Lactobacillus plantarum CAU1055 ameliorates inflammation in lipopolysaccharide-induced RAW264.7 cells and a dextran sulfate sodium–induced colitis animal model

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Received December 21, 2018.
Accepted April 5, 2019.
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ABSTRACT

This study aimed to screen lactic acid bacteria (LAB) for their anti-inflammatory activity by using RAW264.7 cells and dextran sulfate sodium (DSS)-induced colitis. In all, 192 LAB strains were isolated from healthy human feces, of which 8 strains showed excellent nitric oxide (NO) inhibitory activity. Peptidoglycan extracts of these 8 LAB strains were subjected to NO assay, Western blot, and ELISA. Among the 8 tested strains, extracts of 4 strains significantly inhibited the production of NO, related enzyme activities such as inducible nitric oxide synthase and cyclooxygenase 2, and key cytokines such as tumor necrosis factor-α and IL-6 in RAW264.7 cells. The 4 strains belonged to Lactobacillus (CAU1054, CAU1055, CAU1064, and CAU1301). Oral administration of the 4 strains significantly inhibited DSS-induced body weight loss, colon shortening, and colon damage in ICR mice. The colon tissue of the mice treated with Lactobacillus plantarum strain CAU1055 had significantly reduced levels of inducible nitric oxide synthase, cyclooxygenase 2, tumor necrosis factor-α, and IL-6 in RAW264.7 cells. The 4 strains could be used as a candidate probiotic strain for the prevention and treatment of inflammatory bowel disease. Further studies are warranted to confirm the mechanisms of interaction between peptidoglycan of L. plantarum strain CAU1055 and upstream cellular signaling mediators. Key words: lactic acid bacteria, peptidoglycan, RAW264.7 cells, dextran sulfate sodium, anti-inflammatory effect

INTRODUCTION

Inflammation is a host defense mechanism against tissue injury caused by bacterial infections and chemical or physical damage; it is mainly regulated by proinflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and enhanced by proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and IL-6 (Dinarello, 2006; Medzhitov, 2008). Inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn’s disease are chronic inflammatory diseases of the gastrointestinal tract characterized by various symptoms, including rectal bleeding, diarrhea, abdominal pain, fever, and weight loss. Although the etiology of IBD is not yet known, many studies have identified therapeutic molecular targets to treat IBD. For example, Atreya et al. (2000) reported that the soluble IL-6 receptor signaling pathway is involved in inducing resistance to mucosal T-cell apoptosis in patients with Crohn’s disease. Similarly, neutrophil transmigration through the apical junction complex was suggested as a potential strategy to regulate mucosal inflammation (Chin and Parkos, 2006). Monoclonal antibodies against TNF-α or integrin and anti-inflammatory drugs such as 5-aminosalicylates and corticosteroids have been approved for the treatment of IBD by the US Food and Drug Administration. However, the development of safe and economical agents without adverse effects is required to prevent IBD.

Probiotics are defined as living microorganisms that provide health-beneficial functions to hosts; they play critical roles in maintaining the intestinal ecosystem, preventing diarrhea, lowering serum cholesterol, and modulating the immune system. Lactic acid bacteria (LAB) are gram-positive, nonsporulating rod- or cocci-shaped bacteria. Among the LAB, Lactobacillus spp. are widely used in the food and dairy industry and have immunomodulatory functions such as anticancer and anti-inflammatory activities. Among different probiotics, the physiological health-beneficial effects of Lactobacillus and Bifidobacterium have been widely elucidated in diverse chronic diseases, including inflammation. Kim et al. (2015) reported that Lactobacillus helveticus regulated inflammatory T-cell activity and...
suppressed rheumatoid arthritis. *Lactobacillus rhamnosus* GG was found to modulate the immune responses of dendritic cells, macrophages, and monocytes, and to ameliorate inflammatory diseases (Fong et al., 2015). In clinical trials, *Lactobacillus* and *Bifidobacterium* strains were shown to maintain remission and prevent relapse in patients with Crohn’s disease (Hijova and Soltesova, 2013).

Several strains of *Lactobacillus plantarum* have been reported to exhibit some ameliorative effects on inflammation in LPS-induced cell lines and a dextran sulfate sodium (DSS)-induced colitis animal model (Liu et al., 2011; Cui et al., 2016; Wang et al., 2019). However, different *L. plantarum* strains may exert different positive effects on ulcerative colitis in vivo. It is well known that many probiotic properties are strain-specific, meaning that different strains of the same bacterial genus and species may exert diverse probiotic properties. This is why the specific probiotic effects of each individual LAB strains should be well defined, and the effect of each strain should be demonstrated case by case (Campana et al., 2017). Accumulating evidence firmly indicates that probiotics might prevent inflammatory immune malfunctions, including IBD. Thus, significant efforts have been made to screen intestinal microorganisms and identify novel strains that can modulate inflammation.

Although *Lactobacillus* is known to regulate immunity, the molecular mechanisms are not yet elucidated (Weiss et al., 2010). Microbe-associated molecular patterns (MAMP) such as peptidoglycans (PGN) and teichoic acid of *Lactobacillus* are associated with the reaction of pattern recognition receptors (PRR) such as toll-like receptors (TLR) in intestinal epithelial cells; these interactions act to regulate immune functions (Ebrahimi and Jafarei, 2011).

Dairy products such as yogurts and cheeses are considered among the most suitable carriers of probiotic strains for human consumption (Lourens-Hattingh and Viljoen, 2001). Because of its dual role as an indigenous human gut microbiota as well as a safe starter culture in food fermentation, *L. plantarum* exhibits great potential to be applied in dairy systems, including development of various functional dairy products (Li et al., 2017). Potential probiotic strains of *L. plantarum* could be used in starter cultures or used as adjunct bacteria without adverse effects on product quality or sensory properties of fermented milk products (Dan et al., 2019). Many recent studies have reported that consuming *L. plantarum*-containing fermented milk exerts in vivo functionality to the host (Nasrabadi et al., 2011; Kim et al., 2018).

In our previous study, we screened candidate probiotic strains for anti-inflammatory activity. As a result, 4 candidate probiotic strains (*L. plantarum* CAU1054, CAU1055, CAU1064, and CAU1106) were selected based on inhibition of NO in LPS-induced RAW264.7 cells, the results of probiotic property assessments, as well as ability to ferment milk, which is a necessary feature for application in dairy systems (Lee et al., 2015). Herein, we investigated and compared the in vitro anti-inflammatory activity of the PGN fraction of novel strains of LAB in murine macrophage RAW264.7 cells and confirmed our findings in a dextran sulfate sodium (DSS)-induced colitis animal model. We also determined the regulatory effect of molecular markers known to mediate inflammatory processes.

**MATERIALS AND METHODS**

**Reagents**

RAW264.7 cells (murine macrophages) were purchased from Korean Cell Line Bank (KCLB; Seoul, Korea). Dulbecco’s modified Eagle’s medium and fetal bovine serum were purchased from Gibco (Grand Island, NY). Griess reagent was obtained from Sigma-Aldrich (St. Louis, MO). Goat anti-rabbit IgG-horseradish peroxidase (HRP), donkey anti-goat IgG-HRP, and goat anti-mouse IgG-HRP antibodies, iNOS, and COX-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and β-actin antibody was purchased from Thermo Scientific Fermentas (Vilnius, Lithuania) and Raybiotech (Norcross, GA), respectively. Dextran sulfate sodium was from MP Biomedicals (Illkirch, France). Five-week-old male ICR mice were obtained from Young Bio (Seongnam, Korea).

**Strains Used for In Vitro and In Vivo Experiments**

*Lactobacillus plantarum* (CAU1045, CAU1054, CAU1055, CAU1064, and CAU1106), *Weissella viridescens* CAU1224, *Lactobacillus sakei* ssp. sakei CAU1273, and *Lactobacillus salivarius* CAU1301 were obtained from healthy adults (Table 1) and from our laboratory stocks.

For in vitro assays, bacterial cultures were boiled at 95°C for 30 min in 4% SDS (Amresco, Solon, OH). To extract the PGN fraction from the selected 8 novel strains (Table 1), the insoluble materials were washed 5 times with distilled water, suspended in 10 mL of 100 mM Tris-HCl (pH 7.5), and treated with DNase I (5
µL, 1 mg/mL), RNase A (5 µL, 5 mg/mL), and MgSO4 (20 mM) at 37°C for 2 h. Next, trypsin (10 µL, 10 mg/mL) and CaCl2 (10 mM) were added, and incubation was continued at 37°C for 12 h. After centrifugation (12,000 × g for 15 min at room temperature), the pellets were boiled in 1% SDS for 10 min, suspended in 10% trichloroacetic acid at 4°C for 24 h, and washed several times with distilled water until the pH was neutral. The PGN was stored at −20°C (Wu et al., 2013).

For in vivo assays, Lactobacillus strains were incubated in de Man, Rogosa, and Sharpe broth at 37°C and resuspended in PBS at a concentration of 4 × 10^10 cfu/mL.

Cell Culture and LAB Treatment

As described previously (Lee et al., 2015), RAW264.7 cells were incubated in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% streptomycin and penicillin at 37°C in a humidified 5% CO2 incubator. The cells were counted using Trypan blue (Amresco) and a hemocytometer. The RAW264.7 cells were plated at a density of 1.5 × 10^6 cells/well in a 60-mm dish for 24 h; after LPS (100 ng/mL) and PGN (1,000 µg/mL) were added, the incubation was continued for 24 h.

NO Assay

The production of NO in culture supernatant was measured using Griess reagent, as previously described (Green et al., 1982). About 50 µL of the culture supernatant was mixed with equal volumes of Griess reagent for 15 min. Absorbance at 540 nm was measured using a microplate reader. The concentration of nitrite was determined using a standard curve generated using sodium nitrite (Kim et al., 2007).

Western Blot and ELISA

Western blotting was conducted as described previously (Bao et al., 2014). Briefly, cell and tissue lysates were separated on 8% SDS polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. The membrane was incubated with primary antibodies of iNOS, COX-2, and β-actin and then with goat anti-rabbit IgG-HRP, donkey anti-goat IgG-HRP, and goat anti-mouse IgG-HRP secondary antibodies for 1 h. Enhanced chemiluminescence detection reagent (ATTO, Tokyo, Japan) was used to measure the expression levels of the proteins.

The concentrations of the cytokines TNF-α and IL-6 in the cell culture or tissue lysates were measured using ELISA kits, according to the procedure described by the manufacturers.

Animal Experiment

Five-week-old male ICR mice were housed in a room with a 12-h light/dark cycle and a temperature of 22 ± 2°C; they were provided free access to diet and water. The mice were acclimated to the laboratory conditions for 4 d and used for the animal experiment for 3 wk.

The mice were divided into 6 groups of 8 animals each: healthy control group (negative control), DSS-treated group (positive control), and Lactobacillus-treated groups with DSS. During the animal experiment, a DSS-induced mouse model was used. The IBD control and Lactobacillus groups were fed with 1.5% DSS instead of drinking water. The healthy control and IBD control were provided 0.2 mL of PBS, and the Lactobacillus-treated groups received 0.2 mL of Lactobacillus. Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee at Chung-Ang University (Approval number: 2017-00103, Seoul, Korea).

Evaluation of IBD and Histological Evaluation

The BW of the mice was measured at the same time daily to evaluate the degree of IBD. After induction of colitis with DSS, mice were killed under anesthesia. The length of colon from the cecum to the anus was measured. Colon tissues were fixed in 10% formaldehyde (Duksan, Seoul, Korea) and embedded in paraffin. Sections of the colon tissue were stained with hematoxylin and eosin (H&E staining), and stained sections were observed using a light microscope (100×).

Statistical Analysis

Data were analyzed using t-test by using SPSS statistical software (version 12.0, SPSS Inc./IBM Corp., Chicago, IL). All data are expressed as mean ± standard error of 3 independent experiments. Differences were considered significant at P < 0.05.

Table 1. Lactic acid bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
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<tr>
<td>Lactobacillus plantarum CAU1045</td>
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<td>Lactobacillus plantarum CAU1106</td>
<td>Lee et al., 2015</td>
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<tr>
<td>Weissella viridescens CAU1224</td>
<td>Lee et al., 2015</td>
</tr>
<tr>
<td>Lactobacillus sakei ssp. sakei CAU1273</td>
<td>Lee et al., 2015</td>
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<tr>
<td>Lactobacillus salivarius CAU1301</td>
<td>Lee et al., 2015</td>
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RESULTS AND DISCUSSION

Effects of Different Strains of Probiotics on NO Synthesis

Probiotics, including lactobacilli, have been shown to regulate inflammation in a strain-specific manner (Christensen et al., 2002; Foligne et al., 2007). In our previous study, we found that the identified strains had differing efficacies in inhibiting NO synthesis induced by LPS (Lee et al., 2015). Interestingly, Lactobacillus salivarius Ls33 was recently reported to recover 2,4,6-trinitrobenezene sulfonic acid-induced colitis symptoms, where the protective effect was derived from the gram-positive cell wall component PGN (Macho Fernandez et al., 2011), suggesting that muropeptides from strain-specific PGN play key roles in modulating intestinal inflammation (Macho Fernandez et al., 2011). Therefore, we first selected 8 strains (CAU1045, CAU1054, CAU1055, CAU1064, CAU1106, CAU1224, CAU1273, and CAU1301) out of 192 fecal isolates and extracted their PGN fractions to identify the strains with the most significant anti-inflammatory efficacy. After PGN at 500 and 1,000 µg/mL was found to not affect the viability of RAW264.7 cells (data not shown), we investigated the effects of PGN on inhibiting LPS-induced NO production in RAW264.7 cells. All strains except CAU1224 strain (W. viridescens) significantly suppressed NO synthesis compared with the LPS-stimulated NO control (Figure 1A). Of the 7 active strains, the inhibitory activity was as follows: CAU1064 (46% inhibition), CAU1055 (44.8%), CAU1054 (41.4%), CAU1045 (38.2%), CAU1106 (32.3%), CAU1055 (31%), and CAU1045 (23.5%), indicating that the NO inhibitory effect is strain specific even within the same species (L. plantarum).

Effects of Different Strains of Probiotics on iNOS and COX-2 Expression

Induction of iNOS by cytokines leads to the production of NO. High concentrations of NO produced by iNOS can become cytotoxic to cells and are involved in inflammatory diseases (Korhonen et al., 2005). To investigate the role of PGN in iNOS expression, we performed Western blot analysis and found that the protein level (expression) of iNOS induced by LPS treatment was suppressed as follows: CAU1055 (45.8% inhibition), CAU1031 (44.8%), CAU1273 (41.4%), CAU1054 (38.2%), CAU1106 (32.3%), CAU1055 (31%), and CAU1045 (23.5%), indicating that the NO inhibitory effect is strain specific even within the same species (L. plantarum).

Figure 1. Effects of different strains of probiotics (Lactobacillus plantarum CAU1045, CAU1054, CAU1055, CAU1064, CAU1106; Weissella viridescens CAU1224; Lactobacillus sakei CAU1273; and Lactobacillus salivarius CAU1301) on (A) nitric oxide (NO) synthesis, (B) inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) expression, and (C) cytokines IL-6 and tumor necrosis factor (TNF)-α in LPS-induced RAW264.7 cells. PGN = peptidoglycan extracts. Data are expressed as mean ± SE of 3 independent experiments. **0.001 < P < 0.001, and ***P < 0.001 compared with positive group by t-test.
which play critical roles in acute and chronic inflammation and carcinogenesis (Müller-Decker, 2011). Peptidoglycan significantly inhibited COX-2 expression in LPS-induced RAW264.7 cells; CAU1064 (40% inhibition), CAU1106 (39.5%), CAU1055 (33.7%), CAU1054 (33.3%), CAU1045 (20.6%), CAU1301 (20.3%), and CAU1273 (19.6%; Figure 1B). The strains having NO inhibitory activity (Figure 1A) had similar suppressive activity on LPS-induced iNOS and COX-2 expression in RAW264.7 cells. Consistent with our results, Wu et al. (2013) reported that PGN from Lactobacillus acidophilus showed anti-inflammatory activity by inhibiting expression of iNOS and COX-2.

**Effects of Different Strains of Probiotics on IL-6 and TNF-α**

Cytokines, mainly produced by different cell types of the immune system, including macrophages and dendritic cells, play critical roles in regulating the initial immune response by a pathogen (Arango Duque and Descoteaux, 2014). Among them, TNF-α is reported to be highly expressed in the colonic tissue and serum of IBD patients and is known to be involved in the regulation of tight junctions and induction of apoptosis in intestinal cells (Ślebioda and Kmiec, 2014). In addition, IL-6 is a proinflammatory cytokine, which was shown to be overexpressed in patients with Crohn’s disease (Nishimoto and Kishimoto, 2004). These findings suggest that TNF-α and IL-6 might be practical molecular targets for treating inflammatory diseases, including IBD. In this study, the expression of LPS-induced TNF-α and IL-6 was decreased by PGN treatment in the following order for TNF-α: CAU1055 (83% inhibition), CAU1064 (83%), CAU1054 (77.7%), CAU1106 (74.6%), CAU1301 (67.2%), CAU1224 (55.7%), CAU1273 (47.2%), CAU1045 (37.8%), and for IL-6: CAU1106 (92.8% inhibition), CAU1055 (91%), CAU1064 (90%), CAU1054 (89%), CAU1045 (80.5%), CAU1301 (76.6%), and CAU1273 (49.8%; Figure 1C), indicating that PGN of probiotics characterized herein, especially that of CAU1055, CAU1064, and CAU1106, had high efficacy in regulating inflammatory processes in macrophage cells.

**Effects of Different Strains of Probiotics on DSS-Induced Colitis Animal Model**

To further investigate in vivo anti-inflammatory activities, we chose 4 strains, CAU1054, CAU1055, CAU1064, and CAU1106, to test a DSS-induced colitis model. As an model to mimic human IBD, DSS is the most commonly used chemical irritant to induce intestinal colitis, resulting in symptoms such as BW loss, bloody diarrhea, colon shortening, and mucosal inflammation, which are similar to those found in humans (Okayasu et al., 1990; Elson et al., 2005). We first tested different concentrations of DSS (1.5% and 2.5%) to find the optimal concentration of DSS to induce colitis and found that 2.5% DSS for 3 wk was too toxic in ICR mice. After treatment with 1.5% DSS, we noted significant decreases in BW (from 32.35 ± 0.98 g to 30.53 ± 0.87 g) and colon length (from 10.13 ± 0.36 cm to 6.7 ± 0.19 cm) in ICR mice (Figure 2A). Oral administration of L. plantarum CAU1055 significantly increased BW (32.78 ± 0.41 g) compared with the DSS-treated group (30.53 ± 0.87 g), but the effects of other strains on BW were minimal (Figure 2A). The reduced colon length induced by DSS was most strongly recovered by L. plantarum CAU1055 (from 6.7 ± 0.19 cm to 9.27 ± 0.08 cm; Figure 2B), indicating that this strain is an important candidate to modulate intestinal colitis induced by DSS.

**Effects of L. plantarum CAU1055 on Inflammatory Mediators in DSS-Induced Colitis Model**

To investigate the regulatory mechanisms of L. plantarum strain CAU1055 in inflammatory colon tissues, we performed H&E staining and protein expression analysis. The colonic tissue in the control group showed normal epithelium and mucosal layer, but extensive disruption of the epithelium (indicated by arrows) and infiltration of inflammatory cells (indicated by stars) were observed in the DSS-treated group (Figure 3A). However, after treatment with L. plantarum strain CAU1055, the mucosal damage was relieved and infiltration of immune cells was rarely observed (Figure 3A), suggesting that L. plantarum strain CAU1055 protects against intestinal inflammatory symptoms induced by DSS.

Several studies on transcriptome analysis have revealed that DSS induces proinflammatory cytokines and their receptors, matrix metalloproteinase family members, cell proliferation–related genes, and well-known mediators such as iNOS and COX-2 (Lee et al., 2009; Cho et al., 2011). The enhanced expression of iNOS and COX-2 by DSS in colon tissues was suppressed by up to 43.4 and 42.7%, respectively, by the administration of the L. plantarum strain CAU1055 (Figure 3B). In addition, the production of proinflammatory cytokines IL-6 and TNF-α in tissue lysates following induction by DSS was decreased from 65.60 ± 1.96 to 1.95 ± 1.48 pg/240 ng of lysate and from 581.5 ± 28.28 to 378.17 ± 18.00 pg/240 ng of lysate, respectively, after administration of L. plantarum CAU1055 (Figure 3C). Considering
the results of in vitro and in vivo studies, we propose that *L. plantarum* strain CAU1055 ameliorates colonic inflammation by modulating iNOS, COX-2, IL-6, and TNF-α. In agreement with the findings of the present study, several studies have indicated that strain-specific probiotics, including *Lactobacillus fermentum* BR11 and *L. rhamnosus* GG (Geier et al., 2007), *L. plantarum* K68 (Liu et al., 2011), and *L. plantarum* Lp91 (Duary et al., 2012), show significant immunomodulatory effects in DSS-induced animal models, indicating that the novel strain of *L. plantarum* isolated from human feces in the current study may also play a crucial role in modulating inflammatory progression in the colon.

The molecular mechanism of immune modulation by microorganisms is not yet clearly elucidated (Weiss et al., 2010). Peptidoglycan, the main component of gram-positive bacteria cell wall, consists of alternating residues of β-(1,4)-linked N-acetylg glucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) and L- and D-amino acids. The short peptide chain is cross-linked to the peptide chain of another strand and attached to MurNAc. Generally, the peptide chain is L-Ala-γ-D-Glu-β-L-Lys-γ-D-Ala, but this varies depending on microbial species (Chaput and Boneca, 2007). Immunity is controlled by the third amino acid of the peptide chain and the variation in cross-linking to the peptide. Thus, the signaling pathway induced by the interaction of microbe-associated molecular patterns and pattern recognition receptors (such as TLR2/TLR6; Ren et al., 2016) can be controlled variably depending on the microorganism. Diseases such as IBD and allergic reactions can be treated and prevented by this interaction.

**CONCLUSIONS**

Among the 8 identified strains of 192 fecal isolates, we found that PGN from *L. plantarum* CAU1054, CAU1055, CAU1064, and CAU1301 had a significant inhibitory effect on LPS-induced inflammation by regulating iNOS, COX-2, IL-6, and TNF-α in RAW264.7 murine macrophage cells. In the DSS-induced inflammatory animal model, we confirmed that *L. plantarum* strain CAU1055 ameliorated intestinal inflammatory symptoms such as BW loss and colon shortening by modulating the same inflammatory markers in colon tissue. These results indicate that *L. plantarum* strain CAU1055 can act as a potent and safe probiotic with beneficial effects on inflammatory diseases in the colon, although further studies are necessary to clarify the exact mechanisms of interaction between PGN of *L. plantarum* CAU1055 and upstream cellular signaling mediators. Based on the functional properties shown in this study as well as the milk-fermenting characteristic reported in our previous study (Lee al., 2015), we suggest that *L. plantarum* CAU1055 is a potential probiotic candidate for application in the development of functional dairy products.
ACKNOWLEDGMENTS

This work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through the Agri-Bio Industry Technology Development Program, funded by the Ministry of Agriculture, Food and Rural Affairs (MAFRA) (318091-03-1-HD030). This research was also supported by the Chung-Ang University Graduate Research Scholarship in 2017.

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