DR. MARY O'CONNELL MOTHERWAY (Orcid ID : 0000-0001-9164-9320)

Article type : Research Article

A Bifidobacterial pilus-associated protein promotes colonic epithelial proliferation

Mary O'Connell Motherway[a,b,#] , Aileen Houston[a,c,], Grace O'Callaghan[a,c,], Justus Reunanen[d*], Frances O'Brien[a], Tara O'Driscoll[a], Patrick G. Casey[a], Willem M. de Vos[d,e], Douwe van Sinderen[a,b,] and Fergus Shanahan[a,c,#]

[a]APC Microbiome Ireland, National University of Ireland, Cork, Western Road, Cork, Ireland.
[b]School of Microbiology, National University of Ireland, Cork, Western Road, Cork, Ireland.
[c]Department of Medicine, National University of Ireland, Cork, Cork University Hospital, Cork, Ireland.
[d]Research Programme Unit Immunobiology, Department of Bacteriology and Immunology, Helsinki University, Helsinki, Finland.
[e]Laboratory of Microbiology, Wageningen University, The Netherlands.

Running Head: Bifidobacterial proliferation factor

Address correspondence to Mary O'Connell Motherway, m.oconnellmotherway@ucc.ie or Fergus Shanahan, f.shanahan@ucc.ie

*JR Current Affiliation: Cancer and Translational Medicine Research Unit and Biocenter Oulu, University of Oulu, Oulu, Finland.

MOCM and AH contributed equally to this work.
DvS and FS contributed equally.

Keywords: pili, gut epithelium, growth factor, fimbriae, gut colonization, bifidobacteria

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/mmi.14155
This article is protected by copyright. All rights reserved.
Accepted Article

Accepted Article

Abstract

Development of the human gut microbiota commences at birth, with certain bifidobacterial species representing dominant and early colonizers of the newborn gastrointestinal tract. The molecular basis of *Bifidobacterium* colonization, persistence and presumed communication with the host has remained obscure. We previously identified tight adherence (Tad) pili from *Bifidobacterium breve* UCC2003 as an essential colonization factor. Here, we demonstrate that bifidobacterial Tad pili also promote *in vivo* colonic epithelial proliferation. A significant increase in cell proliferation was detectable 5 days post administration of *B. breve* UCC2003. Using advanced functional genomic approaches, bacterial strains either (a) producing the Tad_{2003} pili or (b) lacking the TadE or TadF pseudopilins were created. Analysis of the ability of these mutant strains to promote epithelial cell proliferation *in vivo* demonstrated that the pilin subunit, TadE, is the bifidobacterial molecule responsible for this proliferation response. These findings were confirmed *in vitro* using purified TadE protein. Our data imply that bifidobacterial Tad pili may contribute to the maturation of the naïve gut in early life through the production of a specific scaffold of extracellular protein structures, which stimulate growth of the neonatal mucosa.

Importance

Bifidobacteria are among the first colonizers of the newborn gastrointestinal tract where they remain dominant until weaning. Despite general appreciation of the positive impact on host health, the precise molecular mechanisms underlying the health benefits of a microbiome rich in bifidobacteria in early life remain poorly understood. Previously we identified bifidobacterial Tight adherence (Tad) pili as an essential and conserved colonization factor. Here we show that bifidobacterial Tad pili are also involved in host signalling, leading to *in vivo* colonic epithelial proliferation. Furthermore, we have deduced that a pseudopilin subunit, TadE, is responsible for the pilin-mediated proliferation response. These findings could have several therapeutic implications, particularly in relation to the management of preterm babies with sub-optimal mucosal maturation.

This article is protected by copyright. All rights reserved.

Accepted Article

Introduction

Colonisation of the neonatal gut has important trophic, metabolic and protective functions. Although the intestinal epithelium exhibits self-renewal kinetics from stem cells in the mucosa (1), the microbiota is an important environmental driver of the renewal of the intestinal epithelium, influencing mucosal morphology and homeostasis (2). Epithelial cell turnover in the colonised murine gut is about twice that of germ-free controls (3). While some of the innate sensory pathways involved in bi-directional communication between the microbiota and the epithelium are known (4, 5), the full molecular repertoire of signals from the microbiota to the epithelium is incompletely understood.

Signalling with the host may be a property common to many constituents of the microbiota, but we have focussed on bifidobacteria for the following reasons. First, bifidobacteria are among the earliest colonisers of the neonatal gut and remain dominant until weaning. Second, comparative studies have shown that colonisation during early life when development and maturation is underway represents a critical window of opportunity where the microbiota can imprint host mucosal immunity and homeostasis (6). Thus, colonisation in later life does not confer the same developmental benefit as colonisation in the neonate (6). Third, increasing evidence suggests that the early life microbiota is a critical determinant of the risk of immune and metabolic diseases in later life (7, 8). Fourth, work from several research groups has demonstrated a diversity of bifidobacterial-derived protein and carbohydrate structures which elicit distinct host immune responses in the gut (9-14).

Previously we showed that bifidobacterial tight adherence pili (Tad pili) are required for *in vivo* colonization (15). The Tad pilus fiber consists of multiple copies of one or more different pilin proteins, where the Flp pilin forms the pilin shaft which is decorated with pseudopilins, TadE and TadF. We now report that Tad pili also participate in microbe-host communication, specifically by delivering a proliferative signal to the host epithelium. We show Tad pilus-mediated epithelial proliferation is mediated by the TadE pseudopilin. Conservation of the *tad* gene cluster in all *B. breve* strains and all currently available *Bifidobacterium* genomes supports the concept of ubiquitous pili-

This article is protected by copyright. All rights reserved.

mediated host-microbe signalling, and represents a step towards understanding the molecular detail of microbe-host dialogue in early life.

Accepted Article

Results

B. breve UCC2003 Tad pili promote colonic epithelial proliferation.

We previously established that *B. breve* UCC2003 Tad (designated here as Tad_{2003}) pili represent a colonisation factor in conventional animals that is specifically produced under *in vivo* conditions. We also determined using osmium tetroxide staining of intestinal sections, that *B. breve* UCC2003 is present in close proximity to the intestinal epithelia (Fig. 1). The Tad_{2003} gene cluster comprises genes encoding the pilin proteins, and the mechanisms required for the processing and assembly of the pilus structure (Fig. 2A). In order to establish if Tad_{2003} pili have an effect on the host, 1 x 10^9 cells of *B. breve* UCC2003PK1 or a pilus-negative *B. breve* strain, UCC2003-tadAPK1 (15), were administered by oral gavage to eight week old germ-free (GF) Swiss Webster mice (n=7) for five days. Fecal samples were collected twice weekly for the ensuing three weeks to quantify bacterial shedding in the feces. Each strain monoassociated the GF mice to a similar level of 10^9 cfu $gram^{-1}$ of feces (Fig. 2B). Animals were also weighed weekly, with no difference in weight change seen between the groups. At the end of the trial period samples of intestinal tissue were retained for immunohistochemistry analysis. As proliferating cells express Ki67 in their nuclei which can be detected by immunohistochemistry, sections were immunostained for Ki67 (Fig. 2C), and the percentage of Ki67 positive cells per crypt enumerated (Fig. 2D). Ki67 immunohistochemistry demonstrated that in the presence of the Tad_{2003} pili (Fig. 2C i), proliferation of the epithelial cells was similar to that seen in specific pathogen free (SPF) animals (Fig. 2C iv) (Fig. 2D). Moreover, mice monoassociated with *B. breve* UCC2003PK1 (Fig. 2C i) showed increased proliferation relative to the epithelia of mice monoassociated with *B. breve* UCC2003-tadAPK1 (Fig. 2C ii) (Fig. 2D). Indeed the level of proliferation observed in mice monoassociated with *B. breve* lacking the Tad_{2003} pili, *B. breve* UCC2003-tadAPK1, was similar to that seen in the epithelia of the GF control mice (Fig. 2C iii) (Fig.

This article is protected by copyright. All rights reserved.

2D). These data indicate that Tad_{2003} pili are involved in host signalling and contribute to gut epithelia proliferation and development.

Tad_{2003} mediated epithelial proliferation is detectable 5 days post administration of *B. breve* UCC2003.

In order to establish when Tad_{2003} pili initiate colonic epithelial proliferation, a longitudinal study was performed. Groups of 16 female, 8 week old, GF Swiss Webster mice were administered PBS (carrier), a single dose of 1 x 10^9 cells of *B. breve* UCC2003PK1, or the Tad pili-negative strain *B. breve* UCC2003-tadAPK1. One group of 16 mice was administered homogenised murine feces (20 µl of a 20 mg ml^{-1} suspension of feces from conventional mice) by oral gavage and this group of mice was housed in the conventional animal rooms. Four mice from each group were sacrificed 2, 5, 9 or 15 days after the initial dosing (designated day 1), and samples of the colon retained for immunohistochemistry analysis (Figure 3A; FigS1). Quantification of the level of proliferation at the different time points demonstrated that Tad_{2003} pili-induced colonic epithelial proliferation commenced within 24h of administration of *B. breve* UCC2003PK1 (Fig. 3B), with full proliferation comparable to that found in the conventionalised animals observed within 5 days (Fig 3C). Although significance was lost at 9 days, the level of proliferation seen in the colon of mice monoassociated with *B. breve* UCC2003PK1 was greater than that of the GF controls (Fig. 3D; FigS1). The level of colonic epithelial proliferation seen in animals administered the Tad_{2003} pilus-negative mutant strain, *B. breve* UCC2003-tadAPK1, was lower at all time points relative to mice colonised with the Tad_{2003}-producing strain (Fig. 3B-E), with significance seen at 15 days (Fig. 3A and E). Indeed, the level of proliferation seen in mice administered the strain lacking Tad_{2003} pili was similar to that of the germ-free control animals (Fig. 3B – E; FigS1).

This article is protected by copyright. All rights reserved.

Accepted Article

Lactococcus lactis expressing Tad$_{2003}$ pili promotes epithelial proliferation under *in vitro* conditions

We next sought to confirm that the Tad$_{2003}$ pili was responsible for the colonic epithelial proliferation observed *in vivo*. However, we previously determined that *B. breve* UCC2003 does not produce pili under *in vitro* conditions (15). Thus, the *tad*$_{2003}$ pili cluster was cloned and expressed in *Lactococcus lactis* under the control of nisin-inducible promoters (designated *L. lactis* NZ9000 pNZ-tadZ-spk-pPTPI-tadV) (16). A corresponding control strain, *L. lactis* NZ9000 pNZ8150-pPTPI, harboring empty plasmids was also created. Pili production following nisin induction by *L. lactis* NZ9000 pNZ-tadZ-spk-pPTPI-tadV was confirmed using immunogold electron microscopy (Fig. 4A). Both the *L. lactis* producing and control strains were nisin induced prior to incubation *in vitro* with three different colonic epithelial cell lines, namely HT29, HCT116 or SW480. Cell proliferation was measured by resazurin assay. Proliferating cells reduce resazurin to resorufin, which can be quantified fluorometrically. Cells incubated with *L. lactis* NZ9000 pNZ-tadZ-spk-pPTPI-tadV showed significantly increased resazurin reduction relative to cells incubated with the control strain (Fig. 4B). This analysis demonstrated that Tad$_{2003}$ pili significantly increase proliferation of colonic epithelial cell lines *in vitro*, thereby demonstrating that the proliferation phenotype observed *in vivo* is likely attributable to the Tad$_{2003}$ pili.

The *B. breve* UCC2003 TadE pseudopilin confers Tad$_{2003}$ epithelial proliferation under *in vivo* conditions

We next sought to investigate which component of the Tad$_{2003}$ pilus was responsible for the promotion of colonic cell proliferation. To achieve this, *B. breve* UCC2003 isogenic mutant strains lacking the pseudopilins, TadE (UCC2003ΔtadE) or TadF (UCC2003ΔtadF), were constructed. 1 x 10^9 cells of *B. breve* UCC2003, *B. breve* UCC2003ΔtadE or UCC2003ΔtadF were administered by oral inoculation to eight week old germ-free C57 black mice (n=7) for three days. Fecal samples were collected twice weekly for the following four weeks and bacterial numbers were enumerated. Findings demonstrated

This article is protected by copyright. All rights reserved.

Accepted Article

that each strain colonised the monoassociated murine gut at equivalent levels of 10^9 cfu g^{-1} feces. At day 30, the mice were sacrificed and samples of the intestine retained for immunohistochemistry. Cell proliferation was detected by immunohistochemical staining for both Ki67 (Fig. 5A) and proliferating cell nuclear antigen (PCNA) (Supplementary Fig. S2A), as both of these proteins are only detectable in the nuclei of proliferating cells. Quantification of cell proliferation demonstrated that mice monoassociated with *B. breve* UCC2003 or *B. breve* UCC2003 lacking TadF (UCC2003ΔtadF) showed similar levels of proliferation as seen in the conventional C57 black mouse controls. (Fig. 5B; Supplementary Fig. S2B). Intriguingly, the level of proliferation in the colonic epithelia of mice that had been monoassociated with *B. breve* UCC2003ΔtadE was similar to that of germ-free control animals (Fig. 5B; Supplementary Fig. S2B).

We next verified that the reduced level of proliferation seen in mice monoassociated with *B. breve* UCC2003ΔtadE was not due to the absence of pili production by this strain under *in vivo* conditions. Bacteria were recovered from the caecum of monoassociated animals, and the presence of Tad_{2003} pili containing either TadE and/or TadF detected by immunogold electron microscopy (Fig. 6A). Pili containing both TadE and TadF were recovered from mice monoassociated with *B. breve* UCC2003 (Fig. 6A). However, only pili containing TadF could be recovered from mice monoassociated with *B. breve* UCC2003ΔtadE (Fig. 6B).

Finally, we confirmed the role of TadE in cell proliferation *in vivo* by reintroduction of the *tad*E gene *in trans* on plasmid pBC1.2, under the transcriptional control of the P44 promoter in the UCC2003ΔtadE mutant strain. GF mice were monoassociated with *B. breve* UCC2003, *B. breve* UCC2003 lacking TadE (UCC2003ΔtadE) or the complementation strain, designated UCC2003ΔtadE-pBC1.2-tadE. Intestinal tissue from these mice were immunostained for Ki67 (Fig. 7A) or PCNA (supplemental Fig. S2A), and the level of proliferation enumerated (Fig. 7B and supplemental Fig. S2B). The proliferative phenotype was restored in mice monoassociated with the complementation strain (Fig.7; supplemental Fig. S2). We also confirmed by immunogold electron microscopy that the complementation strain produced pili containing both TadE and TadF

This article is protected by copyright. All rights reserved.

(supplemental Fig. S3). Collectively, these findings demonstrate that the pseudopilin TadE, but not TadF, promotes colonic epithelial cell proliferation and growth.

Purified $TadE_{2003}$ elicits epithelial proliferation under *in vitro* conditions

To confirm that TadE is crucial for the observed Tad_{2003} pilus-induced proliferation response, we heterologously expressed and purified $TadE_{2003}$ or $TadF_{2003}$ proteins as TrxA fusions. We first confirmed that the amino acid sequence of TadE bears no identity to any eukaryotic growth factors or proteins. HT29 cells were then stimulated with 200 ng ml^{-1} of the purified fusion proteins TrxA-TadE, TrxA-TadF or control TrxA, and cell proliferation determined by resazurin assay (Fig. 8A). This concentration of protein was selected following stimulation of the cells with a serial dilution of the proteins. The TrxA-TadE fusion protein significantly increased cell proliferation in HT29 cells, comparable to the level of proliferation induced by epidermal growth factor, (EGF) (Fig. 8A). In contrast, TrxA and TrxA-TadF failed to augment the proliferation of the cells (Fig. 8A). We confirmed these findings using a BrdU incorporation assay (Fig. 8B). BrdU is a synthetic thymidine nucleoside that is incorporated into DNA by proliferating cells. Cells incubated with TrxA-TadE fusion protein showed significantly increased BrdU incorporation relative to control TrxA, consistent with the findings obtained by resazurin assay.

Our preliminary investigations into the host receptor responsible for mediating this proliferative effect focussed on toll-like receptors (TLRs). The TLR family plays an important role in the detection of pathogens and commensals, responding to specific microbial ligands and to danger signals produced by the host during infection, as well as dampening responses to commensals and maintaining tissue homeostasis in physiologic conditions. TLRs are expressed by professional immune cells and by the large majority of non-hematopoietic cells, including epithelial cells. In humans, 10 TLRs have been identified to date and comprise both extracellular and intracellular receptors. In epithelial cells, constitutive and inducible expression of TLR2, TLR4 and TLR5 mRNA and protein has been shown in response to bacterial components and whole organisms. Given the important role played by TLRs

This article is protected by copyright. All rights reserved.

in the intestine, we investigated whether the TadE proteins altered expression of the surface-expressed TLRs. However, no change in expression was detected (data not shown).

Discussion

We have identified the bifidobacterial-derived protein, TadE, as one of the signalling molecules within the microbiota which promote epithelial proliferation in the host. TadE is a constituent protein decorating the multi-molecular pili on the surface of bifidobacteria. As with the other pilus proteins, TadE is not constitutively expressed *in vitro* and is evident only *in vivo* within the host. This is consistent with a specialised function and with a high degree of regulation. The proliferative effect on epithelial cells was, however, demonstrable and quantifiable on different epithelial cell lines *in vitro* when the tad_{2003} cluster was expressed in *L. lactis*. Likewise, purified TadE elicits epithelial cell proliferation *in vitro*. Thus, the proliferative effect of TadE is a true signalling event and not solely attributable to another bifidobacterial effector molecule facilitated by pilus-mediated adhesion to the epithelia.

Since the microbiota may be a source of multiple distinct proliferative signals, the maximal level of epithelial proliferation must be constrained by strict host regulation. Indeed, it is noteworthy that the TadE-mediated epithelial proliferation was sufficient to mimic that seen in conventionally colonised animals, because the colonic epithelial proliferation evident in germ-free animals mono-associated with pilus-bearing *B. breve* UCC2003 was comparable with that of conventionally colonised mice. Other investigators have reported the morphological similarities of the intestinal mucosa of mono-associated experimental animals and that of fully colonised animals (reviewed in 17). In addition, exposure to an enteric virus has been reported to replace some of the beneficial functions of bacteria including reconstitution of mucosal architecture (18). Of course, mono-association studies must be interpreted with caution, results are strain-dependent, restoration of morphology does not imply full immunologic function, and outcome may differ when compared with mice raised with a complex microbiota (17).

This article is protected by copyright. All rights reserved.

Although microbially-induced epithelial proliferation is not exclusive to bifidobacteria, the TadE-mediated signal may have particular relevance in early life, because the Bifidobacteria are among the earliest and dominant colonizers of the neonatal gut. In this respect, it is noteworthy that we observed a significant increase in epithelial proliferation as early as 5 days post administration of *B. breve* UCC2003. Moreover, it has been shown that the timing of colonisation is an important determinant of homeostasis in the host. Comparative analyses of animals colonised during development and at a later age have shown that colonisation at birth is required to optimally imprint the host's mucosal homeostasis (6).

Our preliminary investigations into the host receptor responsible for mediating this proliferative effect focussed on toll-like receptors (TLRs). The TLR family plays an important role in the detection of pathogens and commensals, responding to specific microbial ligands and to danger signals produced by the host during infection, as well as dampening responses to commensals and maintaining tissue homeostasis in physiologic conditions (19). TLRs are expressed by professional immune cells and by the large majority of non-hematopoietic cells, including epithelial cells. In humans, 10 TLRs have been identified to date and comprise both extracellular and intracellular receptors. In epithelial cells, constitutive and inducible expression of TLR2, TLR4 and TLR5 mRNA and protein has been shown in response to bacterial components and whole organisms (19). Given the important role played by TLRs in the intestine, we investigated whether the TadE protein altered expression of these surface-expressed TLRs. However, no change in expression was detected (data not shown).

Prospects for translating the TadE proliferative signal to a clinical arena have several challenges, but one of the clinical unmet needs for an agent such as this may be the immature mucosal barrier function of pre-term infants, including susceptibility to necrotizing enterocolitis (NEC). NEC is the commonest gastrointestinal disease in low-weight premature neonates and is associated with high mortality rates. Clinical trials will be required to test whether the ability of TadE pili in promoting intestinal growth and maturation of the intestinal barrier can protect against pathogenic

This article is protected by copyright. All rights reserved.

Accepted Article

infection. Likewise, TadE might have application in mitigating the epithelial injury associated with non-steroidal anti-inflammatory drugs and cancer chemotherapy.

Several mechanisms by which bifidobacteria modulate host mucosal homeostasis in a strain-specific manner have previously been identified. Firstly, certain strains can exclude or inhibit pathogens, either through direct action or through modulation of the commensal microbiota. Secondly, epithelial barrier function may be enhanced by modulation of signalling pathways such as nuclear factor κB (NF-kB), Akt and mitogen activated protein kinase (MAPK)-dependent pathways, which lead to the induction of mucus or increased tight junction functioning. Thirdly, many strains modulate host immune responses, exerting strain specific local and systemic effects (20-22).

The role of pili in mediating adhesion and virulence of pathogens has been well studied in the intestine and urogenital tract using different organisms (20, 23, 24). More recently, the sortase-dependent pili encoded by *Lb. rhamnosus* GG and *B. bifidum* PRL2010 or type IV pili of *A. muciniphila* have also been shown to be essential for bacterial adhesion. (11, 14, 25, 26). While adhesion to the epithelium and colonisation may facilitate secondary host immune responses (26-28), the present findings highlight the multi-functional nature of the multi-molecular Tad pili of bifidobacteria in which adherence/colonisation and epithelial proliferative signalling are distinct molecular processes.

The full spectrum of Tad E signalling with the host epithelium and whether it could be deployed at extra-intestinal sites remains to be established. Future work will address the Tad_{2003} receptor distribution, on the one hand, and will identify the host-derived signals which influence the expression of Tad pili by bifidobacteria *in vivo*. Whether TadE has a role in microbe-microbe signalling is also an intriguing prospect. Although we have no experimental evidence to implicate TadE or Tad pili in any form of microbe-microbe interaction, it is known that bifidobacteria engage in microbial metabolic co-operation through cross-feeding strategies that allow resource sharing of available carbohydrates (29-31).

Accepted Article

This article is protected by copyright. All rights reserved.

In conclusion, the findings show that bifidobacteria, the predominant colonizers of the infant gastrointestinal tract, produce specific surface structures in response to *in vivo* conditions that are not only essential for efficient colonisation, but also promote intestinal proliferation, and thereby may influence early host development. The work may have therapeutic relevance to the management of preterm neonates in whom intestinal barrier function is immature.

Materials and Methods

Bacterial strains, and culture conditions. Bacterial strains used in this study are listed in Table 1. *Bifidobacterium breve* UCC2003 was routinely cultured in reinforced clostridial medium (RCM; Oxoid Ltd, Basingstoke, Hampshire, United Kingdom) or in de Man Rogosa and Sharpe Medium (MRS) (31) prepared from first principles (33). Prior to inoculation the MRS was supplemented with cysteine-HCl (0.05 %). Bifidobacterial cultures were incubated at 37 ^{o}C under anaerobic conditions which were maintained using an anaerobic chamber (Davidson and Hardy, Belfast, Ireland). *Escherichia coli* was cultured in Luria Bertani broth (LB) (34) at 37 ^{o}C with agitation. *Lactococcus lactis* strains were cultivated in M17 broth containing 0.5% glucose (35) at 30 ^{o}C. Where appropriate growth media contained tetracycline (Tet; 10 μg ml^{-1}), chloramphenicol (Cm; 5 μg ml^{-1} for *E. coli* or *L. lactis*, or 2.5 μg ml^{-1} for *B. breve*), spectinomycin (Spec; 100 μg ml^{-1} for *E. coli* or *B. breve*) or kanamycin (Km; 50 μg ml^{-1} for *E. coli*). Recombinant *E. coli* cells containing pBS424Δrep were selected on LB agar containing Spec, while recombinant *E. coli* cells containing pORI19 or pBC1.2 were selected on LB agar containing Em or Ampicillin (Amp: 50 μg ml^{-1}), respectively, and supplemented with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg ml^{-1}) and 1 mM IPTG (isopropyl-μ-D-galactopyranoside).

Nucleotide sequence analysis. Sequence data were obtained from the Artemis-mediated (36) genome annotations of the *B. breve* UCC2003 sequencing project (15). Database searches were performed using non-redundant sequences accessible at the National Centre for Biotechnology Information

This article is protected by copyright. All rights reserved.

Accepted Article

internet site (http://www.ncbi.nlm.nih.gov) using Blast. Sequence alignments were performed using the Clustal Method of the MEGALIGN program of the DNASTAR software package (DNASTAR, Madison, WI, USA).

DNA manipulations. Chromosomal DNA was isolated from *B. breve* UCC2003 as previously described (37). Minipreparation of plasmid DNA from *E. coli* or *L. lactis* was achieved by using the Qiaprep spin plasmid miniprep kit (Qiagen GmBH, Hilden, Germany) as described previously (38). Single stranded oligonucleotide primers used in this study were synthesized by Eurofins (Ebersberg, Germany). Standard PCRs were performed using TaqPCR mastermix (Qiagen), while high fidelity PCR was achieved using Q5 DNA polymerase (New England Biolabs, Darmstadt, Germany). *B. breve* colony PCRs were performed as described previously (38). PCR fragments were purified using the Roche High pure PCR purification kit (Roche). Electroporation of plasmid DNA into *E. coli* was performed as described by Sambrook et al. (34) and into *L. lactis* as described by Wells et al. (39). Electrotransformation of *B. breve* UCC2003 was performed as described by O'Connell Motherway et al. (38).

Plasmid Constructions. For the construction of plasmid pNZ-tadZ-spk, DNA fragments encompassing the *tad*Z (Bbr_0132) to *tad*C (Bbr_0135), or *flp* (Bbr_0136) to *spk* (Bbr_0139) genes were generated by PCR amplification from chromosomal DNA of *B. breve* UCC2003 using Q5 DNA polymerase and primer combinations tadZF and tadCR, and flpF and spkR, respectively. To facilitate translational fusion to the nisin promoter on pNZ8150 SmaI and XbaI or HindIII restriction sites were incorporated at the 5' ends of each forward and reverse primer combination, respectively, (Table S1). The two generated amplicons, TadZ-TadC or flp-spk, were digested with SmaI and XbaI or HindIII, and ligated into ScaI and XbaI or HindIII-digested nisin-inducible translational fusion plasmid pNZ8150 (16). The ligation mixtures were introduced into *L. lactis* NZ9000 (Table 1) by electrotransformation and transformants selected based on chloramphenicol resistance. The plasmid

This article is protected by copyright. All rights reserved.

Accepted Article

content of a number of Cm^r transformants was screened by restriction analysis and the integrity of positively identified clones was verified by sequencing. To create the final construct pNZ-tadZ-spk, the pNis promoter plus the flp-spk fragment were amplified from pNZ-flp-spk using pNisF and spkR. The XbaI and HindIII restriction sites incorporated at the 5' ends of the forward and reverse primer combination facilitated cloning of the resultant fragment in the corresponding restriction sites of pNZ-tadZ-tadC. The plasmid content of a number of Cm^r transformants was screened by restriction analysis and the integrity of positively identified clones was verified by sequencing. The resulting construct was designated pNZ-tadZ-spk. For the construction of plasmid pPTPI-tadV a DNA fragment encompassing *tad*V (Bbr_0901) and its predicted Shine Dalgarno sequence were generated by PCR amplification from chromosomal DNA of *B. breve* UCC2003 using Q5 DNA polymerase and primer combinations tadVF and tadVR. AflIII and BamHI restriction endonuclease sequences incorporated in the forward and reverse primers, respectively, facilitated cloning in corresponding NcoI and BamHI sites of the nisin inducible transcriptional fusion vector pPTPI (40). The ligation mixture was introduced into *E. coli* EC101 (Table 1) by electrotransformation and transformants selected based on tetracycline resistance. The plasmid content of a number of tet^r transformants was screened by restriction analysis and the integrity of positively identified clones was verified by sequencing. One verified plasmid designated pPTPI-tadV was introduced by electroporation into *L. lactis* NZ9000 pNZ-tadZ-spk to generate *L. lacti*s NZ9000 pNZ-tadZ-spk-pPTPI-tadV.

Heterologous expression of Tad pili in *L. lactis*. To induce expression of Tad pili in *L. lacti*s NZ9000 pNZ-tadZ-spk-pPTPI-tadV and compare to the control strain *L. lactis* NZ9000 harbouring the empty plasmids pNZ8150 and pPTPI 50 ml of M17 broth supplemented with 0.5 % glucose was inoculated with a 2 % inoculum of a particular *L. lactis* strain, followed by incubation at 30 ^oC until an Optical Density (O.D. at wavelength 600 nm) of 0.5 was reached, at which point protein overexpression was induced by the addition of the cell free supernatant of a nisin producing strain followed by continued incubation at 30 ^oC for 90 minutes. Cells were harvested by centrifugation,

This article is protected by copyright. All rights reserved.

washed and suspended in 50 ml of PBS. Enumeration of bacteria cells was performed by plate counting.

Cell lines and Culture conditions. HT29, HCT116 and SW480 colonic epithelial cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM containing 10% FCS and penicillin–streptomycin at 37 C with 5% CO_2 in a fully humidified atmosphere. Cells were seeded at 1 x 10^5 cells per milliliter unless otherwise stated and cultured overnight prior to use in the resazurin or brdU prolifearion assays.

Resazurin assay. Epithelial cells were seeded at 1 x 10^5 cells per milliliter in 6-well plates, cultured overnight, and then treated with 10^6 cfu of nisin-induced *L. lactis* NZ9000 pNZ8150, pPTPI, *L. lactis* NZ9000 pNZ-tadZ-spk-pPTPI-tadV for 8 h, or treated with purified TrxA, TrxA-TadE or TrxA-TadF (200 ng ml^{-1}). Cells were washed, media supplemented with 44µM resazurin was added, and resazurin reduction to resorufin measured fluorometrically using a GENios plate reader (TECAN, Grodig, Austria) and Xfluor spreadsheet software. Results obtained were expressed in fluorescence units (FU) and the percentage viability calculated as follows: (FU treated/ FU control) x 100. Values were normalized relative to the untreated cells.

BrdU Proliferation Assay. HT29 cells were seeded at 2 x 10^5 cells per milliliter in 96-well plates. Following overnight culture, cells were treated with purified TrxA or TrxA-TadE (200 ng ml^{-1}). 48h later, cell proliferation was measured through BrdU incorporation using the BrdU Cell Proliferation Assay Kit (Cell Signalling Technology, Beverly, MA), according to the manufacturer's protocol.

Accepted Article

This article is protected by copyright. All rights reserved.

Cloning, overproduction and purification of the hydrophilic domains of TadE and TadF For the construction of plasmids pNZtrxA-tadE and pNZtrxA-tadF DNA fragments encompassing the truncated *tad*E (from codon 61 to 127) and *tad*F genes (from codon 47 to codon 130) were generated by PCR amplification from chromosomal DNA of *B. breve* UCC2003 using Q5 DNA polymerase and primer combinations TadEHF and TadEHR, and TadFHF and TadFHR, respectively. BamHI and XbaI restriction sites were incorporated at the 5' end of each forward and reverse primer combination, respectively, (Table S1). The generated amplicons were digested with BamHI and XbaI, and ligated into similarly digested nisin-inducible thioredoxin fusion plasmid pNZ-trxA (41). The ligation mixtures were introduced into *L. lactis* NZ9000 (Table 1) by electrotransformation and transformants selected based on chloramphenicol resistance. The plasmid content of a number of Cm^r transformants was screened by restriction analysis and the integrity of positively identified clones was verified by sequencing. For protein overproduction and purification 400 ml of M17 broth supplemented with 0.5 % glucose was inoculated with a 2 % inoculum of a particular *L. lactis* strain, followed by incubation at 30 ^oC until an Optical Density (O.D. at wavelength 600 nm) of 0.5 was reached, at which point protein overexpression was induced by the addition of the cell free supernatant of a nisin producing strain, followed by continued incubation at 30 ^oC for 90 minutes. Cells were harvested by centrifugation, washed and concentrated 40-fold in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole; pH 8.0). Cell extracts were prepared using 106 μm glass beads and the mini-bead-beater-8 cell disrupter (Biospec Products, Bartville, Oklahoma, USA). After homogenization the glass beads and cell debris were removed by centrifugation, while the supernatant containing the cytoplasmic fractions was retained. Protein purification from the cytoplasmic fraction was performed using Ni-NTA matrices in accordance with the manufacturers' instructions (Qiagen). Elution fractions were analysed by SDS polyacrylamide gel electrophoresis on a 12.5 % polyacrylamide gel. After electrophoresis the gels were fixed and stained with Commassie Brilliant blue to identify fractions containing the purified protein. Rainbow prestained low molecular weight protein markers (New England Biolabs, Herdfordshire, UK) were used to estimate the molecular weight of the purified proteins.

This article is protected by copyright. All rights reserved.

Accepted Article

Generation of Bbr_0137 ($TadE_{2003}$) and Bbr_0138 ($TadF_{2003}$) -specific antibodies. Antibodies against purified TrxA-TadE or TrxA-TadF were raised in rabbits by Eurogentec. An initial immunization with the TrxA-TadE or TrxA-TadF conjugated to a carrier and Freund's complete adjuvant was followed by three subsequent boost injections. A final serum sample was acquired 11 weeks after the initial immunization and the anti- Bbr_0137 ($TadE_{2003}$) and Bbr_0138 ($TadF_{2003}$) antibodies affinity purified.

Construction of *B. breve* UCC2003ΔtadE or UCC2003ΔtadF deletion mutant strains. Isogenic non-polar deletion mutants of *tad*E (Bbr_0137), or *tad*F (Bbr_0138), with 276 bp of the 381 bp of *tad*E, or 327bp of the 390bp of *tad*F deleted, were created using pBS423Δrep constructs generated by the splicing by overlap extension (SOEing) PCR procedure (42). In each case primers SOE AB and SOE CD (Table S1) were used to amplify regions flanking the sequence to be deleted using genomic DNA of *B. breve* UCC2003 as template. The resulting PCR products, designated I or II were purified, mixed in a 1:1 ratio and used as template with primers SOE EF. The resulting product was digested with Pst1 and ligated to similarly digested pBS423Δrep (43) prior to transformation into *E. coli* EC101 by electroporation. Transformants were selected based on resistance to Kn and Spec and screened by colony PCR using primers pBSF and pBSR to identify clones harbouring the correct insert. The presence of the correct insert in a number of positive clones was confirmed by plasmid isolation and restriction analysis, while the sequence integrity of the cloned DNA fragment, and the orientation of the insert in the pBS423Δrep vector was confirmed by sequencing. First crossover insertion mutations were generated essentially as described previously (38) to produce *B. breve* UCC2003 derivatives that were designated UCC2003-tadE-(I) or UCC2003-tadE-(II), or UCC2003-tadF-(I) or UCC2003-tadF-(II), respectively, where I or II indicate that the first crossover occurred via fragment I or II (described above). Site-specific recombination in potential spec-resistant mutant isolates was confirmed by colony PCR using primer combinations specFw and specRv to verify spectinomycin gene integration, and primers tadESOE A or tadF2SOE A (positioned upstream of the selected flanking regions of *tad*E_{2003} or *tad*$F_{2003,}$ respectively), each in combination with pBSR or to

This article is protected by copyright. All rights reserved.

Accepted Article

confirm integration at the correct chromosomal location. To promote pBS423Δrep plasmid excision in UCC2003-tadE-(I) or UCC2003-tadE-(II), or UCC2003-tadF-(I) or UCC2003-tadF-(II), plasmid pRTB101-CM was introduced into each strain and transformants were selected on RCA supplemented with CM. CM resistant colonies were subcultured for eight transfers to promote loss of integrated pBS423Δrep. Cells which had excised pBS423Δrep and had either reverted to the wild type genotype, or harboured a *tad*E or *tad*F deletion, were selected based on a Cm^r and $Spec^s$ phenotype. Screening of $Spec^s$ colonies for UCC2003 derivatives harbouring *tad*E or *tad*F deletion was performed by colony PCR using primer pairs tadESOEA and tadESOEB or tadF2SOEA and tadF2SOEB, respectively, and sequencing of the PCR products to confirm the in frame deletion. Curing of pRTB101-CM from *B. breve* deletion mutant strains was performed by subculturing at 42 ^oC for 8 transfers followed by plating on RCA and screening for CM sensitive strains by replica plating.

Complementation of UCC2003ΔtadE. A DNA fragment encompassing *tad*E_{2003} (Bbr_0137) was generated by PCR amplification from *B. breve* UCC2003 chromosomal DNA using Q5 High-Fidelity Polymerase (New England BioLabs, Herefordshire, United Kingdom) and primer pairs: tadEpNZ44F and tadEpNZ44R. The resulting fragment was digested with AflIII and XbaI, and ligated to the similarly digested pNZ44. The ligation mixture was introduced into *L. lactis* NZ9000 by electrotransformation and transformants were then selected based on Cm resistance. The plasmid content of a number of Cm-resistant transformants was screened by restriction analysis. The integrity of the cloned insert of one of the recombinant plasmids, designated pNZ44-tadE, was confirmed by sequencing. The *tad*E-coding sequence, together with the constitutive p44 lactococcal promoter, specified by pNZ44, was amplified by PCR from pNZ44-tadE using Q5 High-Fidelity DNA polymerase and primer combination P44 Forward and tadEpNZ44R (Table S1). The resulting DNA fragment was digested with HindIII and XbaI, and ligated to the similarly digested pBC1.2. The ligation mixture was introduced into *E. coli* XL1-blue by electrotransformation and transformants selected based on Tet and Cm resistance. Transformants were checked for plasmid content using colony PCR, restriction analysis of plasmid DNA, and verified by sequencing. pBC1.2-tadE was

This article is protected by copyright. All rights reserved.

introduced into the deletion mutant *B. breve* UCC2003ΔtadE by electrotransformation and transformants were selected based Cm resistance. The resulting strain was designated *B. breve* UCC2003ΔtadE- pBC1.2-tadE.

Colonization of Germ Free Mice. All experiments using mice were approved by the University College Cork animal ethics committee. Eight-week-old female, germ-free C57Bl/6 or Swiss Webster mice were housed in flexible film gnotobiotic isolators under a strict 12 h light cycle. Mice were fed an autoclaved standard polysaccharide-rich mouse chow diet. Mice (n = 7 or 16 per group) were inoculated with 1 x10^9 cfu of a particular *B. breve* strain (UCC2003PK1, UCC2003-tadA, UCC2003ΔtadE, UCC2003ΔtadF or UCC2003ΔtadE- pBC1.2-tadE) in 20 µl of PBS by oral pipetting whereby the inoculums are delivered by positioning a micropipette tip immediately behind the incisors. Five mice were maintained as uninoculated controls to monitor the germ free status of the facility. Fecal pellets were collected twice weekly to determine the number of each strain present. At day 30 the animals were sacrificed and their intestinal tracts quickly dissected. Samples were retained for bacterial enumeration, immunohistochemistry analysis and immuno-gold electron microscopy analysis. To ensure that changing the murine strain did not impact the findings obtained, mice mono-associated with wildtype *B. breve* UCC2003 as well as GF animals were included as controls in all studies, with similar findings obtained.

Immunohistochemistry analysis Immunohistochemical staining was performed on 4µM thick formalin-fixed, paraffin-embedded (FFPE) tissue sections. Sections were deparaffinized in xylene and rehydrated prior to analysis. Antigen retrieval was performed by microwave irradiation in 0.01 M citrate buffer, pH 6.0. Slides were washed twice for 5 min in a wash buffer containing 50 mM Tris-Cl, pH 7.6; 50 mM NaCl; 0.001% saponin. Endogenous peroxidase was quenched with 3.0% hydrogen peroxide in methanol for 10 min. Non-specific binding was blocked using 5% normal serum in wash buffer for 1 h. Sections were incubated overnight at 4˚C with anti-Ki67 primary Ab (Thermo Fisher

This article is protected by copyright. All rights reserved.

Accepted Article

Scientific, Fermont, CA), or with anti-PCNA primary antibody (Abcam, Cambridge, UK). Primary Ab binding was localized using a biotinylated secondary Ab, and visualized using avidin-conjugated HRP and diaminobenzidine (DAB) substrate, contained within the Vectastain avidin–biotin complex (ABC) detection kit (Vector Laboratories, Burlingame, CA, USA). Slides were counterstained with hematoxylin. A parallel negative control was also performed, using rabbit IgG instead of the primary Ab. The number of Ki67-positive cells, or PCNA-positive cells per total number of cells within a crypt was enumerated by counting the number of Ki67 positive cells per crypt, with a minimum of 10 crypts per tissue section counted. The proliferative index is expressed as the percentage $Ki67^{+}$ cells, or percentage $PCNA^{+}$ cells per number of cells within a crypt. All immunohistochemical analysis and cell counting was performed in a blinded manner.

Immuno-Gold and Osmium Tetroxide Transmission Electron Microscopy (TEM) Analysis. For the examination of *B. breve* cells recovered from the murine caecum, samples of 10 mg of caecal contents from animals monoassociated with either *B. breve* UCC2003, or UCC2003ΔtadE were suspended in 1 ml of fixative solution (0.5 M sucrose in 0.1 M Na-phosphate buffer, 2% paraformaldehyde and 0.16 % glutaraldehyde) and fixed for 2 hours at room temperature. Subsequently, the fixed caecal chyme underwent 3 low-speed centrifugation steps (100 rpm x 1min) to remove particulate material, and the fixed bacterial cells in the supernatant were retained. For the examination of nisin-induced *L. lactis* cultures the bacterial cultures were diluted 1:5 in fixative solution and fixed for 2 hours at room temperature. Thereafter Formvar-carbon-coated copper grids were floated on 100 µl droplets of the *B. breve* or *L. lactis* bacterial suspensions for 1 hour, washed three times with 0.02 M glycine in PBS and blocked 15 minutes with 1 % BSA in PBS. For the first labelling step, the grids were incubated for 1 hour on a droplet of 1:50 diluted anti-$TadE_{2003}$, washed 4 times for 2 minutes with 0.1 % BSA in PBS, and incubated for 20 minutes with protein A conjugated to 5 nm gold particles diluted 1:55 in blocking solution. The grids were washed four times with PBS and fixed for 5 minutes with 1 % glutaraldehyde, followed by 8 washes, each for 2 minutes, with distilled water. For the second labelling step the grids were incubated for 1 hour on a droplet of 1:50

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

diluted anti-TadF_{2003}, the same procedure was followed but this time adopting protein A conjugated to 10 nm gold particles diluted 1:55 in blocking solution. Finally negative staining with a mixture of 1.8 % methylcellulose-0.4 % uranyl acetate was performed at 4°C. The grids were examined, and micrographs visualized, using a JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan).

For osmium tetroxide staining, the intestinal tissue was washed once with 0,1 M phosphate buffer and fixed 2 h at RT with 2 % glutaraldehyde in 0,1 M phosphate buffer. After fixation the sample was washed 3 times with 0,1 M phosphate buffer and post-fixed with 1% osmiumtetroxide for 1 h at RT, dehydrated with graded series of ethanol, and incubated with transitional solvent acetone, after which the sample was gradually embedded in Epon and thin sectioned. The thin sections visualized using Jeol JEM-1400 transmission electron microscope.

Statistical analysis Results are presented as mean +/- SEM. Findings were statistically evaluated using one-way Anova with Dunnett's post-test. The p value, $p<0.001$, $p<0.01$ or $p<0.05$ is indicated by three, two or one stars (*), respectively.

Declarations

Ethics approval All experiments using mice were approved by the University College Cork animal ethics committee.

Competing Interests The authors declare that they have no competing interests.

Acknowledgements This work was supported by Science Foundation Ireland (SFI) (grant no. SFI/12/RC/2273) awarded to the APC Microbiome Institute, and HRB postdoctoral fellowship (grant no. PDTM/20011/9) awarded to MOCM. WMdV is supported by a Gravitation grant (024.002.002

This article is protected by copyright. All rights reserved.

SIAM) and Spinoza Award of the Netherlands Organization for Scientific Research and the Finland Academy of Sciences (Grant 141130 to WMdV) and JR by the Academy of Finland (Grant number 299749). The authors acknowledge the assistance of the staff, and the facilities provided at the Electron Microscopy Unit of the Institute of Biotechnology at the University of Helsinki.

References

1. Clevers H. 2013. The Intestinal Crypt, A Prototype Stem Cell Compartment. Cell 154(2):274-284.
2. Hooper L.V. 2015. Epithelial cell contributions to Intestinal Immunity. Adv Immunol. Chapter 3:130-162
3. Savage DC, Siegel JE, Snellen JE, Whitt DD. 1981. Transit time of epithelial cells in the small intestines of germfree mice and ex-germfree mice associated with indigenous microorganisms. Appl Environ Microbiol. 42(6):996-1001.
4. Hörmann N, Brandão I, Jäckel S, Ens N, Lillich M, Walter U, Reinhardt C. 2014. Gut microbial colonization orchestrates TLR2 expression, signaling and epithelial proliferation in the small intestinal mucosa. PLoS One. 14;9(11).
5. El Aidy S, Derrien M, Merrifield CA, Levenez F, Doré J, Boekschoten MV, Dekker J, Holmes E, Zoetendal EG, van Baarlen P, Claus SP, Kleerebezem M. 2013. Gut bacteria-host metabolic interplay during conventionalisation of the mouse germfree colon. ISME J. 7(4):743-55.
6. El Aidy S, Hooiveld G, Tremaroli V, Bäckhed F, Kleerebezem M. 2013. The gut microbiota and mucosal homeostasis: colonized at birth or at adulthood, does it matter? Gut Microbes. 4(2):118-24.
7. Shanahan F. 2012. The gut microbiota-a clinical perspective on lessons learned. 2012. Nat Rev Gastroenterol Hepatol. 9(10):609-614.
8. Gensollen T, Blumberg RS. 2017. Correlation between early-life regulation of the immune system by microbiota and allergy development. J Allergy Clin Immunol 139:1084-1091.

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

9. Tan TG, Sefik E, Geva-Zatorsky N, Kua L, Naskar D, Teng F, Pasman L, Ortiz-Lopez A, Jupp R, Wu HJ, Kasper DL, Benoist C, Mathis D. 2016. Identifying species of symbiont bacteria from the human gut that, alone, can induce intestinal Th17 cells in mice. Proc Natl Acad Sci U S A 113(50):E8141-E8150.
10. Schiavi E, Gleinser M, Molloy E, Groeger D, Frei R, Ferstl R, , Rodriguez-Perez N, Ziegler M, Grant R, Moriarty TF, Plattner S, Healy S, O'Connell Motherway M, Akdis CA, Roper J, Altmann F, van Sinderen D, O'Mahony L. 2016. The Surface-Associated Exopolysaccharide of *Bifidobacterium longum* 35624 plays an Essential Role in Dampening Host Proinflammatory Responses and Repressing Local TH17 Responses. Appl Environ Microbiol 82(24):7185-7196.
11. Turroni F, Serafini F, Foroni E, Duranti S, O'Connell Motherway M, Taverniti V, Mangifesta M, Milani C, Viappiani A, Roversi T, Sánchez B, Santoni A, Gioiosa L, Ferrarini A, Delledonne M, Margolles A, Piazza L, Palanza P, Bolchi A, Guglielmetti S, van Sinderen D, Ventura M. 2013. Role of sortase-dependent pili of *Bifidobacterium bifidum* PRL2010 in modulating bacterium-host interactions. Proc Natl Acad Sci U S A 110:11151-11156.
12. Nishiyama K, Ueno S, Sugiyama M, Yamamoto Y, Mukai T. 2016. *Lactobacillus rhamnosus* GG SpaC pilin subunit binds to the carbohydrate moieties of intestinal glycoconjugates. Anim Sci J 87(6):809-815.
13. Ardita CS, Mercante JW, Kwon YM, Luo L, Crawford ME, Powell DN, Jones RM, Neish AS. 2014. Epithelial adhesion mediated by pilin SpaC is required for *Lactobacillus rhamnosus* GG-induced cellular responses. Appl Environ Microbiol 80(16):5068-5077.
14. Ottman N, Reunanen J, Meijerink M, Pietilä TE, Kainulainen V, Klievink J, Huuskonen L, Aalvink S, Skurnik M, Boeren S, Satokari R, Mercenier A, Palva A, Smidt H, de Vos WM, Belzer C. 2017. Pili-like proteins of *Akkermansia muciniphila* modulate host immune responses and gut barrier function. PLoS One 12:e0173004.
15. O'Connell Motherway M, Zomer A, Leahy SC, Reunanen J, Bottacini F, Claesson MJ, O'Brien F, Flynn K, Casey PG, Munoz JA, Kearney B, Houston AM, O'Mahony C, Higgins DG, Shanahan F, Palva A, de Vos WM, Fitzgerald GF, Ventura M, O'Toole PW, van

This article is protected by copyright. All rights reserved.

Accepted Article

Sinderen D. 2011. Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. Proc Natl Acad Sci U S A 108:11217-11222.

16. Mierau I, Kleerebezem M. 2005. 10 years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. Appl Microbiol Biotechnol 68:705-717.
17. Ericsson AC, Franklin CL. 2015. Manipulating the Gut Microbiota: Methods and Challenges. ILAR J 56(2):205-17.
18. Kernbauer E, Ding Y, Cadwell K. 2014 An enteric virus can replace the beneficial function of commensal bacteria. Nature 516(7529):94-8.
19. McClure R, Massari P. 2014. TLR-Dependent Human Mucosal Epithelial Cell Responses to Microbial Pathogens. Front Immunol. 2014; 5: 386.
20. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, Torrents D, Ugarte E, Zoetendal EG, Wang J, Guarner F, Pedersen O, de Vos WM, Brunak S, Doré J; MetaHIT Consortium, Antolín M, Artiguenave F, Blottiere HM, Almeida M, Brechot C, Cara C, Chervaux C, Cultrone A, Delorme C, Denariaz G, Dervyn R, Foerstner KU, Friss C, van de Guchte M, Guedon E, Haimet F, Huber W, van Hylckama-Vlieg J, Jamet A, Juste C, Kaci G, Knol J, Lakhdari O, Layec S, Le Roux K, Maguin E, Mérieux A, Melo Minardi R, M'rini C, Muller J, Oozeer R, Parkhill J, Renault P, Rescigno M, Sanchez N, Sunagawa S, Torrejon A, Turner K, Vandemeulebrouck G, Varela E, Winogradsky Y, Zeller G, Weissenbach J, Ehrlich SD, Bork P. 2011. Enterotypes of the human gut microbiome. Nature 473:174-180.
21. Fanning S, Hall LJ, Cronin M, Zomer A, MacSharry J, Goulding D, Motherway MO, Shanahan F, Nally K, Dougan G, van Sinderen D. 2012 Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. Proc Natl Acad Sci U S A 109(6):2108-13.

This article is protected by copyright. All rights reserved.

Accepted Article

22. Konieczna P, Akdis CA, Quigley EM, Shanahan F, O'Mahony L. 2012Portrait of an immunoregulatory Bifidobacterium. Gut Microbes.3(3):261-6.
23. Wurpel DJ, Totsika M, Allsopp LP, Hartley-Tassell LE, Day CJ, Peters KM. 2014. F9 fimbriae of uropathogenic Escherichia coli are expressed at low temperature and recognise Galβ1-3GlcNAc-containing glycans.PLoS One 9(3):e93177.
24. Chessa D, Winter MG, Jakomin M, Bäumler AJ. 2009. Salmonella enterica serotype Typhimurium Std fimbriae bind terminal alpha(1,2)fucose residues in the cecal mucosa. Mol Microbiol 71(4):864-75.
25. Von Ossowski I, Reunanen J, Satokari R, Vesterlund S, Kankainen M, Huhtinen H, Tynkkynen S, Salminen S, de Vos WM, Palva A. 2010. Mucosal adhesion properties of the probiotic *Lactobacillus rhamnosus* GG SpaCBA and SpaFED pilin subunits. Appl Environ Microbiol 76:2049-2057.
26. Tytgat HL, Douillard FP, Reunanen J, Rasinkangas P, Hendrickx AP, Laine PK, Paulin L, Satokari R, de Vos WM. 2016. *Lactobacillus rhamnosus* GG Outcompetes *Enterococcus faecium* via Mucus-Binding Pili: Evidence for a Novel and Heterospecific Probiotic Mechanism. Appl Environ Microbiol 82:5756-5762.
27. Vargas García CE, Petrova M, Claes IJ, De Boeck I, Verhoeven TL, Dilissen E, von Ossowski I, Palva A, Bullens DM, Vanderleyden J, Lebeer S. 2015. Piliation of *Lactobacillus rhamnosus* GG promotes adhesion, phagocytosis, and cytokine modulation in macrophages. Appl Environ Microbiol 81:2050-2062.
28. Segers ME, Lebeer S. 2014. Towards a better understanding of *Lactobacillus rhamnosus* GG-host interactions. Microb Cell Fact Suppl 1,S7.
29. O'Connell Motherway M, O'Brien F, O'Driscoll T, Casey PG, Shanahan F, van Sinderen D. 2018. Carbohydrate Syntrophy enhances the establishment of Bifidobacterium breve UCC2003 in the neonatal gut. Sci Rep. 13;8(1):10627.
30. Milani C, Lugli GA, Duranti S, Turroni F, Mancabelli L, Ferrario C, Mangifesta M, Hevia A, Viappiani A, Scholz M, Arioli S, Sanchez B, Lane J, Ward DV, Hickey R, Mora D, Segata N,

This article is protected by copyright. All rights reserved.

Accepted Article

Margolles A, van Sinderen D, Ventura M. 2015. Bifidobacteria exhibit social behavior through carbohydrate resource sharing in the gut. Sci Rep. 5:15782.

31. Egan M, O'Connell Motherway M, Kilcoyne M, Kane M, Joshi L, Ventura M, van Sinderen D. 2014. Cross-feeding by Bifidobacterium breve UCC2003 during co-cultivation with Bifidobacterium bifidum PRL2010 in a mucin-based medium. BMC Microbiol. 14:282.
32. De Man JC, Rogosa A, Sharpe ME. 1960. A medium for the cultivation of lactobacilli. J Appl Bacteriol 23:130-135.
33. O'Connell Motherway M, Kinsella M, Fitzgerald GF, van Sinderen D. 2013. Transcriptional and functional characterization of genetic elements involved in galacto-oligosaccharide utilization by *Bifidobacterium breve* UCC2003. Microb Biotechnol 6:67-79.
34. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y 1989.
35. Terzaghi BE, Sandine WE. 1975. Improved Medium for Lactic Streptococci and their Bacteriophages. Appl Microbiol 29:807-813.
36. Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B. 2000. Artemis: sequence visualization and annotation. Bioinformatics 16:944-945.
37. O'Riordan K. Studies on antimicrobial activity and genetic diversity of Bifidobacterium species: molecular characterization of a 5.75 kb plasmid and a chromosomally encoded recA gene homologue from *Bifidobacterium breve*. Ph.D. National University of Ireland, Cork 1998.
38. O'Connell Motherway M, O'Driscoll J, Fitzgerald GF, van Sinderen D. 2009. Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in *Bifidobacterium breve* UCC2003. Microbial Biotechnology 2:321-332.
39. Wells JM, Wilson PW, Le Page RW. 1993. Improved cloning vectors and transformation procedure for *Lactococcus lactis*. J Appl Bacteriol 74:629-636.
40. O'Driscoll J, Glynn F, Cahalane O, O'Connell-Motherway M, Fitzgerald GF, Van Sinderen D. 2004. Lactococcal plasmid pNP40 encodes a novel, temperature-sensitive restriction-modification system. Appl Environ Microbiol 70:5546-56.

This article is protected by copyright. All rights reserved.

Accepted Article

41. Douillard FP, O'Connell-Motherway M, Cambillau C, van Sinderen D. 2011. Expanding the molecular toolbox for *Lactococcus lactis*: construction of an inducible thioredoxin gene fusion expression system. Microb Cell Fact 10:66.
42. Horton RM, Cai ZL, Ho SN, Pease LR. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. Biotechniques 8:528-535.
43. Hirayama Y, Sakanaka M, Fukuma H, Murayama H, Kano Y, Fukiya S, Yokota A. 2012. Development of a double-crossover markerless gene deletion system in Bifidobacterium longum: functional analysis of the α-galactosidase gene for raffinose assimilation. Appl Environ Microbiol 78:4984-4994.
44. Law J, Buist G, Haandrikman A, Kok J, Venema G, Leenhouts K. 1995. A system to generate chromosomal mutations in *Lactococcus lactis* which allows fast analysis of targeted genes. J Bacteriol 177:7011-7018.
45. de Ruyter PG, Kuipers OP, de Vos WM. 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl Environ Microbiol 62:3662-3667.
46. Cronin M, Knobel M, O'Connell Motherway M, Fitzgerald GF, van Sinderen D. 2007. Molecular dissection of a bifidobacterial replicon. Appl Environ Microbiol 73:7858-7866.
47. Alvarez-Martín P, O'Connell Motherway M, van Sinderen D, Mayo B. 2007. Functional analysis of the pBC1 replicon from *Bifidobacterium catenulatum* L48. Appl Microbiol Biotechnol 76:1395-1402.

This article is protected by copyright. All rights reserved.

Accepted Article

Figure Legends

Figure 1. Osmium tetroxide staining of intestinal sections of mice monoassociated with *B. breve* UCC2003. GF mice were monoassociated with *B. breve* UCC2003. 72 days later, mice were sacrificed, the caecum removed, fixed with 2 % glutaraldehyde, and then post-fixed with 1% osmiumtetroxide. Samples were embedded in Epon, sectioned and visualized using a Jeol JEM-1400 transmission electron microscope. Bifidobacterial cells in proximity to the epithelial cells are indicated by red arrowheads.

Figure 2. *B. breve* UCC2003 promotes colonic epithelial proliferation. A, Schematic representation of the tad_{2003} gene cluster. **B,** GF mice were monoassociated with *B. breve* UCC2003PK1 or *B. breve* UCC2003-tadAPK1, and bacterial shedding enumerated over 30 days. **C**, To detect the presence of proliferating cells, paraffin-embedded colonic tissue sections from mice monoassociated with (i) *B. breve* UCC2003PK1, (ii) *B. breve* UCC2003-tadAPK1, (iii) untreated GF or (iv) SPF controls were immunostained for Ki67. Ki67 positive cells stain brown. (v) Isotype negative controls were performed using rabbit IgG instead of the primary Ab. Scale bars, 100μM. Images shown are representative of the findings obtained for colonic tissue. **D,** The number of Ki67-positive cells per total number of cells within a crypt was enumerated for each specimen and the proliferative index determined. Statistically significant differences between the groups are indicated by stars (*$p < 0.05$).

Figure 3. *B. breve* Tad$_{2003}$ significantly increases intestinal epithelial cell proliferation by 5 days post colonisation. A, GF mice were administered a single dose of (i) *B. breve* UCC2003PK1, (ii) *B. breve* UCC2003-tadAPK1, (iii) untreated GF or (iv) administered homogenised murine feces. Mice were sacrificed at the indicated time points and immunohistochemical staining for Ki67 was performed on paraffin-embedded colonic tissue sections to detect the presence of proliferating cells. (v) Isotype negative controls were performed using rabbit IgG instead of the primary Ab. Scale bars,

This article is protected by copyright. All rights reserved.

100μM. Images shown are representative of the findings obtained at day 15. **B - E,** The number of Ki67-positive cells per total number of cells within a crypt was enumerated for each specimen and the proliferative index determined for each time point. Statistically significant differences between the groups are indicated by stars (*$p < 0.05$), (**$p < 0.01$).

Figure 4. The proliferation of colonic epithelial cells is increased *in vitro* following co-culture with *L. lactis* expressing *B. breve* Tad_{2003} pili. A, *L. lactis* were engineered to express the *B. breve* Tad_{2003} pili under *in vitro* conditions and immunogold transmission electron microscopy performed on bacterial cells to detect Tad_{2003} pili. TadE and TadF are represented by the immunoreactive 5 nm (red arrow) and 10 nm (yellow arrow) gold particles, respectively. **B,** HT29, HCT116 or SW480 epithelial cells were either untreated or cultured for 8hrs with *L. lactis* control (*L. lactis* NZ9000 pNZ8150 pPTPI) or *L. lactis*-expressing Tad_{2003} pili *(L. lactis* NZ9000 pNZ-tadZ-spk-pPTPI-tadV*).* Alterations in cell proliferation were determined by resazurin assay. Values are shown as mean +/- SEM and are representative of 3 independent experiments. Statistically significant differences between the groups are indicated by stars (***$p < 0.001$).

Figure 5. The pseudopilin TadE promotes colonic epithelial proliferation. A, GF mice were administered (i) *B. breve* UCC2003PK1, (ii) *B. breve* UCC2003ΔtadE, (iii) *B. breve* UCC2003ΔtadF, (iv) untreated GF or (v) SPF controls. Ki67 immunohistochemical was performed on colonic tissue obtained from these mice. (vi) Isotype negative controls were performed using rabbit IgG instead of the primary Ab. Scale bars, 100μM. Images shown are representative of the findings obtained. **B,** The number of Ki67-positive cells per total number of cells within a crypt was enumerated for each specimen and the proliferative index determined. Statistically significant differences between the groups are indicated by stars (** $p < 0.01$; *** $p < 0.001$).

This article is protected by copyright. All rights reserved.

Figure 6. Tad_{2003} Pili from *B. breve* UCC2003ΔtadE monoassociated mice lack TadE but not TadF. Caecum contents were retained from mice monoassociated with **A,** *B. breve* UCC2003 or **B,** *B. breve* UCC2003ΔtadE cells. Samples were immunogold labelled with anti-TadE and anti-TadF antibodies and visualised by transmission electron microscopy. TadE and TadF are represented by the immunoreactive 5 nm (red arrow) and 10 nm (yellow arrow) gold particles, respectively.

Figure 7. Complementation of *B. breve* UCC2003ΔtadE restores the epithelial proliferation phenotype. A, GF mice were administered (i) *B. breve* UCC2003PK1, (ii) *B. breve* UCC2003ΔtadE or (iii) *B. breve* UCC2003ΔtadEpBC1.2tadE**.** Ki67 immunohistochemistry was performed on colonic tissue obtained from these mice. Scale bars, 100μM. Images shown are representative of the findings obtained. **B,** The number of Ki67-positive cells per total number of cells within a crypt was enumerated for each specimen and the proliferative index determined. Statistically significant differences between the groups are indicated by stars (* $p < 0.05$; ** $p < 0.01$).

Figure 8. Purified TrxA-TadE fusion protein significantly increases colonic epithelial cell proliferation *in vitro*. **A**, **B** HT29 cells were stimulated with purified control TrxA, TrxA-TadE fusion protein or TrxA-TadE fusion protein as indicated, and changes in cell proliferation assessed by **B,** Resazurin reduction and **C,** BrdU incorporation. EGF, epidermal growth factor, is included as a positive control. Values are shown as mean +/- SEM and are representative of 3 independent experiments. Statistically significant differences between the groups are indicated by stars (** $p < 0.01$, ns non-significant).

Accepted Article

This article is protected by copyright. All rights reserved.

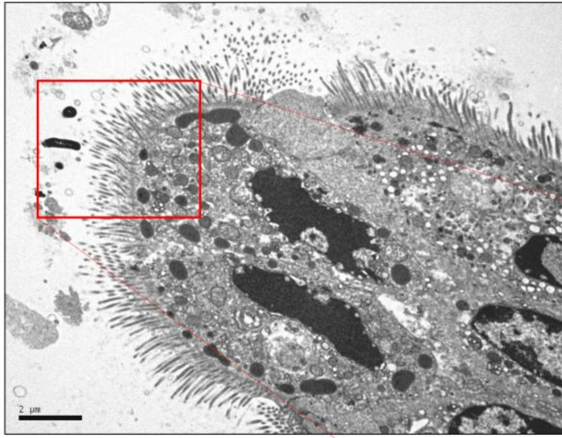
Table 1. Bacterial strains used in this study

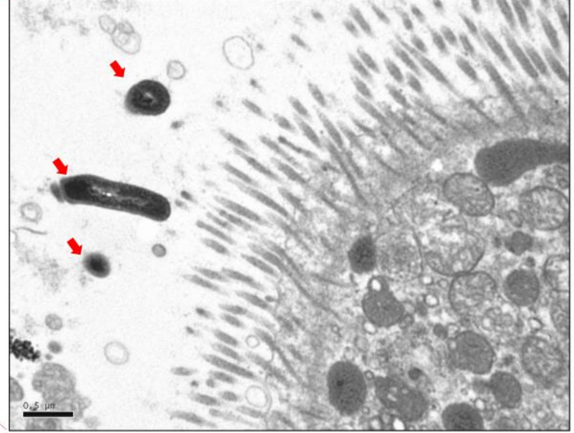
Strains and plasmids	Relevant features	Reference or source
Strains		
Escherichia coli strains		
E. coli EC101	Cloning host, repA$^+$ km^r	(44)
E. coli EC101-pNZ-M.BbrII + M.BbrIII	EC101 harbouring pNZ8048 derivative containing *bbrIIM* and *bbrIIIM*.	(38)
E. coli EC101pBS423Δrep	EC101 harbouring pBS423Δrep	(43)
E. coli EC101pBS423TadE1+2	EC101 harbouring pBS423Δrep + tadE soeing fragments 1+2	This study
E. coli EC101pBS423TadF1+2	EC101 harbouring pBS423Δrep + tadF soeing fragments 1+2	This study
Lactococcus lactis strains		
L. lactis NZ9000	MG1363, *pep*N::*nis*RK, nisin inducible overexpression host	(45)
L. lactis NZ9000 pNZ-tadZ-tadC	NZ9000 containing pNZ-tadZ-tadC	This study
L. lactis NZ9000- pNZ-flp-spk	NZ9000 containing pNZ-flp-spk	This study
L. lactis NZ9000- pNZ-tadZ-spk	NZ9000 containing pNZ-tadZ-spk	This study
L. lactis NZ9000- pPTPI-tadV	NZ9000 containing pPTPI-tadV	This study
L. lactis NZ9000 pNZtrxA-tadE	NZ9000 containing pNZtrxA-tadE	This study
L. lactis NZ9000 pNZtrxA-tadF	NZ9000 containing pNZtrxA-tadF	This study
L. lactis NZ9000 pNZ-tadZ-spk-pPTPI-tadV	NZ9000 containing pNZ-tadZ-spk and pPTPI-tadV	This study
L. lactis NZ9000 pNZ8150 pPTPI	NZ9000 containing pNZ8150 pPTPI	This study
Bifidobacterium sp. strains		
B. breve UCC2003	Isolate from nursling stool	(15)
B. breve UCC2003-tadA	tadA insertion mutant of *B. breve* UCC2003	(15)
B. breve UCC2003ΔtadE	tadE deletion mutant of UCC2003	This study
B. breve UCC2003ΔtadF	tadF deletion mutant of UCC2003	This study
B. breve UCC2003ΔtadE-pBC1.2-tadE	complemented *B. breve* UCC2003ΔtadE	This study
Plasmids		
pBS423Δrep	4.4 kb, *E. coli*- vector, ΔpMB1, ori pTB4 ori repA Spec^r	(43)
pRTB101	7.3 kbp, *E. coli*-Bifidobacterium shuttle vector, pMB1 ori pTB4 ori repA	(43)
pPKCM	pCIBA089-pSK-Cm^r	(46)
pAM5	pBC1-puC19-Amp^r-Tet^r	(47)
pORI19	Em^r, repA$^-$, ori$^+$, cloning vector	(44)
pNZ8150	Nisin inducible expression vector Cm^r	(27)
pPTPI	Nisin inducible expression vector Tet^r	(40)

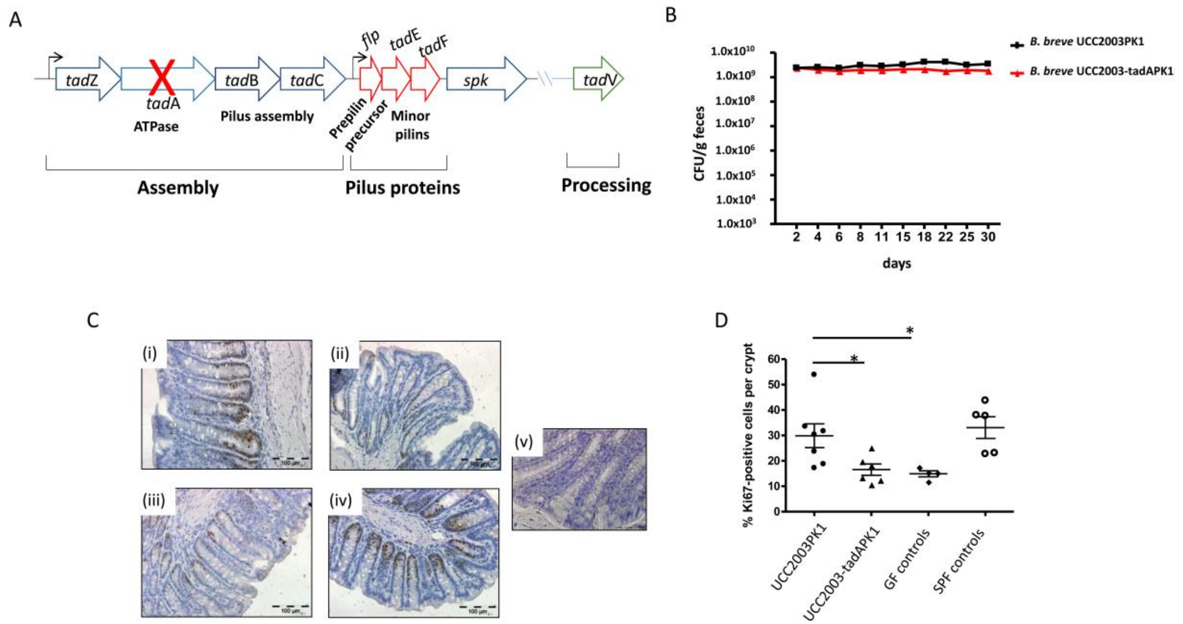
Accepted Article

This article is protected by copyright. All rights reserved.

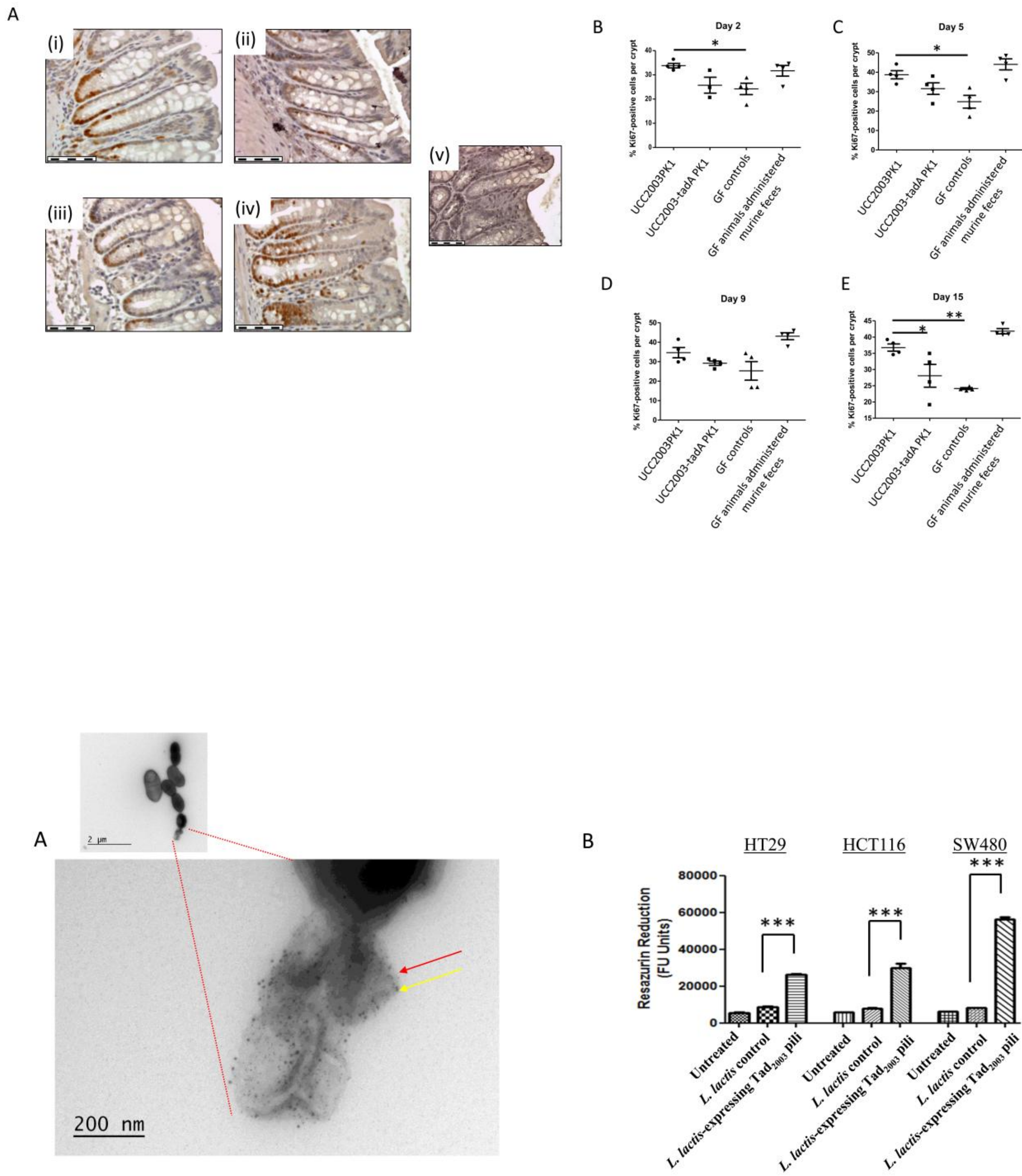
Accepted Article







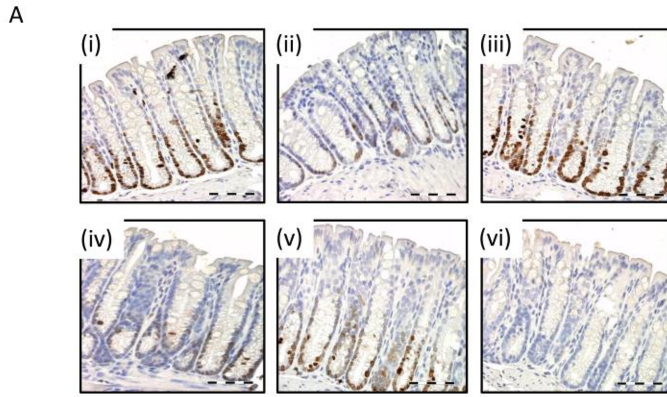
This article is protected by copyright. All rights reserved.

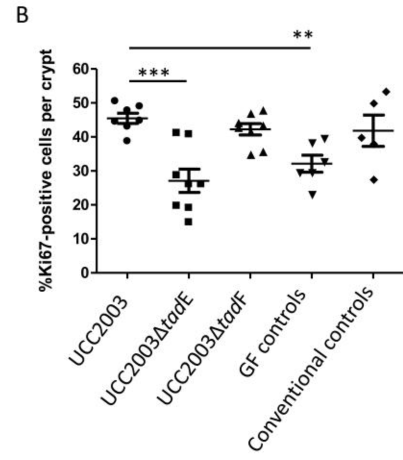


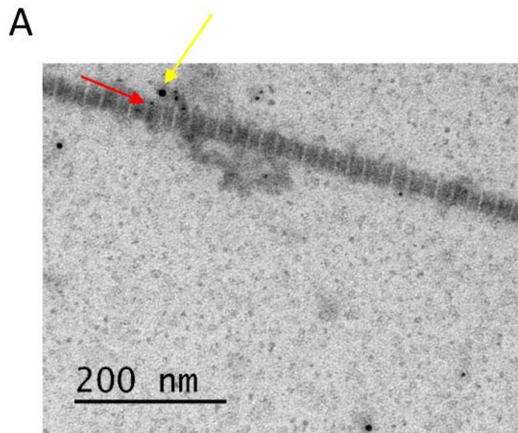
Accepted Article

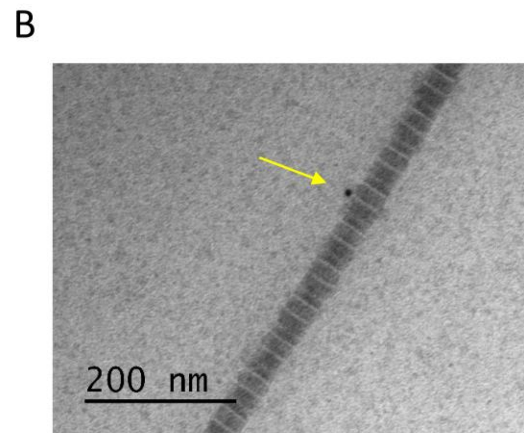
This article is protected by copyright. All rights reserved.

Accepted Article





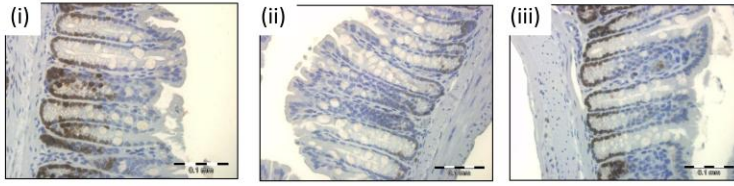




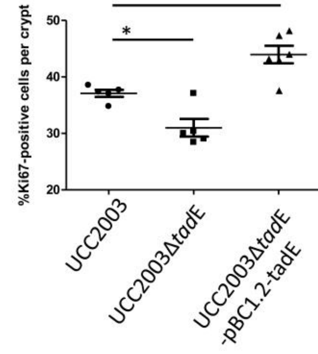
This article is protected by copyright. All rights reserved.

Accepted Article

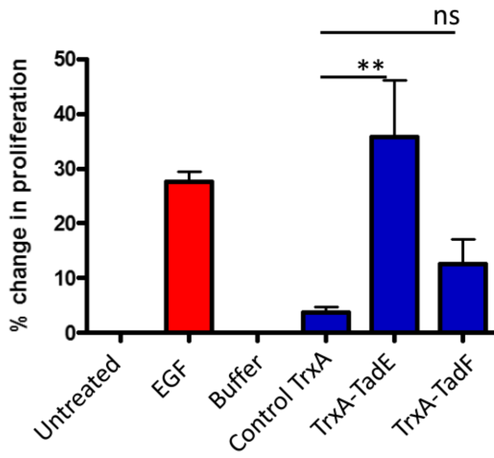
A



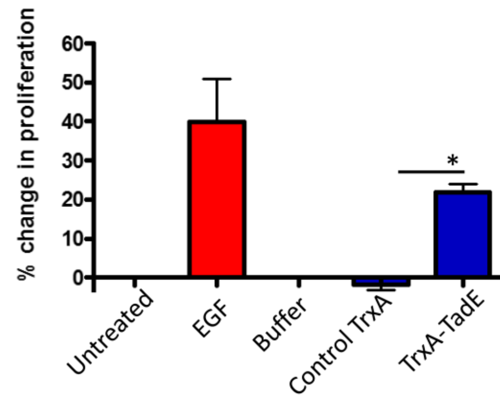
B



A



B



This article is protected by copyright. All rights reserved.