Applied and Environmental Microbiology, July 2011, p. 4681–4684 Vol. 77, No. 13
0099-2240/11/$12.00 doi:10.1128/AEM.03021-10

Inhibition of the NF-κB Pathway in Human Intestinal Epithelial Cells by Commensal *Streptococcus salivarius*▽†

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Received 24 December 2010/Accepted 9 May 2011

Streptococcus salivarius **exhibited an anti-inflammatory effect on intestinal epithelial cells (IECs) and monocytes. Strains were screened using a reporter clone, HT-29/kB-luc-E, induced by tumor necrosis factor alpha (TNF-α). Supernatant from each strain downregulated NF-κB activation. The two most efficient strains produced an active metabolite (<3 kDa) which was able to downregulate the secretion of the proinflammatory chemokine interleukin-8 (IL-8).**

The intestinal microbiota consists of more than 10^{14} bacteria living in a symbiotic relationship with their host. Commensal microorganisms contribute to host health by supplying nutriment, preventing pathogen colonization, and maintaining intestinal homeostasis. The microbial inhabitants contribute to the maturation of the gastrointestinal tract and its immune system by shaping and maintaining normal mucosal immunity (20). These properties underline the existence of an extensive cross talk between the commensal bacteria and the gut mucosae to maintain beneficial relationships and tolerogenic host response (21). The intestinal epithelial cells (IECs) represent the first point of contact for bacteria within the gut, preventing microbial penetration and eliciting first communication for immune recognition of commensal bacteria. Keeping a balance between tolerant response and aberrant inflammation is the main goal of the cross talk between commensal bacteria and IECs in the digestive tract (1, 20).

In an inflammatory context, several commensal and probiotic bacteria have been shown to modulate mucosal innate immune response and reduce the inflammatory signaling cascade (7). *Lactobacillus* and *Bifidobacterium* species have been shown to reduce inflammatory responses, including NF-κB activation and interleukin-8 (IL-8) production, in various models of intestinal epithelial cells (2, 16, 28). Interestingly, the immunomodulation capacities of commensal species from the intestinal microbiota established on IECs are correlated with anti-inflammatory effects *in vivo* (6, 9, 12, 13, 25, 29). Although close contact between live commensal bacteria and eukaryotic cells leads to many biological activities, some secreted bacterial factors have been characterized as responsible for anti-inflammatory effect (8, 17). These bacterial products may be proposed as tools for prevention and/or treatment of human inflammatory bowel diseases.

Several mechanisms underlying the beneficial effects of commensal and probiotic bacteria identified *in vitro* involve a downregulation of the NF-κB-dependent transcriptional activity. NF-κB is a dimeric transcription factor whose activation is connected by signaling cascade to several receptors, including Toll-like receptors (TLRs). The different steps of the NF-κB signaling pathway represent potential targets for anti-inflammatory probiotic and commensal bacteria to weaken NF-κB activation and thereby prevent its transcriptional activity. An inhibition of the NF-κB pathway is observed with targeting genes involved in ubiquitination and proteasome processes by *Lactobacillus casei* anti-inflammatory effect (28), through interference with IκBα degradation of nonpathogenic *Salmonella* strain effect (14, 18), in the cellular phosphorylation step of the NF-κB pathway by interference with soluble factor of *Bifidobacterium breve* (9), and finally, in the nuclear export of NF-κB subunit RelA by *Bacteroides thetaiotamicron* (12).

The commensal bacterium *Streptococcus salivarius* is one of the early colonizers of oral mucosal surfaces a few hours after birth. This species remains prevalent in the oral cavity and subprevalent in the digestive tract throughout the life span and plays an important role in oral ecology. *S. salivarius* displays protective effects against pathogens involved in development of tooth decay and periodontitis (26, 27). It influences the inflammatory responses triggered by periodontopathogens and enteric pathogens (8, 24). Recently, the commensal strain *Streptococcus salivarius* K12 was shown to attenuate NF-κB activation, suggesting a role of this bacterium in inflammation (3). We investigated the regulatory effects of *S. salivarius* strains on the NF-κB pathway in human IECs. We used NF-κB reporter systems stably expressed in HT-29 (ATCC HTB-38) to analyze the effects of different strains of *S. salivarius* on NF-κB activation. Five repeats of the NF-κB binding site were cloned in the luciferase reporter plasmid pGL3 enhancer vector (Promega). The obtained plasmid was cotransfected with pTK-Hyg plasmid, a hygromycin selection vector (Clontech),

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 20 May 2011.

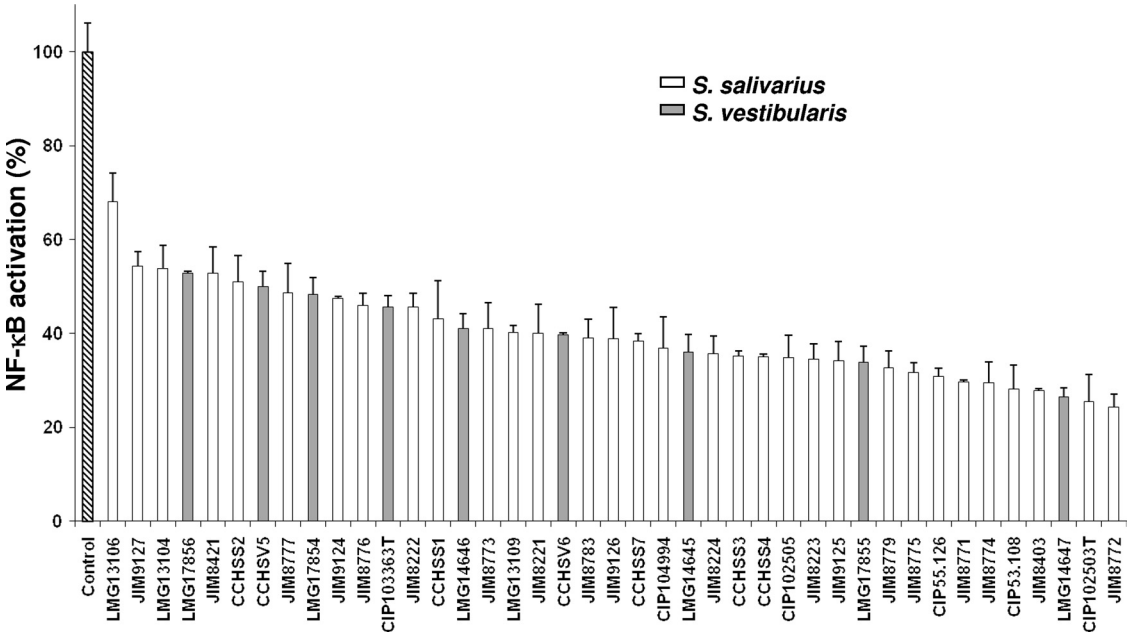

FIG. 1. Effect of the supernatants (Sn) of different strains of *S. salivarius* and *S. vestibularis* on TNF-α-induced NF-κB activation in HT-29/kB-luc-E. Luciferase activity was measured after addition of Sn to HT-29/luc-E reporter cells in the presence of TNF-α (10 ng/ml). The control assay corresponds to luciferase activity obtained with HT-29/kB-luc-E reporter cells in the presence of M17 bacterial growth medium with adjusted pH and TNF-α (10 ng/ml). Results are expressed as relative percentages of NF-κB activation compared to the control assay (100%). Results are represented by means ± standard deviations of triplicate measurements from one representative experiment out of a minimum of three independent experiments performed. Data were analyzed by Student's *t* test ($P < 0.05$ compared to control for all tested supernatants). Results from *S. salivarius* (white bars) and *S. vestibularis* (gray bars) are classified by increasing inhibition strength.

in HT-29 cells using TFX-50 (Promega) according to the manufacturer's instructions. After 3 weeks under hygromycin (200 μg/ml), the HT-29/kB-luc-E clone was selected for its response to tumor necrosis factor alpha (TNF-α; 10 ng/ml; Peprotech). For each experiment, HT-29/kB-luc-E reporter cells were seeded at 50,000 cells per well into 96-well plates and incubated for 48 h in RPMI (Sigma) supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal calf serum (FCS; Lonza) in a humidified 5% CO_2 atmosphere at 37°C, before stimulation (6 h) with TNF-α (10 ng/ml). The supernatants (Sn) of 9 strains of *Streptococcus vestibularis* and 32 strains of *S. salivarius* were tested to determine whether the property to modulate inflammatory response was widely distributed in these species. The strains used in this work were previously described (5) and were grown on M17 (at 37°C) supplemented with glucose (0.5 g/liter). *S. salivarius* and *S. vestibularis* bacterial supernatants were collected by centrifugation of cultures and filtered through 0.22-μm filters. Stimulated cells were tested with each supernatant (10%, vol/vol) or with M17 medium adjusted to pH ~5.5 (pH at the end of the bacterial culture) as a control. Luciferase activity was measured using the luciferase assay system (Promega) and a microplate reader (Infinite 200; Tecan). Relative luminescence units (RLU) are expressed as relative percentage of NF-κB activation compared to positive control, i.e., cells stimulated with growth medium plus the NF-κB activator.

Supernatants of *S. salivarius* and *S. vestibularis* strains markedly inhibited TNF-α-induced NF-κB activation (Fig. 1). The inhibition rate, ranging from 30 to 70%, suggested that an active metabolite modulating the inflammatory response was produced and released in the culture medium. The supernatants of JIM8772 and CIP102503^T strains, presenting higher activity, were selected to characterize the bacterial molecular factor involved in this modulation. We demonstrated by dilution of these two supernatants that the effect is dose dependent. Indeed, compared to the 70% inhibition obtained with the CIP102503^T supernatant, inhibitions of 60%, 30%, and 20% of NF-κB were observed when the supernatant was diluted to 3/4, 1/2, and 1/4, respectively. Similar results were obtained using dilutions of JIM8772 supernatant (data not shown).

Several experiments were then performed to characterize the nature of the active compound(s) released by *S. salivarius*. First, we ruled out that the inhibitory effect was due to bacterial metabolites such as butyric and lactic acid that have previously been shown to regulate the NF-κB pathway in several cell lines, including human IECs (10, 11, 22). High-pressure liquid chromatography (HPLC) measurement of organic acids revealed 80 mM lactic acid and the absence of butyrate in *S. salivarius* supernatant (data not shown). Consequently, we tested the effect of a wide range of concentrations of lactic acid (20 to 120 mM). At these concentrations, lactic acid did not affect NF-κB activation (data not shown). Lastly, *S. salivarius* supernatants had no effect on the baseline NF-κB activity and did not affect cell viability as controlled using the MTS assay (Promega) (data not shown).

To determine the size and the nature of the active component(s) present in *S. salivarius* supernatant, we tested the inhibitory activity after passage through 10-kDa- and 3-kDa-

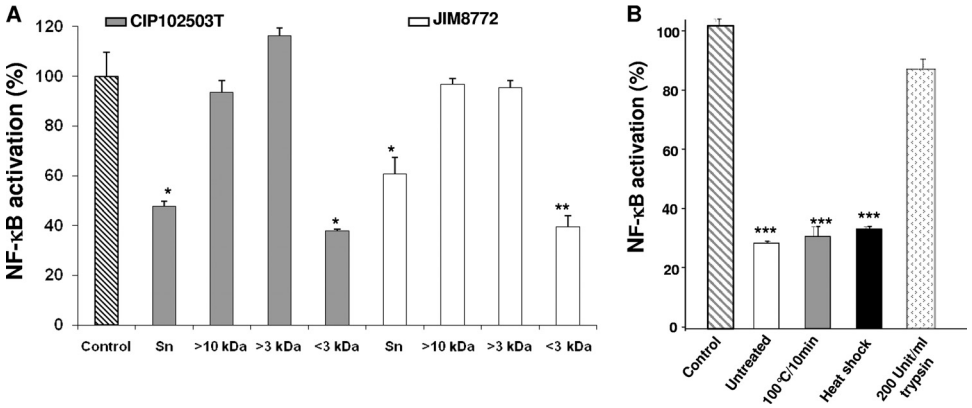

FIG. 2. Determination of molecular mass and nature of the active component secreted by *S. salivarius*. (A) Fractions were obtained after filtrations of *S. salivarius* CIP102503^T Sn (gray bars) and JIM8772 Sn (white bars) after CDM growth through selective membranes. Retained fractions containing metabolites of >10 kDa and >3 kDa and filtered fraction containing metabolites of <3 kDa were tested for inhibition of NF-κB transcription activity in HT-29/kB-luc-E cells. (B) *S. salivarius* JIM8772 <3-kDa Sn was treated by exposure to high temperature (100°C for 10 min), to heat shock (100°C for 10 min before freezing in liquid nitrogen), or to trypsin hydrolysis (200 U/ml). Results are from one representative out of at least three independent experiments. *P* values were <0.05 (*), <0.025 (**), or <0.01 (***) compared to control for the two tested strains. Bars represent standard deviations of the means.

cutoff columns. To facilitate the purification process, strains were grown on chemically defined medium (CDM) (23) supplemented with 0.5 g/liter ascorbic acid, 0.1 mM $MgCl_2$, β-glycerophosphate (disodium salt, 6 g/liter), and glucose (0.5 g/liter). Bacterial supernatants were submitted to ultrafiltration through Centriplus YM-3 or YM-10 membranes that retain molecules larger than 3 or 10 kDa, respectively. For both strains, the <3-kDa fraction inhibited NF-κB activity by 60% while retained fractions (>10 kDa and >3 kDa) displayed no effect (Fig. 2A). Furthermore, a trypsin treatment (200 U/ml; Mag-Trypsin; Ozyme) of the <3-kDa fraction resulted in a drastic loss of the inhibitory effect, with only ~15% remaining inhibition (Fig. 2B). These results suggest that the partially purified active compound of a molecular mass lower than 3 kDa contained peptidic bonds. Exposure to high temperatures (100°C for 10 min) or to heat shock (100°C for 10 min before freezing in liquid nitrogen) did not affect the inhibitory potential. The compound's resistance to heat treatments confirmed that it is a small molecule that may not display a complex folding. Taken together, these results suggest that *S. salivarius* strains mediated their anti-inflammatory effects through the release of a low-molecular-weight component of peptidic nature.

The inhibitory effects of the *S. salivarius* supernatant on the NF-κB pathway were confirmed on the production of interleukin-8 (IL-8) as assayed by enzyme-linked immunosorbent assay (ELISA; Eli-Pair; Diaclone). Both JIM8772 and CIP102503^T supernatants and their corresponding <3-kDa fractions provoked a drastic decrease of IL-8 secretion induced by TNF-α (Fig. 3). The inhibitory effects were similar between raw and fractionated supernatants, with inhibition rates of 75% and 55% for CIP102503^T and JIM8772, respectively.

JIM8772 and CIP102503^T <3-kDa supernatants were also tested on NF-κB activation in the monocyte-like cell clone THP-1 blue (Invivogen) and the colonic epithelial cell clone Caco-2/kB-seap-7 (15), both bearing an NF-κB reporter system with secreted alkaline phosphatase (SEAP) as reporter gene. SEAP was revealed using Quanti-Blue reagent (Invivogen). JIM8772 and CIP102503^T <3-kDa fraction supernatant led to 40% and 20% inhibition of NF-κB activity on THP-1 reporter cells after activation with lipopolysaccharide (LPS) or TNF-α, respectively (see Fig. S1A in the supplemental material). Similarly, the two fractions induced a 60% inhibition of NF-κB activity in Caco-2/kB-seap-7 cells stimulated with IL-1β (see Fig. 1B in the supplemental material). Thus, the effect of *S. salivarius* supernatant is not restricted to epithelial cells but is also observed on monocytic cells. We confirmed the recent studies that have highlighted anti-inflammatory properties of live *S. salivarius* strains *in vitro*. An *S. salivarius* strain was shown to weaken IL-8 production induced by the periodontopathogen *Aggregatibacter actinomycetemcomitans* on human oral epithelial cells (24). Furthermore, *S. salivarius* reduced NF-κB activation and IL-8 production triggered by *Yersinia*

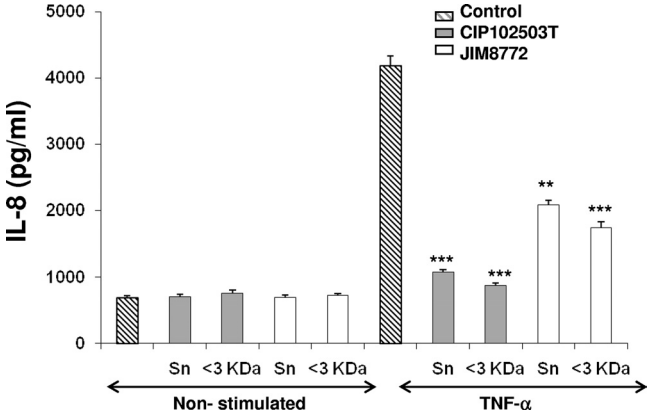

FIG. 3. Effect of *S. salivarius* supernatant (Sn) on IL-8 secretion by HT-29/kB-luc-E cells. HT-29 reporter cells were incubated with *S. salivarius* CIP102503^T and JIM8772 Sn and the corresponding <3-kDa Sn of both strains after CDM growth, in the presence or absence of TNF-α (10 ng/ml). Results are from one representative out of three independent experiments. *P* values were <0.05 (*), <0.025 (**), or <0.01 (***) compared to control for the two tested strains. Bars represent standard deviations of the means.

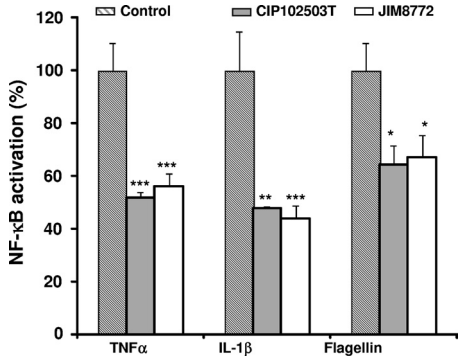

FIG. 4. Effect of *S. salivarius* Sn on NF-κB activation in HT-29/kB-luc-E induced by different inducers. Sn fractions (<3 kDa) from *S. salivarius* JIM8772 (white bars) and CIP102503^T (gray bars) after CDM growth were added to the HT-29/kB-luc-E cells induced by TNF-α (10 ng/ml), IL-1β (10 ng/ml), or flagellin (10 μg/ml). Results are from one representative out of three independent experiments. *P* values were <0.05 (*), <0.025 (**), or <0.01 (***) compared to control for the two tested strains. Bars represent standard deviations of the means.

enterocolitica in HT-29 cells (8) and by *Pseudomonas aeruginosa* or flagellin in a human bronchial epithelial cell line as well as primary cultures of keratinocytes (3). Beneficial effects on inflammatory response and maintenance of intestinal homeostasis were reported for *Streptococcus thermophilus*, a dairy probiotic bacterium and the third known species belonging to the salivarius group (17, 19). Therefore, these results together with our study suggest that species belonging to the streptococcal salivarius group are presenting beneficial effects on host inflammatory processes. Their phylogenetic nearness suggests that they may share a related metabolite production pathway inherited from their common ancestor (4).

Finally, we showed that the supernatant of *S. salivarius* also inhibited NF-κB activity induced in HT-29 cells with another proinflammatory cytokine, IL-1β, and the TLR5 ligand, flagellin (Fig. 4). Thus, the target of the inhibitory compound seemed localized downstream of the receptors and at a step common to all tested TNF, IL-1, and TLR5 receptors.

In summary, we have shown that all strains from a representative collection of *S. salivarius* and *S. vestibularis* strains inhibited the activation of NF-κB. We partially purified a low-molecular-weight metabolite from *S. salivarius* supernatant that harbored anti-inflammatory properties *in vitro* on IECs as well as immune cells. Thus, *S. salivarius* strains are involved in the molecular cross talk with beneficial potential for the host mucosal immune system. The precise characterization of the active molecule and its mechanism of action will facilitate the development of promising therapeutic strategies for inflammatory disorders, such as the safe use of this metabolite as a drug or the use of *S. salivarius* as a probiotic in oral and intestinal pathologies.

This work was supported by the Institut National de la Recherche Agronomique and by the EU MetaHit (Metagenomics of the Human Intestinal Tract) project HEALTH-F4-2007-201052.

We thank Pascal Courtin for the HPLC analysis of organic acids.

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