to the media. Inhibition occurred in an approximately linear fashion over a range of 0–15 μg α^{43} mL^{-1} for Ag43 Phase-ON variants and the level of inhibition did not increase at significantly higher concentrations (Fig. 2b). Similar experiments conducted with an Ag43 Phase-OFF population only showed a marginal

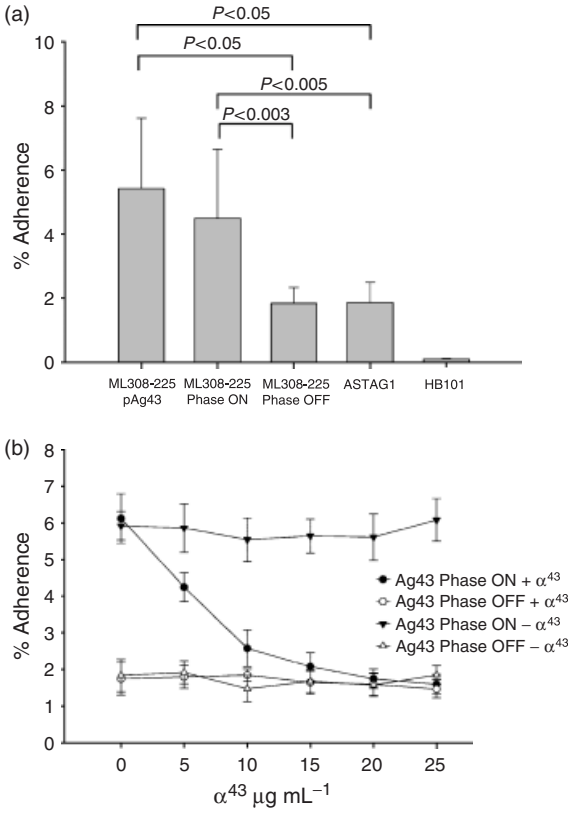

Fig. 2. Quantification of *Escherichia coli* ML308-225 adherence to epithelial cell lines. The numbers of viable bacteria were calculated by serial dilution and plate counts. (a) The figure depicts the percentage of viable bacteria from *E. coli* ML308-225 and its derivatives that remain adherent to HEp-2 cells at the end of incubation. Data are displayed as a percentage of the total number of viable bacteria present at the end of incubation that remain adherent to cells or substratum after repeated washing. In all cases initial inocula were *c.* 10^8 bacterial cells. In both cases, Phase-ON populations of *E. coli* ML308-225 and those harbouring an overexpressing plasmid demonstrate higher levels of adherence that Phase-OFF populations, the null mutant and the negative control, *E. coli* HB101. (b) The figure depicts the levels of adherence of *E. coli* ML308-225 in the presence and absence of purified α^{43}. The figure depicts levels of bacterial adherence to Hep-2 cells incubated with purified α^{43} for 3 h before the addition of bacteria (Ag43 Phase-ON+α^{43} Ag43 Phase-OFF+α^{43}). The presence of α^{43} in the media inhibited the adherence of bacteria from Phase-ON populations in a dose-dependent fashion but Phase-OFF populations are unaffected. However, replacement of the α^{43} containing media with fresh media before the addition of the bacteria (Ag43 Phase-ON – α^{43} and Ag43 Phase-OFF – α^{43}) had no effect on the levels of adherence of Phase-ON or Phase-OFF populations indicating that the increased levels of adhesion are due to α^{43}-mediated interactions between bacteria and not due to α^{43}-mediated direct interactions with host cells.

reduction of adherence. In contrast, no inhibition of adherence was observed when the media containing α^{43} was removed and replaced with fresh media without α^{43} (Fig. 2b) indicating α^{43} does not promote interaction with host cells.

In an attempt to detect possible receptors for α^{43}, HEp-2 cell membranes (*c.* 25 μg) were screened by affinity immunoblotting for interaction with purified α^{43} by subjecting the membranes to SDS-PAGE and Western affinity blotting then using purified α^{43} as the potential primary ligand. Rabbit anti-α^{43} and goat anti-rabbit-horse raddish peroxidase (HRP) were used as secondary and tertiary probes, respectively. However, no specific intereactions were detected with solubilized HEp-2 cell membranes (data not shown). In addition, Western affinity blotting was performed on purified α^{43} (10 μg) using a variety of purified extracellular matrix proteins (*c.* 25 μg; see Materials and methods) as primary ligands and also showed no reaction following probing with their respective HRP-conjugated antibodies. The converse experiment using α^{43} as the primary ligand also failed to demonstrate any interactions. In further attempts to elucidate an α^{43} receptor HEp-2, HeLa, HCT8, HCT116 and Int407 cells were incubated with purified α^{43}. Immunofluorescence studies using anti-α^{43} failed to detect any foci of fluorescence. Furthermore, the total level of fluorescence for control cells was statistically indistinguishable from the fluorescence intensities for cells treated with α^{43}, indicating purified α^{43} does not interact specifically with receptors on these mammalian cells (data not shown).

The failure to inhibit bacterial adhesion to α^{43}-treated mammalian cells in the absence of α^{43} and the failure to detect any α^{43} interactions with host-cell receptors strongly indicates that the increased adherence observed for Phase ON cells is not due to Ag43-mediated interactions with host cells but rather Ag43-mediated interactions with other Ag43-expressing bacterial cells. Indeed, the ability of Ag43 to promote bacterial cell–cell contact has previously been documented and demonstrated to be due to α^{43}–α^{43}-mediated interactions (Owen *et al.*, 1996; Henderson *et al.*, 1997b; Klemm *et al.*, 2004).

Ag43 does not contribute to mouse gastrointestinal colonization

If Ag43 were a *bona fide* adhesin, bacteria expressing Ag43 would be expected to colonize the intestine to greater levels than those not expressing the protein and/or colonize the gut for a longer period of time than those not expressing the protein. Indeed, ShdA, a homologue of Ag43, had been shown previously to play a role in prolonging fecal shedding of *Salmonella* in mouse colonization studies (Kingsley *et al.*, 2000, 2002, 2003). Previous studies had demonstrated that the streptomycin-treated mouse could be used to assess the contribution of putative colonization factors to gastrointestinal colonization and/or fitness for survival during passage through the intestine (Sweeney *et al.*, 1996; Moller *et al.*, 2003; Miranda *et al.*, 2004). Thus, we investigated whether Phase-ON populations of *E. coli* ML308 225S were better adapted to colonization of the mouse intestine than Phase-OFF populations and the null mutant ASTAG1. We could not test the overexpressing strain *E. coli* pAG43 as the plasmid was quickly lost in the absence of antibiotic pressure. In addition, we monitored the bacteria, which were shed in the faeces to determine whether passage through the intestinal tract stimulated a switch from Phase OFF to Phase ON or selected for Phase-ON populations.

An inoculum of *c.* 2×10^8 CFU of each strain was introduced individually to groups of 6–12 mice and faecal colonization was quantified for up to 8 days by estimating the viable number of bacteria per gram of faeces in aseptically collected stool samples. All mutants were able to colonize streptomycin-treated mice when fed individually at levels not significantly different from each other. Phase-ON populations of *E. coli* did not colonize to a greater extent than Phase-OFF populations or the Ag43 null mutant ASTAG1. Furthermore, after 8 days there was a decline in the numbers of bacteria being shed in the faeces from all groups, but with actual levels of CFUs from each group remaining similar (Fig. 3a).

The numbers of Phase-ON and Phase-OFF cells present in the stool samples from each group were calculated by colony blotting using anti-α^{43} antisera. These data demonstrated that in the group of mice to which Phase-ON variants were fed the numbers of Phase-ON CFUs declined significantly over the 8 days of the study. In contrast, the levels of Phase-ON cells shed in the stools from mice fed Phase-OFF variants remained constant (Fig. 3b). Taken together these data strongly suggest that Ag43 does not play a significant role in promoting intestinal colonization.

Discussion

Pathogenic bacteria must develop adhesion mechanisms to effectively colonize the host. The binding of bacteria to mucosal cells generally requires interaction of specific ligands and receptors. Bacterial ligands may take the form of fimbriae but there are documented cases of outer membrane proteins acting as adhesins. Examples include AIDA-I, TibA and the pertactins (Benz & Schmidt, 1989; Leininger *et al.*, 1991; Elsinghorst & Weitz, 1994). The observations that the outer membrane protein Ag43 had N-terminal amino acid sequence homology with fimbriae and known autotransporter adhesins, and that it underwent phase variation prompted the investigations described here on adherence and invasion of Ag43-producing populations of bacteria.

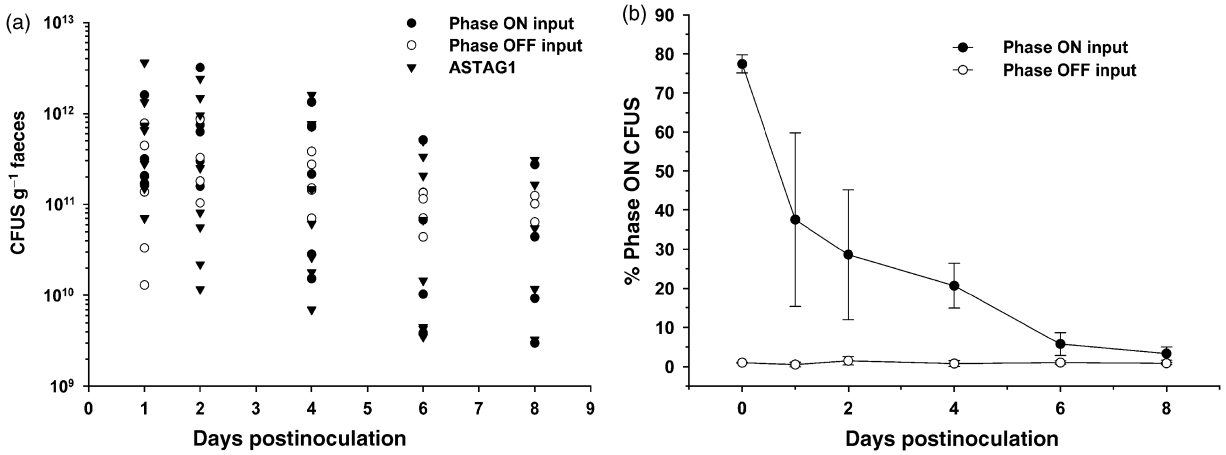

Fig. 3. Mouse colonization studies with *Escherichia coli* ML308-225S. (a) Depicts the numbers of bacteria per gram recovered from faeces in the different batches of mice. Each population of ML308-225S colonized the mice to statistically similar levels. The numbers of bacteria recovered in faeces declined at similar rates for all three populations over the 8-day period indicating that Ag43 does not promote a significant colonization advantage or prolonged shedding of *E. coli* ML308-225S. (b) Indicates the percentage of Phase-ON CFUs recovered in faeces over the 8-day period. The percentage of Phase-ON and Phase-OFF CFUs were enumerated using colony blotting and anti-α^{43} antiserum. The numbers of Phase-ON CFUs shed in faeces from mice which received a Phase-ON inoculum declines to a level similar to that observed for mice fed the Phase-OFF inoculum indicating Ag43 expressing organisms are not selected for during passage through the mouse intestine.

In assays performed with Ag43 Phase-ON and Ag43 Phase-OFF populations of *E. coli* ML308-225 the localized pattern of adherence was distinguished. Initial adhesion experiments indicated that populations expressing Ag43 demonstrated greater adherence than either Ag43 Phase-OFF populations or the null mutant, although the differences did not reach orders of magnitude as observed for other *bonafide* adhesins. Furthermore, inhibition experiments demonstrated that adherence of Ag43 Phase-ON populations of *E. coli* ML308-225 to HEp-2 cells was significantly reduced when cell lines were incubated with purified α^{43} prior to the addition of bacteria. These experiments appear to support the notion that Ag43 acts as a specific adhesion. However, microcolonies formed on cell lines by Ag43 Phase-OFF populations contained a low percentage of cells expressing Ag43, which in many instances do not appear to be in direct contact with epithelial cells implying that factors other than Ag43 are involved in adherence of *E. coli* ML308-225 to the cell lines. Furthermore, inhibition of adherence to cells was abolished when purified α^{43} was removed from the media. These data, in conjunction with the inability to detect any receptor binding with the affinity blots, strongly indicate that Ag43 does not act as a specific adhesin in tissue culture assays and that the increased adhesion observed for Ag43 Phase-ON populations and those overexpressing Ag43 is simply reflective of enhanced cell–cell interactions mediated by Ag43. Indeed, this hypothesis is supported by immunofluorescence and light photomicrographs, which clearly demonstrate that Ag43 Phase-ON populations adhere as compact microcolonies whereas those not expressing Ag43 adhere in a more diffuse manner.

Such a hypothesis is in obvious contrast to the adhesive nature described for a glycosylated form of Ag43 described previously by Sherlock *et al.* (2006). However, this discrepancy can be explained by the fact that in this set of experiments Ag43 was heterologously glycosylated by TibC and Aah, the glycosyltransferases for TibA and AIDA-I, respectively. In contrast to the loci encoding AIDA-I and TibA, no gene for a glycosyltransferase has been detected in association with *agn43* in any of the genome sequenced varieties of *E. coli*. Furthermore, electrospray mass spectroscopy studies performed previously on α^{43} isolated from *E. coli* ML308-225 did not demonstrate the addition of any sugar moieties (Henderson & Owen, 1999). Finally, recent data for AIDA-I has demonstrated that glycosylation is required for the stability of AIDA-I production rather than for mediating the adhesive interactions between AIDA-I and eukaryotic cells, whereas Ag43 is clearly expressed on the cell surface in a stable conformation in the absence of glycosylation (Charbonneau *et al.*, 2007). Taken together these data support the notion that Ag43 does not act as a significant adhesin in these *in vitro* assays and any increase in adhesion is simply a reflection of Ag43 mediated cell–cell interactions.

In support of the conclusions derived from the *in vitro* studies, the mouse colonization studies also demonstrated no significant contribution of Ag43 to colonization of the intestine by *E. coli*, or persistence of *E. coli* within the intestinal tract, and indeed expression was readily lost after only a few days within the intestinal tract. However, several

studies have demonstrated that Ag43 exists as multiple alleles and that several alleles may occur within one strain of *E. coli* (Henderson, 1996; Roche *et al.*, 2001; Klemm *et al.*, 2004; Restieri *et al.*, 2007). Recent investigations demonstrated that at least one allele from uropathogenic *E. coli* CFT073 contributed to long-term persistence of *E. coli* within the mouse bladder (Ulett *et al.*, 2007). However, in the same study, a closely related allele from the same strain did not prolong colonization and over expression of this second allele had a detrimental effect of the ability of the strain to colonize the mouse. Interestingly, a recent phylogenetic study of *agn43* alleles indicated that the *E. coli* K12 allele, which is closely related to the alleles from *E. coli* ML308-225 and the alleles from *E. coli* CFT073, was widely distributed amongst *E. coli* but in contrast a more distantly related allele from *E. coli* O157:H7 was almost exclusively found in diarrhoeagenic *E. coli* strains (Restieri *et al.*, 2007). Thus, it appears that different alleles, even closely related alleles, may have distinct functions and it remains possible that specific alleles are associated with colonization of different sites within the host, i.e. the intestine or the urinary tract. A valid explanation for the findings of Ulett *et al.* may be that Ag43 plays no role in colonization of the urinary tract by *E. coli* but rather promotes survival of *E. coli* once it has established an infection. In support of this hypothesis, recent data has demonstrated that Ag43 expression increases uptake by and survival of *E. coli* within polymorphonuclear neutrophils, and that Ag43 expressing organisms are protected from bactericidal activity within the neutrophils (Fexby *et al.*, 2007). Furthermore, Ag43-mediated aggregation was also shown previously to enhance survival of *E. coli* when exposed to other bactericidal agents in a manner similar to organisms embedded within biofilms (Schembri *et al.*, 2003). Such Ag43-mediated survival characteristics may be responsible for the prolonged shedding of *E. coli* CFT073 observed by Ulett *et al.* (2007). Interestingly, Ag43 mediated internalization of *E. coli* into the neutrophils was not reliant upon the RGD amino acid motifs within Ag43 suggesting an alternate mode of adherence yet to be identified (Fexby *et al.*, 2007).

In conclusion, we have demonstrated that Ag43 plays a role in enhancing bacterial adhesion in *in vitro* assays but that the *E. coli* ML308-225 alleles of Ag43 play no role in intestinal colonization. While the precise role of Ag43 remains to be unequivocally established it appears that Ag43 may play a role in late stages of infection, after the initial colonization event has occurred. In addition, Ag43 may play subtle roles in the infectious process, which are not detectable by our intestinal colonization studies. Furthermore, it is clear that the functions of the various allelic variants of *E. coli* remain to be determined and such investigations may reveal novel phenotypes for this multifunctional protein.

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