Induction of Human β-Defensin 2 by the Probiotic Escherichia coli Nissle 1917 Is Mediated through Flagellin

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Human β-defensin 2 (hBD-2) is an inducible antimicrobial peptide synthesized by the epithelium to counteract bacterial adherence and invasion. Proinflammatory cytokines, as well as certain bacterial strains, have been identified as potent endogenous inducers. Recently, we have found that hBD-2 induction by probiotic Escherichia coli Nissle 1917 was mediated through NF-κB- and AP-1-dependent pathways. The aim of the present study was to identify the responsible bacterial factor. E. coli Nissle 1917 culture supernatant was found to be more potent than the pellet, indicating a soluble or shed factor. Chemical analysis demonstrated the factor to be heat resistant and proteinase digestible. Several E. coli Nissle 1917 deletion mutants were constructed and tested for their ability to induce hBD-2 expression in Caco-2 cells. Deletion mutants for flagellin specifically exhibited an impaired immunostimulatory capacity. Reinsertion of the flagellin gene restored the induction capacity to normal levels. Isolated flagellin from E. coli Nissle 1917 and from Salmonella enterica serovar Enteritidis induced hBD-2 mRNA significantly in contrast to the flagellin of the apathogenic E. coli strain ATCC 25922. H1 flagellin antiserum abrogated hBD-2 expression induced by flagellin as well as E. coli Nissle 1917 supernatant, confirming that flagellin is the major stimulatory factor of E. coli Nissle 1917.

Probiotics are microbial organisms beneficial to health (3, 15). The spectrum of probiotics, including Escherichia coli, lactobacilli, bifidobacteria, and streptococci, as well as yeasts, is as complex as their clinical applications. For example, the duration of traveler’s diarrhea and other self-limited gastrointestinal infections is shortened by probiotics (24, 34), especially in infants (9, 33). Allergies seem to be reduced, and the incidence of infections is decreased after administration of the probiotic E. coli strain PZ 720 to newborns (18). However, the best evidence-based indication is the remission maintenance of ulcerative colitis by E. coli Nissle 1917 (16, 17, 31). Using this strain experimental murine colitis has been ameliorated (36). Also, a probiotic mixture composed of eight different strains, named VSL#3, is effective in primary and secondary prevention of pouchitis after proctocolectomy in ulcerative colitis (11, 12). The numerous effects of probiotics are difficult to explain by a unifying hypothesis that is based on a single quality or mechanism. The mechanisms of action proposed thus far include an alteration of the host’s cytokine repertoire (21), increased immunoglobulin A secretion (22, 26), and mucus formation (20), lymphocyte or macrophage activation (13, 26, 35), or an inhibition of epithelial adhesion and invasion (4, 21).

The genome of E. coli Nissle 1917 (serotype O6:K5:H1) has recently been sequenced (14, 39) and contains many characteristic fitness factors that promote its competitiveness (e.g., six iron uptake systems) and highly effective colonization (e.g., several types of adhesins) of the host (19). Recent in vitro data have demonstrated that E. coli Nissle 1917 induces intestinal epithelial cells to block adherence and inhibit invasion of various pathogenic strains (1, 5). These observations proposed a soluble factor responsible for the inhibition of the pathogen itself or engagement of an epithelial defense mechanism (1) apart from anti-inflammatory influences on T-cell proliferation and function (38). We have recently provided a possible explanation for the probiotic effect of E. coli Nissle 1917 as it induces human β-defensin 2 (hBD-2) expression in cell culture in a time- and dose-dependent manner (41). Since this antimicrobial peptide, produced by the mucosal epithelium, has a broad antibiotic spectrum against gram-negative and -positive bacteria, as well as against fungi and viruses, it may reinforce the mucosal barrier, thereby limiting bacterial adherence and invasion. E. coli Nissle 1917, in contrast to most other E. coli strains, induces hBD-2 expression via nuclear factor-κB (NF-κB)-and, to a smaller extent, activator protein-1 (AP-1)-dependent pathways. The aim of the present study was to identify the factor(s) of E. coli Nissle 1917 responsible for hBD-2 induction.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in the present study are shown in Table 1. Bacteria were grown overnight at 37°C under gentle agitation at 200 rpm in Trypticase soy broth (TSB). To obtain bacteria in a linear growth phase, 100 μl of the bacterial suspension was added to 10 ml of fresh TSB medium and grown under permanent shaking for 5 h. Heat inactivation was carried out in a water bath at 65°C for 1 h. Bacterial culture supernatants were collected by centrifugation at 4,000 × g for 10 min. Bacteria were adjusted to a density of 3 × 108 cells/ml, and the supernatant was diluted equally in fetal calf serum (FCS)- and antibiotic-free culture medium.

Cell culture. Caco-2 cells (German Collection of Microorganisms and Cell cultures [DSMZ] ACC 169) and SW-620 (American Type Culture Collection [ATCC] CCL-227) were grown in Dulbecco modified Eagle medium (DMEM)
### TABLE 1. Bacterial strains used in this study

| Strain | Serotype | Relevant characteristic | Source
<table>
<thead>
<tr>
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<tr>
<td>E. coli Nissle 1917 wild type</td>
<td>O6:K5:H1</td>
<td>Probiotic strain</td>
<td>ACS</td>
</tr>
<tr>
<td>E. coli Nissle mutant strain</td>
<td>O6:K2:H1</td>
<td>Intestinal isolate</td>
<td>IMBI</td>
</tr>
<tr>
<td>E. coli JM109</td>
<td>O4:H-</td>
<td>Clinical isolate</td>
<td>ACS</td>
</tr>
<tr>
<td>E. coli ATCC 35292</td>
<td>O4:H-</td>
<td>Intestinal isolate</td>
<td>RBS</td>
</tr>
<tr>
<td>E. coli ATCC 5323</td>
<td>O4:H-</td>
<td>Intestinal isolate</td>
<td>RBS</td>
</tr>
<tr>
<td>S. enterica serovar Enteritidis</td>
<td>O4:H-</td>
<td>Intestinal isolate</td>
<td>RBS</td>
</tr>
</tbody>
</table>

*a Deletion of toxin alpha-hemolysin.
b ACS, Ardeypharm Collection of Strains (focal isolates); DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; IMBI, strain collection of the Institute of Molecular Biology of Infection, University of Würzburg, Würzburg, Germany; RBS, clinical isolate from the Robert Bosch Hospital, Stuttgart, Germany.

**Note**

E. coli Nissle 1917 (EcN) mutant strains, i.e., the ΔHPI (yersinia-inactin-gene cluster), Δfim (type 1 pili), Δfoc (FIC pilin), Δmfd/mce (type 1 pili plus FIC pilin), Δsfa/SfaA (curli-negative), ΔfliB (cellulose-synthesis-gene cluster), Δactn,ΔMAB (micronuclear-negative), ΔK5 (5′-gene cluster), Δβc2 (beta-lactamase), ΔfliC (flagellin filament), and ΔΔH (hook) mutant strains, were constructed according to a previously described method (6). EcNaeC (E. coli Nissle 1917 cured of both plasmids) was generated according to the German patent 103 28 669 and lacks its strain-specific plasmids. The single gene clusters were replaced by a specific plasmid and 0.05 μg of streptomycin/ml. T84 cells (ATCC CCL-248) were maintained in a 1:1 mixture of DMEM and Ham F-12 medium supplemented with 15 m M HEPES and 2 mM glutamine supplemented with 10% FCS. 50 μg of gentamicin/ml, and 5% nonessential amino acids. T84 cells (ATCC CCL-248) were maintained in a 1:1 mixture of DMEM and Ham F-12 medium supplemented with 15 m M HEPES, 14 mM NaHCO3, 5% FCS, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. For stimulation experiments, cells were seeded into 12-well culture plates (Becton Dickinson GmbH, Heidelberg, Germany). After an overnight culture in FCS- and antibiotic-free DMEM, ~10% confluent cells were incubated with bacterial culture supernatant, pellet, or flagellin for 6 h at 37°C. For signaling pathway studies, specific mitogen-activated protein (MAP) kinase inhibitors AG126 (Calbiochem, Darmstadt, Germany) and 3-4-dimethyl sulfoxide (DMSO). Cells were pretreated with the specific ERK1/2 (for extracellular signal-regulated kinase 1/2), p38, and JNK (for c-Jun N-terminal kinase) inhibitors 1 h prior to stimulation experiments. Cell viability was assessed by the MTT cell proliferation assay (ATCC, Manassas, VA) according to the manufacturer’s protocol.

### Construction of bacterial mutants

The E. coli Nissle 1917 (EcN) mutant strains, i.e., the ΔHPI (yersinia-inactin-gene cluster), Δfim (type 1 pili), Δfoc (FIC pilin), Δmfd/mce (type 1 pili plus FIC pilin), Δsfa/SfaA (curli-negative), ΔfliB (cellulose-synthesis-gene cluster), Δactn,ΔMAB (micronuclear-negative), ΔK5 (5′-gene cluster), Δβc2 (beta-lactamase), ΔfliC (flagellin filament), and ΔΔH (hook) mutant strains, were constructed according to a previously described method (6). EcNaeC (E. coli Nissle 1917 cured of both plasmids) was generated according to the German patent 103 28 669 and lacks its strain-specific plasmids. The single gene clusters were replaced by a specific plasmid and 0.05 μg of streptomycin/ml. T84 cells (ATCC CCL-248) were maintained in a 1:1 mixture of DMEM and Ham F-12 medium supplemented with 15 m M HEPES, 14 mM NaHCO3, 5% FCS, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. For stimulation experiments, cells were seeded into 12-well culture plates (Becton Dickinson GmbH, Heidelberg, Germany). After an overnight culture in FCS- and antibiotic-free DMEM, ~10% confluent cells were incubated with bacterial culture supernatant, pellet, or flagellin for 6 h at 37°C. For signaling pathway studies, specific mitogen-activated protein (MAP) kinase inhibitors AG126 (Calbiochem, Darmstadt, Germany) and 3-4-dimethyl sulfoxide (DMSO). Cells were pretreated with the specific ERK1/2 (for extracellular signal-regulated kinase 1/2), p38, and JNK (for c-Jun N-terminal kinase) inhibitors 1 h prior to stimulation experiments. Cell viability was assessed by the MTT cell proliferation assay (ATCC, Manassas, VA) according to the manufacturer’s protocol.

### Purification of flagellin

Flagellin of diverse bacterial strains was isolated as previously described (25). Briefly, 1 liter of TSB was inoculated with 10 ml of the bacteria and cultured for 16 h at 37°C, pelleted by centrifugation (13,000 × g, 30 min, 4°C), and resuspended in 20 ml of phosphate-buffered saline (PBS). For the flagellin isolation from bacterial supernatant, bacteria were heat-killed, and the supernatant was separated by centrifugation. The precipitated bacteria suspension or the supernatant was adjusted to pH 2 with 1 M HCl and maintained at that pH under constant stirring at room temperature for 30 min. After centrifugation (100,000 × g, 4°C, 1 h), the supernatant containing soluble monomeric flagellins was adjusted to 7.2 with 1 M NaOH. Solid (NH4)2SO4 was added slowly with constant stirring to obtain 65% saturation. After an overnight incubation at 4°C, the precipitate was pelleted by centrifugation (15,000 × g, 15 min). The precipitate was dissolved in distilled water and dialyzed against distilled water for 16 h with three changes of water. To remove heat-labile proteins, the dihydrate was heated at 65°C for 15 min, placed on ice, and centrifuged (100,000 × g, 4°C, 1 h). Then, 0.7 M solid (NH4)2SO4 was added to the supernatant, which contained depolymerized flagellin. After incubation and depolymerization, flagellin was polymerized for another night at 4°C. polymerized flagellin was collected by centrifugation (100,000 × g, 4°C, 1 h) and dissolved in PBS. The protein concentration of isolated flagellin was determined by using the Bradford assay (Bio-Rad) and bovine serum albumin as a standard. The molecular size of the flagellin was identified by mass spectrometry. The samples were diluted 1:10 in 50% acetonitrile containing 0.02% formic acid and analyzed by electrospray mass spectrometry in the positive ionization mode with a quadrupole-orthogonal accelerating-time-of-flight mass spectrometer (Micromass, Manchester, United Kingdom). The spectra were evaluated by using the program MassLynx 3.5. (Micromass).

### Coomassie staining and Western blotting of flagellin

To verify the purity of the flagellin, the isolated protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel (InVitrogen, Carlsbad, CA). The gels were stained for protein with Coomassie brilliant blue. For Western blotting, isolated protein was transferred to 0.45-μm-pore-size nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were incubated with 5% skimmed milk powder in TBST (20 mM Tris-Base [pH 7.4], 0.14 M NaCl, 0.1% Tween 20) for 1 h. E. coli H1-antisera (Statens Serum Institute, Copenhagen, Denmark) served as primary antibody and was diluted 1:500 in TBST. After overnight incubation, strips were washed and incubated with horse- radish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Dianova, Hamburg, Germany) diluted 1:5000 in TBST for 1 h. The membranes were washed in TBST, followed by incubation for 5 min in SuperSignal West Dura extended duration substrate (Pierce, Rockford, IL). Developing bands were detected with a chemiluminescence camera charge-coupled device LAS-1000 (Fuji), and analysis was performed with the software AIDA 2.1 (Raytest, Straubenhardt, Germany).

### Luciferase reporter gene assay

To assess hBD-2 promoter activity, Caco-2 cells were seeded into 12-well culture plates (2.8 × 10^4 cells/ml) and transfected with 107% confluence. The luciferase reporter constructs for hBD-2-2338-luc, D301C (FUGENE 6 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s protocol. At the end of the stimulation experiment, Renilla luciferase activity from the hBD-2-pGL3 reporter vector and Renilla luciferase activity were analyzed with the Dual-Luciferase reporter assay system (Promega) using a luminometer (Berthold). Promoter activity was normalized to the activity of the internal Renilla luciferase control.

### RNA isolation and CDNA synthesis

At the end of the stimulation experiment, cells were washed twice with PBS and harvested with TRIzol reagent (Invitrogen), and RNA was isolated according to the supplier’s protocol. RNA quality and quantity were determined by gel electrophoresis and photometry. Subse-
ently, 1 µg of total RNA was reverse transcribed into cDNA with oligo(dT) primers and 15 U of avian myeloblastosis virus reverse transcriptase (Promega)/µg according to standard procedures.

**Real-time RT-PCR.** Real-time reverse transcription PCR (RT-PCR) analyses were performed in a fluorescence temperature cycler (LightCycler; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. This technique continuously monitors the cycle-by-cycle accumulation of fluorescently labeled PCR product. cDNA corresponding to 10 ng of RNA served as a template in a 10-µl reaction mixture containing 3 mM MgCl2, 0.5 µM concentrations of each primer, and 1× LightCycler-FastStart DNA Master SYBR green 1 mix (Roche Diagnostics GmbH). Samples were loaded into capillary tubes and placed in the fluorescence thermocycler (LightCycler). Initial denaturation at 95°C for 10 min was followed by 45 cycles of 95°C for 30 s, the primer-specific annealing temperature for 30 s, and elongation at 72°C for 30 s.

**For hBD-2 (sense, 5'-ATACGCAGTGGGTCCTTG-3'; antisense, 5'-GAG ACCACAGTGGCAAATT-3')** the annealing temperature was set at 62°C. Amplification using these primers resulted in a 172-bp fragment. For the housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase; sense, 5'-CAGGCGGACCACCACCTGC-3'; antisense, 5'-ATGAGCCCCAGCTTCTC CAT-3'), we used a touchdown protocol with a primary temperature of 66°C and a target temperature of 60°C. At the end of each run, melting-curve profiles were achieved by cooling the sample to 65°C for 15 s and then heating the sample slowly to 90°C with 9°C steps to confirm the amplification of specific transcripts. Cycle-to-cycle fluorescence emission readings were monitored and analyzed by using LightCycler software (Roche Diagnostics GmbH). Melting curves were generated after each run to confirm the amplification of specific transcripts. The specificity of the amplification products was verified by subjecting the amplification products to electrophoresis on a 2% agarose gel. Obtained fragments were visualized by ethidium bromide staining. All quantifications were normalized to the housekeeping GAPDH gene. Relative expression is given as a ratio between target gene and GAPDH gene expression.

**Swarm plate assay.** Swarm agar (0.3%; containing 10 g of NaCl, 5 g of yeast extract, 10 g of peptone, and 3 g of agar per liter) was used to evaluate the motility of *E. coli* Nissle 1917 wild type and the ΔfliC, ΔflgE, ΔflaA, and complemented ΔfliCΔfliB2 and ΔflgEΔflpB3 mutant strains. Small wells were punched out in semisolid LB agar plates, loaded with 5 µl of each strain, and incubated for 10 h at 37°C.

**IL-8 ELISA.** Interleukin-8 (IL-8) secretion in response to IL-1β and *E. coli* Nissle 1917 supernatant was measured in cell culture supernatants from Caco-2, T84, and SW620 cells by enzyme-linked immunosorbent assay (ELISA; OfectEA human IL-8 ELISA kit II; BD Biosciences, San Diego, CA) according to the supplier’s protocol.

**Statistics.** Statistical evaluation was performed with the software package GraphPad Instat (version 3.1 for Windows; GraphPad Software, San Diego, CA). For the description of random samples, the arithmetic mean ± standard deviation (SD) or the median with minimum and maximum values was used. To test for normality, we used the Kolmogorov-Smirnov test. When data failed to follow a Gaussian distribution, nonparametric analysis of variance was performed by using Kruskal-Wallis or Mann-Whitney test.

**RESULTS**

Caco-2 cells have the highest potential to express hBD-2. In order to identify the most suitable model for studying the hBD-2-inducing effect of *E. coli* Nissle 1917, various intestinal epithelial cell lines (Caco-2, T84, and SW620 cells) were stimulated with IL-1β and *E. coli* Nissle 1917 supernatant. SW620 cells did not express hBD-2 mRNA after stimulation with IL-1β and expressed only a small amount of hBD-2 after stimulation with the bacterial supernatant (Table 2). In contrast, IL-1β as well as *E. coli* Nissle 1917 supernatant strongly induced hBD-2 in Caco-2 cells. This effect was less pronounced in T84 cells especially regarding IL-1β as stimulant. The spread of GAPDH mRNA expression levels of the different cell types and between the experiments was narrow, indicating a low variability. IL-8 served as a control response gene measured in culture supernatants by ELISA after 6 h of stimulation. As shown in Table 2, IL-1β as well as *E. coli* Nissle 1917 stimula-

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Mean (ng/ml) ± SD</th>
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</thead>
<tbody>
<tr>
<td>Caco-2</td>
<td>Unstimulated</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>E. coli Nissle supernatant</td>
<td>0 ± 0</td>
<td>820.5 ± 510.2 ± 1865.1</td>
</tr>
<tr>
<td>T84</td>
<td>Unstimulated</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>IL-1β</td>
<td>48.4 ± 18.7</td>
<td></td>
</tr>
<tr>
<td>EcNsn</td>
<td>28.6 ± 17.2</td>
<td></td>
</tr>
<tr>
<td>SW620</td>
<td>Unstimulated</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>EcNsn</td>
<td>13.8 ± 4.4</td>
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*EcNsn, E. coli Nissle supernatant.*

<table>
<thead>
<tr>
<th>TABLE 2. Response of intestinal cell lines to IL-1β (5 ng/ml) and <em>E. coli</em> Nissle supernatant exposure</th>
</tr>
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</table>

*NF-κB.*

The induction of hBD-2 in Caco-2 cells, whereas SW620 cells were expressing IL-8 only after incubation with the bacterial supernatant. The degree of differentiation might also influence hBD-2 expression; hence, we tested hBD-2 transcription levels of cells grown for different time intervals before stimulation. The relative expression of hBD-2 mRNA upon treatment with IL-1β was inferior in confluent versus subconfluent Caco-2 cells (data not shown). Thus, we preferred subconfluent cell layers for our experiments.

**Nonpathogenic *E. coli* Nissle 1917 induces hBD-2 by a supernatant factor.** To analyze the origin of the unknown hBD-2-inducing factor of *E. coli* Nissle 1917, Caco-2 cells were incubated with either resuspended bacterial pellet or supernatant. hBD-2 promoter enhancing activity was predominant in the supernatant (92.7%) compared to the bacterial pellet (7.3%). The other strains tested also displayed most of the activity in the supernatant (Fig. 1). Induction of hBD-2 mRNA by *E. coli* Nissle 1917 was distributed similarly (84.1% activity) in the supernatant. In addition, we tested the stability of the hBD-2-inducing factor in the supernatant of *E. coli* Nissle 1917. Exposure to 100°C for 20 min was unable to abrogate hBD-2 induction compared to *E. coli* Nissle 1917 heated to 65°C (the hBD-2 promoter activity of *E. coli* Nissle supernatant at 100°C was 91.1% ± 18.2% of that of the *E. coli* Nissle supernatant at 65°C). However, the supernatant from unheated bacteria exhibited a diminished promoter inducing capacity (38.1% ± 8.3%).

**T84.**

The remaining supernatant-mediated hBD-2 induction depends on NF-κB and AP-1. The importance of NF-κB and AP-1 binding sites in the promoter region of hBD-2 was ana-

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alized by transfecting Caco-2 cells with hBD-2 promoter luciferase expression constructs encompassing the 5’-untranslated region upstream of the start of gene transcription (2,338 bp). Binding sites for the two 5’ upstream NF-κB sites (positions −205 to −186 and −596 to −572) and AP-1 (positions −127 to −121) were mutated separately or in combination. Consistent with previous findings regarding the bacterial pellet (41), promoter activation by the hBD-2-inducing factor in the supernatant was both NF-κB and AP-1 dependent (Fig. 2). This was confirmed by the complete abrogation of hBD-2 promoter activity after the mutation of both NF-κB and the AP-1 binding sites. Mutation of the AP-1 site alone decreased supernatant-mediated hBD-2 promoter activation by ca. 30%. Promoter activity was almost completely lost after the deletion of both NF-κB sites.

ERK1/2, JNK, and p38 are essential for the E. coli Nissle 1917 supernatant-mediated induction of hBD-2. To gain more insight into the signaling events of the hBD-2-inducing supernatant factor of E. coli Nissle 1917 via the three main MAP kinase pathways, we pretreated Caco-2 cells with specific inhibitors: AG126 for ERK1/2, SB203580 for p38, and SP600125 for JNK. The inhibition of p38 by SB203580 reduced hBD-2 promoter activation by at least 50%; higher doses of the inhibitor were more effective (Fig. 2). Inhibition of ERK1/2 and JNK by AG126 or SP600125, respectively, blocked hBD-2 induction almost completely. An MTT cytotoxicity assay revealed that the decrease in hBD-2 induction was not related to cytotoxic side effects of the inhibitors (data not shown).

Flagellin mutants lose their capacity to induce hBD-2. To clarify which factor in the bacterial culture supernatant is responsible for hBD-2 induction, several E. coli Nissle 1917 strains with mutations of known fitness factors (e.g., adhesins, microcins, siderophores, and flagellin) were constructed and added to Caco-2 cells. The three different mutants of the flagellin gene (Fig. 3a and b) [the ΔfliC, ΔfliE, and ΔfliA mutants] lost their inducing capacity completely, while the other mutants displayed no change compared to the wild-type E. coli Nissle 1917. In order to verify that this loss of hBD-2 induction by the flagellin mutants was specifically attributable to the lack of flagellin, deleted genes were complemented in trans by transforming the respective mutant with a recombinant plasmid harboring the corresponding gene. After complementation, the ΔfliC and ΔfliE mutants regained their capacity to induce hBD-2 mRNA (Fig. 3b). As further proof for the loss of
flagellin synthesis, LB swarm agar plates were inoculated with the different strains. The nonmotile colonies of the *E. coli* Nissle 1917 Δ*fliA*, Δ*fliC*, and Δ*fliE* mutant strains appeared as dots, whereas the wild-type colonies spread out in larger spots. Complementation of the Δ*fliC* and Δ*fliE* mutants only partially restored their ability to swarm (Fig. 3c). This shows that the complementation with the flagellin or the hook gene, respectively, was successful regarding the regain of hBD-2 inducing capacity but failed to accomplish the full motility function of the flagella. The lack of flagellin synthesis after the mutation was also confirmed by Western blotting. Anti-H7 flagellin antiserum showed only a positive band of the proteins of wild-type or complemented *E. coli* Nissle 1917 strains (Fig. 3d).

**Isolated *E. coli* Nissle 1917 flagellin induces hBD-2 mRNA expression in Caco-2 cells.** To determine whether *E. coli* Nissle 1917 H1 flagellin induces hBD-2 expression in intestinal epithelial cells, Caco-2 cells were stimulated with flagellin isolated from wild-type *E. coli* Nissle 1917 (Fig. 4a and b). The molecular mass of the flagellin, determined by mass spectrometry, was 60.81 kDa (Fig. 4c). As shown in Fig. 4d, flagellin induced a dose-dependent increase of hBD-2 promoter activation. Moreover, the preincubation of *E. coli* Nissle 1917 culture supernatants with H1 flagellin antiserum inhibited hBD-2 mRNA expression by almost 70%. Consistent with this observation, the hBD-2 induction by isolated H1 flagellin was inhibited by 50% in the presence of the corresponding antiserum (Fig. 4e).
Since flagellins from different Salmonella species have been shown to induce hBD-2 expression in epithelial cells (25, 27), we isolated flagellin from Salmonella enterica serovar Enteritidis, as well as from the apathogenic E. coli strain ATCC 25922 and the E. coli strain JM109 as controls. The genome structure of E. coli Nissle 1917 was shown to resemble closely that of the uropathogenic E. coli CFT073/hly strain (14). Hence, we compared the hBD-2-inducing capacity of the serotype-identical CFT073Δhly with that of the E. coli Nissle 1917 flagellin. Only flagellin from serovar Enteritidis induced hBD-2 mRNA significantly (Fig. 5a and b) in contrast to the flagellin of the apathogenic E. coli strain ATCC 25922 that lacked an induction. As expected, the E. coli JM109 isolate hardly induced hBD-2, whereas CFT073Δhly was able to induce hBD-2 but not as strongly as the flagellin of E. coli Nissle 1917 (Fig. 5b). The isolated flagellins were also subjected to Western blot analysis with an anti-H1 flagellin antiserum. Since serologically distinct flagella possess a highly conserved primary sequence of flagellin monomers within the exposed central region, all flagellins could be detected by Western blotting despite their different flagellin serotypes (Fig. 5c).

DISCUSSION

The present study suggests that flagellin is the major factor expressed by the probiotic bacterium E. coli Nissle 1917 responsible for the induction of hBD-2 based on the following findings: (i) the factor is released into bacterial supernatant
and is digestible by proteinase, (ii) genetically manipulated E. coli deficient in flagellin or the hook protein fails to induce the defensin expression, (iii) isolated flagellin from the E. coli Nissle 1917 strain is effective through the same signaling pathway as intact E. coli Nissle 1917, and (iv) the effect of isolated flagellin and of E. coli Nissle 1917 supernatant on hBD-2 induction is abrogated by H1 flagellin antiserum. Thus, the Nissle 1917 flagellin appears to be unique among most E. coli strains and mimics the similar induction capacity of flagellin from pathogenic strains, including Salmonella sp. (25).

In order to characterize the responsible factor for hBD-2 induction, we tested known genes, which code for surface expressed or secreted proteins. The genes of known fitness factors, differing from other strains, were therefore deleted to evaluate their relevance for hBD-2 expression. Among the constructed deletion mutants were flagellin-negative mutants, which turned out to be the only strains with a diminished induction potential of bacterial suspension supernatant compared to the pellet. The flagellin might be shed in the supernatant; this was probably triggered by heat treatment.

The next step was the investigation of the signaling pathway used by the stimulatory factor in the E. coli Nissle 1917 supernatant to induce hBD-2 expression. The transcription factors NF-κB and AP-1 appeared to both be necessary for full hBD-2 promoter activation by the bacterial supernatant, similar to our previous findings with the bacterial pellet (41). AP-1 might serve thereby in principal as a synergistic factor, not being able to elicit hBD-2 gene activation without the contribution of NF-κB. An NF-κB-dependent hBD-2 induction through flagellin as the assumed main stimulatory factor in the supernatant seems to be most probable since Salmonella flagellin was shown to induce excessive NF-κB activation in Caco-2BBe cells (8).

Previous experiments regarding signaling events upstream of NF-κB mediated by whole bacteria (41) revealed the explicit involvement of the JNK, whereas the supernatant factor transmitted its signal also via ERK 1/2 and to a smaller extent via p38. Our results indicate that E. coli Nissle 1917 shares with several bacterial pathogens, including Helicobacter pylori and enteropathogenic and enterohemorrhagic E. coli, the ability to activate MAP kinase signaling pathways and epithelial proinflammatory responses such as IL-8 but in a noninvasive manner. In contrast to pathogens, E. coli Nissle 1917 might induce a protective response, including hBD-2 as well as IL-8 but just below the threshold level of an active inflammation.

The isolated flagellin protein was able to induce hBD-2 in a dose-dependent fashion, further substantiating that flagellin alone is a sufficient stimulant. This effect is also not a secondary artifact due to LPS contamination, since this endotoxin lacks any hBD-2-inducing effect in Caco-2 cells (41). Moreover, different flagellin mutant strains failed to show hBD-2 gene induction. The anti-H1 flagellin antiserum displayed also no immunoreactivity against the flagellin isolates of the reference strains. This serological cross-reaction might be explained by the remarkable conservation of the sequences that mediate filament assembly, which indicates that all bacterial flagellins are likely to be packed into filaments in a comparable way (2).

The reduced activity of CFT073Δhly H1 flagellin to induce hBD-2 expression compared to that of E. coli Nissle 1917 may be based on posttranslational modifications, i.e., glycosylation resulting in a different electrophoretic mobility of the CFT073Δhly flagellin in the Western blot.
Similar to our observations, the filament protein flagellin (flfC gene), isolated from Salmonella, has been demonstrated to induce hBD-2 (25). However, the missing pathogenicity of E. coli Nissle 1917 favors positive effects by this strain rather than by Salmonella, despite the defensin induction characteristic of the latter. Finally, the addition of anti-H1 to Caco-2 cells suppressed flagellin-mediated hBD-2 induction by ca. 50%. This abrogation was probably incomplete because the polyclonal H1 flagellin antibody likely had a relatively low binding affinity. We conclude that flagellin is the responsible factor of E. coli Nissle 1917-mediated hBD-2 induction in Caco-2 cells. This finding is remarkable since in another probiotic bacterial mix (VSL#3) the active factor was bacterial CpG-DNA (deoxyadenylate-phosphate-deoxyguanylate), which has been demonstrated elegantly in various experimental animal models (29). Interestingly, the oral route of irradiated bacteria, as well as isolated CpG-DNA from VSL#3, was equally effective (30). Thus, the relevant factors in probiotics appear to differ from strain to strain.

Next to its main function in providing bacterial motility, the flagellum also plays a role in adhesion and biofilm formation on the mucosa (28). Flagellin, the flagellum filament structural protein, triggers further native immune responses since flagellins from Pseudomonas aeruginosa (7), S. enterica serovar Typhimurium (10), and enteraggregative E. coli (37) have been shown to stimulate epithelial cells to express IL-8 or nitric oxide (8). Further, flagellin-deficient enteropathogenic E. coli failed to induce IL-8 secretion (43), and this capacity was restored after complementation of the flfC gene.

The signal might be transmitted by TLR5 (Toll-like receptor), which is expressed in Caco-2 cells, but other pathways might be involved as well (38). Further, flagellin-deficient enteropathogenic E. coli failed to induce IL-8 secretion (43), and this capacity was restored after complementation of the flfC gene.

REFERENCES


