Impact of biofilm matrix components curli fimbriae and cellulose on interaction of commensal *Escherichia coli* with gastrointestinal cell line HT-29

Running title: interaction of commensal *Escherichia coli* with HT-29 cell line

Xiaoda Wang[1#], Maike Rochon[1#], Agaristi Lamprokostopoulou[1], Heinrich Lünsdorf[2], Manfred Nimtz[3], Ute Römling[1*]

[1] Microbiology and Tumor Biology Center, Box 280, Karolinska Institutet, SE-17177 Stockholm, Sweden;

[2] Department of Microbiology, Gesellschaft für Biotechnologische Forschung (GBF), Braunschweig, D-38124 Germany;

[3] Department of Structural Biology, Gesellschaft für Biotechnologische Forschung (GBF), Braunschweig, D-38124 Germany;

#Contributed equally to this work.

***** Corresponding author

Tel: +46 8 5248 7319; Fax: +46 8 330 744; email: ute.romling@ki.se.

Abstract. Commensal *Escherichia coli* form biofilms at body temperature by expressing the extracellular matrix components curli fimbriae and cellulose. The role of curli fimbriae and cellulose in the interaction of commensal *E. coli* with the intestinal epithelial cell line HT-29 was investigated. Expression of curli fimbriae by the typical commensal isolate *E. coli* TOB1 caused adherence and internalization of the bacteria and triggered IL-8 production in HT-29 cells. In particular, induction of IL-8 production was complex and involved curli-bound flagellin. While cellulose alone had no effect on the interaction of TOB1 with HT-29 cells, co-expression of cellulose with curli fimbriae decreased adherence to, internalization and IL-8 induction of HT-29 cells. Investigation of a panel of commensal isolates showed a partial correlation between expression of curli fimbriae and enhanced internalisation and IL-8 production. Thus, the consequences of expression of extracellular matrix components on commensal bacterial-host interactions are complex.

Key words. Intestinal microflora; adherence; invasion; IL-8; flagellin; proinflammatory response; actin cytoskeleton.

Introduction

The mucosal lining of the intestine provides the largest surface area in the adult human. Covered by a single layer of epithelial cells, the intestinal mucosa plays an important role in host defence through the development of tolerance to commensal flora and immediate response to pathogens [1]. The epithelial cells recognize intestinal inhabitants and invaders by expressing specific receptors that evolved to recognize structurally conserved microbial molecules, which have been termed pathogen associated molecular patterns (PAMPs). The pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) recognize, for example, peptidoglycan, lipid A part of the lipopolysaccharide and flagellin [1, 2]. While differentiated gastrointestinal epithelial cell lines have been demonstrated to be insensitive to lipid A stimulation due to the lack of TLR4 expression [3-5], proinflammatory response to flagellin, the structural subunit of the flagellar filament produced by pathogenic and commensal *Escherichia coli strain*s has been demonstrated [6-9]. In addition, flagellin is a major antigen in Crohn's disease, an inflammatory bowel disease [10] and contributes to the activation of systemic inflammation in LPS-resistant mice [11].

E. coli is one of the first colonizers of the intestinal microflora of most humans where it later accounts for 1% of the total bacterial biomass, with up to 10^8 cells/ml [12, 13]. The faecal enterobacterial flora is variable and consists of transient and persistent strains; potentially pathogenic strains harbouring virulence factors and harmless commensals. Persistence of *E. coli* in the gastrointestinal tract has been associated with the expression of adhesins [14, 15]. P-fimbriae enhance the colonization capacity of *E. coli* in the intestine [16]. Other adhesins like Type 1 fimbriae do not affect gut colonization [17, 18], but play a role in adherence and invasion of intestinal epithelial cells by *E. coli* strains isolated from lesions of Crohn´s disease [19].

Expression of curli fimbriae has been demonstrated in pathogenic and commensal isolates of *E. coli* [20-24]. A variety of virulence properties had been assigned to curli fimbriae [25], for example, binding to extracellular matrix proteins of the host such as fibronectin and laminin [22, 26]. Curli fimbriae also mediated invasion of epithelial cells [27, 28] via curli-bound fibronectin [29] and trigger an innate immune response in macrophages and vascular smooth muscle cells [20, 30]. In addition, curli fimbriae produced by *E. coli* contributed to adherence, colonization and persistence in the avian gut [31, 32] and were virulence factor of enterohaemmorhagic *E. coli* in a model of streptomycin-treated mice [28].

The exopolysaccharide cellulose, which is a component of the extracellular matrix of biofilm-forming bacteria, is frequently co-expressed with curli fimbriae by commensal *E. coli* strains [21, 33]. Actually, a typical commensal isolate expressed curli fimbriae and cellulose at 28 and 37 °C. However, since biofilm formation has been considered a virulence factor, the role of cellulose and curli fimbriae in the interaction with intestinal epithelial cells has never been investigated in commensal bacteria.

In the present study, the impact of expression of the extracellular matrix components in the representative commensal isolate *E. coli* TOB1 on the interaction with the intestinal epithelial cell line HT-29 was investigated. Production of curli fimbriae by *E. coli* TOB1 enhanced adherence and internalization of the bacteria and IL-8 production of HT-29 cells. IL-8 induction by *E. coli* TOB1 expressing curli fimbriae was complex and involved expression of flagellin. In contrast, co-expression of cellulose counteracted curli fimbriae mediated interactions of *E. coli* TOB1 with HT-29 cells. To generalize the findings made with *E. coli* TOB1, internalization and IL-8 induction properties of a panel of commensal isolates was investigated. In general,

expression of curli fimbriae leads to a higher level of internalization and IL-8 induction in combination with flagellin.

Materials and methods

Bacterial strains

E. coli TOB1 and derivatives and plasmids used are listed in Table 1. The fecal strains FEC6, FEC9, FEC10, FEC23, FEC32, FEC41, FEC51, FEC53, FEC55, FEC61, FEC65, FEC75, FEC81, FEC93, FEC101 and FEC108 have been described recently [21]. Bacteria were grown in Luria-Bertani (LB) broth without salt or on LB without salt agar plates for 24 hours at 37ºC.

Genetic manipulations

One step knock-out of *fliC*, the gene encoding the subunit of the flagellar filament, was carried out according to the protocol of Datsenko and Wanner [34], with the exception that 0.7-1.2 µg PCR product was electroporated into the target strain to achieve the gene knock-out. Primers used were: EC_*fliC*_Start: ATGGCACAAGTCATTAATACCAACAGCCTCTCGCTGATCACTCAAAATAA TATCAACAAGGTGTAGGCTGGAGCTGCTTC, EC_*fliC*_Stopp: CTGCGCT-TTCGACATGTTGGACACTTCGGTCGCATAGTCGG CGTCCTGAATACGGG-ACTGCATATGAATATCCTCCTTAGT. Underlined the sequence required to amplify the chloramphenicol (Cm) cassette of pKD3. The gene knock-out was verified by PCR using a primer in the Cm cassette (ATCACTGGATATACCACCGTT) and a primer flanking the *fliC* gene (TTACGACAGACGATAACAGG).

Plasmid pQE60F was constructed by cloning *Salmonella enterica* serovar Typhimurium *fliC* into plasmid pQE60. Primer used to amplify *fliC* were: CCCCCATGGATGGCACAAGTCATTAATACAAA (*Nco*I restriction site

underlined) and CCCGGATCCACGCAGTAAAGAGAGGAC (*Bam*HI restriction site underlined). Subsequently, the PCR product was cut by restriction enzymes *Nco*I and *Bam*HI and cloned into pQE60.

Sequencing of *fliC*

Sequencing of flagellin was performed with primers up- and down-stream of *fliC* (Ag-start1: ATTAGTGGGTGAAATGAGGG; Ag-stop1: ACAAGTCATTAAT-ACCAACAGCC; Ag-stop2: GACTCCCAGCGATGAAATA). In addition, Start10101: GTCTGCGCAACAGAAATACC was used for sequencing of FEC10 and FEC101 *fliC* and F10stop1520: GTAACGCGAATGATGGTATT and F10stop1120: CCTTTACTATTGATGCGACA were used for sequencing of FEC10. The *fliC* sequences of FEC10 and FEC32 were submitted to the database under the accession number AM231154 and AM231155, respectively.

Adhesion studies

HT-29 cells were grown to confluency on glass coverslips deposited into a 24-well plate. Prior to infection the bacteria were resuspended in 1mg/ml Fluorescein isothiocyanate (FITC) (FITC buffer: 0.05 M Na_2CO_3, 0.1 M NaCl) and kept on ice for 1 hour. After excessive washing of the FITC labelled bacterial cells with PBS epithelial cells were infected with approximately 10^8 bacteria (multiplicity of infection (MOI): 17). After 1.5 h of incubation, the glass cover slips were rinsed three times with 2 ml of PBS and fixed with 4% formaldehyde 10 to 15 minutes. The number of bacteria that adhered to HT-29 cells was determined by fluorescence microscopy. For each adhesion assay, at least 4000 epithelial cells were observed. Each experiment was done twice.

Scanning electron microscopy (SEM)

After the adhesion assay, samples were prepared as described [35].

Internalization assay

Detection of internalization of intestinal epithelial cells by *E. coli* strains was essentially performed as described [36]. HT-29 cells were seeded into 24-well tissue culture plates at 1.2 x 10^6 cells per well and incubated until full confluency was reached. Prior to infection the medium was exchanged and each well was infected with the respective bacteria at a MOI of 17. After 3 or 6 hours incubation, the cells were washed three times with PBS and fresh medium containing 100 µg/ml gentamicin sulfate (Sigma) was added to kill extracellular bacteria. After incubation for an additional hour, monolayers were washed three times with PBS and 1 ml of 1% Triton X-100 in deionized water was placed into each well for 5 minutes to lyse eukaryotic cells. Samples were removed and appropriate dilutions were plated on LB agar plates. The internalization level was defined as the percentage of the original inoculum that resisted treatment of gentamicin. *S.* Typhimurium UMR1 (ATCC14028 Nal^r) grown in standing culture until o.D. 0.6 was used as a positive control for invasion. Every experiment was performed at least twice in duplicates.

Purification of flagellin

E. coli strains were precultured in 5 ml LB medium overnight at 37ºC. 0.5 ml of each culture was inoculated into 500 ml fresh LB medium and incubated overnight with shaking at 37ºC. The bacterial suspension was pelleted by centrifugation and the bacterial pellet was resuspended in 30 ml 0.5 M Tris-HCl, pH 8 and flagella were sheared off by pressing the suspension through a syringe. After centrifugation for 20

min, 8000g, at 4ºC to remove the cells, the supernatant was filtered through a 0.22 µm filter and centrifuged again at 106000g at 4ºC for 1h to recover the flagella. The pellet was resuspended in approximately 300 µl PBS, and analysed by SDS-PAGE on 12% acrylamide gels. The identity of expressed flagellin proteins with the protein sequence encoded by the *fliC* locus was verified by MALDI-TOF and MS/MS analysis.

Flagellar filaments were depolymerised by adjusting the pH to 3.5 with 3 M HCl, and restoration to a neutral pH by addition of 10 M NaOH after 5 min. Depolymerisation of flagella was analysed by native gel electrophoresis using 12% acrylamide gels, which were stained with colloidal Coomassie. Quantification of the protein amount of monomerized flagellin was performed using the Bradford Protein Determination Assay (Biorad) and SDS-PAGE gels.

Interleukin 8 (IL-8) activation by bacteria

HT-29 cells were prepared in 24-well tissue culture plates in the same way as for the internalization assay. Prior to infection the medium was changed and each well was infected with the respective bacterial strain at a MOI of 17. After 3 or 6 hours incubation, bacteria-cell culture supernatants were collected by centrifugation and stored at -20ºC until used for the measurement of IL-8 concentration. Each experiment was performed at least twice in duplicates.

The ability of purified monomeric flagellin to induce an IL-8 response was assessed by co-incubation of HT-29 cell with appropriate concentrations of flagellin for 5 hours.

IL-8 measurement

IL-8 concentration of the cell culture supernatant was measured by using the Human IL-8 Eli-pair kit exactly as recommended by the manufacturer (Nordic BioSite AB, Stockholm).

Swimming assay

The agar plate included LB medium and 0.3% agar and was freshly prepared for the swimming assay. 10 µl of a bacterial suspension of an OD_{600}4.0 was inoculated into the agar. The swimming zone was observed after 6 hours incubation at 37ºC. A final statement about swimming motility was done after 24 h of incubation.

Results

Recently, commensal *E. coli* strains have been demonstrated to express the extracellular matrix components cellulose and curli fimbriae in vitro [21]. Since nothing is known about the role of the two components in commensal-host interaction, the impact of curli fimbriae and cellulose on the interaction of commensal bacteria with intestinal epithelial cells was addressed. A representative faecal commensal isolate, TOB1 that expressed cellulose and curli fimbriae at 37ºC [21] was chosen to investigate the interaction with the intestinal colonic carcinoma cell line HT-29.

Role of cellulose and curli fimbriae on adherence to HT-29 cells

Since *E. coli* has been found in close association with the mucosal epithelial cell surface [37], adhesion to gastrointestinal cells has been considered to be an indicator of colonization of the gastrointestinal tract. Therefore, as a first assay, the adherence of *E. coli* TOB1 and its mutants with defects in the expression of the extracellular matrix components (Table 1) to the colonic carcinoma cell line HT-29 was determined. The wild type strain TOB1 showed intermediate adherence to the intestinal colonic carcinoma cell line HT-29, while the mutant expressing only cellulose and the mutant expressing neither cellulose nor curli fimbriae did not significantly adhere (figure 1). TOB3 that expressed only curli fimbriae adhered significantly to the HT-29 cell line. Those results showed that under the growth conditions used curli fimbriae, but not cellulose mediated adherence. On the contrary, when cellulose was co-expressed with curli fimbriae, adhesion to epithelial cells was decreased.

To visualize how TOB1 and its derivatives adhere, the adherence pattern of ToB1 and its derivatives to the epithelial cell line HT-29 was examined by fluorescence microscopy and scanning electron microscopy. When foci of adherence were found, TOB1 and mutants expressing one individual matrix component were usually found not as single cells but in bigger cell clumps or chains consistent with their multicellular phenotype. Only TOB2, which does not express matrix components, was found as single cells (figure 2).

Role of cellulose and curli fimbriae in the internalization of TOB1 into HT-29 cells

The internalization capability of TOB1 and its respective mutants were tested after growth on agar plates for 24 hours, conditions where the known invasion genes and type 1 fimbriae were repressed [38], but expression of cellulose and curli fimbriae was optimal [21]. The rate of internalisation of the individual mutants basically correlated with amount of adherence (figure 3A). TOB1, which expressed curli fimbriae and cellulose showed almost no internalization, while TOB3, which expressed only curli fimbriae, showed 100-fold higher internalization rate than TOB1, although still no high-level internalization was reached (0.013%). Internalization by the cellulose-producing mutant and the mutant with no expression of extracellular matrix components was not significant. We concluded that cellulose expression *per se* did not influence the internalization process, but prevented effective internalization by curli fimbriae. Preliminary experiments with polarized and unpolarized T-84 intestinal cells gave similar results although the differences between mutants were not as dramatic. Internalization was enhanced 7-fold by curli fimbriae expression as compared to TOB2 expressing no matrix components and 3-fold when cellulose was

co-expressed with curli fimbriae (data not shown). The role of curli fimbriae in internalization was confirmed by use of the commensal isolate MC4100, a derivative of the laboratory strain *E. coli* K-12 and its isogenic deletion mutant of the *csg* operon (figure 3A).

Role of host factors on internalisation

To gain entry into the host, microbial pathogens, among them pathogenic *E. coli*, manipulate the actin cytoskeleton of non-phagocytic cell [39, 40]. In order to test whether TOB1 and its mutants are internalised by HT-29 cell in an actin dependent process, HT-29 cells were treated with cytochalasin D (1 µg/ml) and subsequently infected with TOB1 and its respective mutants. While the number of internalised wild type TOB1 and TOB2, which does not express extracellular matrix components, were too low to achieve statistically significant results, treatment of HT-29 cell with cytochalasin D blocked internalization of TOB3 expressing curli fimbriae by 86% demonstrating that actin microfilaments are required for uptake of curli fimbriae producing bacteria (Figure 3B).

The microtubule system plays a role in the entry of *E. coli* into epithelial cells [41]. To address whether the internalization of *E. coli* TOB1 and its mutants required the microtubule system, HT-29 cells were treated with colchicine (1 µg/ml) prior to infection. The number of internalised TOB2, which does not express extracellular matrix components, was too low to achieve statistically significant results. Internalization of TOB1 and TOB3 expressing curli fimbriae was not affected by colchicines (Figure 3C) indicating that microtubule function is not required for the internalization of commensal *E. coli* TOB1.

Curli fimbriae have been shown to bind fibronectin [22] and mediated internalization of pathogenic *E. coli* strain via the RGD domain of fibronectin with integrin receptors on the cell surface [29]. Incubation of HT-29 cells with RGDS peptide for 1 hour prior to infection largely prevented internalization of the commensal curli-expressing mutant TOB3 in contrast to the control peptide RGES (figure 3D). A similar trend was seen with TOB1, although the low bacterial counts prevented statistically significant results.

Besides the capability to invade the capability of TOB1 and its matrix mutants to persist or multiply inside intestinal epithelial cells was examined. Strains were able to persist or even to significantly multiply in HT-29 cells up to 72 hours when the medium was changed every 6 or 24 hours (data not show).

Internalization of fecal isolates by HT-29 cells

The previous experiments have shown that curli fimbriae trigger internalization of TOB1 into HT-29 cells, but presence of cellulose does prevent the internalization. In order to investigate whether internalization of various commensal *E. coli* strains is correlated with the expression of extracellular matrix components, 11 fecal isolates of different genetic backgrounds that produced various combinations of extracellular matrix components were chosen from a strain collection (Table 1, [21]). Internalization of the fecal isolates by HT-29 cells showed that most strains had very low internalization rates from 0,00001% to 0.0003%. The exception were two strains with bdar morphotype (indicating strong expression of curli fimbriae, [21] which were internalized at 10 and 1000-fold higher rate (0.003% and 0.082%) than the other strains (Figure 4).

Role of cellulose and curli fimbriae on stimulation of the proinflammatory cytokine IL-8

Commensal *E. coli* stains are able to trigger a proinflammatory response in gastrointestinal epithelial cells [6]. Thus we tested whether the interaction of TOB1 and its mutants with intestinal epithelial cells triggered the production of the proinflammatory cytokine IL-8 (Figure 5A). After three hours of co-incubation with bacteria, IL-8 production was highest in HT-29 cells co-incubated with TOB3 producing curli fimbriae only. TOB1 producing both matrix components, its mutant TOB2p producing only cellulose or TOB2 producing no extracellular matrix induced a significantly lower IL-8 response. Similar results were gained after 6 h of coincubation. In conclusion, these results showed that curli fimbriae were able to trigger or enhance a proinflammatory response when expressed by the fecal isolate TOB3.

Although components of adhesive fimbriae of *E. coli* were shown to elicit an immune response [7], flagellin has been recognized as a major determinant of IL-8 induction from gastrointestinal epithelial cells triggered by pathogenic and commensal *E. coli* [6, 9, 42, 43]. In order to elucidate whether flagellin was involved in IL-8 induction of HT-29 cells by TOB1 and its mutants, *fliC*, encoding flagellin the structural subunit of flagella was knocked out in TOB1 and TOB3 to create TOB5 and TOB6, respectively. The IL-8 activation of HT-29 cells by TOB5 and TOB6 was compared to their respective wild types. After 3 and 6 hours co-incubation with HT-29, TOB5 induced only insignificantly less IL-8 production than TOB1 suggesting that the presence of flagellin did not contribute to the immune response triggered by TOB1. In contrast, the IL-8 level induced by TOB6 was significantly decreased compared to its parent strain TOB3 suggesting that the presence of flagellin contributed substantially to the

immune response triggered by TOB3 (Figure 5B and data not shown). Basically, the IL-8 induction was at the same low level in TOB5 and TOB6. Consequently, neither flagellin nor curli fimbriae alone triggered a substantial IL-8 response in HT-29 cells. However, the significantly decreased IL-8 induction in TOB6, but not TOB5 suggested that flagellin bound to curli fimbriae contributed to the high level of IL-8 production seen in TOB3.

Complementation of the *fliC* knock-out in TOB5 and TOB6 with *fliC* of *Salmonella enterica* serovar Typhimurium induced a high level of IL-8 production in HT-29 cells. This finding indicated that the flagellin derived from *S.* Typhimurium is able to cause an immune response in *E. coli* TOB1 and its mutants independent of curli fimbriae.

Induction of a proinflammatory response by a panel of fecal isolates

In order to investigate the capability of commensal *E. coli* to induce a proinflammatory response, 16 fecal *E. coli* isolates were selected according to different H serotypes in combination with various expression patterns of cellulose and curli fimbriae [21]. Expression of flagella was verified by the ability of the strains to swim (data not shown). Flagella negative and most of the flagellin positive commensal *E. coli* isolates induced a low level of IL-8 secretion < 0.2 ng/ml after 3 hours of co-incubation with HT-29 cells. However, 3 flagella positive isolates elicited an IL-8 induction >0.4 ng/ml; thereby Fec101 (0.98 ng/ml) and Fec108 (0.47 ng/ml) showed strong expression of curli fimbriae, while Fec10 (0.91 ng/ml) did not express any matrix components (Figure 6). Consequently, the ability of commensal *E. coli* to cause a high immune response can be, but is not necessarily coupled to the co-expression of curli fimbriae and flagellin.

Induction of a proinflammatory response by flagellin

To directly address the role of flagellin in triggering the IL-8 production in HT-29 cells, flagella were isolated from 7 isolates, which had shown to trigger a low, intermediate and high immune response, and coincubated with HT-29 cells. Since intact flagella show 100-fold lower immune response than monomeric flagellin [44], depolymerization of flagella by acid treatment was performed as described in Materials and methods. Most flagellin monomers, including flagellin isolated from FEC101 and FEC108, induced an IL-8 production between 4-12 fold over basal level when 10 ng (approx. 0.2 nM) flagellin was added for 5 h (see FliC from TOB1 as an example in figure 7). This result indicated that the immune response to flagellin alone could not explain the high immune response observed by the application of whole cells of FEC101 and FEC108. Most likely the combined expression of curli fimbriae and flagellin is responsible for the high immune response of FEC101 and FEC108 whole cells. The flagellin produced by FEC10 showed an exceptionally high immune response since it induced IL-8 production approximately 30 fold over background level when 10 ng (approx. 0.2 nM) flagellin was added (Figure 7). Since FEC10 does not express curli fimbriae at 37˚C, a synergistic effect of curli fimbriae and flagellin can be excluded. Most likely, the high IL-8 production induced by whole bacterial cells is solely caused by the flagellin variant, which is highly immunogenic (Figure 7).

Sequence comparison of flagellin

To investigate the molecular basis of the differential immunity of the flagellin proteins, *fliC*, the gene encoding flagellin was sequenced from the strains investigated. Thereby, TOB3, FEC9, FEC101 and FEC108 had a FliC protein

identical to the respective reference H serotype in the database (data not shown). FEC32 harboured a novel FliC protein sequence, while the FliC sequence of FEC10 deviated in three amino acids from the published serotype H27 sequence. Flagellin sequences of *E. coli* have been classified in two major groups according to their sequence homology [45]. While H27 belongs to Ec2, all other FliC sequences belong to group Ec1.

Investigation of the N- and C-terminal regions of FliC showed that most amino acids demonstrated to be required for stimulation are conserved between *S.* Typhimurium and *E. coli* sequences of the Ec1 and Ec2 group or subject to conservative amino acid exchange. Thus, the molecular basis of the differential stimulation of TLR5 mediated IL-8 response by various *E. coli* flagellins remains to be explored.

Discussion

In a previous study, it was reported that 44% of commensal *E. coli* isolates expressed the extracellular matrix component curli fimbriae at 37ºC, while in 48 % of those strains the exopolysaccharide cellulose was coexpressed with the curli fimbriae [21]. In this communication we demonstrate the impact of the expression of curli fimbriae and cellulose on the interaction between commensal isolates of *E. coli* and the colonic carcinoma cell line HT-29. Using the representative commensal isolate *E. coli* TOB1 and its isogenic mutants we showed that the extracellular matrix components curli fimbriae and cellulose affect adherence, internalization and IL-8 production in HT-29 cells. In addition, we show that flagellin interacts with curli fimbriae to mediate IL-8 production.

Pathogenicity of *E. coli* is associated with the ability to adhere to gastrointestinal epithelial cells in the small intestine [46]. Most commensal *E. coli* isolates are not associated with disease and common virulence genes are mainly absent [16, 21]. However, the ability to adhere to intestinal epithelial cells via P-fimbriae [47] is one of the factors associated with the persistence in the gastrointestinal tract [48, 49]. Here it is demonstrated that curli fimbriae mediate adherence of *E. coli* commensal isolates to gastrointestinal epithelial cells. Based on previously carried out epidemiological studies [21] the expression of curli fimbriae does not seem to be a prerequisite for colonization of the gastrointestinal tract, however might be one factor contributing to intestinal persistence.

High level of invasion of the intestinal epithelia is performed by enteric pathogens such as *Yersinia*, *Shigella*, *Salmonella* spp. and *E. coli* [50-52]. Previously, curli-mediated invasion of pathogenic *E. coli* and *S. enterica* strains was observed with strains, which showed an (in their context) atypical expression of curli fimbriae or the

rdar morphotype at 37ºC [27, 28, 53, 54]. The invasion rate (0.013% of inoculum number) of the commensal strain *E. coli* TOB1 mediated by curli fimbriae was approximately 10-fold lower than curli-mediated invasion of pathogenic *E. coli* [27, 28], but similar to other low level invasion rates [55]. Consistently, evaluation of the internalization rate of a panel of commensal *E. coli* with different genetic backgrounds demonstrate that stains with bdar morphotype (strong expression of curli fimbriae) were internalized at 10 and 1000-fold higher rate compared to the strains with other morphotypes (no expression of extracellular matrix, expression of both matrix components or only expression of cellulose) (Figure 4).

Whether curli-mediated adherence and invasion of commensal *E. coli* can be considered a virulence factor remains to be shown. The intestinal flora, in particular members of the family of enterobacteriaceae, play an etiological role in triggering disease in the immunocompromised host such as inflammatory bowel disorders, cancer and sepsis caused by translocation over the epithelial barrier [56, 57].

Translocating strains and *E. coli* strains from patients with Crohn's disease tightly interacted with epithelial cells [58-60] and modulated epithelial barrier function by cytoskeleton rearrangement [58] . An involvement of the actin cytoskeleton in invasion of curli-expressing TOB1 was also found in this study.

A possible physiological role of intestinal epithelial cell invasion by commensal isolates might be translocation over the epithelial cell lining thereby stimulating the immune system at a low level. The rate of invasion in such a system should certainly not exceed a certain threshold to keep a balance with the subsequent elimination of bacterial cells. In this context, it is interesting to note that TOB1 and its matrix mutants were able to persist or even to significantly multiply in HT-29 cells up to 72 hours (data not shown).

Besides as acting as a physical barrier the intestinal epithelial cells integrate host innate and adaptive immune responses [61]. Thereby, the intestinal commensal microflora plays a role in stimulating a background inflammatory infiltrate that maintains the host immune defence [6, 62]. It has been shown before that commensal E. coli strain are able to elicit a proinflammatory response [6, 56, 58]. In this study, TOB1 and its isogenic mutants triggered a differential IL-8 response in HT-29 cells. This finding indicate that the differential interaction of the bacteria with host cells, such as adhesion and invasion modulate the immune response, since cellulose and curli fimbriae are not immunologic themselves (see below).

Cellulose per se did not seem to have an influence on interaction of bacteria with epithelial cells, since cellulose expressing TOB2p had the same low adherence, invasion and IL-8 induction rate as TOB2 which did not express any of the two matrix components. However, the interaction of cellulose with curli fimbriae on the bacterial surface significantly prevented curli fimbriae to function as an adhesin, invasin or immunogen. This finding is in agreement with previous results in *S.* Typhimurium, where expression of cellulose did not influence the expression of curli fimbriae, but diminished fibronectin binding which is a characteristic of curli fimbriae [63].

It has been demonstrated in numerous studies that flagellin from pathogenic and commensal *E. coli* caused IL-8 induction in a variety of intestinal epithelial cell lines and murine ileal biopsies [6, 9, 42, 43, 58, 64]. Investigation of the role of flagellin in the stimulation of HT-29 cells revealed a complex pattern of events. While flagellin contributed only slightly to the induction of IL-8 production by TOB1, the IL-8 production was dramatically reduced in the flagellin knock-out mutant of TOB3 expressing curli fimbriae. imilar low IL-8 induction was achieved in the flagellin knock-out mutant of TOB1 and TOB2 without matrix components, but expressing

flagellin. Therefore, binding of flagellin to curli fimbriae is responsible for the elevated IL-8 response of TOB3. Synergistic effect of flagellin bound to curli fimbriae on IL-8 induction has been recently demonstrated for *Salmonella enterica* serovar Typhimurium [65].

Recently, it has also been shown that curli fimbriae is a PAMP recognized by TLR2 [66]. In the system used in this study, no evidence for a PAMP function of curli fimbriae could be detected. It is not known which toll-like receptor, if any, recognizes the flagellin/curli fimbriae complex. Although TLR5 is the primary receptor for flagellin recognition and signalling, TLR2, TLR4 and gangliosides cooperate with TLR5 as receptors for flagellin binding and signalling [67]. Comparison of the IL-8 induction in a panel of commensal isolates with the IL-8 induction of purified flagellin indicated that cooperativity in induction of IL-8 between flagellin and curli fimbriae occurred more frequently.

We also identified the flagellin sequence of serotype H27 as being particular immunostimmulatory. Flagellin of H27 serotype belongs to Ec2 group of flagellin sequences, which is distinct from the majority of flagellin sequences of *E. coli* since the protein is more closely related to flagellin from *S. enterica* [45]. However, examination of the amino acids that were previously demonstrated to be required for TLR5 mediated IL-8 stimulation did not reveal any conspicuous features.

In any case, almost nothing is known about the features of commensal *E. coli* strains. Investigation of this strain population will contribute to our understanding of the important role the commensal flora plays in human health and disease.

Acknowledgements

This work was supported by the Karolinska Institutet (Elitforskartjänst to U.R.), Vetenskapsradet (K2002-06X-14232-01A) and the European Commission (Early Stage Research Training Programme ‘EU’s IMO-Train; contract number MEST-CT-2004-008475).

References

1 Collier-Hyams, L.S. and A.S. Neish. (2005) Innate immune relationship between commensal flora and the mammalian intestinal epithelium. Cell Mol Life Sci. **62**: 1339-1348.

2 Saitoh, S., S. Akashi, T. Yamada, N. Tanimura, M. Kobayashi, K. Konno, F. Matsumoto, K. Fukase, S. Kusumoto, Y. Nagai, Y. Kusumoto, A. Kosugi, and K. Miyake. (2004) Lipid A antagonist, lipid IVa, is distinct from lipid A in interaction with Toll-like receptor 4 (TLR4)-MD-2 and ligand-induced TLR4 oligomerization. Int Immunol. **16**: 961-969.

3 Haller, D., L. Holt, A. Parlesak, J. Zanga, A. Bauerlein, R.B. Sartor, and C. Jobin. (2004) Differential effect of immune cells on non-pathogenic Gram-negative bacteria-induced nuclear factor-kappaB activation and pro-inflammatory gene expression in intestinal epithelial cells. Immunology. **112**: 310-320.

4 Abreu, M.T., P. Vora, E. Faure, L.S. Thomas, E.T. Arnold, and M. Arditi. (2001) Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. J Immunol. **167**: 1609-1616.

5 Hornef, M.W., B.H. Normark, A. Vandewalle, and S. Normark. (2003) Intracellular recognition of lipopolysaccharide by toll-like receptor 4 in intestinal epithelial cells. J Exp Med. **198**: 1225-1235.

6 Bambou, J.C., A. Giraud, S. Menard, B. Begue, S. Rakotobe, M. Heyman, F. Taddei, N. Cerf-Bensussan, and V. Gaboriau-Routhiau. (2004) In vitro and ex

vivo activation of the TLR5 signaling pathway in intestinal epithelial cells by a commensal *Escherichia coli strain*. J Biol Chem. **279**: 42984-42992.

7 Harrington, S.M., M.C. Strauman, C.M. Abe, and J.P. Nataro. (2005) Aggregative adherence fimbriae contribute to the inflammatory response of epithelial cells infected with enteroaggregative *Escherichia coli*. Cell Microbiol. **7**: 1565-1578.

8 Khan, M.A., J. Kang, and T.S. Steiner. (2004) Enteroaggregative *Escherichia coli* flagellin-induced interleukin-8 secretion requires Toll-like receptor 5-dependent p38 MAP kinase activation. Immunology. **112**: 651-660.

9 Steiner, T.S., J.P. Nataro, C.E. Poteet-Smith, J.A. Smith, and R.L. Guerrant. (2000) Enteroaggregative *Escherichia coli* expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells. J Clin Invest. **105**: 1769-1777.

10 Vernier, G., B. Sendid, D. Poulain, and J.F. Colombel. (2004) Relevance of serologic studies in inflammatory bowel disease. Curr Gastroenterol Rep. **6**: 482-487.

11 Eaves-Pyles, T., K. Murthy, L. Liaudet, L. Virag, G. Ross, F.G. Soriano, C. Szabo, and A.L. Salzman. (2001) Flagellin, a novel mediator of *Salmonella*-induced epithelial activation and systemic inflammation: I kappa B alpha degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction. J Immunol. **166**: 1248-1260.

12 Hartl, D.L. and D.E. Dykhuizen. (1984) The population genetics of *Escherichia coli*. Annu Rev Genet. **18**: 31-68.

13 Leclerc, H., D.A. Mossel, S.C. Edberg, and C.B. Struijk. (2001) Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. Annu Rev Microbiol. **55**: 201-234.

14 Grozdanov, L., C. Raasch, J. Schulze, U. Sonnenborn, G. Gottschalk, J. Hacker, and U. Dobrindt. (2004) Analysis of the genome structure of the nonpathogenic probiotic *Escherichia coli strain* Nissle 1917. J Bacteriol. **186**: 5432-5441.

15 Le Bouguenec, C. (2005) Adhesins and invasins of pathogenic *Escherichia coli*. Int J Med Microbiol. **295**: 471-478.

16 Wold, A.E., D.A. Caugant, G. Lidin-Janson, P. de Man, and C. Svanborg. (1992) Resident colonic *Escherichia coli strain*s frequently display uropathogenic characteristics. J Infect Dis. **165**: 46-52.

17 Bloch, C.A. and P.E. Orndorff. (1990) Impaired colonization by and full invasiveness of *Escherichia coli* K1 bearing a site-directed mutation in the type 1 pilin gene. Infect Immun. **58**: 275-278.

18 McCormick, B.A., D.P. Franklin, D.C. Laux, and P.S. Cohen. (1989) Type 1 pili are not necessary for colonization of the streptomycin-treated mouse large intestine by type 1-piliated *Escherichia coli* F-18 and *E. coli* K-12. Infect Immun. **57**: 3022-3029.

19 Boudeau, J., N. Barnich, and A. Darfeuille-Michaud. (2001) Type 1 pili-mediated adherence of *Escherichia coli strain* LF82 isolated from Crohn's disease is involved in bacterial invasion of intestinal epithelial cells. Mol Microbiol. **39**: 1272-1284.

20 Bian, Z., A. Brauner, Y. Li, and S. Normark. (2000) Expression of and cytokine activation by *Escherichia coli* curli fibers in human sepsis. J Infect Dis. **181**: 602-612.

21 Bokranz, W., X. Wang, H. Tschape, and U. Romling. (2005) Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. J Med Microbiol. **54**: 1171-1182.

22 Olsen, A., A. Jonsson, and S. Normark. (1989) Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature. **338**: 652-655.

23 Patri, E., Szabo, E., Pal, T., Emody, L., (2000) Thin aggregative fimbriae on urinary *Escherichia coli* isolates, in Genes and proteins underlying microbial urinary tract virulence, L. Emody, Editor., Kluwer Academic/Plenum Publishers: AA Dordrecht. pp. 219-224.

24 Collinson, S.K., L. Emody, T.J. Trust, and W.W. Kay. (1992) Thin aggregative fimbriae from diarrheagenic *Escherichia coli*. J Bacteriol. **174**: 4490-4495.

25 Römling, U., Bokranz, W., Gerstel, U., Lünsdorf, H., Nimtz, M., Rabsch, W., Tschäpe, H., Zogaj, X., (2003) Dissection of the genetic pathway leading to multicellular behaviour in *Salmonella* typhimurium and other Enterobacteriaceae in Medical implications of biofilms, in Cellulose, D. M. Wilson and Devine, Editor., Cambridge University Press, Cambridge, England. . pp. 231-261.

26 Olsen, A., A. Arnqvist, M. Hammar, S. Sukupolvi, and S. Normark. (1993) The RpoS sigma factor relieves H-NS-mediated transcriptional repression of

csgA, the subunit gene of fibronectin-binding curli in *Escherichia coli*. Mol Microbiol. **7**: 523-536.

27 Gophna, U., M. Barlev, R. Seijffers, T.A. Oelschlager, J. Hacker, and E.Z. Ron. (2001) Curli fibers mediate internalization of *Escherichia coli* by eukaryotic cells. Infect Immun. **69**: 2659-2665.

28 Uhlich, G.A., J.E. Keen, and R.O. Elder. (2002) Variations in the csgD promoter of *Escherichia coli* O157:H7 associated with increased virulence in mice and increased invasion of HEp-2 cells. Infect Immun. **70**: 395-399.

29 Gophna, U., T.A. Oelschlaeger, J. Hacker, and E.Z. Ron. (2002) Role of fibronectin in curli-mediated internalization. FEMS Microbiol Lett. **212**: 55-58.

30 Bian, Z., Z.Q. Yan, G.K. Hansson, P. Thoren, and S. Normark. (2001) Activation of inducible nitric oxide synthase/nitric oxide by curli fibers leads to a fall in blood pressure during systemic *Escherichia coli* infection in mice. J Infect Dis. **183**: 612-619.

31 La Ragione, R.M., W.A. Cooley, and M.J. Woodward. (2000) The role of fimbriae and flagella in the adherence of avian strains of *Escherichia coli* O78:K80 to tissue culture cells and tracheal and gut explants. J Med Microbiol. **49**: 327-338.

32 La Ragione, R.M., A.R. Sayers, and M.J. Woodward. (2000) The role of fimbriae and flagella in the colonization, invasion and persistence of *Escherichia coli* O78:K80 in the day-old-chick model. Epidemiol Infect. **124**: 351-363.

33 Römling, U. (2002) Molecular biology of cellulose production in bacteria. Res Microbiol. **153**: 205-212.

34 Datsenko, K.A. and B.L. Wanner. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A. **97**: 6640-6645.

35 Lunsdorf, H., C. Strompl, A.M. Osborn, A. Bennasar, E.R. Moore, W.R. Abraham, K.N. Timmis, B. Middleton, H.C. Rodgers, M. Camara, A.J. Knox, P. Williams, and A. Hardman. (2001) Approach to analyze interactions of microorganisms, hydrophobic substrates, and soil colloids leading to formation of composite biofilms, and to study initial events in microbiogeological processes. Methods Enzymol. **336**: 317-331.

36 Boudeau, J., A.L. Glasser, E. Masseret, B. Joly, and A. Darfeuille-Michaud. (1999) Invasive ability of an *Escherichia coli strain* isolated from the ileal mucosa of a patient with Crohn's disease. Infect Immun. **67**: 4499-4509.

37 Hartley, C.L., C.S. Neumann, and M.H. Richmond. (1979) Adhesion of commensal bacteria to the large intestine wall in humans. Infect Immun. **23**: 128-132.

38 Blomfield, I.C. (2001) The regulation of pap and type 1 fimbriation in *Escherichia coli*. Adv Microb Physiol. **45**: 1-49.

39 Rottner, K., S. Lommel, J. Wehland, and T.E. Stradal. (2004) Pathogen-induced actin filament rearrangement in infectious diseases. J Pathol. **204**: 396-406.

40 Selbach, M. and S. Backert. (2005) Cortactin: an Achilles' heel of the actin cytoskeleton targeted by pathogens. Trends Microbiol. **13**: 181-189.

41 Guignot, J., M.F. Bernet-Camard, C. Pous, L. Plancon, C. Le Bouguenec, and A.L. Servin. (2001) Polarized entry of uropathogenic Afa/Dr diffusely adhering *Escherichia coli strain* IH11128 into human epithelial cells: evidence

for alpha5beta1 integrin recognition and subsequent internalization through a pathway involving caveolae and dynamic unstable microtubules. Infect Immun. **69**: 1856-1868.

42 Sierro, F., B. Dubois, A. Coste, D. Kaiserlian, J.P. Kraehenbuhl, and J.C. Sirard. (2001) Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells. Proc Natl Acad Sci U S A. **98**: 13722-13727.

43 Berin, M.C., A. Darfeuille-Michaud, L.J. Egan, Y. Miyamoto, and M.F. Kagnoff. (2002) Role of EHEC O157:H7 virulence factors in the activation of intestinal epithelial cell NF-kappaB and MAP kinase pathways and the upregulated expression of interleukin 8. Cell Microbiol. **4**: 635-648.

44 Smith, K.D., E. Andersen-Nissen, F. Hayashi, K. Strobe, M.A. Bergman, S.L. Barrett, B.T. Cookson, and A. Aderem. (2003) Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat Immunol. **4**: 1247-1253.

45 Wang, L., D. Rothemund, H. Curd, and P.R. Reeves. (2003) Species-wide variation in the *Escherichia coli* flagellin (H-antigen) gene. J Bacteriol. **185**: 2936-2943.

46 Law, D. (2000) Virulence factors of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. J Appl Microbiol. **88**: 729-745.

47 Adlerberth, I., L.A. Hanson, C. Svanborg, A.M. Svennerholm, S. Nordgren, and A.E. Wold. (1995) Adhesins of *Escherichia coli* associated with extra-intestinal pathogenicity confer binding to colonic epithelial cells. Microb Pathog. **18**: 373-385.

48 Nowrouzian, F.L., A.E. Wold, and I. Adlerberth. (2005) *Escherichia coli strain*s belonging to phylogenetic group B2 have superior capacity to persist in the intestinal microflora of infants. J Infect Dis. **191**: 1078-1083.

49 Herias, M.V., T. Midtvedt, L.A. Hanson, and A.E. Wold. (1995) Role of *Escherichia coli* P fimbriae in intestinal colonization in gnotobiotic rats. Infect Immun. **63**: 4781-4789.

50 Luck, S.N., V. Bennett-Wood, R. Poon, R.M. Robins-Browne, and E.L. Hartland. (2005) Invasion of epithelial cells by locus of enterocyte effacement-negative enterohemorrhagic *Escherichia coli*. Infect Immun. **73**: 3063-3071.

51 Adam, T., M. Giry, P. Boquet, and P. Sansonetti. (1996) Rho-dependent membrane folding causes *Shigella* entry into epithelial cells. Embo J. **15**: 3315-3321.

52 Chen, L.M., S. Hobbie, and J.E. Galan. (1996) Requirement of CDC42 for *Salmonella*-induced cytoskeletal and nuclear responses. Science. **274**: 2115-2118.

53 Dibb-Fuller, M.P., E. Allen-Vercoe, C.J. Thorns, and M.J. Woodward. (1999) Fimbriae- and flagella-mediated association with and invasion of cultured epithelial cells by *Salmonella enteritidis*. Microbiology. **145 (Pt 5)**: 1023-1031.

54 Sukupolvi, S., R.G. Lorenz, J.I. Gordon, Z. Bian, J.D. Pfeifer, S.J. Normark, and M. Rhen. (1997) Expression of thin aggregative fimbriae promotes interaction of *Salmonella* typhimurium SR-11 with mouse small intestinal epithelial cells. Infect Immun. **65**: 5320-5325.

55 Plancon, L., L. Du Merle, S. Le Friec, P. Gounon, M. Jouve, J. Guignot, A. Servin, and C. Le Bouguenec. (2003) Recognition of the cellular beta1-chain integrin by the bacterial AfaD invasin is implicated in the internalization of afa-expressing pathogenic *Escherichia coli strain*s. Cell Microbiol. **5**: 681-693.

56 Clark, E., C. Hoare, J. Tanianis-Hughes, G.L. Carlson, and G. Warhurst. (2005) Interferon gamma induces translocation of commensal *Escherichia coli* across gut epithelial cells via a lipid raft-mediated process. Gastroenterology. **128**: 1258-1267.

57 Nazli, A., P.C. Yang, J. Jury, K. Howe, J.L. Watson, J.D. Soderholm, P.M. Sherman, M.H. Perdue, and D.M. McKay. (2004) Epithelia under metabolic stress perceive commensal bacteria as a threat. Am J Pathol. **164**: 947-957.

58 Zareie, M., J. Riff, K. Donato, D.M. McKay, M.H. Perdue, J.D. Soderholm, M. Karmali, M.B. Cohen, J. Hawkins, and P.M. Sherman. (2005) Novel effects of the prototype translocating *Escherichia coli*, strain C25 on intestinal epithelial structure and barrier function. Cell Microbiol. **7**: 1782-1797.

59 Darfeuille-Michaud, A., C. Neut, N. Barnich, E. Lederman, P. Di Martino, P. Desreumaux, L. Gambiez, B. Joly, A. Cortot, and J.F. Colombel. (1998) Presence of adherent *Escherichia coli strain*s in ileal mucosa of patients with Crohn's disease. Gastroenterology. **115**: 1405-1413.

60 Darfeuille-Michaud, A., J. Boudeau, P. Bulois, C. Neut, A.L. Glasser, N. Barnich, M.A. Bringer, A. Swidsinski, L. Beaugerie, and J.F. Colombel. (2004) High prevalence of adherent-invasive *Escherichia coli* associated with ileal mucosa in Crohn's disease. Gastroenterology. **127**: 412-421.

61 Berkes, J., V.K. Viswanathan, S.D. Savkovic, and G. Hecht. (2003) Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation. Gut. **52**: 439-451.

62 Farthing, M.J. (2004) Bugs and the gut: an unstable marriage. Best Pract Res Clin Gastroenterol. **18**: 233-239.

63 Römling, U., M. Rohde, A. Olsen, S. Normark, and J. Reinköster. (2000) AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella* typhimurium regulates at least two independent pathways. Mol Microbiol. **36**: 10-23.

64 Zhou, X., J.A. Giron, A.G. Torres, J.A. Crawford, E. Negrete, S.N. Vogel, and J.B. Kaper. (2003) Flagellin of enteropathogenic *Escherichia coli* stimulates interleukin-8 production in T84 cells. Infect Immun. **71**: 2120-2129.

65 Rochon, M., and Römling, U. (2006) Flagellin bound to curli fimbriae elicits an immune response in the gastrointestinal epithelial cell line HT-29. Microbes Infection, accepted.

66 Tukel, C., M. Raffatellu, A.D. Humphries, R.P. Wilson, H.L. Andrews-Polymenis, T. Gull, J.F. Figueiredo, M.H. Wong, K.S. Michelsen, M. Akcelik, L.G. Adams, and A.J. Baumler. (2005) CsgA is a pathogen-associated molecular pattern of *Salmonella enterica* serotype Typhimurium that is recognized by Toll-like receptor 2. Mol Microbiol. **58**: 289-304.

67 Ramos, H.C., M. Rumbo, and J.C. Sirard. (2004) Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa. Trends Microbiol. **12**: 509-517.

68 McClelland, M., K.E. Sanderson, J. Spieth, S.W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S.

Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R.K. Wilson. (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature. **413**: 852-856.

Table 1. Strain constructs and genes studied

Strains, plasmids, Genes				Reference
Stain	Genotype	Morphotype	Relevant features	
TOB1	Wild type	$rdar_{28^oC}/rdar_{37^oC}$	cellulose$^+$; curli fimbriae$^+$; flagella$^+$	[21]
TOB2	TOB1 Δ*csgD*::Cm	saw_{28^oC}/saw_{37^oC}	cellulose$^-$; curli fimbriae$^-$; flagella$^+$	[21]
TOB2P	TOB1 Δ*csgD*::Cm; CsgD$^+$	$pdar_{28^oC}/pdar_{37^oC}$	cellulose$^+$; curli fimbriae$^-$; flagella$^+$	[21]
TOB3	TOB1 Δ*bcsA*::Cm	$bdar_{28^oC}/bdar_{37^oC}$	cellulose$^-$; curli fimbriae$^+$; flagella$^+$	[21]
TOB5	TOB1 Δ*fliC*::Cm	$rdar_{28^oC}/rdar_{37^oC}$	cellulose$^+$; curli fimbriae$^+$; flagella$^-$	This study
TOB6	TOB3 Δ*fliC*::Cm	$bdar_{28^oC}/bdar_{37^oC}$	cellulose$^-$; curli fimbriae$^+$; flagella$^-$	This study
Plasmid	Construct	Antibiotic resistance	Relevant features	
pUMR15	pBAD30::*csgD*	Amp	Arabinose dependent expression of *csgD*	(Römling et al., 2000)
pQE60	pQE60	Amp	high copy number expression vector	This study
pQE60F	pQE60::*fliC*	Amp	IPTG dependent expression of *fliC*	This study
Gene	Description			
bcsA	Catalytic subunit of the cellulose synthase			(Zogaj et al., 2001)
csgA	Curli fimbriae subunit gene A			(Römling et al., 1998b)
csgD	Transcriptional regulator of LuxR family, activates biosynthesis of cellulose and curli fimbriae			(Römling et al., 1998b)
fliC	Structural gene of flagellin			[68]

Figure legends:

Figure 1. Adherence of TOB1 and isogenic mutants to intestinal epithelial HT-29 cells: the mutant expressing curli fimbriae (TOB3) adhered significantly to HT-29 cells.

Figure 2. Interaction of TOB1 and its isogenic mutants with HT-29 cells. (A,E) TOB1 (cellulose+/curli+), (B,F) TOB2 (cellulose-/curli-), (C,G) TOB3 (cellulose-/curli+) and (D,H) TOB2p (cellulose+/curli-) were observed by fluorescence microscopy (A-D; Magnification 1000x) or scanning electron microscopy (E-H).

Figure 3. Internalization of TOB1 and isogenic mutants by HT-29 cells and the impact of host factors on the internalization. TOB3 (cellulose-/curli+) showed highest invasion rate (A). The actin cytoskeleton is required for internalization of *E. coli* TOB3. Preincubated with cytochalasin D (1 µg/ml) for 1 hour, HT-29 cells were infected with TOB1 and its isogenic mutants (B). The microfilament system is not required for internalization of *E. coli* TOB3. HT-29 cells were preincubated for 1 hour with colchicine (1 µg/ml), subsequently infected with TOB1 and its isogenic mutants (C). The fibronectin-like peptide RGDS impairs internalization of HT-29 cells by TOB1 and TOB3. HT-29 cells were preincubated for 1 hour with synthetic peptide RGDS (dark bars) or RGES (light bars), which served as a negative control, at a final concentration of 60 µg/ml, and then infected with TOB1 and its isogenic mutant TOB3 (D). B, C and D: Percent internalization relative to control without agents.

Figure 4. Investigation of internalization in a panel of commensal *E. coli* isolates. Morphotype are indicated: saw, indicates no expression of cellulose and curli fimbriae; bas, indicates weak expression of curli fimbriae; bdar, indicates strong expression of curli fimbriae; rdar, indicates expression of both matrix components, pdar, indicates cellulose expression; m, mucoid. The bacterial isolates were co-incubated with HT-29 cell for 3 hours. n.d.=not determined

Figure 5. IL-8 release of HT-29 cells during 3 hours infection with TOB1 and its isogenic mutants. *S.* Typhimurium UMR1 (ATCC14028 Nal^r) grown under conditions that promote invasion served as a positive control (A). Effects of flagellin expression on IL-8 induction from HT-29 cells by *E. coli* TOB1 and isogenic mutant TOB3 (B). TOB5, the *fliC* knockout of TOB1, stimulated IL-8 release in the range of the wild type strain after 3 hours of infection. In contrast, TOB6, the *fliC* knockout of TOB3, led to a dramatically decreased IL-8 release after 3h of infection compared to wild type TOB3. Complementation of *fliC* mutants with flagellin from *S.* Typhimurium restored or enhanced IL-8 production of HT-29 cells. u=unstimulated control; VC=vector control pQE60; $FliC^+$=pQE60F (B).

Figure 6. Investigation of IL-8 induction of HT-29 cells in a panel of commensal *E. coli* isolates. Morphotype and flagellin expression are indicated. For morphotype: saw, indicates no expression of cellulose and curli fimbriae; bas, indicates weak expression of curli fimbriae; bdar, indicates strong expression of curli fimbriae; rdar, indicates expression of both matrix components, pdar, indicates cellulose expression; m, mucoid. The bacterial isolates were co-incubated with HT-29 cell for 3 hours.

Figure 7. IL-8 induction of HT-29 cells by purified flagellin monomers from TOB1 and FEC10. Flagellin from FEC10 had a higher immunostimulatory capacity than flagellin isolated from TOB1.

Figure 8. Alignment of FliC from *E. coli* TOB3 and FEC10 with FliC from *S.* Typhimurium. The immunostimulatory regions at the N- and C-terminus of the protein [44] are shown in bold. Underlined are the amino acids investigated by alanine scanning for TLR5 stimulated IL-8 production. Dark grey background, significant reduction of TLR5 stimulated IL-8 production by 76-97%; light grey background, reduction by 50-75%; no background colour, no effect.

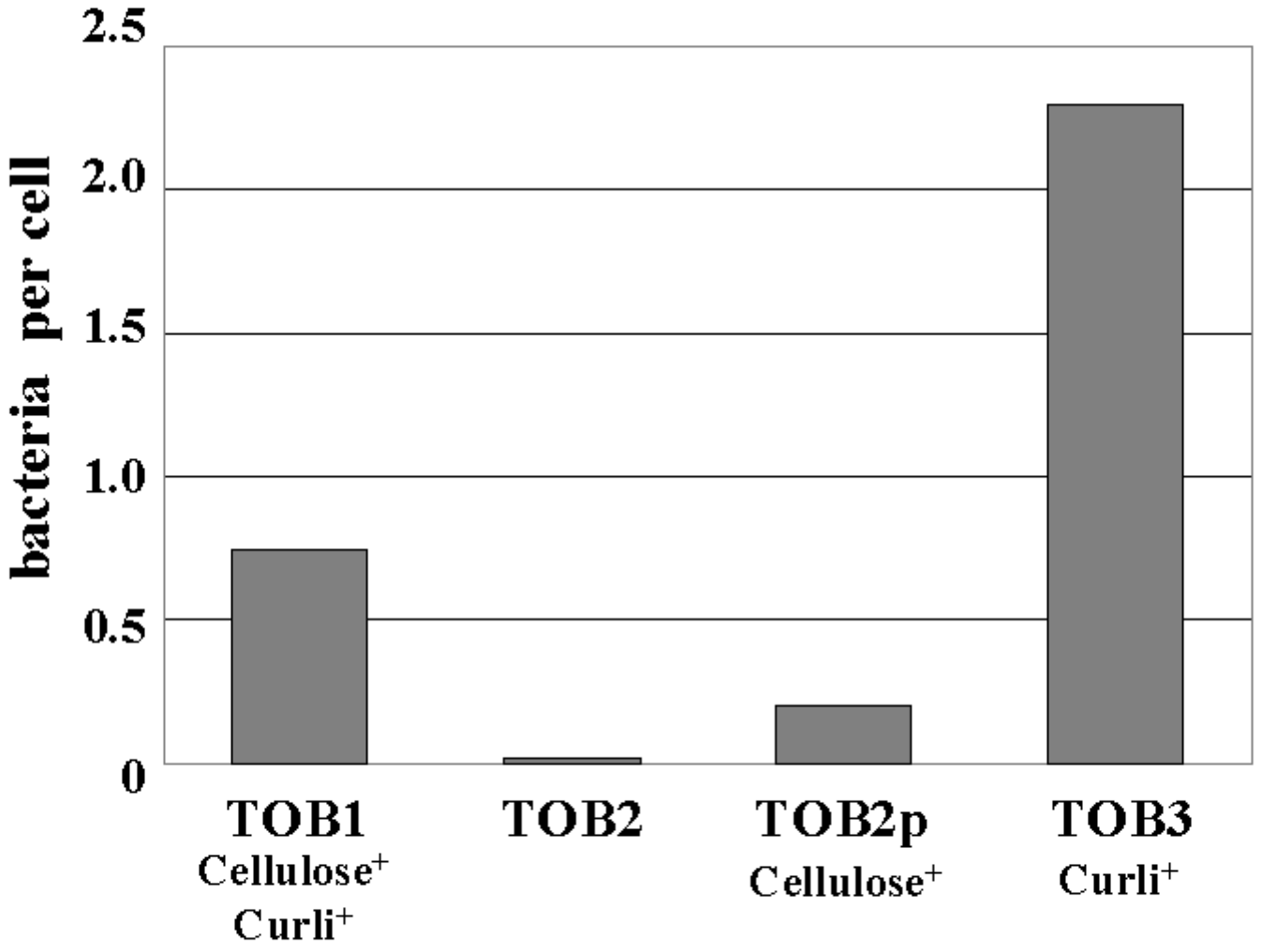
bacteria per cell
2.5
2.0
1.5
1.0
0.5
0
TOB1
Cellulose+
Curli+
TOB2
TOB2p
Cellulose+
TOB3
Curli+

Figure 1

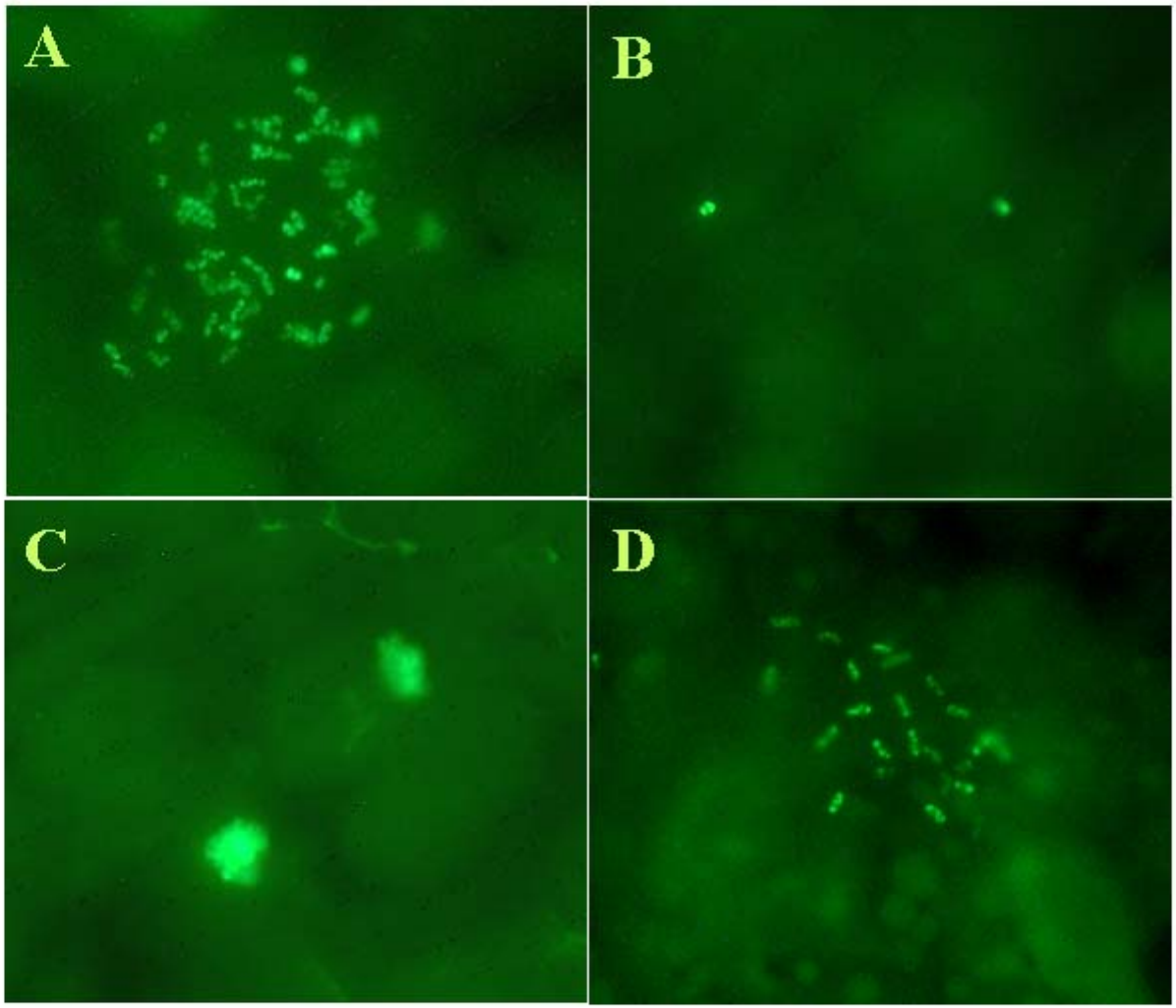

Figure 2

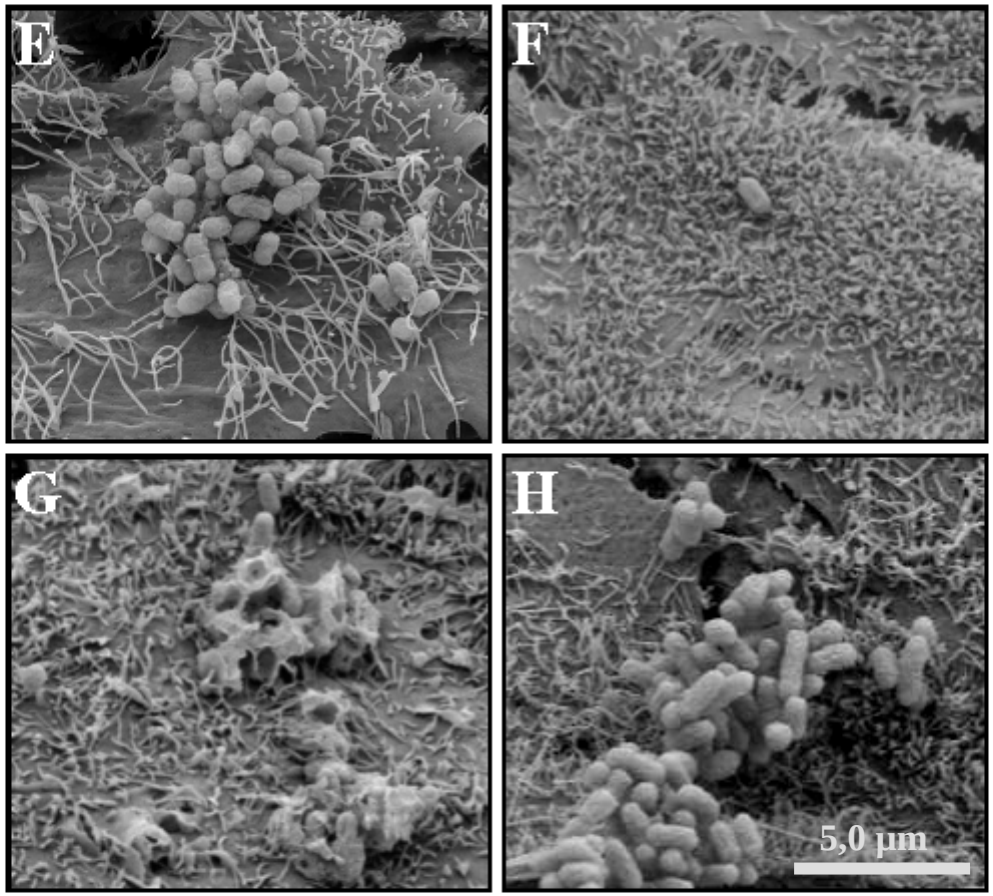

Figure 2

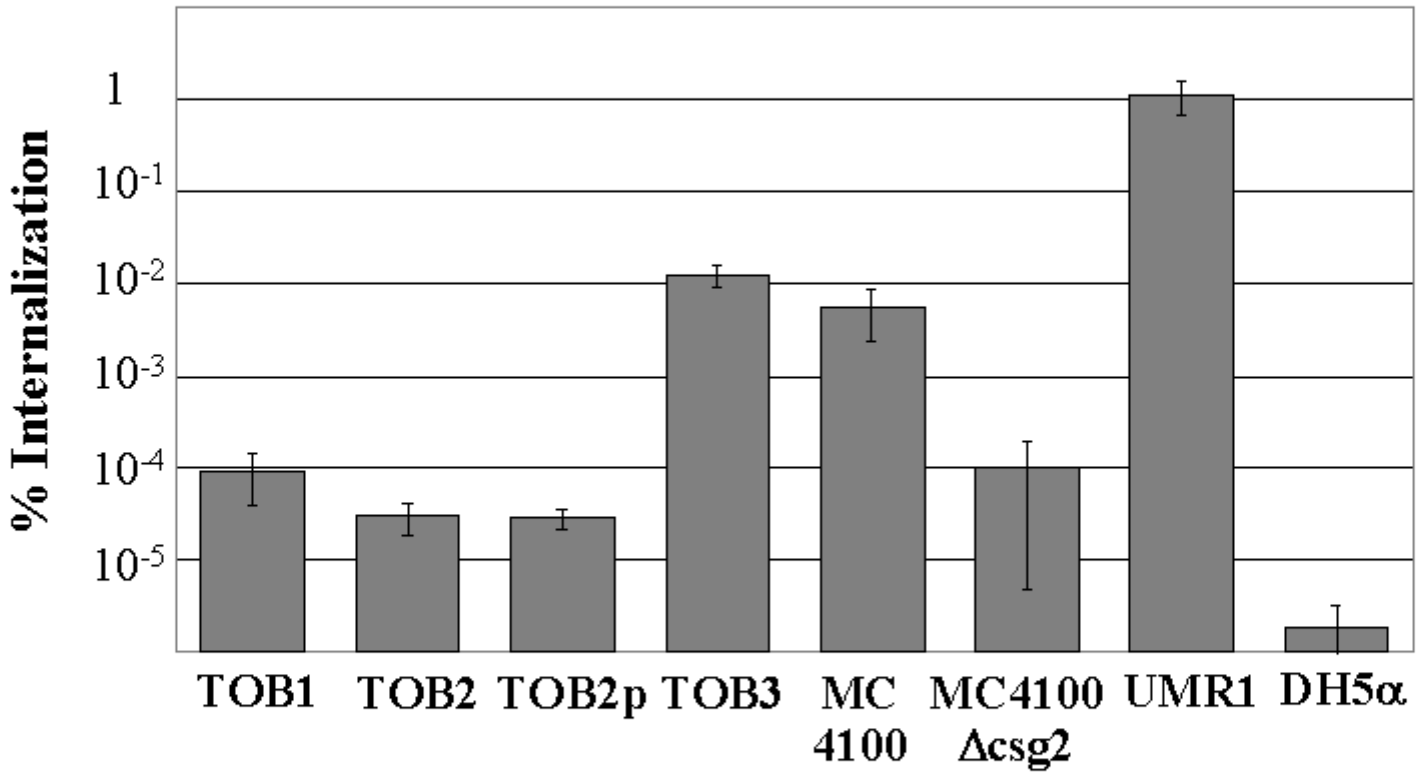
% Internalization
1
10-1
10-2
10-3
10-4
10-5
TOB1
TOB2
TOB2p
TOB3
MC 4100
MC4100 Δcsg2
UMR1
DH5α

Figure 3A

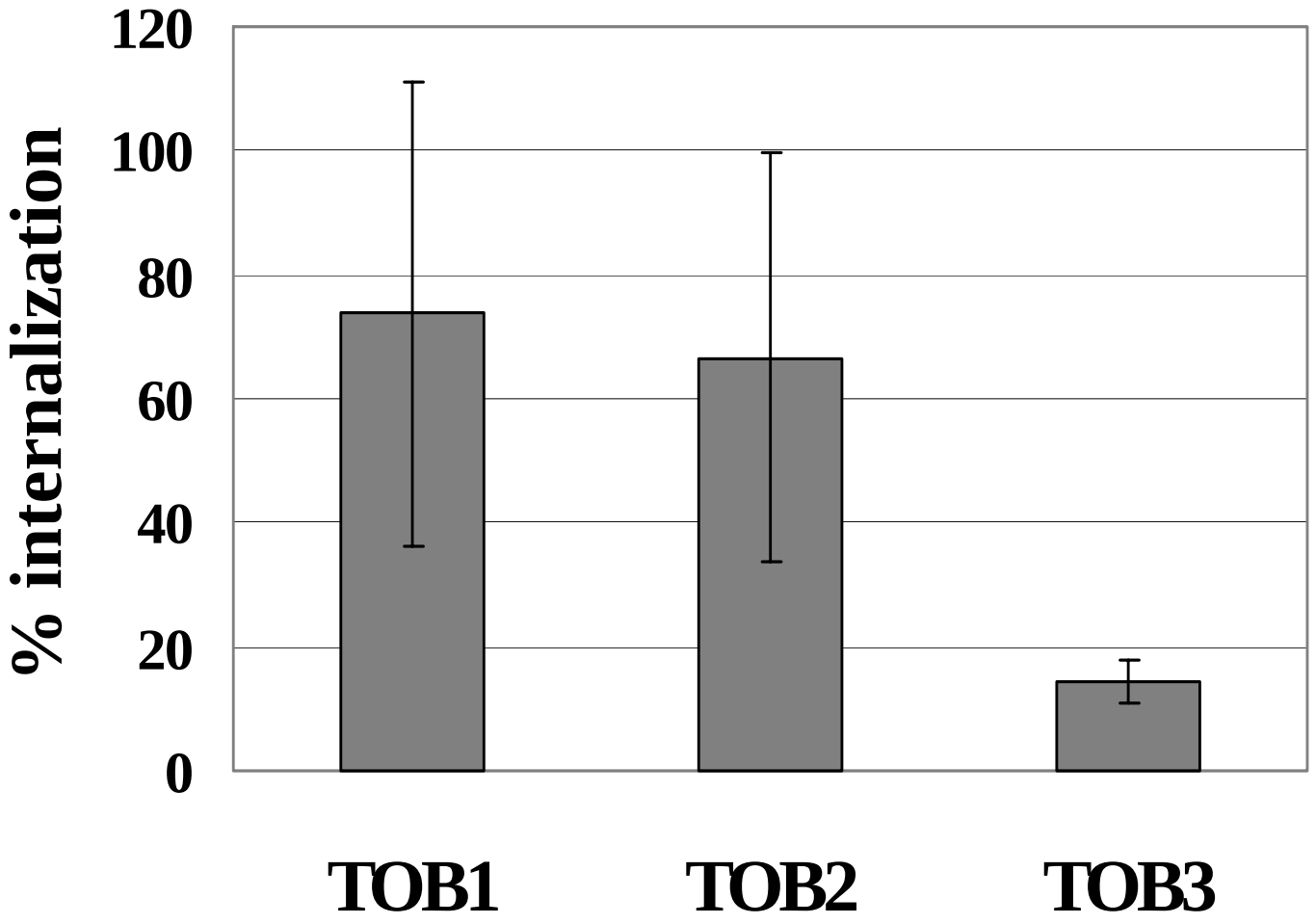

Figure 3B

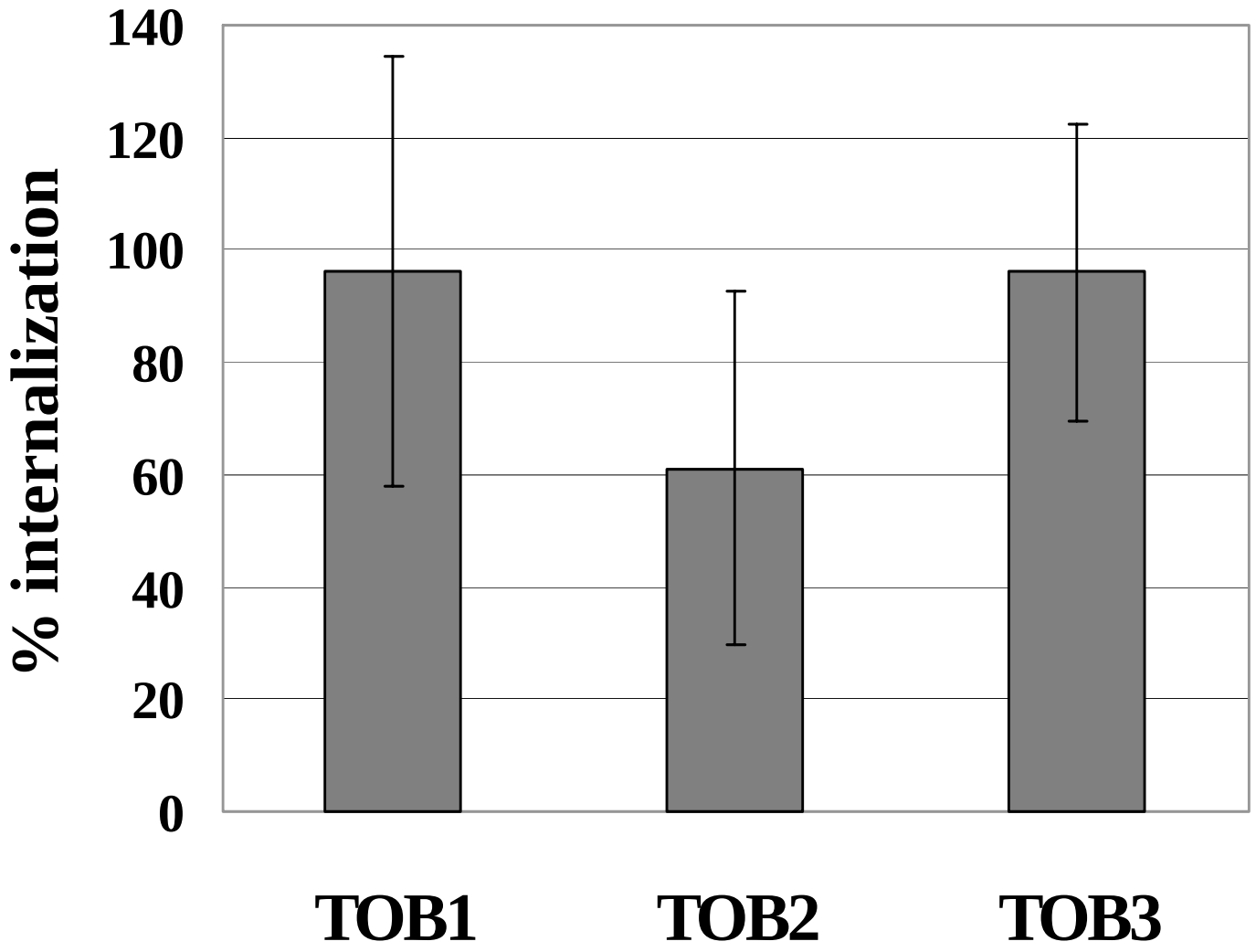

Figure 3C

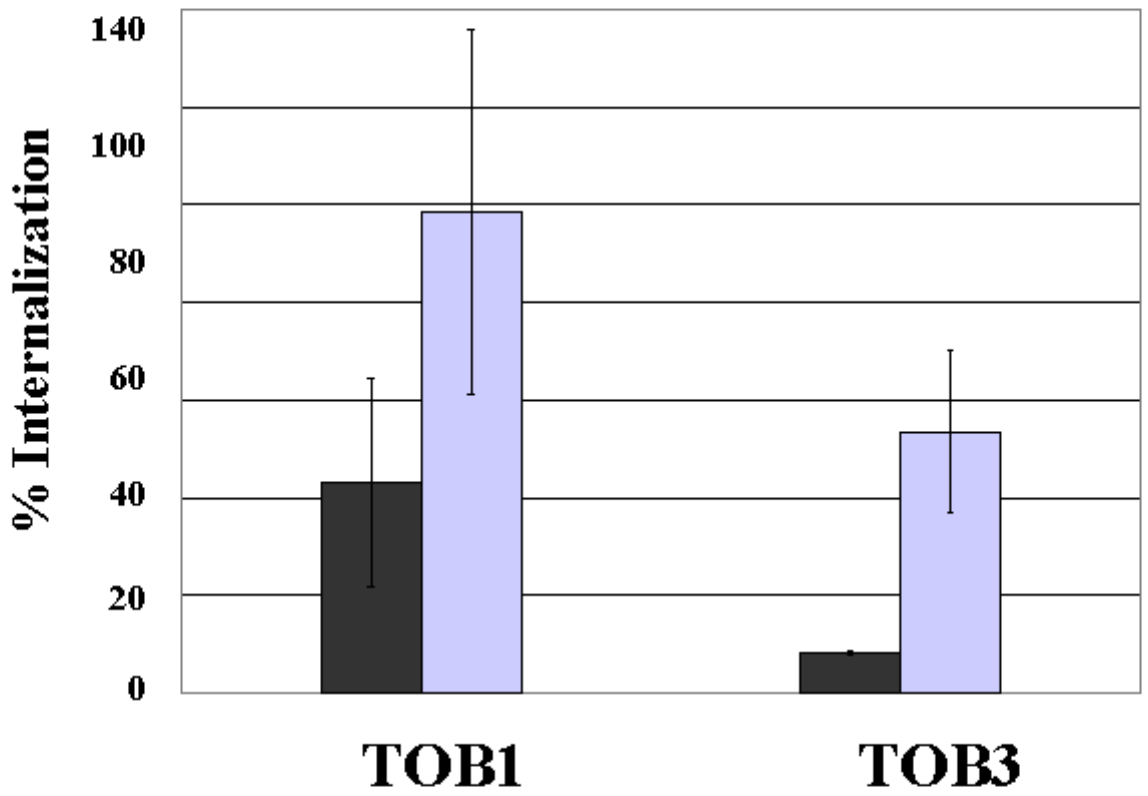

Figure 3D

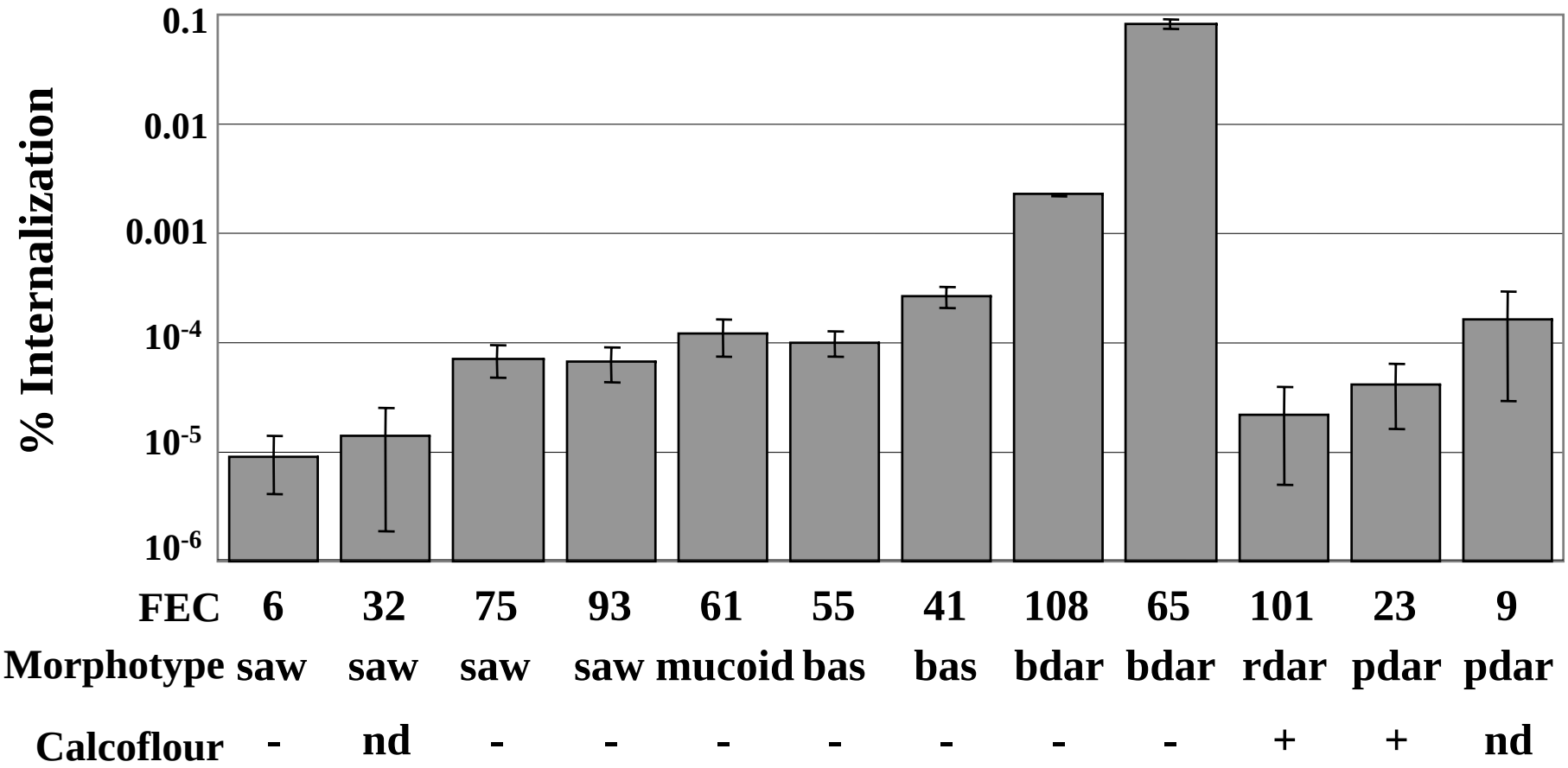
% Internalization
0.1
0.01
0.001
10-4
10-5
10-6
FEC 6 32 75 93 61 55 41 108 65 101 23 9
Morphotype saw saw saw saw mucoid bas bas bdar bdar rdar pdar pdar
Calcoflour - nd - - - - - - - + + nd

Figure 4

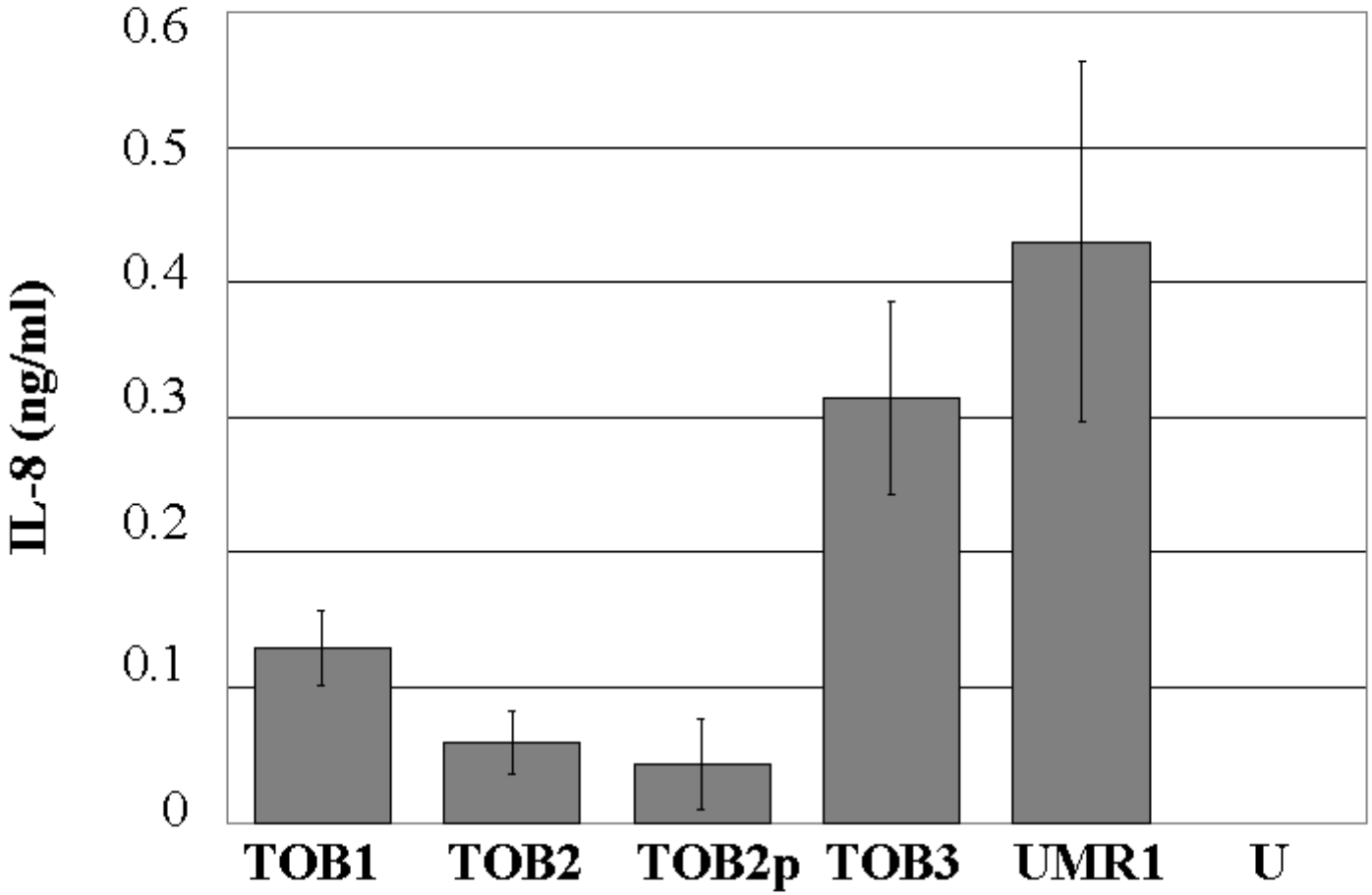

Figure 5A

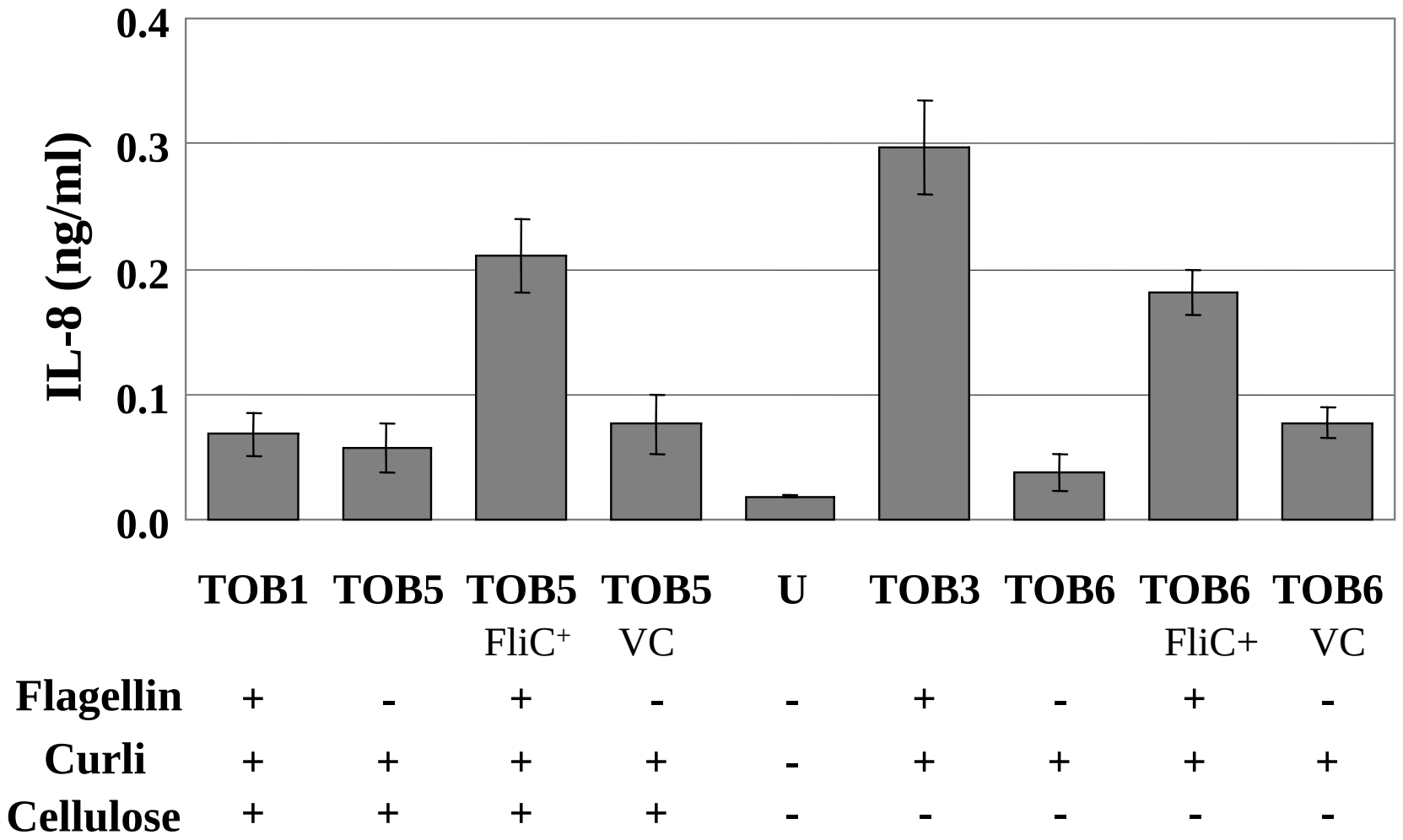
IL-8 (ng/ml)
0.4
0.3
0.2
0.1
0.0
TOB1
TOB5
TOB5 FliC+
TOB5 VC
U
TOB3
TOB6
TOB6 FliC+
TOB6 VC
Flagellin + - + - - + - + -
Curli + + + + - + + + +
Cellulose + + + + - - - - -

Figure 5B

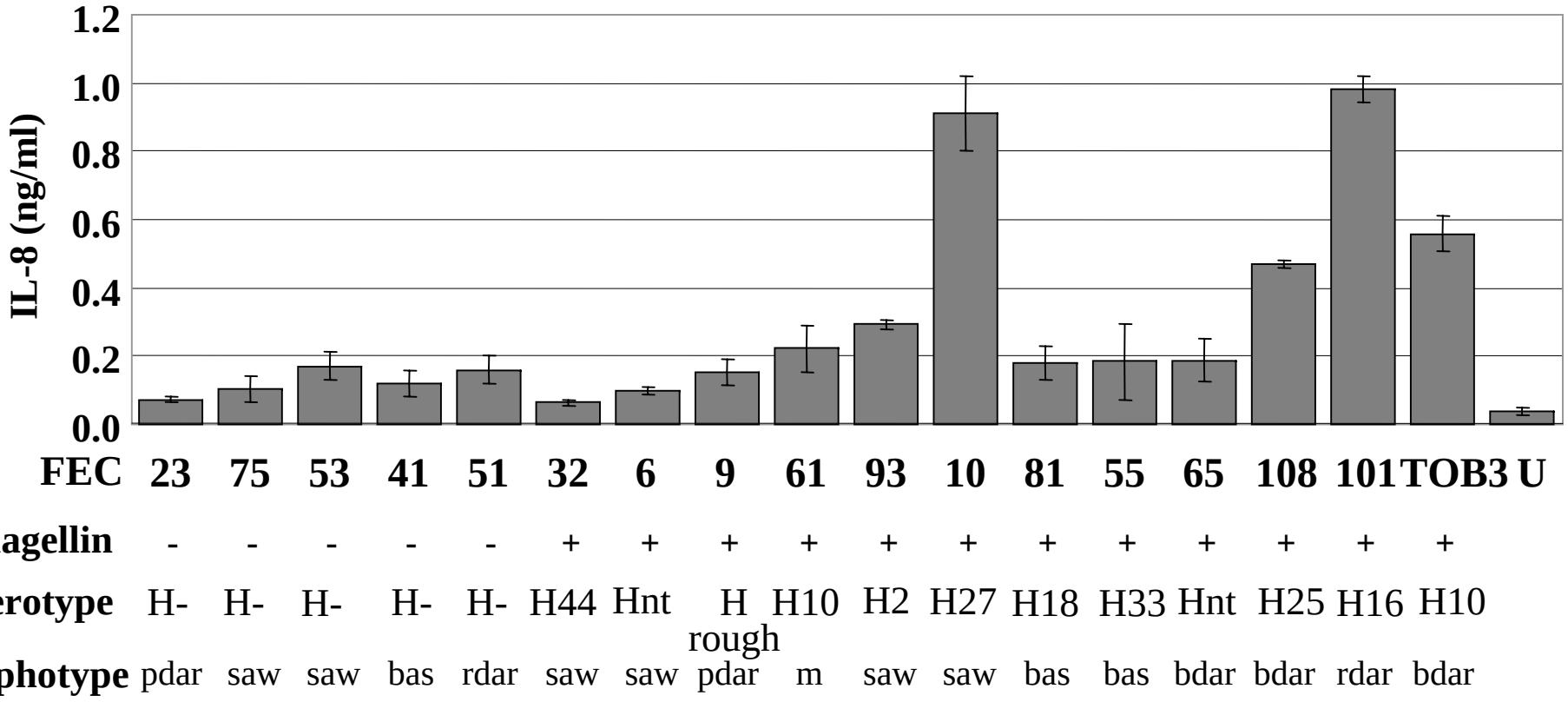
IL-8 (ng/ml)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
FEC 23 75 53 41 51 32 6 9 61 93 10 81 55 65 108 101 TOB3 U
Flagellin - - - - - + + + + + + + + + + + +
Serotype H- H- H- H- H- H44 Hnt H rough H10 H2 H27 H18 H33 Hnt H25 H16 H10
Morphotype pdar saw saw bas rdar saw saw pdar m saw saw bas bas bdar bdar rdar bdar

Figure 6

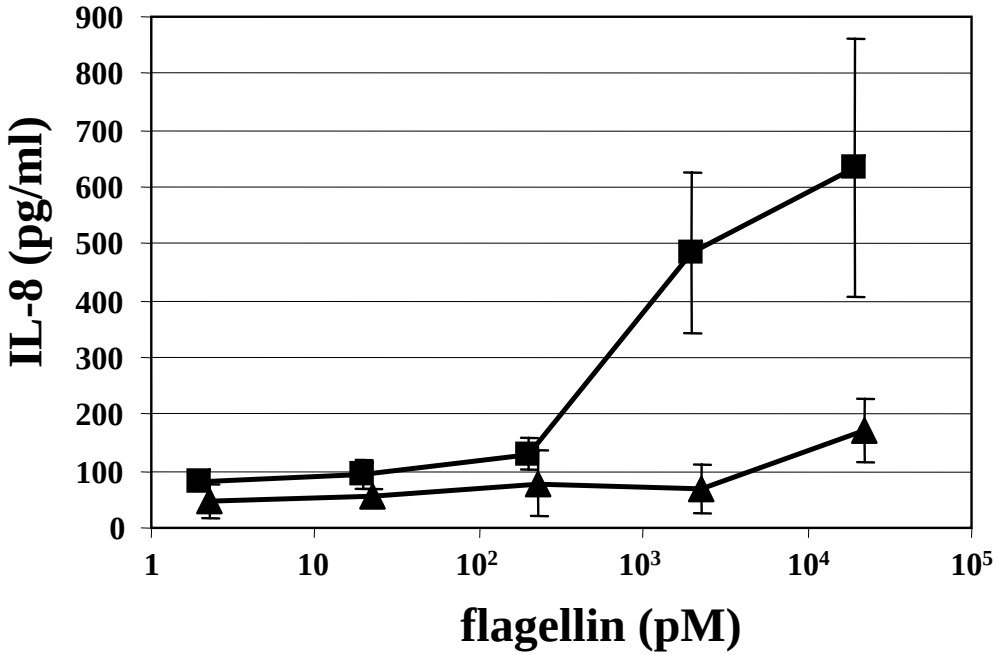
900
800
700
600
500
400
300
200
100
0
IL-8 (pg/ml)
1
10
10^2
10^3
10^4
10^5
flagellin (pM)

Figure 7

```
FLIC_SALTY          MAQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKG 60
FLIC_ECOLI_TOB3     MAQVINTNSLSLITQNNINKNQSALSSSIERLSSGLRINSAKDDAAGQAIANRFTSNIKG 60
FLIC_ECOLI_FEC10    MAQVINTNSLSLITQNNINKNQSALSSSIERLSSGLRINSAKDDAAGQAIANRFTANIKG 60
                    ************:****:**.****.::****************************:****

FLIC_SALTY          LTQASRNANDGISIAQTTEGALNEINNNLQRVRELAVQSANSTNSQSDLDSIQAEITQRL 120
FLIC_ECOLI_TOB3     LTQAARNANDGISVAQTTEGALSEINNNLQRVRELTVQATTGTNSESDLSSIQDEIKSRL 120
FLIC_ECOLI_FEC10    LTQASRNANDGISVAQTTEGALNEINNNLQRVRELTVQATNGTNSDSDLSSIQAEITQRL 120
                    ****:********:********.************:**::..***:***.*** **..**

FLIC_SALTY          NEIDRVSGQTQFNGVKVLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDTLNVQ--QK 178
FLIC_ECOLI_TOB3     EEIDRVSSQTQFNGVNVLAKDGKMNIQVGANDGQTITIDLKKIDSSTLNLSSFDAT--NL 178
FLIC_ECOLI_FEC10    EEIDRVSEQTQFNGVKVLAENNEMKIQVGANDGETITINLAKIDAKTLGLDGFNIDGAQK 180
                    :****** *******:***::. :.********:** *:* :*::.**.*. ::    :

FLIC_SALTY          YKVSDTAATVTGYADTTIALDNSTFKASATGLGGTDQKIDGDLKFDDTTGKYYAKVTVTG 238
FLIC_ECOLI_TOB3     GTSVKDGATIN----KQVAVGAGDFKDKASGSLGT-------LKLVEKDGKYYVNDTTN- 226
FLIC_ECOLI_FEC10    ATGSDLISKFKATGTDNYQINGTDNYTVNVDSGAVQNEDGDAIFVSATDGSLTTKSDTKV 240
                     .  .  :...        :.         .  ..       : .  . *.  .:  ..

FLIC_SALTY          G---TGKDGYYEVSVDKTNGE------VTLAGGATSPLTGGLPATATEDVKNVQVANADL 289
FLIC_ECOLI_TOB3     -------SKYYDAEVDTSKGE------INFN----STNESGTPPTAATEVTTVGRD---- 265
FLIC_ECOLI_FEC10    GGTGIDATGLAKAAVSLAKDASIKYQGITFTNKGTGAFDGSSNGTLIANIDGKDVT---- 296
                              .. *. ::.       :.:     ..  ..   *   ::

FLIC_SALTY          TEAKAALTAAGVTGTASVVKMSYTDNNGKTIDGGLAVKVGDDYYSATQNKDGSISINTTK 349
FLIC_ECOLI_TOB3     ----VKLDASALKANQSLV--VYKD------------KSGNDAY-IIQTKD--VTTNQST 304
FLIC_ECOLI_FEC10    ----FTIDATGKDATLKTSDPVYKN------------SAGQFTTTKVENKA-ATASDLDL 339
                         : *:.  .. .     *.:            . *:      :.*    : :

FLIC_SALTY          YTADDGTSKTALNKLGGADGKTEVVSIG-GKTYAASKAEGHNFKAQPDLAEAAATTTENP 408
FLIC_ECOLI_TOB3     FNAAD------ISDAG-------VLSIG-ASTTAPS-----------------NLTADP 332
FLIC_ECOLI_FEC10    NNAKKVGSSLVVNGADYEVSADGKTVTGLGKTMYLSKSEGGSPILVKEDAAKSLQSTTNP 399
                     .* .      :.  .           * ..*   *                    * :*

FLIC_SALTY          LQKIDAALAQVDTLRSDLGAVQNRFNSAITNLGNTVNNLTSARSRIEDSDYATEVSNMSR 468
FLIC_ECOLI_TOB3     LKALDDAIASVDKFRSSLGAVQNRLDSAIANLNNTTTNLSEAQSRIQDADYATEVSNMSK 392
FLIC_ECOLI_FEC10    LETIDKALAKVDNLRSDLGAVQNRFDSAITNLGNTVNNLSSARSRIEDADYATEVSNMSR 459
                    *: :* *:*.**.:**.*******::***:**.**..**:.*:***:*:**********:

FLIC_SALTY          AQILQQAGTSVLAQANQVPQNVLSLLR- 495
FLIC_ECOLI_TOB3     AQIIQQAGNSVLAKANQVPQQVLSLLQG 420
FLIC_ECOLI_FEC10    AQILQQAGTSVLAQANQTTQNVLSLLR- 486
                     ***:****.****:***..*:*****:
```

Figure 8