Immunobiology 219 (2014) 208–217

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Molecular identification of potential Th1/Th2 responses-modulating bacterial genes using suppression subtractive DNA hybridization

Darab Ghadimi[a,*], Patrisio Njiru Njeru[c], Claudia Guigas[b], Mohammed Hassan Farghaly[c], Regina Fölster-Holst[d], Arnold Geis[a], Michael de Vrese[a,c], Jürgen Schrezenmeir[c,e], Knut J. Heller[a]

[a] Department of Microbiology and Biotechnology, Max Rubner-Institut, Hermann-Weigmann-Str. 1, D-24103 Kiel, Germany
[b] Department of Microbiology and Biotechnology, Max Rubner-Institut, Haid-und-Neu-Str. 9, D-76131 Karlsruhe, Germany
[c] Department of Physiology and Biochemistry of Nutrition, Max Rubner-Institut, Hermann-Weigmann-Str. 1, D-24103 Kiel, Germany
[d] Clinic of Dermatology, University Hospital Schleswig-Holstein, Schittenhelmstr. 7, D-24105 Kiel, Germany
[e] Clinical Research Center, Schauenburgerstr. 116, D-24118 Kiel, Germany

ARTICLE INFO

Article history:
Received 19 June 2013
Received in revised form
25 September 2013
Accepted 7 October 2013
Available online 14 October 2013

Keywords:
Cytokine
Lactobacillus fermentum
Optical density
PBMCs
Probiotics
Suppression subtractive hybridization

ABSTRACT

Background and objectives: We characterized the immunomodulating potential of a number of lactobacilli isolated from an African fermented food by co-incubation with peripheral blood mononuclear cells (PBMCs). Two strains with different immune modulating properties were genetically compared by suppression subtractive hybridization (SSH).
Methods: From 48 *Lactobacillus* strains isolated from *Kimere*, *African* fermented pearl millet dough, 10 were selected based on their bile salt tolerance. Their effects on secretion by PBMCs of the T-helper cells Th1- and Th2-cytokines IFN-γ and IL-4, respectively, in the presence or absence of staphylococcal enterotoxin A (SEA) were assessed. To study the genetic basis of different immune-modulating properties, a subtracted cDNA library for *L. fermentum* strains K1-Lb1 (Th1 inducer) and K8-Lb1 (Th1 and Th2 suppressor) was constructed using SSH. Finally, adhesion of these strains to hydrocarbons (relative hydrophobicity) and to human HT-29 colonic epithelial cell line was assessed.
Results: Two strains, K1-Lb1 and K4-Lb6, induced basal IFN-γ secretion. Four strains, K1-Lb6, K6-Lb2, K7-Lb1, and K8-Lb1 diminished INF-γ secretion by SEA-stimulated PBMCs. All strains, except K1-Lb1, K2-Lb4, and K9-Lb3, inhibited SEA-stimulated IL-4 secretion. Comparing the genomes of K1-Lb1 and K8-Lb1 by SSH indicated that K1-Lb1 is able to synthetize polysaccharides, for the synthesis of which K1-Lb8 appears to lack enzymes. A difference in the hydrophobicity properties of the surfaces of both strains indicated that this has impact on their surface.
Conclusion: The K1-Lb1-specific sequences encoding putative glycosyltransferases and enzymes for polysaccharides synthesis may account for the observed differences in immunomodulation and surface properties between the two strains and for mediating potential probiotic effects.

Introduction

Aberrant gut microbiota and allergic and other inflammatory disorders can shift the Th1/Th2 cytokine balance towards a Th2 response, leading to activation of Th2 cytokines and the release of interleukin-4 (IL-4), IL-5, and IL-13 as well as IgE production (Michail 2009). Accordingly, oral intake of probiotics is suggested to prevent or alleviate allergic and other inflammatory disease, specifically those related to inappropriate immune functions associated with Th1/Th2 immune responses (Kuitunen 2009; Lee and Bak 2011). Probiotic bacteria and their components have been shown to modulate Th1/Th2 immune response(s) of antigen/alergen-stimulated immune cells (*e.g.* PBMCs) both *in vitro* and *in vivo* (Foligne et al. 2007; Forsythe et al. 2007; Ghadimi et al. 2008; Helwig et al. 2006; Pochard et al. 2002). Probiotic bacteria can enhance IFN-production and decrease IgE and antigen-induced TNFα, IL-5, and IL-10 secretion (Michail 2009). They and their components, respectively, can potentially modulate the Toll-like receptors and ameliorate inflammatory status (Foligne et al. 2007; Winkler et al. 2007; Zakostelska et al. 2011).

Abbreviations: CFU, colony-forming units; EPS, exopolysaccharide; GTF, glucosyltransferase; LTA, lipoteichoic acid; MAMPs, microbe-associated molecular patterns; NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid; OD, optical density; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PGN, peptidoglycan; PPT, pyruvyltransferase; SEA, staphylococcal enterotoxin A; Th1, T helper cell type 1; Th2, T helper cell type 2; WTA, wall teichoic acid.

* Corresponding author. Tel.: +49 04316092512; fax: +49 0431 609 2472.
E-mail address: darab.ghadimi@mri.bund.de (D. Ghadimi).

0171-2985/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.imbio.2013.10.005

Selection of strains for application as probiotics focuses on two main properties: (i) adaptability to the gastrointestinal environment and (ii) health promoting or functional properties. These selection schemes include survival at low pH and in the presence of bile salts, adhesion to intestinal epithelial cells, colonization of the gut, maintenance of microbial balance, non-pathogenicity to the host, resistance to technological challenges such as processing and distribution, and, last but not least, ability to confer health benefits to the host (FAO/WHO 2002; Heller 2001; Schrezenmeir and deVrese 2001). It should be noted that not every single probiotic strain needs to possess all these characteristics. According to recent scientific evidence, bacteria need not necessarily be 'live' to exert immunomodulation effects as both live and dead cells as well as bacterial DNA were shown to exert some potential health benefits (Ghadimi et al. 2008, 2011; Laudanno et al. 2006; Winkler et al. 2007). Depending on functional targets like colonization of the gut, exclusion of putrefactive bacteria or pathogens, and modulation of microbial metabolism, survival in the gut, however, may be essential in order to ensure that probiotics reach the intended site in an active state (Schrezenmeir and deVrese 2001; Salminen and Isolauri 2006).

As the functional properties of probiotic bacteria are known to be strain-specific, selection and assessment of potential probiotic isolates are important for development of new efficient probiotic preparations (Larsen et al. 2009). With this respect, previous comparative *in vitro* and *in vivo* studies on the effects of probiotic strains on immune function of a range of immune cells have shown significant differences in the cytokine profiling of the *Lactobacillus acidophilus* strains. For example, Holvoet et al. (2013) determined the effects of probiotics on Th2-skewed cells, classified probiotic strains with anti-allergic potential and showed that cytokine profiles induced by probiotics were strain specific. Dong et al. (2012), Drago et al. (2010), Pérez-Cano et al. (2010), Snel et al. (2011) and Vissers et al. (2010, 2011) compared the immunomodulatory effects of different probiotic strains and showed that modulation of expression of some cytokines like IFN-γ and IL-4 was strongly strain-specific. Donkor et al. (2012) showed that although all tested strains of probiotic bacteria had the capacity to induce pro- and anti-inflammatory cytokine production by cell lines and PBMCs, the magnitude of production of each cytokine varied depending on the strain. This strain-specific modulation of expression of cytokines has been attributed to differences in bacterial genomes (van Hemert et al. 2010; Meijerink et al. 2010).

Regarding probiotic characteristics like acid and bile tolerance, antibiotic susceptibility, antimicrobial activity, cell adhesion and antioxidant activity, Dixit et al. (2013), Jamaly et al. (2011), Larsen et al. (2009), Lewandowska et al. (2005) and Venkatesan et al. (2012) have shown significant differences in such characteristics when comparing different strains.

In Africa, several fermented cereal products are produced and consumed. Communities around Mount Kenya have fermented cereal gruel as their daily fermented food. *Kimere* is a traditional fermented pearl millet (*Pennisetum glaucum*) gruel, which is consumed among the Mbeere community. This diet plays a major role in nutrition of the society and may also be considered a possible vehicle for delivery of probiotics to these communities and a source of genetically diverse strains with probiotic properties in general. *Kimere* is consumed without heat treatment after fermentation and hence contains 'live' bacteria. We have shown 10^8 colony forming units (CFU) of lactobacilli to be present per gram of *Kimere* (Njeru et al. 2010), corresponding to a daily intake of approximately 5×10^{10} lactobacilli, assuming a daily intake of 500 g of gruel. Using molecular biology methods including PFGE we have isolated and characterized 48 *Lactobacillus* strains from *Kimere*. Our previous *in vitro* study (Njeru et al. 2010) showed that some of the *Lactobacillus fermentum* strains isolated were resistant to low pH and bile salts. Based on these results, in the first part of the present study we evaluated the immune-modulating effects of these strains on the expression levels of the Th1 and Th2 cytokines IFN-γ and IL-4, respectively, in human PBMCs in response to SEA superantigen. Although previous *in vitro* and *in vivo* studies have shown that modulation of production of cytokines by probiotics in human and animal models is dose, time, and particularly strain dependent (FAO/WHO 2002; Fujiwara et al. 2004; Ghadimi et al. 2008; Kekkonen et al. 2008; Kopp et al. 2008; Luyer et al. 2005; Maassen and Claassen 2008; Pochard et al. 2002; Ryan et al. 2008), there seems to be no study so far that has compared *L. fermentum* strains. In the second part of the study the molecular basis for induction of the Th1-response (IFN-γ) was investigated by suppression subtractive hybridization (SSH) (Annunziato et al. 2007; Ghadimi et al. 2011). We constructed subtracted cDNA libraries for *L. fermentum* strains K1-Lb1 (Th1-stimulating strain) and K8-Lb1 (Th1-nonstimulating strain) in order to identify genes potentially involved in modulation of the Th1 response in PBMCs. Finally, we assessed the hydrophobicities of the cell surfaces of strains K1-Lb1 and K8-Lb1, respectively, in order to verify whether genetic differences of these strains resulted in alterations of their surface structures. In this context, adhesion of both strains to intestinal HT-29 cells was assessed, too.

Materials and methods

Bacterial strains

47 strains of *L. fermentum* and one *Lactobacillus plantarum* strain were isolated from various *Kimere* samples, collected from 11 different homesteads in the Mbeere community of Kenya and characterized using classical microbiological and molecular biology methods (Njeru et al. 2010). Briefly; *Lactobacillus* isolates were characterized and identified using biochemical methods like carbohydrate fermentation patterns, API 50 CHL, growth temperatures, and Gram and catalase reaction, as well as molecular methods like species-specific polymerase chain reaction (PCR), amplified rDNA restriction analysis (ARDRA) and partial sequencing of 16S rDNA. To study strain diversity, 46 *L. fermentum* isolates were subjected to pulsed-field gel electrophoresis (PFGE) analysis, using AscI as restriction enzyme. Analysis of *L. fermentum* strains with PFGE indicated different profiles and relatively large biodiversity within that species for 38 strains. Eight strains were excluded from further evaluation due to unsatisfactory PFGE profiles. Remaining bacterial strains were maintained at −80 °C in MAST Cryobank™ (Mast Diagnostic-Reinfeld Germany) and among them, based on bile salt tolerance, the following strains were selected and used in this study: *L. fermentum K1-Lb1*, *L. fermentum K1-Lb6*, *L. fermentum K2-Lb4*, *L. fermentum K6-Lb2*, *L. fermentum K6-Lb4*, *L. fermentum K7-Lb1*, *L. fermentum K8-Lb1*, *L. fermentum K8-Lb3*, *L. fermentum K9-Lb3*, and *L. plantarum K4-Lb6*.

Propagation of bacteria

Bacterial strains were propagated according to previously published procedures (Ghadimi et al. 2008). Briefly, using a 0.02% inoculum from bacterial stocks stored at −80 °C in 30% glycerol, lactobacilli were grown in MRS broth medium (according to de Man, Rogosa, Sharpe; Merck, Darmstadt, Germany) anaerobically (The Modular Atmosphere Controlled System, MACS-VA500 workstation with airlock, Don Whitley Scientific Limited, UK) at 37 °C overnight. Bacterial cultures were centrifuged at 14,000 × *g* (approximately 14,500 rpm in Eppendorf Minispin-plus centrifuge) for 2 min. Bacterial pellets were washed two times with phosphate-buffered saline (PBS), suspended in 1 ml PBS containing 20%