Molecular mechanisms underlying the probiotic effects of *Escherichia coli* Nissle 1917 involve ZO-2 and PKCζ redistribution resulting in tight junction and epithelial barrier repair

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Summary

The probiotic *Escherichia coli* strain Nissle 1917 (EcN) has been used for decades in human medicine in Central Europe for the treatment and prevention of intestinal disorders and diseases. However, the molecular mechanisms underlying its beneficial effects are only partially understood. To identify molecular responses induced by EcN that might contribute to its probiotic properties polarized T84 cells were investigated employing DNA microarrays, quantitative RT-PCR, Western blotting, immunofluorescence and specific protein kinase C (PKC) inhibitors. Polarized T84 epithelial cell monolayers were used as a model to monitor barrier disruption by infection with the enteropathogenic *E. coli* (EPEC) strain E2348/69. Co-incubation of EPEC with EcN or addition of EcN following EPEC infection abolished barrier disruption and, moreover, restored barrier integrity as monitored by transepithelial resistance. DNA-microarray analysis of T84 cells incubated with EcN identified 300+ genes exhibiting altered expression. EcN altered the expression, distribution of zonula occludens-2 (ZO-2) protein and of distinct PKC isotypes. ZO-2 expression was enhanced in parallel to its redistribution towards the cell boundaries. This study provides evidence that EcN induces an overriding signalling effect leading to restoration of a disrupted epithelial barrier.

This is transmitted via silencing of PKCζ and the redistribution of ZO-2. We suggest that these properties contribute to the reported efficacy in the treatment of inflammatory bowel diseases and in part rationalize the probiotic nature of EcN.

Introduction

The intestinal ecosystem is characterized by dynamic and reciprocal interactions among its microflora, the epithelium and the immune system (Macpherson and Harris, 2004; Yan and Polk, 2004; Xavier and Podolsky, 2005). The composition of the intestinal microflora is central to normal immune development and epithelial function and protects mucosal surfaces from pathogens. Intestinal barrier integrity is one prerequisite for maintaining the stability of the intestinal ecosystem. Acute diarrhoea and relapses of inflammatory bowel disease (IBD) are among the well-known consequences of a disturbed epithelial barrier. In the maintenance of barrier integrity tight junction (TJ) complexes play a decisive role (Mitic *et al*., 2000; Schneeberger and Lynch, 2004). Intestinal infections by pathogenic microorganisms often affect the tightly controlled interactions of TJ proteins resulting in barrier disruption which in turn promotes inflammatory processes in the gut (Spitz *et al*., 1995; Berkes *et al*., 2003; Fasano and Nataro, 2004).

Probiotics are defined as non-pathogenic microorganisms which, when given in adequate numbers, exhibit beneficial effects on the host (FAO/WHO, 2001; Reid *et al*., 2001; Sanders, 2003). These effects include the balancing and restoration of the intestinal microflora, protection against pathogens, modulation of the immune system and the maintenance and repair of intestinal barrier functions (e.g. Czerucka *et al*., 2000; Madsen *et al*., 2001; Sartor, 2003; Sherman *et al*., 2005; Paton *et al*., 2006).

The probiotic *Escherichia coli* Nissle 1917 strain (Nissle, 1916; 1919; 1925) (EcN) is a human faecal isolate which despite exhibiting a serotype (O6:K5:H1) characteristic of *E. coli* strains associated with urinary tract infections, is completely non-pathogenic (Gunzer *et al*., 2002; Grozdanov *et al*., 2004). It has been shown in...
several recent studies: EcN is a safe probiotic microorganism (Westendorf et al., 2005). Its biosafety is also underlined by its long medical history in Central Europe as a microbial remedy (Mutaflor®, since 1917) and the use has been recommended for the prevention of diarrhoeal diseases caused by pathogens such as Shigella, Salmonella or E. coli and for treatment of functional bowel disorders (Mollenbrink and Bruckschen, 1994) and IBDs, i.e. Ulcerative colitis and Crohn’s disease (Kruis et al., 1997; 2004; Rembacken et al., 1999; Isolauri et al., 2002; Poldolsky, 2002; Kruis, 2004; Grabig et al., 2006). Furthermore, EcN has been used for intentional colonization of the gut of newborns in order to suppress the acquisition of pathogenic and multiresistant microorganisms (Lodinova-Zadnikova and Sonnenborn, 1997; Westendorf et al., 2005). However, only a few experimental studies on the underlying molecular and cellular mechanisms for these beneficial effects on the host have been published to date (Wehkamp et al., 2004; Sturm et al., 2005).

Important components of the epithelial barrier regulating the passage of fluid, ions, lipids and other substances via the paracellular pathway are TJ which represent the uppermost basolateral connection between neighbouring enterocytes (Mitic et al., 2000; Schneeberger and Lynch, 2004). TJ are composed of the transmembrane proteins occludins, claudins, and the junctional adhesion molecules. The cytosolic domains of these proteins interact with the peripheral junctional proteins of the zona occludens family (ZO-1, -2, -3 proteins) and also include protein kinases, especially protein kinase C (PKC) isoforms for regulatory purposes (González-Mariscal et al., 2000; Schneeberger and Lynch, 2004). TJ assembly and paracellular permeability are regulated by a network of signalling pathways that involves different PKC isoforms (Avila-Flores et al., 1990; Stuart and Nigam, 1995; Dodane and Kachar, 1996; Suzuki et al., 2001; Schneeberger and Lynch, 2004). The 11 currently known PKC isoforms are usually divided into three distinct subtypes: conventional (cPKC): α, βⅠ, βⅡ, γ, novel (nPKC): δ, ε, η, μ, θ, and atypical (aPKC) isoforms: ζ, η, λ (Avila-Flores et al., 1990). PKCs are located intracellularly, at or near the zona occludens complex. Therefore, activation of selective subsets of PKC isoforms might differentially affect the cellular properties of transport and barrier function of epithelia (Song et al., 2001). PKCζ is the only PKC isoform also present at the cellular border (Dodane and Kachar, 1996), and thus may be part of the zona occludens complex at the intercellular contact sites. In uninfected intestinal epithelial cells the majority of PKCζ is found in the cytosol, but after enteropathogenic E. coli (EPEC) infection, PKCζ is redistributed to the periphery of the cells (Savkovic et al., 2003; Tomson et al., 2004).

Different PKC isoforms appear to be complexed with ‘zonula occludens protein-2’ (ZO-2) (Tomson et al., 2004). This suggests not only that this protein might be regulated by phosphorylation but also that it might fulfill diverse functions. Conventional and novel PKCs seem to participate in the process of TJ formation, whereas aPKC (λ and ζ) might regulate the disassembly process through their action on ZO-2.

The aim of this study was to investigate the molecular mechanisms underlying the beneficial effects of the probiotic EcN strain, in particular the effects on the epithelial barrier using polarized T84 cells as model system. In the polarized T84 cell model system expression of only five PKC isoforms (α, βⅠ, δ, ε, ζ) has been demonstrated (Tomson et al., 2004). Moreover, as infections with EPEC are accompanied by the disruption of epithelial integrity we also asked whether the presence of EcN would influence the otherwise deleterious barrier disruption of polarized T84 cells caused by EPEC bacteria. DNA-microarrays were used as a tool to search for differences in the expression of genes affected by exposure of T84 cells to EcN or EPEC.

Results
Escherichia coli Nissle 1917-induced enhancement of transepithelial resistance
As probiotics have been reported to strengthen barrier functions (Czerucka et al., 2000; Madsen et al., 2001), we measured the effect of EcN on the transepithelial resistance (TER) of confluent T84 monolayers. As shown in Fig. 1, incubation with EcN alone did not significantly effect the TER of T84 monolayers: TER increased approximately 20% over time from 336.7 ± 11.5 Ω × cm² (0 min) to 402.77 ± 10.5 Ω × cm² (120 min). This is in contrast to infections of T84 cells with the EPEC prototype strain E2348/69 which resulted in an approximately 70% decrease of TER from 331.7 ± 2.1 Ω × cm² (0 min) to 110.0 ± 1.5 Ω × cm² (120 min) confirming previous observations (Spitz et al., 1995; Vandesompele et al., 2002). However, when T84 cells were co-incubated simultaneously with EPEC and EcN (Fig. 1C), the previously observed reduction of TER due to EPEC infection was not found. Moreover, when we incubated T84 cells with EPEC bacteria for 1 h, removed the pathogen and then added EcN with the same multiplicity of infection (moi), the probiotic EcN restored barrier function to nearly the same TER values as had been measured with untreated control cells (Fig. 1C). For further proof of principle and to support our findings with T84 cells we also investigated Caco-2 cell monolayers in coculture experiments. Co-incubation of these cells with EcN either showed no alterations or sometimes resulted in a slight decrease of TER (max. 11.4%). However, this was not surprising as also the replacement of tissue culture medium often resulted in a
similar decrease of TER. In contrast, the infection with EPEC led to a substantial decrease of TER to 62.6% of the control values within 120 min. As expected from the alterations in TER observed with T84 cells after the simultaneous co-incubation of Caco-2 cells with EcN and EPEC, we observed an increase of TER up to 136.7% of the control values which appeared to be linked to the co-incubation of T84 cells with EcN (see Table 1). For the TJ protein ZO-2 we found a slight upregulation of mRNA after incubation with EcN (Fig. 2A) that after 120 min of incubation returned to initial values. In contrast, downregulation of ZO-2 expression was observed during EPEC infection (Fig. 2B). This alteration in gene expression peaked after 60 min of co-incubation and is mirrored by changes of TER. In contrast, co-incubation of T84 cells with EPEC alone led to a continuous decrease of ZO-2 mRNA (Fig. 2B). Co-infection with EPEC and EcN is characterized by an initial ‘burst’ of ZO-2 mRNA after 15 min (Fig. 2C) which is followed by a slow decline of ZO-2 mRNA during the following 2 h approaching normal values and revealing a profile that is quite similar to that found with EcN incubation alone (Fig. 2A).

Monitoring and visualization of ZO-2 expression

The increase in ZO-2 mRNA after incubation of T84 cells with EcN is paralleled by the level of protein expression and alterations in protein localization (Fig. 3A–G). The diffuse staining of ZO-2 at the cell boundaries of non-infected T84 cells (Fig. 3A) changed to a distinct membrane staining upon incubation with EcN (Fig. 3C). This might be attributed to a translocation of cell boundary-associated ZO-2 proteins to the TJ complex putatively leading to an enforcement of cell–cell contacts which would correlate very well with the observed enhancement of TER.

Analysis of Western blotting experiments with cytosolic, membrane and cytoskeletal fractions after incubation with EcN or EPEC also revealed a significant difference between the effects of EcN and EPEC on the distribution of ZO-2 between the various compartments in T84 cells.
(Fig. 3G). When T84 monolayers were infected with EPEC, TER was significantly reduced and ZO-2 was downregulated (Figs 1B and 2B). In addition, the distribution of ZO-2 was also affected as demonstrated by the patchy appearance of the immunofluorescence staining that might indicate a re-location or even exclusion of the protein from TJ complexes (Fig. 3B). In contrast, incubation of T84 cells with EcN induced an enhanced translocation and concentration of ZO-2 to the TJ regions (Fig. 3C). When T84 monolayers were co-incubated with EPEC and EcN for 120 min, ZO-2 expression did not change and when compared with untreated control cells the TJs appeared not to be negatively affected (Fig. 3A and F). Moreover, when T84 cells were first incubated with EPEC for 1 h followed by incubation with EcN for another hour, ZO-2 is again primarily found to decorate the regions of TJs (Fig. 3E). These findings indicate that during co-incubation with EPEC and EcN or during EPEC-infection followed by EcN, ZO-2 is not only upregulated but also more pronounced. In uninfected control cells PKCζ appeared to be present mostly in the cytosol (Fig. 4A). Western blotting analysis indicated that although the amount of PKCζ increased in both cytosol and membrane fractions (Fig. 4B) the increase in the cytosolic compartment was much more pronounced. In uninfected control cells PKCζ appeared to be present mostly in the cytosol (Fig. 4A). Interestingly, the expression of PKCζ mRNA is considerably upregulated after incubation with EcN or EPEC (Fig. 4C left and centre). However, PKCζ mRNA was found to be upregulated only slightly during co-infection with EPEC and EcN (Fig. 4C right) and also the distribution of PKCζ was comparable to the distribution observed in untreated cells.

### Table 1. Microarray derived regulated genes after T₈₄ co-incubation with EcN.

| Gene-symbol | Annotation                                      | Function                                         | Probe set     | Regulation |
|-------------|------------------------------------------------|-------------------------------------------------|---------------|------------|
| CLARP       | Caspase-like apoptosis regulatory protein       | Apoptose inhibitor; inhibition by blocking the receptor | 1868_g_at     | Nissle: ↑   |
| GUCY2C      | Heat-stable enterotoxin receptor               | Transmembrane receptor; interaction with enterotoxin, resulting in increased water- and chloride-secretion | 34450_at     | Nissle: ↓   |
| IKB         | Nuclear factor kappa-B inhibitor               | Inhibitor; prevents the translocation of NFκB to the nucleus; degradation by proteases after phosphorylation and ubiquitination | 1461_at      | Nissle: ↑   |
| IKK1        | IKK-kinase 1                                   | Kinase; phosphorylation of IκB at lysine-residues, marking for degradation | 33770_at     | Nissle: ↓   |
| IL4R        | Interleukin 4 receptor                        | Receptor for interleukin 4, regulation of IgE production | 404_at       | Nissle: ↑   |
| MIF         | Macrophage migration inhibitory factor         | Expression during inflammation, regulation of macrophage-function | 895_at       | Nissle: ↓   |
| MIR7        | Monocyte/macrophage immunoglobulin-like receptor 7 | Receptor for MHC molecules                      | 35926_s_at   | Nissle: ↓   |
| MIP3α       | Macrophage inflammatory protein 3-alpha (exodus) | Chemokine; stimulation of chemotaxis mononuclear blood cells | 40385_at     | Nissle: ↑   |
| NFATC3      | Nuclear factor of activated T cells            | Activation of cytokines after antigen stimulation | 40822_at     | Nissle: ↑   |
| NF-IL6      | Interleukin 6-dependent DNA-binding protein    | Receptor for interleukin 6, modulation of inflammatory response, induction of acute-phase-proteins | 38354_at     | Nissle: ↑   |
| NFκB p65    | Nuclear factor of kappa light chain, p65 subunit | Transcription factor; expression of proinflammatory cytokines (TNF-α, IL1, etc.) | 1295_at      | Nissle: ↑   |
| PSMD12 p55  | Proteasome 26S subunit                        | Protein complex, ATP-dependent degradation of cellular proteins | 1192_at      | Nissle: ↑   |
| TNFAIP3     | Tumour necrosis factor alpha induced protein   | Inhibition of NFκB, limitation of inflammatory reactions | 595_at       | Nissle: ↑   |

Alterations in expression and distribution of PKC isoforms

The results of the DNA-microarray analysis and the PKC-dependent translocation of zonula occludens protein 1 from the membrane after EPEC infection reported previously (Cario et al., 2004) suggest that specific isoforms of PKCs might be involved in the effects observed upon incubation of T₈₄ cells with EcN, e.g. the translocation of ZO-2.

We could confirm earlier findings by Tomson et al. (2004) that PKCζ is translocated to the membrane following an infection of T₈₄ epithelial cells with EPEC (Fig. 4A centre; 4B left). In contrast, after incubation of T₈₄ cells with EcN, immunofluorescence microscopy revealed an increased presence of PKCζ in the cytosol and only a residual and somewhat fuzzy staining at the membrane for PKCζ (Fig. 4A right, 4B centre). Western blotting analysis indicated that although the amount of PKCζ increased in both cytosol and membrane fractions (Fig. 4B) the increase in the cytosolic compartment was much more pronounced. In uninfected control cells PKCζ appeared to be present mostly in the cytosol (Fig. 4A). Interestingly, the expression of PKCζ mRNA is considerably upregulated after incubation with EcN or EPEC (Fig. 4C left and centre). However, PKCζ mRNA was found to be upregulated only slightly during co-infection with EPEC and EcN (Fig. 4C right) and also the distribution of PKCζ was comparable to the distribution observed in untreated cells.
To elucidate whether the effect of EcN on the distribution of ZO-2 might depend on the activity of PKCζ, we co-incubated T84 cells with the specific PKCζ inhibitor (PKCζ-PS). For this we investigated the activity of PKCζ by an in vitro kinase assay (Fig. 5A) as well as the expression of ZO-2 by immunofluorescence and Western blotting (Fig. 5B and C). The activity of PKCζ is reduced during incubation with EcN as well as during co-incubation with EcN and EPEC. In contrast EPEC leads to an activation of PKCζ (Fig. 5A). Apparently the PKCζ-PS inhibitor affects ZO-2 localization to the cellular boundaries as immunofluorescence indicates ZO-2 to be present in the cytosol to a large extent (Figs 5A and 2). Interestingly, PKCζ appears also to be involved in the EPEC-induced rearrangement of ZO-2 as re-localization of this protein is prevented by the PKCζ pseudosubstrate inhibitor (Figs 5A and 4). Moreover, the total amount of protein available in the cells is reduced (Fig. 5C, lane 4) to an even greater extent than with the inhibitor alone (Fig. 5C, lane 2). After incubation with EcN, the amount of ZO-2 is comparable to that of uninfected cells (Fig. 5C, lanes 1 and 3), but due to the inhibition of PKCζ no translocation of ZO-2 to the membrane occurs (Figs 5B and 3).

Immunostaining of ZO-2 and PKCζ in T84 monolayers demonstrated that in non-infected cells PKCζ and ZO-2 are primarily detectable in the cytosol near the cell boundaries with only a limited amount of protein being concentrated at the cellular contact sites and appear to be colocalized (Fig. 6). After EPEC infection staining of ZO-2 exhibits a disorganized pattern and colocalization with PKCζ is reduced as indicated by the merged micrographs. After incubation with EcN double staining for PKCζ and ZO-2 demonstrated that both proteins were colocalized and were present in higher concentrations directly at the cellular contact sites (Fig. 6).

Discussion

The overall importance of commensal and probiotic bacteria for the physiology and immunology of the intestinal tract has been well documented (e.g. FAO/WHO, 2001; Otte and Podolsky, 2004; Yan and Polk, 2004; Xavier and Podolsky, 2005; Liévin-Le Moal and Servin, 2006; O’Hara and Shanahan, 2006; Sekirov and Finlay, 2006) and probiotic bacteria have been regularly employed for therapeutic purposes (e.g. Rembacken et al., 1999; Podolsky, 2002; Schultz et al., 2003; Kruis, 2004; Kruis et al., 2004; Sartor, 2004; Sazawal et al., 2006). However, the mechanisms underlying the beneficial effects observed upon treatment with probiotic bacteria are not well understood.

In this study, mechanisms contributing to the interactions of the probiotic EcN have been investigated using polarized intestinal T84 human epithelial cells as a model system for the intestinal barrier. With a focus on epithelial barrier function the activities of EcN have been compared with the effects induced by the prototype EPEC strain E2348/69. In addition, for profiling host cell responses to probiotic versus pathogenic bacteria we employed DNA microarrays. Our findings demonstrate that the probiotic EcN strain does sustain TER of T84 monolayers and – for comparison – also of Caco-2 monolayers. Moreover, we found that EcN is able to counteract and even abrogate the barrier disrupting activities found upon infection with the EPEC E2348/69. In the following we therefore sought to identify molecular mechanisms that might contribute to the rescue of barrier functions.

One of the prominent members of TJ protein complexes is the ‘zonula occludens protein-2’ (ZO-2) that has been shown previously to be negatively affected by EPEC (Philpott et al., 1998). In contrast to EPEC infections (Fig. 3B), co-incubation with EcN resulted in a close association of ZO-2 with the cytoskeleton and a concentration of ZO-2 at the cellular contact sites (Fig. 3C) that is known to stabilize TJ structures and helps to maintain the cell

Fig. 2. Changes in ZO-2 mRNA of T84 cells as compared with untreated cells (set to 100%) after incubation with EcN (A), EPEC (B), and after co-infection with both bacteria (C) during a time-course of 0–120 min as measured by quantitative RT-PCR.
Fig. 3. ZO-2 distribution after co-incubation of T84 cells with EcN and EPEC. T84 monolayers were infected with bacteria and stained for ZO-2.

A. Control, uninfected cells.
B. Cells infected with EPEC for 120 min.
C. Cells incubated with EcN for 120 min.
D. Cells infected with EPEC then washed and incubated with regular medium for 60 min.
E. Cells incubated with EPEC for 60 min then washed and incubated with EcN for 60 min.
F. Cells co-incubated with EcN and EPEC for 120 min at a 1:1 ratio.
G. T84 cells were incubated with EcN, EPEC or both for 60 and 120 min. Cells were fractionated into cytosolic, membrane and cytoskeletal fractions. Subsequently, proteins were analysed by Western blotting with anti-ZO-2 antibody to examine the subcellular redistribution of ZO-2. Bars = 5 μm.
morphology of T84 cells (Chen et al., 2002). In addition, we found that EcN leads to an increase of ZO-2 mRNA and protein as has been shown by immunofluorescence and Western blotting experiments (Fig. 3C and G top row). This is in contrast to EPEC E2348/69 infection that causes a decrease in ZO-2 mRNA (Fig. 2B) and protein expression (Fig. 3G middle). These results demonstrate that the amount and localization of ZO-2 appear to be crucial for the beneficial effects of EcN. Interestingly, co-incubation experiments of T84 cells with both EcN and EPEC simultaneously demonstrated that EcN abrogates the detrimental effects of EPEC E2348/69 (Fig. 3F and G bottom). Moreover, even when EcN was applied considerably late in the EPEC infection barrier function was restored (Fig. 3E) indicating an important overriding protective effect of the probiotic EcN against the damaging activity of EPEC. When compared with the probiotic effect of Lactobacillus acidophilus (strain ATCC4356) investigated in a previous study by Resta-Lenert and Barrett (2003) that showed that only the pretreatment but not the simultaneous exposure of epithelial cells with L. acidophilus prevents the invasion of an enteroinvasive E. coli strain (EIEC O29:NM), this demonstrates an extended activity of the probiotic EcN. These findings as well as the recent report of Sazawal et al. (2006) on the differential effects of probiotics in the prevention of acute diarrhoea further support the notion that probiotics exert characteristic and strain-specific effects on the intestinal barrier.

The assembly of TJs and the maintenance of barrier functions involve the phosphorylation of key proteins by members of the PKC family (Avila-Flores et al., 1990; Stuart and Nigam, 1995). Inhibition of PKCζ, the only PKC isoform located at the TJ complexes (Dodane and Kachar, 1996), protects against disruption of TJs caused by EPEC infection by preventing the removal of ZO-2 from TJs to the cytoskeleton. This prompted us to further investigate the effect of EPEC and EcN on the expression and distri-
bution of PKCζ. Following the co-incubation of epithelial cells with EcN expression of ZO-2 is upregulated and translocated to the cytoplasmic membrane in a process that also appears to involve PKCζ as inhibition of PKCζ (Fig. 5B3) at least partially inhibits the concentration of ZO-2 at the cellular contact sites (Figs 3C and 5). This suggests that the relocalization of ZO-2 by EcN might be regulated via multiple pathways.

As demonstrated in Fig. 4A and B incubation of T84 monolayers with EPEC resulted in a redistribution of PKCζ from a primarily cytosolic location to the cellular contact sites reflected also by the increase of PKCζ in the membrane fraction (Fig. 4B left). In contrast, incubation with EcN enhanced the expression of PKCζ primarily in the cytosol (Fig. 4B centre) whereas co-incubation of T84 with EPEC and EcN resulted in a distribution of PKCζ reminiscent of untreated control cells (Fig. 4B right). This is in accordance with earlier reports on the TJ-disrupting effects of overexpression of a kinase-dead PKCζ mutant (Suzuki et al., 2001) and the identification of the N-terminal fragment (1–126 aa) of PKCζ as the responsible domain in MDCK II epithelial cells (Gao et al., 2002). These authors further showed that in epithelial cells Cdc42 and Par6 negatively regulate TJ assembly. In light of these observations a possible interpretation of our data for the effect of EPEC is that by concentrating PKCζ at the cellular boundaries the interaction of the regulatory domain of PKCζ with and the subsequent activation of Par6 might be enhanced which in turn inhibits TJ assembly. However, whether this hypothesis is indeed correct will require further investigations.

As only active PKCζ is able to phosphorylate ZO-2 and only the phosphorylated form of ZO-2 can be withdrawn from the TJ complex resulting in destabilization of the TJs (Avila-Flores et al., 1990), alterations in the activity status of PKCζ might also contribute to the relocalization mechanism of ZO-2. To further support this possibility we addressed the activity of PKCζ by in vitro kinase assays.
as described. As depicted in Fig. 5A infection with EPEC enhanced \( \text{PKC}_z \) activity in the cytosol and did not significantly alter its activity in the membrane fraction. This is reminiscent of previous findings where PKC activation has been reported due to infection with EPEC (Crane and Oh, 1997; Savkovic et al., 2003). In contrast, incubation with EcN clearly reduced \( \text{PKC}_z \) activity in both compartments but, interestingly, enhanced the presence of \( \text{PKC}_z \) at the membrane (Fig. 6). Therefore, the beneficial effect of EcN on epithelial barrier function seems to be associated with a block or at least a reduction of \( \text{PKC}_z \) phosphorylation rendering the enzyme partially inactive. This in turn might further enhance the observed concentration of ZO-2 at the cell boundaries.

The beneficial effect of EcN on the formation and stabilization of TJs is a novel mechanism of action of this probiotic strain. As such these findings represent a further piece of a puzzle how to explain the therapeutic efficacy of EcN and potentially also other probiotic bacteria on diarrhoeal diseases and IBD on the molecular level. The results obtained in this study concur very well with recent work from other laboratories in demonstrating an important role for probiotic bacteria in the maintenance and even restoration of barrier function and in the interruption of processes contributing to pathogenicity. Whether for clinical applications the beneficial effects might be enhanced by combining suitable strains of Gram-positive and Gram-negative probiotic bacteria has to await further investigations.

**Experimental procedures**

**Cell culture**

The intestinal epithelial cell line T84 (ECACC, Salisbury, UK, passage 59–71) was grown in a 5% CO\(_2\) humidified incubator at 37°C with medium containing Ham’s F-12 nutrient mixture and DMEM (PAA Laboratories, Cölbe, Germany) supplemented with 10% FCS, antibiotics (PenStrep) (Madara et al., 1987). The human enterocyte cell line Caco-2 cell clone 1 (ECACC, passage 20–30) was cultured in DMEM medium (25 mM glucose) supplemented with 10% FCS, antibiotics (PenStrep) (Madara et al., 1987). The human enterocyte cell line Caco-2 cell clone 1 (ECACC, passage 20–30) was cultured in DMEM medium (25 mM glucose) supplemented with 10% FCS, 1 mg ml\(^{-1}\) transferrin and antibiotics. Cells were passaged and plated on collagen-coated flasks, filters and glass coverslips, as described previously (Yuhan et al., 1997; Hecht and Koutsouris, 1999). On Transwell filters (6.5 mm diameter, polycarbonate membrane, 0.4 \( \mu \)m pore size, Costar, Corning, NY) T84 cells were seeded at a density of \( 5 \times 10^5 \) cells/insert as described (Savkovic et al., 1996; Madsen et al., 2001). Monolayers were fed every other day.

**Bacterial strains and infection**

Overnight cultures of EcN and of EPEC strain E2348/69, grown in Luria–Bertani broth, were diluted (1:33) in serum- and

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Fig. 6. Monolayers were incubated with EcN or EPEC for 120 min and then double-stained for ZO-2 and \( \text{PKC}_z \). Panels in the left column were stained with anti-\( \text{PKC}_z \) antibody (green), those in the middle column were stained with anti ZO-2 antibody (red), and the panels in the right column represent the overlay (merge) of the two images. Bars = 5 \( \mu \)m.
antibiotic-free tissue culture medium containing 0.5% mannose and grown at 37°C to mid-log growth phase. Epithelial cell monolayers were infected as previously described (Tomson et al., 2004) with a moi of 100. In co-infection experiments with EPEC and EcN, both strains were present in a ratio of 1:1 at a combined moi of 100.

**RNA extraction and cRNA preparation**

Total RNA was extracted using the RNeasy Minikit (Qiagen, Hilden, Germany) and cRNA was prepared as described previously (Thykjaer et al., 2001). Briefly, RNA was isolated from tissue culture flasks. Total RNA samples were used to generate biotinylated cRNA targets according to the ‘Affymetrix Microarray Suite 4.0 User Guide’. Enzymes were supplied by Invitrogen (Breda, the Netherlands) and Roche (Mannheim, Germany). The oligo-dT primer containing a T7 RNA polymerase promoter was purchased from Ambion (Huntington, UK). Labelled cRNA was prepared by using the ‘Microarray Target Synthesis Kit’ and biotin labelled UTP from Roche (Mannheim, Germany) and hybridized to Hu U95A chip sets (representing 12 000 human DNA sequences, Affymetrix, Santa Clara, CA) according to the supplier’s instructions.

**Hybridization and scanning of DNA microarrays**

Hybridization and staining was done according to the ‘Affymetrix Microarray Suite 4.0 User Guide’. Quality control and data analysis were performed using the GeneData ‘Expressionist®’ software. For this set-up we considered a 1.8-fold difference in gene expression in log-log plots to be significant, when comparing epithelial cell monolayers with or without the addition of bacteria between co-incubated and not co-incubated cells.

**Confirmation of gene expression data by quantitative RT-PCR**

Changes in gene expression were independently verified by RT-PCR employing the LightCycler amplification and detection system (Roche Molecular Biochemicals, Mannheim, Germany) as described (Li et al., 2002). Gene-specific primer pairs were designed by using the ‘primer3’ software (Whitehead Institute for Biomedical Research, Cambridge, MA) using the HPRT I gene (hypoxanthine phosphoribosyl-transferase I), a low-abundance housekeeping gene, as the reference (Vandesompele et al., 2002). Data were analysed by application of the quantification program software (Version 3.3, Roche Molecular Biochemicals, Mannheim, Germany).

**Transepithelial electrical resistance**

T84 cells were seeded onto Transwell filters at a density of $5 \times 10^5$ cells/insert as described earlier (Li et al., 2002). Monolayer confluency and barrier properties were documented by measuring TER before and after apical exposure to bacteria using an epithelial tissue voltohmmeter (EVOM, World Precision Instruments, Sarasota, FL) and Endohm-6 chambers specifically designed for use with Transwell filters. Data were calculated by subtracting the value of a blank insert and normalized for growth area (ohm cm²).

**Cell fractionation and Western blotting**

After incubation T84 cells were washed three times with ice-cold phosphate-buffered saline (PBS) and immediately scraped into 750 μl of cold Lysis Buffer A (20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 4 mM EDTA, 2 mM EGTA, 1 mM Na$_2$VO$_4$, 50 mM NaF, containing ‘Complete protease inhibitor cocktail’ tablets, Roche, Mannheim, Germany), kept at 4°C. The cells were lysed on ice using a Branson ultra-sonifier (Branson Ultrasoundics, Danbury, CT).

Whole cell lysates and the accompanying cytosolic, membrane and cytoskeletal fraction were prepared as described elsewhere (Smith et al., 2005). ZO-2 was immunoprecipitated from detergent-soluble and -insoluble fractions with rabbit polyclonal ZO-2 or mouse monoclonal PKCζ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) using 50 μl of protein A-coupled microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany).

Immunoprecipitates were analysed by SDS-PAGE and Western blotting and stained for ZO-2, PKCζ (Santa Cruz Biotechnology, CA), or phospho-PKCζ (Cell Signaling Technology, Beverly, MA).

**PKC activity and in vitro kinase assays**

Confluent T84 monolayers were incubated with EPEC or EcN. Infected and control cells were washed three times with ice-cold PBS and scraped into 750 μl of ice-cold ultrasonic buffer: 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 1 mM NaF, 0.4 mM Na$_2$VO$_4$ and ‘Complete protease inhibitor cocktail tablets’ (Roche, Mannheim, Germany).

The cells were lysed on ice as described above. The resulting supernatants represented the membrane fraction. Polyclonal antibodies against PKCζ (3 μg), protein-A microbeads and the lysates were incubated overnight at 4°C. The kinase assay (PepTag non-radioactive protein kinase C assay, Promega, Madison, WI) was carried out as described elsewhere (Cario et al., 2004). All experiments were repeated at least three times.

**Immunofluorescence detection of TJ components**

T84 cells were seeded onto glass coverslips, grown to confluency and treated as described above. Cells were washed three times with PBS and then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After fixation, samples were quenched with 50 mM NH$_4$Cl (in PBS) for 15 min, and again washed with PBS. Cells were blocked with normal serum-blocking solution (2% goat or donkey serum; 1% BSA; 0.1% Triton X-100; 0.05% Tween 20; in PBS, pH 7.2) for 20 min at room temperature and incubated with primary antibodies (ZO-2 and PKCζ 1:100) in blocking buffer for 1 h at room temperature. Cy2-conjugated goat anti-mouse (Sigma, Taufkirchen, Germany) and Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, Dianova, Hamburg, Germany) antibodies were used as secondary antibodies (dilution 1:100, 60 min at room temperature). Samples were mounted and evaluated within the next 24 h by laser-scanning confocal microscopy (magnifica-
tion 63x, Zeiss Axiosvert 100M-LSM 510, Jena, Germany). All images were recorded with identical laser settings and were processed using standardized two-colour or monochannel settings (software LSM 510 v3.2).

**Treatment of T84 cells with inhibitor**

T84 cells were treated with the PKCζ-specific pseudosubstrate inhibitor (10 μM; PKCζ-PS, Tocris, Ellisville, MO) for 1 h prior to co-incubation with EcN or EPEC.

**Statistical analysis**

Experiments have been performed independently three to five times. Statistical analysis was performed using the Student’s t-test. Error bars represent the standard error of the mean.

**Acknowledgements**

This study has been supported by grants from the Deutsche Forschungsgemeinschaft (DFG SFB93 TP B5), the Bundesministerium für Bildung and Forschung [BMBF: Project Network of Competence Pathogenomics Alliance ‘Functional genomics research on enterohaemorrhagic, enteropathogenic and enteroreaggregative Escherichia coli’, PG Karch/Schmidt; Universität Münster (BD No. 119523/207800) PTJ-BIO/ 03U213B VBIIPG3], and from Ardeypharm GmbH (Herdecke). This study is part of the PhD thesis of A.A.Z. A.A. Zyrek has been supported by a research grant of Ardeypharm GmbH. C. Enders and U. Sonnenborn are scientific staff members in the Department of Biological Research of Ardeypharm GmbH in Herdecke.

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