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Genotoxicity of *Escherichia coli* Nissle 1917 strain cannot be dissociated from its probiotic activity

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Abbreviations: ANOVA, analysis of variance; cfu, colony forming unit; DMEM, Dulbecco’s modified Eagle’s medium; DSS, dextran sodium sulfate; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; IBD, inflammatory bowel diseases; IEC, intestinal epithelial cells; IL, interleukin; MPO, myeloperoxidase; UC, ulcerative colitis; WT, wild-type

Oral administration of the probiotic bacterium *Escherichia coli* Nissle 1917 improves chronic inflammatory bowel diseases, but the molecular basis for this therapeutic efficacy is unknown. *E. coli* Nissle 1917 harbors a cluster of genes coding for the biosynthesis of hybrid nonribosomal peptide-polyketide(s). This biosynthetic pathway confers the ability for bacteria to induce DNA double strand breaks in eukaryotic cells. Here we reveal that inactivation of the clbA gene within this genomic island abrogated the ability for the strain to induce DNA damage and chromosomal abnormalities in non-transformed cultured rat intestinal epithelial cells but is required for the probiotic activity of *E. coli* Nissle 1917. Thus, evaluation of colitis severity induced in rodent fed with *E. coli* Nissle 1917 or an isogenic non-genotoxic mutant demonstrated the need for a functional biosynthetic pathway both in the amelioration of the disease and in the modulation of cytokine expression. Feeding rodents with a complemented strain for which genotoxicity was restored confirmed that this biosynthetic pathway contributes to the health benefits of the probiotic by modulating its immunomodulatory properties. Our data provide additional evidence for the benefit of this currently used probiotic in colitis but remind us that an efficient probiotic may also have side effects as any other medication.

Introduction

Probiotics, when administered alive and in adequate amounts, are supposed to be safe and confer health benefits to the host.1 As they are generally marketed as “natural” cures, probiotics benefit also from a positive public image among patients. Nonetheless, physicians need scientific guidance and additional investigations to definitively state on efficacy of probiotics before they can be routinely recommended in clinical practice.2 Several probiotic bacteria have been identified as promising in the management of inflammatory bowel disease (IBD).3,4,5 but only few studies identified the mode of action of these bacteria.6-9 A better understanding of the mechanisms by which probiotics promote health remains critical to fully optimize their safety assessment for human use.10

*Escherichia coli* Nissle 1917 (Mutoxifer®) has been commercially available for almost one century and successfully used in humans as an oral treatment for a number of intestinal disorders.11 A number of studies have shown positive results when this strain is used in conditions such as Crohn disease, pouchitis, irritable bowel syndrome or necrotizing enterocolitis12 but it is especially used in the prevention of relapse in patients with ulcerative colitis (UC). Double blind randomized controlled trials comparing the efficacy of *E. coli* Nissle 1917 to that of mesalazine, which is part of the first-line treatment in patients with UC, have shown that *E. coli* Nissle 1917 is as efficient as mesalazine to prevent relapse of UC.13-15 In addition, the probiotic treatment is associated with a prolonged remission without any reported adverse effects. However, although *E. coli* Nissle 1917 is one of the best studied probiotic bacterial strains, the genetic determinants governing the probiotic nature of *E. coli* Nissle 1917 are yet to be elucidated.16,17

We have previously identified in the genome of *E. coli* Nissle 1917 a cluster of genes named “pks island” that allow production of a hybrid peptide polyketide genotoxin, called Colibactin.18 Up to date, purification of Colibactin has failed and its structure remains unsolved but we previously reported that *E. coli* strains harboring this pks island are able to induce DNA damage in vivo and trigger genomic instability and gene mutations in mammalian cells.19 Given a possible contribution of Colibactin to the development of sporadic colorectal cancer, we wished to explore whether inactivation of this biosynthetic
pathway could improve *E. coli* Nissle 1917 biosafety without altering the health benefits of this probiotic in the management of IBD.

### Results

Construction of an isogenic mutant of *E. coli* Nissle 1917 (Nissle ΔclbA) unable to induce DNA double strand breaks and chromosomal abnormalities in eukaryotic cells. We have previously shown that the clbA gene localized on the pks island codes for a phosphopantetheinyl transferase that catalyzes the essential post-translational activation of polyketide synthases and non-ribosomal polypeptide synthetases involved in *E. coli* essential post-translational activation of polyketide synthases codes for a phosphopantetheinyl transferase that catalyzes the gene localized on the pks previously shown that the clbA gene.

To generate a non-genotoxic isogenic mutant of *E. coli* Nissle 1917, we disrupted clbA by allelic exchange. Whereas the Nissle wild-type (WT) strain was able to inflict high levels of DNA double strand breaks to cultured intestinal crypt cells, the isogenic Nissle ΔclbA mutant was unable to induce such damage, as revealed by analyzing the phosphorylation of the histone γH2AX (Fig. 1A–C). We next assessed chromosomal abnormalities in dividing cells 72 h after infection with a low dose of genotoxic bacteria inducing reversible DNA damage response that did not repair all double strand breaks, and may therefore lead to chromosome abnormalities. Whereas Nissle WT induced chromosomal abnormalities (polyploidy, chromatid break, translocation or ring chromosomes) as previously observed with other pks+ *E. coli* strains,11 the isogenic Nissle ΔclbA mutant was unable to induce such damage (Fig. 1B and C).

The non-genotoxic *E. coli* Nissle 1917 mutant is impaired in prevention of DSS-induced colitis. To examine the probiotic properties of the non-genotoxic Nissle 1917 mutant, we first used a rat model in which colonic inflammation was chemically induced by the administration of Dextran Sodium Sulfate (DSS) in drinking water. DSS induces acute colitis characterized by bloody diarrhea, ulcerations and inflammatory infiltration of the colon. Daily oral administration of PBS, Nissle WT or Nissle ΔclbA was initiated one week before DSS initiation and prolonged during the inflammation. Over the course of the experiments, fecal counts of Nissle WT and Nissle ΔclbA did not differ between groups (Fig. S1). As already observed,12 oral administration of Nissle WT limited the severity of the DSS-induced colitis. Consistent with a significant reduction of the colitis score (Fig. 2B), oral administration of Nissle WT decreased both colonic myeloperoxidase (MPO) activity and pro-inflammatory cytokine IL-1β production and increased production of the regulatory cytokine IL-10 (Fig. 2C and D). Whereas our results support the claim that administration of *E. coli* Nissle 1917 can reduce intestinal inflammation, treatment with the Nissle ΔclbA mutant failed to protect the colon from DSS-induced damage. Indeed, as compared with rats fed with Nissle WT, we observed in rats fed with Nissle ΔclbA mutant...
a significant worsened body weight gain concomitant with enhanced colitis score and MPO activity. The colonic cytokine profiles of rat fed with Nissle ΔclbA mutant were also similar to that seen in DSS-treated rats fed with PBS (Fig. 2).

The non-genotoxic *E. coli* Nissle 1917 mutant exacerbated the mortality of colitic mice. We next examined the probiotic properties of the non-genotoxic *E. coli* Nissle 1917 mutant in a T-cell dependent model of chronic colitis induced by the adoptive transfer of naïve CD4+ CD45RBhigh T cells in immunocompromised SCID mice. Oral treatments with PBS, Nissle WT or Nissle ΔclbA were initiated 3 d after T cell transfer and were repeated every 3 d initially for 8 weeks. The non-transferred control mice remained healthy during the entire observation period. In contrast, the injection of CD4+ CD45RBhigh T cells in PBS-treated SCID mice resulted in discernible symptoms such as hunched posture and weight loss (Fig. 3A), diarrhea, rectal prolapse and anal bleeding. Colitis severity eventually resulted in death of the animal (6.6%) and an increase of mortality especially in animals fed with Nissle ΔclbA (19%) that led us to abort prematurely the experiment at day 45 (Fig. 3B). Body weight loss concomitant with an earlier onset of colitis were also observed in transferred SCID mice fed with both Nissle WT or Nissle ΔclbA (Fig. 3A), but only the treatment with the non-genotoxic Nissle ΔclbA leads to an increased mortality in comparison with mice fed with Nissle WT (5.3%; Fig. 3B). A maximal colitis score of 8 was attributed to mice died from colitis (Fig. 4A) but no further analysis was possible for those animals, leading to underestimation of inflammation assessment particularly for surviving mice fed with the isogenic ΔclbA mutant strain of *E. coli* Nissle 1917.

*E. coli* Nissle 1917 requires a functional pks island for attenuation of CD4+ CD45RBhigh T cell-induced colitis. Among the CD4+ CD45RBhigh T cells transferred SCID mice that survived, those fed with PBS developed inflammation involving the entire colon and resulted in diarrhea, bloody stool and macroscopic damage such as colon wall thickening and colon length shortening (Fig. 4A–C). Microscopically, colonic sections from PBS-treated mice presented prominent epithelial hyperplasia, submucosal edema and massive infiltration in the lamina propria, mainly with mononuclear cells (Fig. 4D). In some diseased mice, crypt abscesses and mucosal ulceration were also observed. All these microscopic and macroscopic changes were accompanied with an increase expression of pro-inflammatory cytokines such as IL-1β and an increase of anti-inflammatory cytokine IL-10 (Fig. 4E). In contrast, oral administration of Nissle WT improved clinical signs of colitis and decreased colonic damage (Fig. 4A and B). Furthermore, Nissle WT treatment improved the shortening and thickening of colonic sections.


the in vitro restoration of genotoxicity displayed by the complemented clbA mutant, we next re-assessed colitis development in CD45RB<sup><small>+</small></sup> T cells injected SCID mice fed with Nissle WT, Nissle ΔclbA at Nissle ΔclbA + clbA. Consistent with our previous data (Fig. 3A), body weight loss kinetics were similar in the three groups (Fig. S3A). Although global colitis severity was exacerbated in this experiment, we re-observed an increased mortality among the group of transferred mice fed with Nissle ΔclbA as compared with the group of transferred mice fed with Nissle WT (Fig. S3B). These results were confirmed by a significantly higher clinical score associated to the treatment of transferred SCID mice with Nissle ΔclbA (Fig. 5A and B). Moreover, restoration of clinical scores similar to those observed with Nissle WT treatment was obtained for mice fed with the complemented clbA mutant. In the same way, as indicated by the measurement of colon weight/length ratio (Fig. 5C) or by the examination of histological sections (Fig. 5D), increased intestinal inflammation resulting from clbA deletion was completely reversed when transferred SCID mice were fed with the Nissle strain in which the wild-type clbA gene was re-introduced within the phi island. These data clearly demonstrate that the immunomodulatory properties of Nissle 1917 required the clbA gene of the phi island and that the probiotic activity of E. coli Nissle 1917 cannot be dissociated from its genotoxicity.

Discussion

Our results together with previous studies<sup>20-23</sup> and clinical trials<sup>15-17</sup> confirm the therapeutic potential of this E. coli Nissle 1917 strain in promoting gut homeostasis upon mucosal injury. This probiotic was regarded, so far, as nonpathogenic,<sup>11</sup> but we have shown that this bacterial strain induce DNA double strand breaks and chromosomal abnormalities in eukaryotic cells. Even if in vivo genotoxicity of this probiotic strain remains to be investigated, we hypothesized that construction of a non-genotoxic mutant of E. coli Nissle 1917 could improve its safety. However, we were unable to dissociate the genotoxic activity of the strain from its probiotic activity since inactivation of the biosynthetic pathway leading to the production of Colibactin abrogated also the probiotic activity in two different models of experimental colitis. Whereas Colibactin appears to be a beneficial compound in the probiotic activity of E. coli Nissle 1917, the nature of this activity remains to be clarified and raises several considerations: Colibactin was first reported to be a genotoxin but our results now suggest that the same molecule may also possess anti-inflammatory activity and could be thus considered as an immune-modulator. This beneficial activity could be directly or indirectly linked to the mechanism triggering DNA damage in the host cell. Alternatively, it is possible that Colibactin may contain of more than one molecule. The biosynthetic gene clusters of the phi island may encode additional compounds that could mediate the probiotic activity observed in the gut.

Since the genotoxic activity of E. coli Nissle 1917 cannot be dissociated from its probiotic activity, safety considerations about large-scale use of this probiotic strain have to be re-evaluated. We have previously shown that the transient DNA damage inflicted
Figure 4. E. coli Nissle 1917 requires a functional pks island for attenuation of CD4+ CD45RBhigh T cell-induced colitis. The extent of colitis and colonic damage were assessed blindly before tissue samples were collected from SCID mice followed as in Figure 3. Error bars represent the SEM and the data shown are the pooled results from duplicate experiments performed in 5–11 mice per group. (A) Plotted data represent pooled colitis scores of each individual mouse and medians are indicated. *p < 0.05 and ** p < 0.01, Kruskal-Wallis test, Dunn’s multiple comparison test. (B) Representative gross organ morphologies of the cecum and colon at day 45 are shown. (C) Samples were measured, rinsed with PBS and weighed. Colon length and mucosa thickening that are dependent of the severity of colitis are modulated according to the oral treatment. Means of colon weight/length ratios are represented. *p < 0.05 and **** p < 0.0001 by one-factor ANOVA analysis, Bonferroni’s multiple comparison test. (D) Colonic sections were stained with H&E to determine disease severity. Left, the scores for several parameters were summed for a total severity score. *p < 0.05, Kruskal-Wallis test, Dunn’s multiple comparison test. Right, representative H&E stained sections from mice colons are shown. Images in each row are the same magnification. Bars = 100 μm (above) or 500 μm (below). The higher magnification below shows massive epithelial hyperplasia and inflammatory cell infiltration into colonic tissues in mice fed with PBS and Nissle ΔclbA compared with those fed with Nissle WT. (E) Colonic samples were homogenized for cytokine expression measurements by q-PCR. Transcriptional expression of IL-1β and IL-10 was normalized to HPRT expression. Represented fold expressions were normalized to PBS-treated colitic mice. *p < 0.05 and ** p < 0.01 by one-factor ANOVA analysis, Bonferroni’s multiple comparison test.
to eukaryotic cells by such a strain at a dose as low as 1 bacterial per cell can lead also to genomic instability, an event associated with the predisposition to colorectal carcinogenesis development. We can hypothesize that the long-term use of E. coli Nissle 1917 to treat patients may significantly increase their risk to develop colorectal cancer. Conversely, the reduced inflammation conferred by clbA-dependent probiotic activity of E. coli Nissle 1917 treatment could decrease this risk, similarly to the reduced colitis-associated cancer observed for patients treated with other anti-inflammatory drugs. Further, DSM 6601 (AIEC) by using the lambda Red recombinease method as described previously. Procedures, primers and templates used for directed-mutagenesis and resultant mutants are given in Supplemental Material and Table S1. Successful mutagenesis was confirmed by PCR using flanking primers and sequencing.

Cell culture, in vitro infection and genotoxicity assay. Non transformed rat intestinal epithelial IEC-6 cells (ATCC CRL-1592) were maintained in Dulbecco’s modified Eagle’s medium (DMEM GlutaMax; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen). 80 μg ml⁻¹ gentamicin, and bovine insulin (0.1 unit ml⁻¹, Sigma) at 37°C in a 5% CO₂ atmosphere. IEC-6 (~75% confluent) were washed four times and incubated in infection medium based on DMEM supplemented with 25 mM Hepes and 10% FBS (Invitrogen). Bacteria were pre-grown in infection medium to the mid-logarithmic phase, and then the infection dose was calculated to a multiplicity of infection of 20 or 100 bacteria per cell. After a 4 h infection at 37°C and 5% CO₂, the cells were washed and grown until analysis in cell culture medium supplemented with 200 μg/ml gentamicin.

DNA double strand breaks were demonstrated by γ-H2AX immunofluorescence analysis. Mouse anti-γ-H2AX (Millipore) was diluted 1:500 in blocking solution and incubated for 1 h. DNA was counterstained for 5 min with TO-PRO-3 (Invitrogen) was diluted 1:100 in blocking solution and incubated overnight at 4°C. FITC-conjugated goat anti mouse secondary antibody (Invitrogen) was diluted 1:500 in blocking solution and incubated overnight. Images were acquired with an Olympus IX70 laser scanning confocal microscope, in sequential mode, with the Fluoview FV500 software. At least 15 random fields have been evaluated blindly for γ-H2AX foci counting under 60x apochromatic objective for each experiment. Pan-nuclear γ-H2AX staining consisted of nuclei with more than 100 γ-H2AX foci.

Metaphasic chromosome spreading, visualization of chromosomal abnormalities and polyplody cells were done as previously described. Chromosomes were stained with TO-PRO-3 (Invitrogen) and examined with an Olympus IX70 laser scanning confocal microscope under a 60x objective. Images were deconvoluted with Isis 5.59 software (Christian Buhl, Isis software). Flow cytometry analyses were done with a FACScalibur flow cytometer (Bekton Dickinson). At least 2x10⁶ cells were acquired per sample and analyzed with FlowJo software (Tree Star).

Animals. Male Wistar rats (180–200 g body weight, Janvier) or male SCID mice (25 g body weight; Charles River) were maintained under specific pathogen-free condition in Animal Care Facility of Neuro-gastroenterology group with free access to food

E. coli

Materials and Methods

Bacterial strains and mutagenesis procedures. In order to investigate the potential role of Colibactin production in protection from colitis, we generated chromosomal isogenic mutants of E. coli Nissle 1917 (Mutagenesis procedures). These opposite effects encountered among closely bacteria from the same species highlights that the crosstalk between probiotics, prebiotics and gut microbiota and host is probably more complex than previously supposed. In conclusion, although intervention studies with probiotics have yielded promising results, it remains difficult to draw general conclusions. This is probably due to the highly variable nature of the probiotic used since health benefit of one probiotic strain cannot be extrapolated to others and bacteria from the same species may have opposite effects. Deciphering the precise mechanisms leading to probiotic action, and potential side effects, are required to delineate the optimal use of probiotics as supplementary therapy. From this perspective, it is time to connect probiotic researchers with approaches used for pathogens in the field of infectious disease.
and water throughout the study. All experiments were performed in accordance with European guidelines and were reviewed by the local ethics committee on animal experiment (Région Midi-Pyrénées). A persistently hunched posture and labored respiration, a weight loss of more than 20% were considered as end-points where animals were euthanized.

DSS-induced acute colitis. Wistar rat received drinking water supplemented with 5% (wt/vol) DSS (molecular weight 36,000–50,000; MP Biomedicals) from day 0 to day 7. Controls were all time-matched and consisted of rats receiving normal tap water (n = 10 per group). Oral administrations of Nissle WT or Nissle ΔclbA were initiated 7 d before inflammation and prolonged during the DSS treatment. Each rat received a daily oral gavage of 10^8 cfu dissolved in PBS. Body weight, stool consistency and bleeding were monitored daily. Clinical scoring was based on the evaluation (0–4) from the last two parameters: 0, formed stool and absence of blood trace; 1, diarrhea and presence of blood trace in stool; 2, diarrhea and rectal bleeding; 4, diarrhea and rectal bleeding with rectal prolapse. Animals were sacrificed on day 7 and distal segments of colon were stored at -80°C until assayed for measurement of myeloperoxidase (MPO) activity as previously described and ELISA. For ELISA, tissue proteins were extracted with RIPA buffer (1% Igepal, 0.5% deoxycholic acid and 0.1% sodium dodecyl sulfate in Tris-buffered saline 0.05 and ***p < 0.001 by one-factor ANOVA analysis, Bonferroni’s multiple comparison test.

Figure 5. Genetic complementation of the clbA mutant of Nissle 1917 restores its probiotic activity. CD4⁺ CD45RBhigh T cells were purified from spleens of BalB/c ANNCrl mice and 2 × 10^5 cells were injected (i.p.) into recipient SCID mice. Groups of 9 mice fed with Nissle WT, Nissle ΔclbA or Nissle ΔclbA + ΔclbA (2.5 × 10^6 cfu/mouse) every 3 d and sacrificed pre-emptively at 56 d after transfer due to global exacerbation of colitis severity. The extent of colitis damage was assessed blindly before tissue samples were collected. Error bars represent the SEM and the data shown result from one experiment. For related data, see Figure S2. (A) Plored data represent colitis scores of each individual mouse and medians are indicated. *p < 0.05, Kruskal-Wallis test, Dunn’s multiple comparison test.

Conventional BalB/c AnNCrl nodeficiency, background C.B-17lcr-Prkdc scid/lcrCrl) were used as recipients of transferred cells. CD4⁺ CD45RBhigh T cells were purified from spleens of BalB/c ANNCrl mice and 2 × 10^5 cells were injected (i.p.) into recipient SCID mice. Groups of 9 mice fed with Nissle WT, Nissle ΔclbA or Nissle ΔclbA + ΔclbA (2.5 × 10^6 cfu/mouse) every 3 d and sacrificed pre-emptively at 56 d after transfer due to global exacerbation of colitis severity. The extent of colitis damage was assessed blindly before tissue samples were collected. Error bars represent the SEM and the data shown result from one experiment. For related data, see Figure S2. (A) Plored data represent colitis scores of each individual mouse and medians are indicated. *p < 0.05, Kruskal-Wallis test, Dunn’s multiple comparison test.

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qRT-PCR assays (see cDNA conversion using iSript cDNA synthesis kit (Bio-rad). Tubes were shaken in CK-MIX beads; Bertin Technologies). Tubes were shaken in a Precellys 24 bead apparatus (Bertin Technologies) at 6,000 rpm for 2 cycles of 10 sec with interval of 10 sec. RNA was then extracted by using Qiagen kit, as recommended by the manufacturer. RNA (1μg) was treated with DNase (Sigma) prior to cDNA conversion using iScript cDNA synthesis kit (Bio-rad). qRT-PCR assays (see Table S2) were performed using IQ SYBR Green Supermix (Bio-rad). Reactions were performed on a Bio-Rad CFX96TM. For cytokine analysis, samples were normalized to the housekeeping gene hypoxanthine guanine phosphoribosyltransferase 1 (HPRT) for intersample variation. Normalized fold expressions analyzed using CFX-manager software (Bio-rad) were done to PBS-treated colitis mice. Histology. Rat or mouse colonic tissues fixed in Dubosq-Brazil were embedded in paraffin, sectioned (7-μm slices) and stained with Hematoxylin and Eosin (H&E). Colonic damage were graded semi-quantitatively in blinded fashion by one or two of the authors (E.M., C.C.). C.C. was blinded to the experimental protocol used. Each of 5 histological parameters was scored as absent (0), mild (1), moderate (2), or severe (3) density of inflammatory cells, epithelial injury, edema, crypt abscess and epithelial hyperplasia. Histological score was the sum of these parameters. Statistical analysis. Results are expressed as mean ± SEM or median unless otherwise stated. For in vivo experiments, N refers to the number of animals per group used for each experiment. Statistical significance of differences between experimental groups was performed using two-way analysis of variance (ANOVA) with Bonferroni multiple-comparison post-test, or Kruskal-Wallis non-parametric ANOVA with Dunn’s multiple-comparison post-test as appropriate (Prism 5, GraphPad Software). Two-side analyses were used throughout and p values < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here: http://www.landesbioscience.com/journals/gutmicrobes/article/21737/

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